Calcium-Independent Activation of Calcium Ion Dependent Cyclic Nucleotide Phosphodiesterase by Synthetic Compounds: Quinazolinesulfonamide Derivatives†

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ABSTRACT: Quinazolinesulfonamides are synthetic compounds which calcium-independently stimulate Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. As this activation was observed with 2,4-dipiperidino-6-quinazolinesulfonamides but not with 4-piperidino-6-quinazolinesulfonamides, the activation seems to be dependent on the piperidine residue at the 2 and 4 position of the quinazoline ring, and the extent of hydrophobicity of each compound was thus enhanced. 2,4-Dipiperidino-6-quinazolinesulfonamide activates Ca^{2+}-dependent phosphodiesterase in the absence of Ca^{2+}–calmodulin (CaM). These quinazolinesulfonamides did not further enhance the activity of Ca^{2+}-dependent phosphodiesterase activated by the Ca^{2+}–CaM complex. These compounds are also potent inhibitors of cyclic AMP and GMP phosphodiesterases. CaM antagonists such as *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), its derivatives, and chlorpromazine and prenylamine inhibited selectively the quinazolinesulfonamide-induced activations of the phosphodiesterase. These quinazolinesulfonamides, in a high concentration, had only a slight stimulatory effect on myosin light chain kinase activity. All these findings suggest that the quinazolinesulfonamides are calcium-independent activators of Ca^{2+}-dependent phosphodiesterase and they are proving to be useful tools for the study of CaM and phosphodiesterase, in vitro.

Calmodulin (CaM)[1] is now well accepted as being a major calcium-binding protein in various biological systems (Means & Dedman, 1980). This ubiquitous, heat-stable multifunctional protein was first discovered to be an activator of Ca^{2+}-dependent cyclic 3′,5′-nucleotide phosphodiesterase (Kakiuchi et al., 1970; Cheung, 1970). Recently, CaM was shown to mediate the calcium regulation of a large number of fundamental intracellular enzyme systems (Klee et al., 1980). The mode of action of CaM with respect to phosphodiesterase has been studied extensively; however, the molecular mechanism of this activation remains obscure. On the other hand, alternate activators of CaM-dependent enzymes have been demonstrated. Activators such as proteinases (Cheung, 1971), phospholipids (Wolff & Brostrom, 1976), gangliosides (Davis & Daly, 1980), and fatty acids (Hidaka et al., 1978a) are naturally occurring substances, and there is apparently no documentation of synthesized compounds which stimulate CaM-dependent enzymes. To elucidate CaM–enzyme interactions and to characterize CaM-dependent enzymes, we synthesized and looked for compounds which stimulate the activity of CaM-dependent enzymes.

We now report newly synthesized activators of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase and the activation mechanism of these compounds, in comparison with findings in case of the Ca^{2+}–CaM complex.

Experimental Procedures

Materials. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) and its derivatives were synthesized by the method of Hidaka et al. (1978b). Chlorpromazine and prenylamine were donated from Yoshitomi Pharmaceutical Industries, Ltd., and Hoechst Japan, Ltd., respectively. Cyclic [^{3}H]adenosine 3′,5′-monophosphate, cyclic [^{3}H]guanosine 3′,5′-monophosphate, and adenosine 5′-[γ-^{32}P]triphosphate were from Amersham International, Ltd. All other chemicals were of reagent grade or better. Calmodulin (CaM) was isolated from bovine brain and purified by the procedure described by Yazawa et al. (1980). Myosin light chain kinase was purified from chicken gizzard by the method of Adelstein & Klee (1981). Myosin light chain of chicken gizzard, used as a substrate for the kinase assay, was prepared by the procedure of Perrie & Perry (1970). The light chain was separated from CaM by DEAE-cellulose chromatography (Adelstein et al., 1978). CaM-deficient cyclic nucleotide phosphodiesterase was purified to apparent homogeneity from bovine heart by the method of LaPorte et al. (1979). Cyclic GMP and cyclic AMP phosphodiesterase were prepared from human platelets, as previously reported (Hidaka & Asano, 1976).

