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Selective Inhibition of Separated Forms of Human Platelet Cyclic Nucleotide Phosphodiesterase by Platelet Aggregation Inhibitors

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SUMMARY

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Human platelets contain three distinct cyclic nucleotide phosphodiesterases which catalyze the hydrolysis of both cyclic 3′,5′-AMP and cyclic 3′,5′-GMP. These are a relatively specific cyclic GMP phosphodiesterase (F I), a cyclic nucleotide phosphodiesterase (F II), and a relatively specific cyclic AMP phosphodiesterase (F III). This study was conducted to evaluate the possibility of selective inhibition of these three forms of the phosphodiesterase by various platelet aggregation inhibitors: papaverine, theophylline, caffeine, EG 626, adenosine, 2-chloroadenosine, and dipyridamole. Selective inhibition was found when the inhibitory potencies of the compounds were compared by means of Dixon plots. All agents tested except dipyridamole showed relatively selective inhibition for F III, and the order of potency of these drugs as inhibitor of the F III enzyme was papaverine, EG 626, dipyridamole, theophylline, 2-chloroadenosine, caffeine, and adenosine. EG 626 was 30 times more potent as an inhibitor of F III than of F I. On the other hand, dipyridamole was 20 times more potent as an inhibitor of F I than of F III. The K_i values of these agents for F I and F III were identical whether cyclic AMP or cyclic GMP was used as substrate. However, these compounds revealed approximately 2 times more affinity for F II using cyclic GMP than with cyclic AMP as substrate. The K_i values of these compounds for inhibition of cyclic AMP hydrolysis by F II decreased by half in the presence of 2 μM cyclic GMP and were found to correspond to the K_i values obtained using cyclic GMP as substrate. Hydrolysis of cyclic AMP by F II was stimulated by 0.1–10 μM cyclic GMP. The decreased K_i values in the presence of a low concentration of cyclic GMP may be due to the effect of cyclic GMP on the active site of F II.

INTRODUCTION

The primary role of platelets in thrombus formation is now firmly established, and platelet function has been investigated in a number of laboratories. Increasing evidence suggests a regulatory role for cyclic 3′,5′-AMP in platelet aggregation. Since stimulation of platelet adenylate cyclase by prostaglandin E_1 (1, 2) and inhibition of phosphodiesterase by papaverine,

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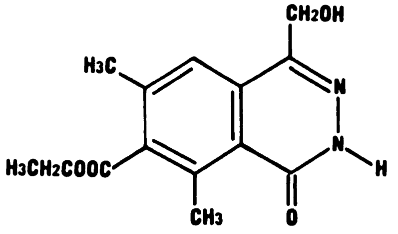
theophylline, dipyridamole (3-6), and other compounds causes inhibition of platelet aggregation, it appears clear that elevated levels of cyclic AMP are associated with inhibition of aggregation (7). Recent studies (8) have also indicated a role for cyclic 3′,5′-GMP in platelet function and suggested that the effects of these two cyclic nucleotides appear to be opposite in nature, as pointed out by Goldberg *et al.* (9). Phosphodiesterase inhibitors that are selective for either cyclic AMP or cyclic GMP hydrolysis could be useful tools in defining the roles of these nucleotides and in understanding the relationship between their roles in platelets and in other tissues. We have separated and partially characterized cyclic nucleotide phosphodiesterase activities from human blood platelets (10). Human platelets contain three distinct forms of cyclic nucleotide phosphodiesterase (F I, F II, and F III) which can be clearly separated by DEAE-cellulose chromatography. F I is a cyclic nucleotide phosphodiesterase with a high K_m for cyclic AMP and a negatively cooperative, low K_m for cyclic GMP (relatively specific cyclic GMP phosphodiesterase). F II hydrolyzes cyclic AMP and cyclic GMP about equally, with a high K_m for both substrates (cyclic nucleotide phosphodiesterase). F III is a low-K_m phosphodiesterase which hydrolyzes cyclic AMP faster than cyclic GMP (relatively specific cyclic AMP phosphodiesterase). This study was conducted to evaluate the possibility of selective inhibition of these three forms of human platelet phosphodiesterases by various platelet aggregation inhibitors. The compounds tested—papaverine, theophylline, caffeine, EG 626, adenosine, 2-chloroadenosine, and dipyridamole—were reported to inhibit platelet aggregation (3-5, 11, 12).

