Inhibition of Cyclic Nucleotide Phosphodiesterases by Reticulol

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Inhibition mechanism of rat cerebral cortex cyclic nucleotide phosphodiesterases (PDE) by reticulol was investigated. The inhibition of PDE by reticulol was not reduced in the presence of excess PDE activating factor (PAF) or/and Ca²⁺ ion. Reticulol showed lower Ki values for Ca²⁺-PAF dependent PDE than for Ca²⁺ independent PDE.

Reticulol was isolated from a culture filtrate of a streptomycetes strain (MD611-C6) as an inhibitor of rabbit brain cyclic nucleotide phosphodiesterases (PDE) [EC 3.1.4.1].¹ Reticulol has no structural resemblance to the substrates (adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP)) of PDE. Therefore, its inhibition mechanism of rat cerebral cortex PDE was studied and reported in this paper.

MATERIALS AND METHODS

Chemicals and their sources are as follows: EGTA (Ethleneglycol-bis (β-aminoethyl ether), N',N'-tetra-acetic acid, Daiichi Pure Chemicals), theophylline·HCl (Wako Chemicals), Tris (hydroxymethyl)-aminomethane (Sigma, Trisma Base). cAMP (Sigma), cAMP-8-¹⁴C (N. E. N. 50mCi/mM), adenosine 5'-monophosphate (5'-AMP) (Sigma), cGMP-8-¹⁴C (N. E. N. 60mCi/mm). All reagents were of analytical grade.

Preparation of enzyme. Cerebral cortex of rat brain (Donryu, 120~150 g, male) was homogenized with 5 times its weight of 10 mm Tris-HCl (pH 7.5)-1 mm MgCl₂ by a Teflon homogenizer (10 strokes) and centrifuged at 100,000 × g for 3 days after preparation. The enzyme concentration used was 1.6~15 μg protein/ml. Enzymes from other tissues of rat were prepared in the same manner as for the brain enzyme. In this study, the enzyme was always freshly prepared because freezing and thawing process partially denatures this enzyme resulting in fluctuation of sensitivity of the enzyme to various inhibitors. For the assay of the activator and Ca²⁺ effect, the fresh enzyme was dialyzed against 300 volumes of 10 mM Tris-HCl (pH 7.5)-1 mM MgCl₂-0.1 mM EGTA and used as Ca²⁺ free PDE.

Preparation and assay of activator. The activator of rat cerebral cortex was prepared according to Chung.² Fresh crude enzyme solution was heated at 85°C for 5 min and chilled quickly in an ice bath. The solution was centrifuged at 100,000 × g for 1 hr and the denatured protein was removed. The supernatant was dialyzed twice against 300 volumes of 10 mM Tris-HCl (pH 7.5)-1 mM MgCl₂-0.1 mM EGTA. The dialyzed supernatant (PDE activating factor, PAF) was stored at -80°C. The assay of PAF was made based on the ability to enhance the activity of Ca²⁺ free PDE in the presence of 100 μM Ca²⁺ ion. Ca²⁺ free PDE was also used for the studies on the Ca²⁺ effect by using Ca²⁺-EGTA buffer according to Kakiuchi et al.³

Assay of enzyme activity. The assay mixture (total volume 0.1 ml) was (unless otherwise cited) consisted of 80 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 0.2~200 μM cAMP-8-¹⁴C or cGMP-8-¹⁴C, and enzyme and ten times concentration 5'-AMP or 5'-GMP of the substrate in order to avoid subsequent degradation of the reaction product (5'-AMP-8-¹⁴C or 5'-GMP-8-¹⁴C) by 5'-nucleotidase, a slight contaminant in the crude enzyme. The assay mixture except for the substrate was preincubated at 30°C for 3 min. The enzyme reaction was started by addition of the substrate, continued at 30°C for 20 min and stopped by heating in a boiling water bath for 3 min. The assay method of cAMP by dry alumina column was described in a previous paper.¹ In the assay of cGMP, 4 ml of 50 mM Tris-HCl (pH 7.5) instead of 2.5 ml of 10 mM Tris-HCl (pH 7.5) was used as the elution buffer of cGMP from dry alumina column.

