



YC-1 inhibited human platelet aggregation through NO-independent activation of soluble guanylate cyclase

Chin-Chung Wu, Feng-Nien Ko, Sheng-Chu Kuo*, Fang-Yu Lee** & ¹Che-Ming Teng

Pharmacological Institute, College of Medicine, National Taiwan University, Taipei; *Graduate Institute of Pharmaceutical Chemistry, China Medical College, Taichung and **Yung-Shin Pharmaceutical Industry Co. Ltd., Taichung, Taiwan

- 1 Our previous study demonstrated that YC-1, a derivative of benzylindazole, is a novel activator of soluble guanylate cyclase (sGC) in rabbit platelets. This work investigated whether the antiplatelet effect of YC-1 was mediated by a nitric oxide (NO)/sGC/cyclic GMP pathway in human platelets.
- 2 In human washed platelets, YC-1 inhibited platelet aggregation and ATP released induced by U46619 (2 μM), collagen (10 $\mu\text{g ml}^{-1}$) and thrombin (0.1 u ml^{-1}) in a concentration-dependent manner with IC_{50} values of (μM) 2.1 ± 0.3 , 11.7 ± 2.1 and 59.3 ± 7.1 , respectively.
- 3 In a 30,000 g supernatant fraction from human platelet homogenate, YC-1 (5–100 μM) increased sGC activity in a concentration-dependent manner. At the same concentration-range, YC-1 elevated cyclic GMP levels markedly, but only slightly elevated cyclic AMP levels in the intact platelets.
- 4 MY-5445, a selective inhibitor of cyclic GMP phosphodiesterase, potentiated the increases in cyclic GMP caused by YC-1, and shifted the concentration-anti-aggregation curve of YC-1 to the left. In contrast, HL-725, a selective inhibitor of cyclic AMP phosphodiesterase, did not affect either the increases in cyclic nucleotides or the anti-aggregatory effect caused by YC-1.
- 5 Methylene blue, an inhibitor of sGC, blocked the increases of cyclic GMP caused by YC-1, and attenuated markedly the anti-aggregatory effect of YC-1. The adenylate cyclase inhibitor, 2',5'-dideoxyadenosine (DDA) did not affect YC-1-induced inhibition of platelet aggregation.
- 6 Haemoglobin, which binds NO, prevented the activation of sGC and anti-aggregatory effect caused by sodium nitroprusside, but did not affect YC-1 responses.
- 7 These results would suggest that YC-1 activates sGC of human platelets by a NO-independent mechanism, and exerts its antiplatelet effects through the sGC/cyclic GMP pathway.

Keywords: YC-1; human platelets; guanylate cyclase; cyclic GMP; nitric oxide

Introduction

Guanosine 3':5'-cyclic monophosphate (cyclic GMP) has been established as an intracellular signalling molecule in a variety of cells and tissues. In platelets, cyclic GMP plays a critical role in the inhibition of platelet aggregation and adhesion (Walter, 1989). The mechanism of action of cyclic GMP is still not well understood, but at least includes the inhibition of agonist-induced calcium elevation via cyclic GMP-dependent protein kinase (Schmidt *et al.*, 1993).

The synergistic anti-aggregatory effect of cyclic GMP and adenosine 3':5'-cyclic monophosphate (cyclic AMP) has been demonstrated (Radomski *et al.*, 1987). This effect could be of physiological importance to the antithrombotic property of vascular endothelium, because stimulated endothelial cells simultaneously release prostacyclin (a cyclic AMP-elevating agent) and NO (a cyclic GMP-elevating agent). In addition, agents which elevate cytosolic calcium and activate platelets are also known to elevate platelet cyclic GMP levels moderately, thus the increase of platelet cyclic GMP may act as a negative feedback mechanism to regulate platelet aggregation (Radomski *et al.*, 1990).

The concentration of cyclic GMP in platelets is regulated by two enzymes, i.e., soluble guanylate cyclase (sGC), which catalyzes the synthesis of cyclic GMP from GTP, and cyclic GMP phosphodiesterase, which degrades cyclic GMP to 5'-GMP. Agents that elevate the cyclic GMP level either by stimulating sGC or inhibiting cyclic GMP phosphodiesterase, are powerful inhibitors of platelet aggregation (Walter *et al.*, 1991; Buechler *et al.*, 1994). Our previous studies (Ko *et al.*, 1994) demonstrated that YC-1, a derivative of benzylindazole, inhibited activation of rabbit platelets induced by various agonists,

and demonstrated that it is an activator of guanylate cyclase. In this study, we investigated whether the antiplatelet effect of YC-1 in human platelets is mediated by the NO/sGC/cyclic GMP pathway.