METHODS

Materials. These were previously described (10). 2-Chloroadenosine was purchased from Sigma. Papaverine, theophylline, caffeine, and adenosine were obtained Katayama Chemical Company. 7-Ethoxycarbonyl-6,8-dimethyl-4-hydroxymethyl-1(2*H*)-phthalazinone (EG 626) (Fig. 1) was a gift from Dr. T. Shimamoto, Department of Internal Medicine, Tokyo Ikashika University, as was one of the phthalazone derivatives, which are potent inhibitors of the cyclic nucleotide phosphodiesterase (12, 13). Dipyridamole was a gift from Nippon C. H. Boehringer Soehne Company.



7-ethoxycarbonyl-6,8-dimethyl-4-hydroxymethyl-1(2H)-phthalazinone

(EG 626)

FIG. 1. *Structure of EG 626*

Phosphodiesterase assay. The assay of cyclic nucleotide phosphodiesterase activity was in principle that described by Butcher and Sutherland (14) with the tritiated substrate modification of Hidaka and Shibuya (15). The reaction mixture (0.5 ml) contained 50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 µg of bovine serum albumin, cyclic [^{3}H]AMP or cyclic [^{3}H]GMP, various concentrations of inhibitors, and an appropriate amount of enzyme, and was incubated at 30° for 10-30 min. The reaction was terminated by boiling for 5 min; 50 µg of snake venom were added, and the mixture was incubated for another 10 min. Then 1 ml of water was added, and denatured protein was removed by centrifugation. The clear supernatant fluid was applied to a small cation-exchange resin column (AG 50W-X4, 200-400 mesh, 0.7 × 1.5 cm). The product, [^{3}H]adenosine or [^{3}H]guanosine, was eluted with 1.5 ml of 3 N ammonium hydroxide after the column had been washed with 15 ml of water. The amount of product was determined in a liquid scintillation counter (Beckman LS-233). More than 95% of adenosine or guanosine could be recovered by this method. About 10% of added cyclic nucleotide was generally hydrolyzed during the incubation. K_i values were estimated by the method of Dixon (16).

Phosphodiesterase preparation. The three forms of human platelet phosphodiesterase were prepared as previously described (10). Human blood samples were collected from normal volunteer donors, and citrated, platelet-rich plasma was prepared by low-speed centrifugation. Careful microscopic checks showed that platelets were virtually free from erythrocyte and leukocyte contamination. Platelets were isolated from the platelet-rich plasma by centrifugation. Platelet homogenates were prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM $MgCl_2$, using homogenizer tubes with Teflon pestles. The homogenized solutions were sonicated (30 sec/ml), and the soluble phosphodiesterase preparation was obtained from the sonicated homogenates by centrifugation at 105,000 × g for 60 min. This preparation, referred to as the sonic extract, was applied to a DEAE-cellulose column. DEAE-cellulose chromatography was performed as described previously (10) on columns (1.5 × 20 cm) with bed volumes of 35 ml. The buffer was 50 mM Tris-acetate (pH 6.0) containing 3.75 mM 2-mercaptoethanol. The enzyme preparation was applied to the column, followed by elution with several column volumes of buffer. The initial wash contained no detectable phosphodiesterase activity. A linear gradient from 0 to 1 M sodium acetate was then applied with a flow rate of 0.5 ml/min and a total gradient volume of 300 ml. Sonic extracts of human blood platelets, when chromatographed on DEAE-cellulose, yielded three active cyclic nucleotide phosphodiesterase fractions, designated F I, F II, and F III, as reported previously (10). F I was eluted from DEAE-cellulose at about 0.08 M sodium acetate and contained about 80% cyclic GMP phosphodiesterase in column eluates assayed at a low substrate level (0.4 μM), and about 55% cyclic GMP hydrolytic activity and about 40% cyclic AMP hydrolytic activity in column eluates assayed at a higher substrate level (100 μM). F II was eluted from DEAE-cellulose at about 0.2 M sodium acetate and contained about 40% each of cyclic AMP and cyclic GMP phosphodiesterase activity when assayed at a high substrate level. F III was eluted at about 0.35 M sodium acetate and contained about 80% cyclic AMP phosphodiesterase activity and 10% cyclic GMP phosphodiesterase activity when assayed at a low substrate level. Since the enzyme activity was more labile in solutions containing a low concentration of protein, the preparation was usually stored at 4° in the presence of 0.1 mg/ml of bovine serum albumin.