¹ Inhibitors of Cyclic Nucleotide Phosphodiesterases Produced by Streptomycetes. Part IV.
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content was determined by the method of Lowry with bovine serum albumin as the standard.4)

RESULTS

Linearity of the enzyme reaction

The linearity of the enzyme reaction with time and enzyme concentration is shown in Fig. 1. At low substrate concentration (0.222 \( \mu \text{M} \)), the enzyme reaction linearly progressed until the ratio of the hydrolyzed cAMP to the total cAMP (the reaction rate) reached 30%. The influence of the preincubation (0~40 min at 30°C) on the reaction velocity was not observed. Therefore, all enzyme reactions in this paper were conducted in the condition that the preincubation time was 3 min and the reaction rate was less than 30% in 20 min where the rate of reaction was a linear function of enzyme amount and of incubation time.

**FIG. 1.** Time Course of Rat Cerebral Cortex cAMP Phosphodiesterase.

Substrate concentration was 0.222 \( \mu \text{M} \). Enzyme concentration was as follows: \( \triangle \): 50 \( \mu \text{g} \) protein/ml, \( \bigcirc \): 25.0 \( \mu \text{g} \) protein/ml, \( \triangle \bigcirc \): 12.5 \( \mu \text{g} \) protein/ml, \( \square \bigcirc \): 6.3 \( \mu \text{g} \) protein/ml.

Effect of PAF on cAMP PDE

The effect of PAF on rat cerebral cortex cAMP PDE at low substrate concentration (1.47 \( \mu \text{M} \)) is shown in Fig. 2. Cerebral cortex enzyme was fully activated by more than 10 \( \mu \text{g} \) protein of PAF in the presence of 100 \( \mu \text{M} \) Ca\(^{2+}\). At high substrate concentration (100 \( \mu \text{M} \)), the necessary amounts of PAF for full activation were the same as those at low substrate concentration.

**FIG. 2.** Effect of Phosphodiesterase Activating Factor on Rat Cerebral Cortex cAMP Phosphodiesterase Activity.

Substrate concentration was 1.47 \( \mu \text{M} \).

\( \bigcirc \bigcirc \bigcirc \): +Ca 0.1 mm, \( \bigcirc \bigcirc \bigcirc \): +EGTA 0.1 mm.

Effect of Ca\(^{2+}\) and Mg\(^{2+}\) ion

The effect of Ca\(^{2+}\) ion on cAMP PDE in the presence of excess amounts of PAF (11 \( \mu \text{g} \) protein/0.1 ml) in the reaction mixture is shown in Fig. 3. At low substrate concentration (1.47 \( \mu \text{M} \)), the activity of cAMP PDE was stimulated 4 to 6 times by Ca\(^{2+}\) ion more than 2.5 \( \mu \text{M} \). At high substrate concentration (100 \( \mu \text{M} \)), it was stimulated about 3 times by the same Ca\(^{2+}\) ion concentration. In this paper, the activity of PDE stimulated by Ca\(^{2+}\) and PAF is called Ca\(^{2+}\)-PAF dependent PDE, and the activity of PDE measured in the presence of 0.1 mm EGTA instead of Ca\(^{2+}\) and PAF is called Ca\(^{2+}\) independent PDE. The effect of Mg\(^{2+}\) ion on Ca\(^{2+}\)-PAF dependent
and Ca\(^{2+}\) independent PDE was examined. In the presence of excess amounts of Ca\(^{2+}\) (0.1 mm) and PAF (20 \(\mu\)g protein/0.1 ml), maximal activity of cAMP PDE was observed at more than 3 mm Mg\(^{2+}\). However, in the absence of Ca\(^{2+}\) and PAF, Ca\(^{2+}\) independent PDE was significantly inhibited by more than 3 mm Mg\(^{2+}\) (data are not shown). High concentration of Mg\(^{2+}\) was already shown to inhibit mammalian PDE.\(^5\) It was considered that the inhibition of PDE by Mg\(^{2+}\) results from the competition of this metal ion with Ca\(^{2+}\). In this paper, 3 mm Mg\(^{2+}\) was contained in the reaction mixture to avoid the effect of Mg\(^{2+}\) under the various condition.

**Effect of PAF on the inhibition of PDE by reticulol**

The effect of PAF on the inhibition of PDE by reticulol is shown in Table I. The inhibition of PDE by reticulol was not reduced by excess amounts of PAF at low and high substrate concentrations. This result means that the inhibition by reticulol is not caused by the disturbance of the action of PAF.

**TABLE I. EFFECT OF PHOSPHODIESTERASE ACTIVATING FACTOR ON THE INHIBITION OF RAT CEREBRAL CORTEX CAMP PHOSPHODIESTERASE BY RETICULOL**

Assay mixture was shown in MATERIALS AND METHODS. The substrate concentration was 1.47 \(\mu\)M of cAMP.