Preparation of Cyclic Nucleotide Phosphodiesterase. Human blood platelets contained three distinct forms of cyclic nucleotide phosphodiesterase. Sonicated extracts of human blood platelets chromatographed on DEAE-cellulose yielded three cyclic nucleotide phosphodiesterase fractions designated as FI, FII, and FIII. FI was a specific cyclic GMP phosphodiesterase at low substrate concentration (Hidaka & Asano, 1976). FIII hydrolyzes cyclic AMP faster than cyclic GMP and with a high affinity. FI and FIII were used for this experiment, as cyclic GMP and cyclic AMP phosphodiesterase, respectively. The properties of cyclic GMP (FI) and cyclic AMP (FIII) phosphodiesterases obtained from human platelets differed with regard to sedimentation coefficient values (for FI, 8.9; for FIII, 4.6), K_m values for each nucleotide, and stability for heat or freezing/thawing treatment (Hidaka & Asano, 1976).

Preparation of Quinazolinesulfonamides. To a mixture of 1-(2-ethoxy-2-phenylethyl)piperazine (0.47 g) and triethylamine (0.32 g) in 20 mL of chloroform, a solution of 2,4-dipiperidino-6-quinazolinesulfonyl chloride (0.62 g) in 10 mL

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[1] Abbreviations: CaM, calmodulin; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; HA-542, 1-(2,4-dipiperidino-6-quinazolinesulfonyl)-4-(2-ethoxy-2-phenylethyl)piperazine; HA-543, 1-(2,4-dipiperidino-6-quinazolinesulfonyl)-4-cinnamylpiperazine; HA-544, 1-(4-piperidino-6-quinazolinesulfonyl)-4-(2-ethoxy-2-phenylethyl)-piperazine; HA-550, 1-(4-piperidino-6-quinazolinesulfonyl)-4-cinnamylpiperazine.

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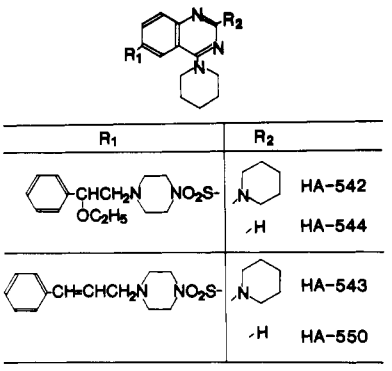

FIGURE 1: Chemical structures of quinazolinesulfonamides. HA-542, 1-(2,4-dipiperidino-6-quinazolinesulfonyl)-4-(2-ethoxy-2-phenylethyl)piperazine; HA-543, 1-(2,4-dipiperidino-6-quinazolinesulfonyl)-4-cinnamylpiperazine; HA-544, 1-(4-piperidino-6-quinazolinesulfonyl)-4-(2-ethoxy-2-phenylethyl)piperazine; HA-550, 1-(4-piperidino-6-quinazolinesulfonyl)-4-cinnamylpiperazine.

of chloroform was added under cooling in an ice bath and the preparation stirred for 5 h at room temperature. After removal of the solvent, under reduced pressure, the residue was purified by silica gel column chromatography (elution with chloroform) to give an oily product which was then dissolved in alcohol (20 mL) containing concentrated hydrochloric acid (2 mL), and the solution was evaporated under reduced pressure. Recrystallization of the residue from alcohol gave 0.90 g of 1-(2,4-dipiperidino-6-quinazolinesulfonyl)-4-(2-ethoxy-2-phenylethyl)piperazine (HA-542, mp 182 °C). 1-(2,4-Dipiperidino-6-quinazolinesulfonyl)-4-cinnamylpiperazine (HA-543, mp 165 °C) was synthesized from 2,4-dipiperidino-6-quinazolinesulfonyl chloride treated with 1-cinnamylpiperazine, in a similar manner as the HA-542 synthesis. The same treatment of 4-piperidino-6-quinazolinesulfonyl chloride with 1-(2-ethoxy-2-phenylethyl)piperazine or 1-cinnamylpiperazine resulted in 1-(4-piperidino-6-quinazolinesulfonyl)-4-(2-ethoxy-2-phenylethyl)piperazine (HA-544, mp 191 °C) or 1-(4-piperidino-6-quinazolinesulfonyl)-4-cinnamylpiperazine (HA-550, mp 247 °C), respectively. Chemical structures of each quinazolinesulfonamide are shown in Figure 1.