RESULTS

Inhibition of chromatographically separated phosphodiesterase activity by various compounds. The effects of papaverine, theophylline, caffeine, EG 626, adenosine, 2-chloroadenosine, and dipyridamole on each form of the DEAE-cellulose-resolved phosphodiesterase were investigated. The inhibitory potencies of the seven drugs were compared by means of Dixon plots (16). The resulting K_i values are shown in Table 1, along with the order of potency of these drugs as inhibitors of the F III enzyme. It was found that these compounds inhibited the three forms of the phosphodiesterase by different degrees. EG 626 was 30 times more potent as an inhibitor of F III than of F I, while dipyridamole was 20 times more potent as an inhibitor of F I than of F III activity and also revealed high affinity for F I. 2-Chloroadenosine, caffeine, and adenosine were 5–20 times more potent as inhibitors of F II and F III than of F I. Papaverine and theophylline inhibited F III more effectively. If the relative selectivity of the drugs for F III (a relatively specific cyclic AMP phosphodiesterase) and F I (a relatively specific cyclic GMP phosphodiesterase) is considered, a clearer picture develops. This relative selectivity may perhaps best be determined by calculating the F I:F III ratios, i.e., K_i for cyclic GMP phosphodiesterase (F I) (M)/K_i for cyclic AMP phosphodiesterase (F III) (M), used by Amer (17). A high F I:F III ratio would indicate a relatively high selectivity for F III (cyclic AMP phosphodiesterase) and hence a tendency for the drug to promote the accumulation of relatively more cyclic AMP than cyclic GMP, whereas a low F I:F III ratio would indicate the opposite effect. The results of this type of calculation for the drugs under study are given in Table

TABLE 1

Inhibition of DEAE-cellulose resolved phosphodiesterases (F I, F II, and F III) by various drugs

K_i values were estimated using the method of Dixon (16). Values are means ± standard errors of four experiments with four different preparations. Each experiment consisted of duplicate determinations. Five inhibitor concentrations were used at each substrate concentration, and three substrate concentrations were used for determination of each K_i value. All the measured K_i values were observed inside the range of inhibitor concentrations used. The K_i values obtained using cyclic AMP and cyclic GMP as substrate were compared by Student's *t*-test. Statistical significance was assumed with $p < 0.05$.

Inhibitor	K_i value					
	F I		II		F III	
	Cyclic AMP, 100-500 µM	Cyclic GMP, 0.1-0.5 µM	Cyclic AMP, 10-50 µM	Cyclic GMP, 10-50 µM	Cyclic AMP, 0.1-0.5 µM	Cyclic GMP, 0.1-0.5 µM
	µM	µM	µM	µM	µM	µM
Papaverine	0.91 ± 0.09	1.18 ± 0.17	4.45 ± 0.09	2.10 ± 0.14^{a}	0.27 ± 0.01	0.21 ± 0.03
EG 626	47.8 ± 2.8	44.0 ± 4.4	20.5 ± 2.2	10.0 ± 1.3^{b}	1.4 ± 0.2	1.3 ± 0.1
Dipyridamole	1.0 ± 0.3^{c}	1.0 ± 0.2^{c}	2.3 ± 0.2	1.0 ± 0.1^{d}	19.3 ± 0.3^{c}	20.7 ± 0.7^{c}
Theophylline	269 ± 28	323 ± 26	286 ± 17	123 ± 3^{a}	45 ± 5	43 ± 1
2-Chloroadenosine	870 ± 15	830 ± 20	81 ± 3	40 ± 2^{d}	70 ± 6	83 ± 3
Caffeine	875 ± 136	888 ± 77	215 ± 14	110 ± 6^{d}	131 ± 17	157 ± 47
Adenosine	3833 ± 498	3567 ± 260	915 ± 95	550 ± 17^{d}	420 ± 49	475 ± 59

[a] Significantly different from K_i value with cyclic AMP as substrate ($p < 0.001$).