<table>
<thead>
<tr>
<th>Addition</th>
<th>Phosphodiesterase activity (a)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.57</td>
<td>0</td>
</tr>
<tr>
<td>PAF 16 (\mu)g</td>
<td>3.40</td>
<td>0</td>
</tr>
<tr>
<td>Reticulol 12.5 (\mu)g</td>
<td>0.55</td>
<td>65.0</td>
</tr>
<tr>
<td>Reticulol 12.5 (\mu)g + PAF 16 (\mu)g</td>
<td>1.25</td>
<td>64.2</td>
</tr>
</tbody>
</table>

\(a\) nmoles of hydrolyzed cAMP/mg protein. min.

**Effect of Ca\(^{2+}\) ion on the inhibition of PDE by reticulol**

As reticulol has a hydroxy residue, it may be considered that this compound has a strong chelating activity with metals. Therefore, it was examined whether reticulol inhibited PDE by chelation with Ca\(^{2+}\) or not. As shown in Table II, reticulol showed stronger inhibition of PDE in the presence of Ca\(^{2+}\) than in the absence of this metal, and the inhibition by reticulol was not reduced by excess amounts of Ca\(^{2+}\). These results indicate that inhibition by reticulol is not due to its chelation with Ca\(^{2+}\). Reticulol inhibited more preferably Ca\(^{2+}\)-PAF dependent PDE than Ca\(^{2+}\) independent PDE.

**Michaelis constant \(K_m\) of cAMP PDE**

As the preliminary experiment to measure inhibitor constants \(K_i\) of reticulol, \(K_m\) values

![FIG. 4. Hofstee Plots of Ca\(^{2+}\)-PAF Dependent and Ca\(^{2+}\) Independent cAMP Phosphodiesterase of Rat Cerebral Cortex.](image-url)

Panel A: Ca\(^{2+}\) independent cAMP phosphodiesterase, B: Ca\(^{2+}\)-PAF dependent cAMP phosphodiesterase. Velocity is expressed in nmols of hydrolyzed cAMP/mg protein min. Ca\(^{2+}\) independent phosphodiesterase was assayed in the presence of 0.1 mm EGTA in the assay mixture. Ca\(^{2+}\)-PAF dependent phosphodiesterase was assayed in the presence of 0.1 mm Ca\(^{2+}\) and PAF (0.1 mg protein/ml).
of rat cerebral cortex cAMP PDE were measured by Hofstee plots.6) As shown in Fig. 4, both Ca\(^{2+}\)-PAF dependent and Ca\(^{2+}\) independent PDE had two KM values. Low and high KM values were about 2 \(\mu\)M and 20 \(\mu\)M respectively.

**Ki of reticulol for cAMP PDE**

From the results described above, the Ki of reticulol for low and high KM enzymes of cAMP PDE were measured using the following substrate concentrations: 0.2~0.5 \(\mu\)M for low KM enzyme, 5~25 \(\mu\)M for high KM enzyme. The sub-saturating substrate concentrations (0.2~0.5 \(\mu\)M) were used for low KM enzyme in order to avoid the effect of high KM enzyme (the contribution of high KM enzyme to reaction velocity (V) were observed with the substrate concentration more than 0.8 \(\mu\)M as shown in Panel B of Fig. 4).

Dixon plot of reticulol for low KM enzyme of cAMP PDE is shown in Fig. 5. Reticulol gave lower Ki values (1/3.4) for Ca\(^{2+}\)-PAF dependent PDE than for Ca\(^{2+}\) independent PDE. In Table III, Ki values of reticulol and theophylline for Ca\(^{2+}\)-PAF dependent and Ca\(^{2+}\) independent PDE in low (0.2~0.5 \(\mu\)M) and high substrate (5~25 \(\mu\)M) concentrations are compared. Reticulol showed lower Ki values for Ca\(^{2+}\)-PAF dependent enzyme than for Ca\(^{2+}\) independent enzyme in both low and high substrate concentrations. Theophylline gave the same Ki values for Ca\(^{2+}\)-PAF dependent enzyme as those for Ca\(^{2+}\) independent enzyme. Kakiuchi et al. showed that Ca\(^{2+}\)-PAF dependent PDE of cerebral cortex might be cGMP PDE.7) From Ki values of reticulol, it may be inferred that reticulol is a comparatively selective inhibitor of cGMP PDE.

