EFFECT OF 8-SUBSTITUTED CYCLIC AMP DERIVATIVES ON SOLUBLE GUANYLATE CYCLASE ACTIVITY FROM HUMAN PLATELETS

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Abstract—Inhibitory effect of 8-alkyl cyclic AMP derivatives on the soluble guanylate cyclase activity from human platelets was investigated. Cyclic AMP derivatives with alkyl chains of 6 or less carbons at 8-position of the purine base were not inhibitory, but the derivatives with alkyl chains of 7 or more carbons produced significant inhibition of the soluble guanylate cyclase activity. The extent of inhibitory effect increased when a longer alkyl chain was introduced at the 8-position of cyclic AMP. In contrast, 8-alkyl adenosine derivatives did not inhibit the cyclase activity. These results suggest that the platelet guanylate cyclase has an affinity for cyclic AMP derivatives with hydrophobic property as well as for several guanine nucleotides such as GTP or cyclic GMP.

In most tissues, the activity of guanylate cyclase is associated with both supernatant and particulate fractions of the homogenate. The soluble form of guanylate cyclase interacts with hydrophobic components of cell such as fatty acids (1), lysolecithin (2), fatty acid peroxides (3, 4). It was also suggested by Garbers that the soluble form of guanylate cyclase of rat lung contains bound fatty acids or other hydrophobic components of cell (5).

On the other hand, Mittal and Murad reported the formation of cyclic AMP from ATP by soluble guanylate cyclase from rat liver after activation with sodium azide and protein activator factor (6). Nakane and Deguchi demonstrated the activation by cyclic AMP of soluble guanylate cyclase activity in rat brain (7). These findings suggested that the soluble guanylate cyclase interacts with not only guanine nucleotides but also with adenine nucleotides.

The objective of the present study was to elucidate how hydrophobic groups of a substance or adenine nucleotide interact with human platelet guanylate cyclase. We investigated the effect of cyclic AMP derivatives with various lengths of alkyl chain at 8-position of purine base on the activity of soluble guanylate cyclase from human platelets.

MATERIALS AND METHODS

8-[^3H]GTP (specific activity, 14 Ci/mmol) was purchased from Radiochemical Centre Ltd. (Amersham, England) and nonlabeled GTP was from Boehringer Mannheim Gbh (W. Germany). DEAE cellulose (DE52) was from Whatman Ltd. (England), AG 1-X2 was from Bio-Rad Laboratories and neutral alumi-
num oxide was from Woelm Pharma Geh & Co. (W. Germany). 8-Substituted cyclic AMP and adenosine derivatives were synthesized according to the method described in previous papers (8, 9).

Human platelets were isolated from the citrated platelet-rich plasma by centrifugation and stored at −70°C until use. Platelets were homogenized in 50 mM Tris-acetate buffer (pH 6.0) containing 0.1 mM EGTA and 20 mM 2-mercaptoethanol (buffer A) with glass-teflon homogenizer, followed by sonication. The resulting sonicated extracts were centrifuged at 105,000×g for 60 min at 4°C.

DEAE cellulose column chromatography was carried out as previously described (10). The 105,000×g supernatant fraction was applied to a DEAE column (1.5×20 cm) equilibrated previously with the buffer A, and then the column was washed with the equilibration buffer and eluted with a linear sodium acetate gradient (0–0.5 M) in the same buffer of 300 ml at flow rate of 30 ml/h and each fraction was collected. The fractions containing guanylate cyclase activity were pooled and used as the enzyme preparation. This preparation was assessed to be free from cyclic GMP phosphodiesterase activity. The maximal velocity of guanylate cyclase activity was 303 pmol/mg of protein/min, and Km value for GTP was 11 μM. The Hill coefficient was 0.98 for the cyclic GMP formation. The standard assay mixture contained 100 μM [3H]GTP (50 Ci/mol), 3 mM MnCl₂, 2 mM dithiothreitol, 50 mM Tris-HCl (pH 7.7), and appropriate amount of the enzyme preparation in a final volume of 0.2 ml. A nucleoside triphosphate regenerating system and cyclic GMP were not added to the reaction mixture, because the enzyme preparation did not contain significant cyclic GMP phosphodiesterase and GTPase activities. Reaction was initiated by the addition of the solution containing GTP and MnCl₂, and incubation was conducted at 30°C for 20 min. Reaction was terminated by the addition of 40 μl of HCl, followed by boiling for 2 min. The radioactive cyclic GMP formed was isolated by serial use of a neutral aluminum oxide and AG 1-X2 column and quantitated by a liquid scintillation counter (11). The activity was linear up to 30 min and the reaction was carried out for 20 min otherwise stated.

Cyclic GMP phosphodiesterase activity was determined by the method of Hidaka and Asano (12).

Circular dichroism determination was performed at appropriate concentrations of derivatives (in the range from 0.08 mM to 0.1 mM) at 24°C as described previously (9).