Retention Indices of Quinazolinesulfonamides. The retention index of each quinazolinesulfonamide was determined by the method of Baker et al. (1979). High-pressure liquid chromatography (HPLC) was carried out by using a Waters Associates, Inc., M-6000 pump, U6K injector, and Model 440 dual-wavelength ultraviolet detector (254 and 280 nm). A C_{18}-reverse-phase column (4.0 mm × 30 cm, μBondapak C_{18}, Waters Associates, Inc.) was used. The mobile phase was composed of a phosphate buffer (pH 7.0) and methanol (3:7), and the flow rate was 1.0 mL/min. The retention index of the drug studied was defined according to eq 1 by using the

$$\text{retention index} = 100\left(\frac{\log K'_D - \log K'_N}{\log K'_{N+1} - \log K'_N}\right) + 100N \quad (1)$$

capacity factor (K') and calculated from the observed capacity factor for the drug (K'_D), the capacity factor for a 2-ketoalkane standard eluting just before the test compound (K'_N), and the capacity factor for higher homologue (K_{N+1}). The capacity factors (K') of the drugs and the standards were determined from the observed retention time (t_R) by using eq 2, where t_0 was the void volume, as detected by the solvent front.

$$K' = \frac{t_R - t_0}{t_0} \quad (2)$$

Assay Procedure. Cyclic nucleotide phosphodiesterase activity was measured as previously described (Hidaka & Asano, 1976). The reaction mixture contained 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.4 μM cyclic [^{3}H]guanosine monophosphate (GMP, 100 000 cpm) or 0.4 μM cyclic [^{3}H]adenosine monophosphate (AMP, 100 000 cpm), 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) or 0.1 mM $CaCl_2$ and 400 ng/mL CaM, and the phosphodiesterase preparation, in a total volume of 0.5 mL. The reaction was initiated by addition of the substrate. HA-542, HA-543, HA-544, and HA-550 dissolved in 0.005 N HCl were individually added to the reaction mixture just before adding the substrate. CaM was assayed for its ability to activate a fixed amount of CaM-deficient phosphodiesterase, under standard conditions (Hidaka et al., 1979). One unit of CaM was defined as the amount activating a maximum of 50% of the phosphodiesterase attainable under standard experimental conditions and was equivalent to 10 ng of protein (Hidaka et al., 1980). Myosin light chain kinase activity was assayed, as previously described (Tanaka et al., 1980). Unless otherwise noted, 0.1 μM CaM was used.

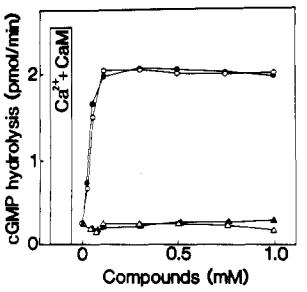

FIGURE 2: Effect of quinazolinesulfonamide derivatives on Ca^{2+}-dependent cyclic nucleotide phosphodiesterase in the absence of calcium. Phosphodiesterase was assayed as described under Experimental Procedures in the presence of HA-542 (○), HA-543 (●), HA-544 (△), or HA-550 (▲) with 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA). The enzyme activity with Ca^{2+}–CaM is given in the column within the figure.

Results

Activation of PDE by Quinazolinesulfonamides. As shown in Figure 2, Ca^{2+}-independent activation of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase was observed with 2,4-dipiperidino-6-quinazolinesulfonamides (HA-542, HA-543) but not with 4-piperidino-6-quinazolinesulfonamides (HA-544, HA-550). HA-542 and HA-543 stimulated Ca^{2+}-dependent cyclic nucleotide phosphodiesterase, to the same extent in the presence or absence of calcium ion, and the concentrations of HA-542 and HA-543 required to give 50% of the maximal activation were approximately 50 μM. The activation by these compounds was comparable to that obtained with the Ca^{2+}–calmodulin (CaM) complex. However, no significant activation by 2-piperidino-6-quinazolinesulfonamides (HA-544, HA-550) was observed with doses up to 1 mM.