[b] Significantly different from K_i value with cyclic AMP as substrate ($p < 0.05$).

[c] Noncompetitive inhibition (the others are competitive inhibition).

[d] Significantly different from K_i value with cyclic AMP as substrate ($p < 0.01$).

2, the drugs being listed in order of decreasing selectivity for cyclic AMP phosphodiesterase. All the agents tested except dipyridamole showed relatively selective inhibition for cyclic AMP phosphodiesterase (F III).

The degree of inhibition of F I and F III by each drug was virtually independent of the substrate used; the K_i values of these agents for F I and F III enzymes were identical whether cyclic AMP or cyclic GMP was used as substrate (Table 1). However, in the case of F II there were significant differences between the K_i values obtained using cyclic AMP and cyclic GMP as substrate. All seven compounds revealed about 2 times more affinity for F II using cyclic GMP than with cyclic AMP as substrate. This difference in K_i values for inhibition of F II may be explained by the presence of two different catalytic sites, which hydrolyze cyclic AMP and cyclic GMP, respectively, or by the presence of a common catalytic site for both cyclic nucleotides with some "specific mechanism." The latter was supported by our previous findings, which suggested that the hydrolysis of both cyclic AMP and cyclic GMP is catalyzed by a single catalytic site on the enzyme (10). In order to study this "specific mechanism," the properties of the F II enzyme were examined further.

Effect of cyclic GMP on cyclic AMP hydrolysis by F II. At a low substrate concentration, cyclic GMP stimulated rather than inhibited cyclic AMP hydrolysis by F II (Fig. 2). The maximum stimulation (130%) occurred between 0.5 and 5 μM cyclic GMP. Because activation was due primarily to an increase in the affinity of the enzyme for cyclic AMP, only a slight effect of cyclic GMP was observed at a substrate concentration above 50 μM. These results suggested that cyclic GMP would probably affect the active site of F II through conformational changes. F II enzyme which had been dialyzed after incubation with 2 μM cyclic GMP did not exhibit any greater activity than enzyme previously incubated in the absence of cyclic GMP; both the treated and the control preparation were stimulated to the same extent by cyclic GMP. These results indicate that the activation of F II enzyme by cyclic GMP is reversible and that the binding of cyclic GMP to F II is dissociable by dialysis.

TABLE 2

Relative selectivity of various drugs for F III (a relatively specific cyclic AMP phosphodiesterase) and F I (a relatively specific cyclic GMP phosphodiesterase)

Inhibitor	F I:F III ratio[a]
EG 626	31.4
2-Chloroadenosine	11.9
Adenosine	8.5
Theophylline	7.2
Caffeine	6.8
Papaverine	4.4
Dipyridamole	0.052

[a] K_i for cyclic GMP phosphodiesterase (F I) (M)/K_i for cyclic AMP phosphodiesterase (F III) (M).

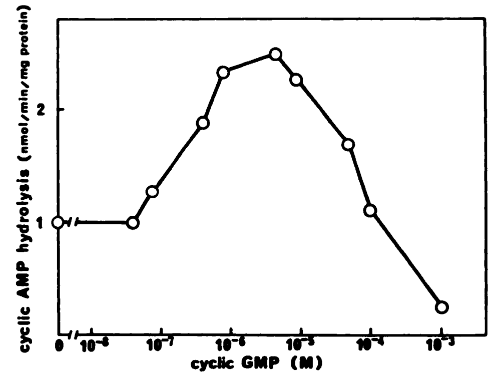


FIG. 2. *Effect of cyclic GMP on cyclic AMP hydrolysis by F II*

The substrate concentration (cyclic AMP) was 5 μM. The concentrations of cyclic GMP shown on the abscissa indicate the average amounts of cyclic GMP present throughout the incubation [(cyclic GMP added + cyclic GMP at end of incubation)/2]. Cyclic GMP at the end of the incubation was determined as follows. Various concentrations of cyclic [^{3}H]GMP were incubated with enzyme in the presence of 5 μM cyclic AMP under the same conditions; cyclic [^{3}H]GMP hydrolysis was measured, and the cyclic GMP remaining at the end of the incubation was calculated. Approximately 50% of the cyclic GMP added was hydrolyzed by the end of incubation when less than 0.5 μM cyclic GMP was added, but less than 10% of the cyclic GMP added was found to be hydrolyzed when more than 1 μM cyclic GMP was used as an effector.