RESULTS

Effects of 8-alkylamino and alkylthio cyclic AMP derivatives on human platelet guanylate cyclase were studied. Cyclic AMP derivatives with alkyl chains of 6 or less at 8-position of purine base did not affect the cyclase activity, and the derivatives with longer alkyl chain inhibited the activity dose-dependently, as shown in Fig. 1. Inhibition by these derivatives increased according to the elongation of alkylamino and alkylthio chains. Kinetic analysis revealed that 8-alkylamino or a alkylthio cyclic AMP derivative was a competitive inhibitor of the cyclase, and typical results are shown in Fig. 2. Effect of 8-substituted cyclic AMP derivatives on the guanylate cyclase activity is summarized in Table 1. Cyclic AMP and its 8-substituted derivatives with shorter alkyl chains did not inhibit the activity significantly, and the derivative with the longest alkyl chain was the most potent inhibitor in each series (Table 1). Since it is well known that several kinds of detergents affect the cyclase activity (2) and longer alkylamino or alkylthio chains of 8-position of purine base may act as detergents, the
effects of the alkylamines and alkylthiols on the cyclase activity were tested. Neither alkylamines nor alkylthiols inhibited the activity at concentrations up to 1,000 μM. Adenosine derivatives such as 8-hexylthio, decylthio, or dodecylthio adenosine derivative did not inhibit the cyclase activity at the concentration up to 1,000 μM.

To investigate the difference between the conformation of cyclic AMP derivative and that of adenosine derivative, the circular dichroism (CD) spectra of derivatives were measured. The CD spectra were obtained for 8-alkylthio cyclic AMP and adenosine. Figure 3 shows the relationship between the magnitude of the Cotton effect and the carbon number of the substituent of derivative. The carbon number corresponds to the methylene number of the substituent and zero indicates 8-mercapto derivatives.
taken for comparison. As shown in Fig. 3-A, the CD spectra of cyclic AMP and adenosine derivatives exhibited similar negative Cotton effects at UV absorption maxima (B2u band), but the magnitude of negative Cotton effect of cyclic AMP derivative was smaller than that of corresponding adenosine derivative (Fig. 3-B). Both cyclic AMP and adenosine derivatives with alkyl chains of 4 or longer carbons became constant in the magnitude of negative Cotton effect; -8,000 [β] for cyclic AMP derivatives and -13,500 [β] for adenosine derivatives.

**DISCUSSION**

As shown in Figs. 1 and 2, cyclic AMP derivatives with alkyl chains of 7 or more carbons at 8-position of purine base produced a significant inhibition of the soluble guanylate cyclase from human platelets, in competition with substrate, but adenosine derivatives with longer alkyl chains at 8-position were not inhibitory. These results suggest that cyclic AMP is necessary for inhibition of soluble guanylate cyclase.

The magnitude of the negative Cotton effect of 8-substituted cyclic AMP derivative was smaller than that of the corresponding 8-substituted adenosine derivative (Fig. 3-B). Ikehara et al. reported that the magnitude of Cotton effect of purine riboside correlates to the torsion angle about N9-C1' glycosyl bond of purine riboside (13). These results suggest that the torsion angle about N9-C1' glycosyl bond of 8-substituted adenosine derivative differs from the angle of 8-substituted cyclic AMP derivative. Moreover, it is reported that the ribose configuration of adenosine differs from that of cyclic AMP which is twisted, so called C3'-end-C4'-exo (Tt) (14). The active site of the guanylate cyclase appears to have an affinity for an appropriate configuration of the ribose, or an appropriate conformation of purine nucleotide such as cyclic AMP.

In contrast, cyclic AMP and adenosine derivatives, which assume a syn conformation in neutral solution, competitively inhibit the phosphodiesterase activity (9). The active site of phosphodiesterase has the affinity for both configurations of ribose of cyclic AMP and adenosine.

**Table 1. Effect of 8-substituted cyclic AMP derivatives on the activity of human platelet guanylate cyclase**

<table>
<thead>
<tr>
<th>Substituent</th>
<th>IC50 value* (μM)</th>
<th>Substituent</th>
<th>IC50 value* (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃(CH₂)₄S-</td>
<td>1000&lt;</td>
<td>CH₃(CH₂)₄NH-</td>
<td>1000&lt;</td>
</tr>
<tr>
<td>CH₃(CH₂)₅S-</td>
<td>1000&lt;</td>
<td>CH₃(CH₂)₅NH-</td>
<td>1000&lt;</td>
</tr>
<tr>
<td>CH₃(CH₂)₆S-</td>
<td>950</td>
<td>CH₃(CH₂)₆NH-</td>
<td>1000&lt;</td>
</tr>
<tr>
<td>CH₃(CH₂)₇S-</td>
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<td>CH₃(CH₂)₇NH-</td>
<td>480</td>
</tr>
<tr>
<td>CH₃(CH₂)₈S-</td>
<td>150</td>
<td>CH₃(CH₂)₈NH-</td>
<td>200</td>
</tr>
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<td>120</td>
<td>CH₃(CH₂)₉NH-</td>
<td>160</td>
</tr>
</tbody>
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*) The concentration producing 50% inhibition of the guanylate cyclase.
The soluble guanylate cyclase from rat lung is reported to bind tightly to alkyl-agarose gel of 6 carbons or longer (5), suggesting that the guanylate cyclase has an affinity for a longer alkyl substance. In fact, elongation of the alkyl chain of cyclic AMP derivatives make the derivatives more potent inhibitors. The soluble form of guanylate cyclase has been reported to interact with hydrophobic compounds, such as fatty acids (1, 15), lysocellatin (2), fatty acid peroxides (3, 4), and Triton X-100 (15), which activate or bind to guanylate cyclase. These data suggest that the hydrophobic region of the cyclase appears to be near the active site.

REFERENCES