The effects of 2,4-dipiperidino-6-quinazolinesulfonamides on the cyclic GMP concentration dependence of the enzyme were determined at saturating concentrations of each quinazolinesulfonamide. The HA-542- and HA-543-dependent phosphodiesterase activity exhibited an apparent K_m of 3.7 and 3.5 μM for cyclic GMP and a V_{max} of 169 and 177 nmol min^{-1} mg^{-1} (Table I). These compounds provided an apparent increase in V of the phosphodiesterase associated with decrease in the apparent K_m for the substrate. The kinetic constants of the HA compounds–activated enzyme were similar to those characteristics of the Ca^{2+}–CaM-dependent phosphodiesterase activity.

Multiple forms of cyclic nucleotide phosphodiesterase have been detected in mammalian tissues (Wells & Hardman,

Table I: Effect of Various Activators on the Kinetic Constants of Ca^{2+}-Dependent Cyclic Nucleotide Phosphodiesterase[a]

agent	apparent K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
none	13.6	0.036
HA-542	3.7	0.169
HA-543	3.5	0.177
Ca^{2+}–CaM	2.6	0.185

[a] Phosphodiesterase activity was measured with and without the addition of saturating concentrations of Ca^{2+} (0.1 mM) and CaM (0.1 μM), HA-542 (200 μM), and HA-543 (200 μM), as described under Experimental Procedures by using cyclic GMP as the substrate. The K_m and V_{max} values were obtained from Lineweaver-Burk plots of the increments of activity in the presence of the added agents.

1977). Three distinct cyclic 3′,5′-nucleotide phosphodiesterase activity peaks (FI, FII, FIII) on DEAE-cellulose chromatography have also been detected in the case of the human heart, lung, liver, kidney, platelets, aorta, and cerebrum (Hidaka & Asano, 1976; Hidaka et al., 1977; Hidaka et al., 1978a). FI and FIII phosphodiesterase from human platelets (Hidaka & Asano, 1976) were used as cyclic GMP and cyclic AMP phosphodiesterase, respectively. Cyclic AMP and cyclic GMP phosphodiesterase and purified bovine CaM-deficient Ca^{2+}–CaM-dependent cyclic nucleotide phosphodiesterase were used as they are most sensitive to inhibition by HA compounds. Investigations undertaken to examine the effects of HA-542 and HA-543 on three types of phosphodiesterase indicated that these compounds are selective activators of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (Figure 3). All quinazolinesulfonamides inhibited both cyclic AMP and cyclic GMP phosphodiesterase, in a concentration-dependent fashion. The IC_{50} values for HA-542, HA-543, HA-544, and HA-550 for human platelet cyclic AMP phosphodiesterase (FIII) were 0.22, 0.58, 0.59, and 0.40 mM and for human platelet cyclic GMP phosphodiesterase (FI) were 0.73, 0.38, 0.80, and 0.03 mM, respectively (Figure 3). Moreover, activators of the phosphodiesterase, HA-542 and HA-543, produced no further stimulation of the enzyme, in the presence of CaM and Ca^{2+}. HA-542 was a weak inhibitor of CaM-induced activation, and HA-543 had no significant effect on the activated enzyme activity (Figure 3).

Limited proteolysis of Ca^{2+}-dependent phosphodiesterase concomitantly activates the enzyme yet desensitizes it to further simultation by CaM (Cheung, 1971). Similarly, the trypsin-treated Ca^{2+}-dependent phosphodiesterase was no longer sensitive to activation by 2,4-dipiperidino-6-quinazolinesulfonyl derivatives (HA-542, HA-543) (data not shown). Although these synthetic activators of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase failed to stimulate significantly myosin light chain kinase from smooth muscle, an extremely high concentration of HA-542 and HA-543 (1 mM) activated the enzyme by 9% and 15%, respectively, of that seen with Ca^{2+}–CaM. This activation was not observed with HA-544 and HA-550.

Mechanism of the Activation. These structure–activity relationships of quinazolinesulfonamides in activation of Ca^{2+}-dependent phosphodiesterase suggest that piperidine at position 2 of the quinazoline ring may play an important role in the stimulatory effect on the phosphodiesterase, such as is the case with the Ca^{2+}–CaM complex. However, the mechanism of this activation is obscure. We have already demonstrated the importance of hydrophobic regions in the enzyme–CaM interaction (Tanaka & Hidaka, 1980); therefore to examine the relationship between the hydrophobicity and