**Ki of reticulol for cGMP PDE**

Rat cerebral cortex cGMP PDE showed one KM value (2.0 \(\mu\)M) in the presence of Ca\(^{2+}\) (0.1 mM) and PAF (11 \(\mu\)g protein/0.1 ml), and two KM values (2.0 and 10 \(\mu\)M) in the presence

### Table III. Ki of Reticulol and Theophylline for Rat Cerebral Cortex CAMP Phosphodiesterase

<table>
<thead>
<tr>
<th>Assay Mixture</th>
<th>Reticulol</th>
<th>Theophylline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ki I(^a)</td>
<td>Ki II(^b)</td>
</tr>
<tr>
<td>No addition</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>+0.1 mM EGTA</td>
<td>54</td>
<td>69</td>
</tr>
<tr>
<td>+0.1 mM Ca(^{2+}) and 10 (\mu)g PAF</td>
<td>16</td>
<td>27</td>
</tr>
</tbody>
</table>

\(^{a}\) Ki values (\(\times 10^{-6}\) M) for low KM cAMP phosphodiesterase (Substrate concentration, 0.2~0.5 \(\mu\)M).

\(^{b}\) Ki values (\(\times 10^{-6}\) M) for high KM cAMP phosphodiesterase (Substrate concentration, 5~25 \(\mu\)M).

**Fig. 5. Ki of Reticulol for Ca\(^{2+}\)-PAF Dependent and Ca\(^{2+}\) Independent cAMP Phosphodiesterase (Dixon Plots) of Rat Cerebral Cortex.**

Panel A: control, B: Ca\(^{2+}\) independent phosphodiesterase, C: Ca\(^{2+}\)-PAF dependent phosphodiesterase. Velocity is expressed in nmols of hydrolyzed cAMP/mg protein min. Control was assayed in no addition of Ca\(^{2+}\), PAF and EGTA to the assay mixture. Ca\(^{2+}\) independent and Ca\(^{2+}\)-PAF dependent phosphodiesterase activities were assayed in the same conditions as shown in Fig. 4. Substrate concentrations (cAMP) were as follows: ○—○: 0.222 \(\mu\)M, △—△: 0.367 \(\mu\)M, □—□: 0.490 \(\mu\)M. Enzyme concentration was 5.0 \(\mu\)g protein/ml.
Inhibitors of Cyclic Nucleotide PDE Produced by Streptomyces. Part IV

FIG. 6. Ki of Reticulol for Ca\textsuperscript{2+}.-PAF Dependent and Ca\textsuperscript{2+} Independent cGMP Phosphodiesterase (Dixon Plots) of Rat Cerebral Cortex.

Panel A: control, B: Ca\textsuperscript{2+} independent phosphodiesterase, C: Ca\textsuperscript{2+}.-PAF dependent phosphodiesterase. Velocity is expressed in nmols of hydrolyzed cGMP/mg protein min. Control, Ca\textsuperscript{2+} independent and Ca\textsuperscript{2+}.-PAF dependent phosphodiesterase activities were assayed in the same conditions as shown in Fig. 5. Substrate concentrations (cGMP) were as follows: ○—○: 0.20 μM, △—△: 0.333 μM, □—□: 0.50 μM. Enzyme concentration was 1.6 μg protein/ml.

TABLE IV. Ki of Reticulol and Theophylline for Rat Cerebral Cortex cGMP Phosphodiesterase

Assay mixture was shown in Materials and Methods. Ki values were determined by Dixon plots.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Reticulol Ki (10^{-6}) M</th>
<th>Theophylline Ki (10^{-6}) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>No addition</td>
<td>15</td>
<td>140</td>
</tr>
<tr>
<td>+0.1 mM EGTA</td>
<td>24</td>
<td>140</td>
</tr>
<tr>
<td>+0.1 mM Ca\textsuperscript{2+} + 10 μg PAF</td>
<td>13</td>
<td>150</td>
</tr>
</tbody>
</table>

\(\text{a)} Ki \text{ values (} \times 10^{-6}\text{ M) for low } KM \text{ cGMP phosphodiesterase (Substrate concentration, } 0.2 \sim 0.5 \text{ μM).}

of EGTA (0.1 mM). As reticulol gave almost the same Ki values in both low and high substrate concentrations for cAMP PDE, Ki values of reticulol for cGMP PDE were measured in low substrate concentrations (0.2 \sim 0.5 μM). As shown in Fig. 6 and Table IV, reticulol showed the lower Ki values for Ca\textsuperscript{2+}.-PAF dependent enzyme than for Ca\textsuperscript{2+} independent enzyme. It was noticeable that reticulol showed lower Ki values (1/2.3) for Ca\textsuperscript{2+} independent PDE against the substrate cGMP than against cAMP. On the other hand, theophylline gave the same Ki values in Ca\textsuperscript{2+}.-PAF dependent and Ca\textsuperscript{2+} independent PDE.