TABLE 3

Effects of various drugs on cyclic AMP hydrolysis by F II in the presence and absence of 2 μM cyclic GMP

K_i values were estimated using the method of Dixon (16). Values are means ± standard errors of four determinations with four different preparations. Each experiment consisted of duplicate determinations. Five inhibitor concentrations were used at each substrate concentration, and three substrate concentrations were used for determination of each K_i value. All the measured K_i values were observed inside the range of inhibitor concentrations used. The K_i values obtained in the presence and absence of 2 μM cyclic GMP were compared by Student's *t*-test. Statistical significance was assumed when $p < 0.05$.

Inhibitor	K_i value	
	−Cyclic GMP	+Cyclic GMP
	μM	μM
Papaverine	4.45 ± 0.09	2.45 ± 0.10[a]
EG 626	20.5 ± 2.2	9.4 ± 1.4[b]
Dipyridamole	2.3 ± 0.2	1.1 ± 0.2[b]
Theophylline	286 ± 17	130 ± 3[a]
2-Chloroadenosine	81 ± 3	42 ± 1[a]
Caffeine	215 ± 14	109 ± 7[c]
Adenosine	915 ± 95	533 ± 31[c]

[a] Significantly different from K_i value in the absence of cyclic GMP ($p < 0.001$).

[b] Significantly different from K_i value in the absence of cyclic GMP ($p < 0.05$).

[c] Significantly different from K_i value in the absence of cyclic GMP ($p < 0.01$).

Effects of inhibitors on cyclic AMP hydrolysis by F II in the presence of cyclic GMP. The inhibitory potencies of the seven drugs toward cyclic AMP hydrolysis by F II in the presence of a low concentration (2 μM) of cyclic GMP were compared by means of Dixon plots (16). The resulting K_i values, as well as the K_i values obtained in the absence of cyclic GMP, are shown in Table 3. The K_i values of all seven compounds for inhibition of cyclic AMP hydrolysis by F II decreased by half in the presence of a low concentration of cyclic GMP and closely corresponded to the K_i values obtained using cyclic GMP as substrate (Tables 1 and 3).

DISCUSSION

Most of the compounds studied showed relatively selective inhibition toward F III. These results suggest that the compounds might increase platelet cyclic AMP levels through inhibition of cyclic AMP phosphodiesterase (F III) and seem to support the hypothesis of Salzman and Levine (7) that platelet aggregation is accompanied by a reduction in the platelet cyclic AMP level, and inhibition of aggregation, by an increase in platelet cyclic AMP. McElroy and Philp (18) also observed relatively selective inhibition of the hydrolysis of cyclic GMP and cyclic AMP in crude human platelet preparations by dipyridamole and related agents and reported a close correspondence between their tendency to promote a relative accumulation of cyclic AMP and their inhibitory effects on platelet adhesion and aggregation. EG 626, which is an effective inhibitor of platelet aggregation (12), was 30 times more potent as an inhibitor of cyclic AMP phosphodiesterase (F III) than of cyclic GMP phosphodiesterase (F I), while dipyridamole was 20 times more potent as an inhibitor of cyclic GMP phosphodiesterase than of cyclic AMP phosphodiesterase. Their highly selective inhibition of cyclic AMP and cyclic GMP phosphodiesterases could be useful in defining the roles of these nucleotides and in understanding the relationship between their roles in platelets and in other tissues.