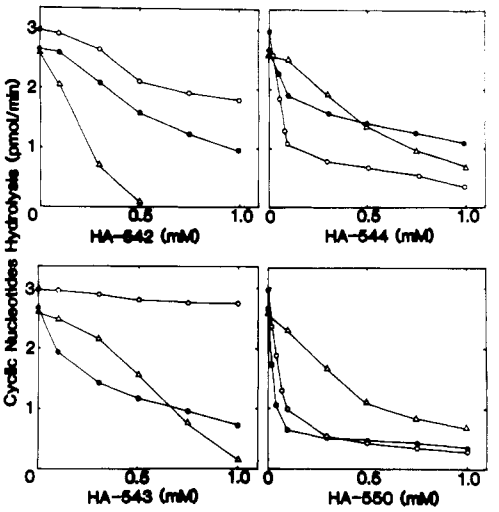

FIGURE 3: Effect of quinazolinesulfonamides on various forms of cyclic nucleotide phosphodiesterase. Phosphodiesterase activity was assayed as described under Experimental Procedures. Data are expressed as the activity of Ca^{2+}-dependent phosphodiesterase with Ca^{2+}–CaM (○), cyclic AMP phosphodiesterase (△), and cyclic GMP phosphodiesterase (●), in the absence and presence of various concentrations of the compounds.

potency in activation of the enzyme, we determined the hydrophobicity for all of the quinazolinesulfonamides.

The use of octanol–water partition coefficients (log P) has become a standard method to estimate the hydrophobicity of a drug. High-pressure liquid chromatography (HPLC) enables determination of log P values of drugs, and this method have several advantages over the classical shake flask method. Furthermore, Baker et al. (1979) reported that the HPLC retention indices showed a higher correlation with biological activity than was found between octanol–water partition coefficients and biological activity. Thus, we measured the retention indices of 2,4-dipiperidino-6-quinazolinesulfonamide derivatives (HA-542, HA-543) and 4-piperidino-6-quinazolinesulfonamide derivatives (HA-544, HA-550) according to the method of Baker et al. (1979). Retention indices of HA-542, HA-543, HA-544, and HA-555 were 769, 748, 584, and 566, respectively. We found that these indices of 2,4-piperidino derivatives were larger than those of 4-piperidino analogues, and these results parallel differences in the calculated partition coefficients (Leo et al., 1975). All these observations suggest that the action of these compounds may depend on their hydrophobicity and the existence of piperidine at position 2 of the quinazoline ring.

Inhibitors of the Activation. *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), chlorpromazine (CPZ), and prenylamine were found to be CaM antagonists (Hidaka et al., 1980; Levin & Weiss, 1979). These compounds also inhibited selectively the HA-542-induced activation of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase, as shown in Figure 4. The addition of 0.2 mM HA-542 to the phosphodiesterase preparation increased the enzymatic activity approximately 8-fold. Increasing the concentration of these CaM antagonists progressively inhibited the activation of phosphodiesterase. The concentrations of W-7, CPZ, and prenylamine which inhibited the activation of phosphodiesterase by 50% [IC_{50}(HA activated)] were 108, 39, and 52 μM, respectively (Table II). Several CaM antagonists with diverse structures, and the systemically synthesized CaM antagonists, naphthalenesulfonamides, were examined with regard to their ability to inhibit phosphodiesterase in the presence and absence of Ca^{2+}–CaM or HA-542. We have

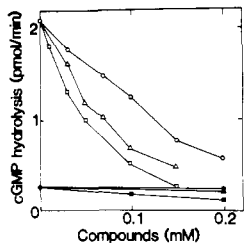

FIGURE 4: Effect of various CaM antagonists on the activation of Ca^{2+}-dependent phosphodiesterase by HA-542 (open symbols) and on the basal activity without Ca^{2+}–CaM (closed symbols). Phosphodiesterase activity was measured at a series of concentrations of *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (○, ●), prenylamine (△, ▲), and chlorpromazine (□, ■).