Inhibition of PDE of other tissues by reticulol

Concentrations of reticulol for 50% inhibition (Ic 50) of cerebral, cerebellum, lung, liver, and aorta PDE are shown in Table V. In all tissues except lung cited in Table V, reticulol showed the lower Ic 50 values for cGMP PDE than for cAMP PDE and the ratios of Ic 50 for cGMP PDE/Ic 50 for cAMP PDE were

TABLE V. Ic 50\(\text{a)}\) of Reticulol and Theophylline for cAMP and cGMP Phosphodiesterases from Various Tissues of Rat

The supernatants centrifuged at 100,000 \times g from various tissue homogenates of rats were used as the enzyme solution. The enzyme from aorta was prepared from rabbit. Assay mixture were contained 80 mM Tris-HCl (pH 7.5), 7 mM MgCl\textsubscript{2}, inhibitors and 1 μM cAMP or cGMP.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>cAMP (10^{-6}) M</th>
<th>Reticulol (63 \times 10^{-6}) M</th>
<th>cGMP (960 \times 10^{-6}) M</th>
<th>Theophylline cAMP (1400)</th>
<th>Theophylline cGMP (1700 \times 10^{-6}) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cerebral</td>
<td>150 (10^{-6}) M</td>
<td>63 (\times 10^{-6}) M</td>
<td>960 (\times 10^{-6}) M</td>
<td>1400</td>
<td>1700 (\times 10^{-6}) M</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>220</td>
<td>77</td>
<td>270</td>
<td>660</td>
<td>250</td>
</tr>
<tr>
<td>Lung</td>
<td>130</td>
<td>170</td>
<td>630</td>
<td>290</td>
<td>300</td>
</tr>
<tr>
<td>Liver</td>
<td>230</td>
<td>95</td>
<td>290</td>
<td>270</td>
<td>270</td>
</tr>
<tr>
<td>Heart</td>
<td>110</td>
<td>54</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aorta</td>
<td>150</td>
<td>63</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

\(\text{a)}\) Inhibitors concentrations in the reaction mixture for 50% inhibition.
0.3~0.4. These results indicate that reticulol may inhibit cGMP PDE more selectively.

DISCUSSION

It was shown that the inhibition of rat cerebral cortex PDE by reticulol was not due to its chelating action or to the disturbance of PAF. The fixed Ki values of reticulol and theophylline were obtained in several experiments. But the inhibition types of PDE by both compounds could not be determined definitely even in the repeated experiments, although it is appeared that reticulol has the different inhibition type with a difference of the substrate (cAMP or cGMP) as shown in Figs. 5 and 6. This is because the enzymes used were not pure and PDE had complicated characters (e.g. negative cooperativity and subunit structures). The remarkable differences between reticulol and theophylline was observed in the Ki values for Ca²⁺-PAF dependent and Ca²⁺ independent PDE, and IC₅₀ values for cAMP and cGMP PDE of several tissues. Namely, reticulol showed lower Ki values for Ca²⁺-PAF dependent PDE than for Ca²⁺ independent PDE in the both substrate (cAMP and cGMP). On the other hand, theophylline showed almost the same values for both cases. If Ca²⁺-PAF dependent enzyme of cerebral cortex was cGMP PDE (Kakiuchi et al.), it can be inferred that reticulol might inhibit cGMP PDE more selectively than cAMP PDE. This might be supported by the fact that reticulol showed lower IC₅₀ values for cGMP PDE than for cAMP PDE in all examined tissues except lung. It might be caused by using rabbit brain enzyme for the screening of inhibitors. Namely, it was proved that the brain enzyme showed 2 or 3 times higher reaction velocity in the substrate of cGMP than in cAMP (our data, data are not shown). The discovery of the comparatively selective inhibitor for cGMP PDE means that inhibitors of cAMP PDE may possibly be found in microbial cultured broths. In view of various physiological actions of cAMP, it will be an important subject to find a selective inhibitor of cAMP PDE.

REFERENCES

5) W. Y. Chung, Biochemistry, 6, 1079 (1967).