The allosteric activation of cyclic AMP hydrolysis by micromolar amounts of cyclic GMP has been reported by other workers (19–21). Although it was shown in our previous report (10) that cyclic GMP does not stimulate cyclic AMP hydrolysis by F II at higher substrate concentrations (100 μM), the cyclic AMP hydrolytic activity of F II was also found to be activated by micromolar amounts of cyclic GMP when a lower substrate concentration was used (Fig. 2). Because this activation was due primarily to an increase in the affinity of the enzyme for cyclic AMP, only a slight effect of cyclic GMP was observed at substrate concentrations above 50 μM. Various drugs used in this study revealed approximately 2 times greater affinity for F II using cyclic GMP than with cyclic AMP as substrate, whereas the K_i values of these drugs for F I and F III were identical whether cyclic AMP or cyclic GMP was used as substrate. The K_i values for inhibition of cyclic AMP hydrolysis by F II decreased by half in the presence of cyclic GMP and were found to correspond closely to the values obtained using cyclic GMP as substrate. The detailed mechanism of this phenomenon in the F II enzyme is not clear, but it is suggested that the decrease in K_i values for cyclic AMP hydrolysis by the addition of cyclic GMP may be related to the stimulation of cyclic AMP hydrolysis by cyclic GMP.

In our previous report (10) it was shown that cyclic AMP hydrolysis by F I was also stimulated by low concentrations of cyclic GMP. However, the K_i values of various inhibitors for F I were identical whether cyclic AMP or cyclic GMP was used as substrate; furthermore, the addition of low concentrations of cyclic GMP failed to affect the K_i values of these compounds for cyclic AMP hydrolysis by F I. These results suggest that cyclic GMP affects cyclic AMP hydrolysis by F I and F II by different mechanisms.

REFERENCES

1. Wolfe, S. M. & Shulman, N. R. (1969) *Biochem. Biophys. Res. Commun.* 35, 265-272.
2. Marquis, N. R., Vigdahl, R. L. & Tavormina, P. R. (1969) *Biochem. Biophys. Res. Commun.*, 36, 965-972.
3. Ardlie, N. G., Schultz, B. G. & Schwartz, C. J. (1967) *Thromb. Diath. Haemorrh.*, 18, 670-673.
4. Vigdahl, R. L., Mongin, J., Jr. & Marquis, N. R. (1971) *Biochem. Biophys. Res. Commun.*, 42, 1088-1094.
5. Horlington, M. & Watson, P. A. (1970) *Biochem. Pharmacol.*, 19, 955-956.
6. Bygdeman, S. & Johnsen, O. (1971) *Acta Med. Scand. Suppl.*, 525, 179-180.
7. Salzman, E. W. & Levine, L. (1971) *J. Clin. Invest.*, 50, 131-141.
8. Haslam, R. J. & McClenaghan, M. D. (1974) *Biochem. J.*, 138, 317-320.
9. Goldberg, N. D., O'Dea, R. F. & Haddox, M. K. (1973) *Adv. Cyclic Nucleotide Res.*, 3, 155-223.
10. Hidaka, H. & Asano, T. (1976) *Biochim. Biophys. Acta*, 429, 485-497.
11. Born, G. V. R. (1964) *Nature*, 202, 95-96.
12. Shimamoto, T. (1974) in *New Horizons in Cardiovascular Practice* (Russek, H. I., ed.), pp. 449-469, University Park Press, Baltimore.
13. Hidaka, H., Asano, T., Shibuya, M. & Shimamoto, T. (1974) *Thromb. Diath. Haemorrh. Suppl.*, 60, 321-327.
14. Butcher, R. W. & Sutherland, E. W. (1962) *J. Biol. Chem.*, 237, 1244-1250.
15. Hidaka, H. & Shibuya, M. (1974) *Biochem. Med.*, 10, 301-311.
16. Dixon, M. (1953) *Biochem. J.*, 55, 170-171.
17. Amer, M. S. (1974) *Am. J. Dig. Dis.*, 19, 71-74.
18. McElroy, F. A. & Philp, R. B. (1975) *Life Sci.*, 17, 1479-1494.
19. Beavo, J. A., Hardman, J. G. & Sutherland, E. W. (1971) *J. Biol. Chem.*, 246, 3841-3846.
20. Franks, D. J. & MacManus, J. P. (1971) *Biochem. Biophys. Res. Commun.*, 42, 844-849.
21. Russell, T. R., Terasaki, W. L. & Appleman, M. M. (1973) *J. Biol. Chem.*, 248, 1334-1340.