Table II: Effect of Calmodulin Antagonists on HA-542-Induced Activation of Ca^{2+}-Dependent Cyclic Nucleotide Phosphodiesterase

	IC_{50} (μM)[f]		
compounds	HA activated	CaM activated	unactivated
W-7[a]	108	28	1200
W-5[b]	300	240	3100
W-9[c]	71	14	820
W-6[d]	250	130	1400
CPZ[e]	39	16	130
prenylamine	52	18	600

[a] *N*-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7). [b] *N*-(6-Aminohexyl)-1-naphthalenesulfonamide (W-5). [c] *N*-(6-Aminohexyl)-5-chloro-2-naphthalenesulfonamide (W-9). [d] *N*-(6-Aminohexyl)-2-naphthalenesulfonamide (W-6). [e] Chlorpromazine (CPZ). [f] The concentrations of a drug which produced 50% inhibition of phosphodiesterase activation in the presence of 400 ng/mL CaM and 100 μM HA-542 are defined as IC_{50}(CaM activated) and IC_{50}(HA activated), respectively. IC_{50}(unactivated) is defined as the concentration of drug which produced 50% inhibition of phosphodiesterase in the absence of CaM.

reported that the actions of naphthalenesulfonamides as CaM antagonists depended on the chlorination of the naphthalene ring (Hidaka et al., 1981). With regard to inhibition of the HA-induced activation, a structure–activating relationship, with or without chlorine, was as shown in Table II. Kinetic analysis of W-7-induced inhibition of activation of phosphodiesterase by HA compounds revealed that this agent inhibited the activity, in a competitive fashion, and K_i values were 0.12 and 0.13 mM, respectively.

Discussion

We found that newly synthesized compounds such as 2,4-dipiperidino-6-quinazolinesulfonamides were calcium-independent activators of Ca^{2+}-dependent cyclic nucleotide phosphodiesterase. Total activity, as measured with saturating Ca^{2+}–CaM, was little changed, at any concentration used (Figure 3), thereby indicating that these synthetic activators and Ca^{2+}–CaM competed for activation of the enzyme. The agents which stimulated the phosphodiesterase had two piperidines at positions 2 and 4 of the quinazoline ring and the inactive derivatives had only one piperidine at position 4. Therefore, these studies on the structural requirements for enzyme activation clearly indicate the importance of piperidine at position 2 of the quinazoline ring. This piperidine may possibly be responsible for increase in the hydrophobicity of the compounds, and stimulation of the enzyme would follow. These results are in agreement with the findings in case of lipid activators (Wolff & Brostrom, 1976) and the Ca^{2+}–CaM complex (Tanaka & Hidaka, 1980). In the presence of micromolar Ca^{2+}, CaM undergoes a change to a more helical structure (Leo et al., 1975) and interacts with the enzymes at hydrophobic regions (Tanaka & Hidaka, 1980; Levin & Weiss, 1979). These compounds differed from sodium dodecyl sulfate which activates the phosphodiesterase at low concentrations and inhibits the enzyme activity at higher concentrations, in the presence or absence of Ca^{2+}–CaM (Wolff & Brostrom, 1976).

Moreover, Ca^{2+}–CaM and these synthetic activators share significant common features as activators of phosphodiesterase. Kinetic studies showed that Ca^{2+}–CaM and the compounds have similar effects on enzyme properties in that they increase the V_{max} and decrease the K_m for the substrate. Although there are conflicting reports regarding the effect of Ca^{2+}–CaM on the cyclic nucleotide substrate concentration dependence of the phosphodiesterase and its V_{max}, our results are similar to the data reported by Kakiuchi et al. (1973). Moreover, these active compounds appear to have the same structure as the active site of CaM since CaM antagonists seem to inhibit competitively these agent-induced activations of Ca^{2+}–PDE. Similar structure–activity relationships of naphthalenesulfonamides were also observed in the inhibition of both HA-induced and Ca^{2+}–CaM-induced activation of the enzyme, suggesting that W-7 and chlorpromazine may function by binding to HA-542 or HA-543. All these results suggest that HA-542 and HA-543 may have physical properties in common with those of Ca^{2+}–CaM.

The modes of activation of the enzyme with the Ca^{2+}–CaM complex and these compounds differ. Maximal stimulation of the phosphodiesterase by HA-542 or HA-543 was slightly less than that by Ca^{2+}–CaM. Moreover, these compounds produced only slight activation of smooth muscle myosin light chain kinase which is regulated by Ca^{2+}–CaM.

We found that phosphatidylinositol, a potent activator of Ca^{2+}-dependent phosphodiesterase (Wolff & Brostrom, 1976), was a weak activator of smooth muscle myosin light chain kinase (Tanaka & Hidaka, 1980). This enzyme from skeletal muscle was not inhibited by myelin basic protein or troponin I, at a concentration which sufficiently reduced Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (Grand & Perry, 1979). Thus, different domains of CaM may be responsible for activation of each enzyme. These newly synthesized compounds seem to possess physical properties required for activation of phosphodiesterase but not the myosin light chain kinase.

In ongoing studies, these compounds are proving to be useful tools for elucidating the mechanism of CaM-induced enzyme activation, and studies on Ca^{2+}-dependent cyclic nucleotide phosphodiesterase will be reported elsewhere.

Acknowledgments

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Registry No. HA-542, 84215-07-6; HA-543, 84215-08-7; HA-544, 84215-09-8; HA-550, 81871-41-2; W-7, 65595-90-6; W-5, 79458-81-4; W-9, 84215-10-1; W-6, 84215-11-2; CPZ, 50-53-3; Ca, 7440-70-2; prenylamine, 390-64-7; 1-(2-ethoxy-2-phenylethyl)piperazine, 6722-51-6; 1-cinnamylpiperazine, 18903-01-0; 2,4-dipiperidino-6-quinazolinesulfonyl chloride, 84215-12-3; 4-piperidino-6-quinazolinesulfonyl chloride, 81870-88-4; cyclic 3′,5′-nucleotide phosphodiesterase, 9040-59-9.

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Specific Labeling of the Active Site of Cytosolic Aspartate Aminotransferase through the Use of a Cofactor Analogue, *N*-(Bromoacetyl)pyridoxamine†

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ABSTRACT: The cofactor analogue *N*-(bromoacetyl)pyridoxamine (BAPM) has been employed to inactivate the cytosolic isozyme of apo-aspartate aminotransferase. Inactivation is the result of covalent bond formation in the (bromoacetyl)-pyridoxamine–transferase complex, via the ϵ-amino group of a lysyl residue at the active site. The stoichiometry of this inactivation is one molecule of (bromoacetyl)pyridoxamine per subunit of the transaminase dimer. Trace amounts of inorganic phosphate protect the enzyme from BAPM inactivation. In the absence of phosphate, inactivation demonstrates time, concentration, and pH dependence with an apparent p*K* for the target group of about 8.5 or higher. A tryptic peptide from the α subform has been obtained containing the carboxymethyl derivative of lysine-258, identifying this particular residue as the reactive group in the region of cofactor binding. Evidence is presented indicating that the p*K* of Lys-258 appears to be highly dependent upon the electrostatic state of neighboring groups in the active site region. Hence, experimentally obtained values vary according to the chemical nature and charge of the modifying agent or probe.

Aspartate aminotransferase (EC 2.6.1.1) is a functional dimer containing 1 mol of pyridoxal 5′-phosphate bound per subunit active site. It catalyzes the following conversion via two half-reactions:

$$\text{glutamate} + E_{\text{pyridoxal-5′-P}} \rightleftharpoons \alpha\text{-ketoglutarate} + E_{\text{pyridoxamine-5′-P}} \quad (1)$$

$$E_{\text{pyidoxamine-5′-P}} + \text{oxaloacetate} \rightleftharpoons \text{aspartate} + E_{\text{pyridoxal-5′-P}} \quad (2)$$

Two natural isozymes occur which are localized within the cytosolic and mitochondrial compartments of the cell and which presumably occur as part of the malate–aspartate shuttle which brings reducing equivalents into the mitochondrium (Dawson, 1979). The cytosolic isozyme consists of 412 amino acid residues having a tertiary structure which has been determined by X-ray crystallography to a resolution of 2.7 Å (Arnone et al., 1982).

In the absence of substrate, the cofactor is covalently bound at the active site via an internal aldimine with Lys-258. During transamination, the amino acid substrate is proposed to form a new aldimine with the aldehyde group of the pyridoxal-5′-P[1]

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[1] Abbreviations: BAPM, *N*-(bromoacetyl)pyridoxamine; Mops, 3-(*N*-morpholino)propanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; [^{14}C]BAPM, *N*-(bromo[^{14}C]acetyl)pyridoxamine; pyridoxal-5′-P, pyridoxal 5′-phosphate; pyridoxamine-5′-P, pyridoxamine 5′-phosphate.

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