Methods

Preparation of washed platelets

Human platelet suspension was prepared according to the procedure previously described (Huang *et al.*, 1993). Fresh whole blood anticoagulated with acid citrate dextrose (ACD) was obtained from healthy human volunteers who had not taken any drugs within the last two weeks. The platelets, after washing, were finally suspended in Tyrode's solution (mM): NaCl 136.9, CaCl₂ 2, KCl 2.7, MgCl₂ 2.1, NaHCO₃ 11.9, glucose 11.1, pH 7.4 containing bovine serum albumin (3.5 mg ml⁻¹) at a concentration of 3×10^8 platelets ml⁻¹.

Platelet aggregation and ATP release reaction

Platelet aggregation was measured by the turbidimetric method (O'Brien, 1962). The platelet suspension was pre-incubated with dimethyl sulphoxide (DMSO, 0.5%, control) or YC-1 at 37°C for 3 min, the U46619, collagen or thrombin was added to induce platelet aggregation. The absorbance of platelet suspension was taken as 0% aggregation and the absorbance of Tyrode's solution as 100% aggregation. ATP released from platelets was detected by the bioluminescence method using the luciferase/luciferin enzyme system described by DeLuca & McElory (1978). Both aggregation and ATP release were simultaneously measured in a Lumi-aggregometer (Chrono-Log Co., U.S.A.). Platelet preparations were stirred at 1,200 r.p.m.

¹ Author for correspondence.

To eliminate the effect of solvent on aggregation, the final concentration of DMSO was fixed at 0.5% (vol/vol).

In some experiments, specific phosphodiesterase inhibitors (MY-5445, HL-725), guanylate cyclase inhibitor (methylene blue), adenylate cyclase inhibitor 2',5'-dideoxyadenosine (DDA) or NO-scavenger (haemoglobin) was used to investigate the mechanism of the anti-aggregatory effect of YC-1. MY-5445, HL-725 or haemoglobin was added to the platelet suspension 1 min before the addition of YC-1. Methylene blue or DDA was added to platelet suspension 4 min before the addition of YC-1.

Estimation of platelet cyclic nucleotides

The platelet suspension was incubated with YC-1, sodium nitroprusside (SNP) or prostaglandin E₁ (PGE₁) at 37°C for 2 min, and the reaction terminated by adding trichloroacetic acid (TCA, final concentration 5%). Cell debris was removed by centrifugation at 10,000 g for 5 min and the TCA in the supernatant was then used to assay for cyclic AMP and cyclic GMP with enzyme immunoassay kits. In some experiments, the platelet suspension was pre-incubated with MY-5445 or HL-725 for 1 min, or with methylene blue or DDA for 4 min before the addition of YC-1, SNP or PGE₁.

Determination of guanylate cyclase activity

Human washed platelets were prepared as described above but finally resuspended in Tris-HCl buffer (mM: Tris-HCl 50, dithiothreitol 1, EDTA 0.2, pH 7.4). Platelets were disrupted by sonication at 4°C (4–6 × 10 s; setting 5, vibra cell, sonics and Materials Inc.), and the homogenate was centrifuged at 30,000 g and 4°C for 15 min. The supernatant was used as a source of crude soluble guanylate cyclase. Protein content was determined with a protein assay kit (Bio-Rad).

Guanylate cyclase activity was determined as previously described (Gerzer *et al.*, 1983). The enzyme preparation was incubated in a final volume (200 μl) with other reactants as follows: GTP (0.2 mM containing 1 × 10⁶ c.p.m. [³²P]-GTP), MgCl₂ (5 mM), cyclic GMP (0.1 mM), IBMX (1 mM), creatine phosphate (5 mM), creatine kinase (0.25 mg ml⁻¹), with or without dithiothreitol (DTT, 10 mM), YC-1, SNP or haemoglobin in Tris-HCl buffer (50 mM, pH 7.4). The reaction was initiated by adding the enzyme preparation and, after incubation at 37°C for 10 min, was terminated by adding HCl (0.5 N, 200 μl). Next, the reaction mixture was heated to 100°C for 2 min and then cooled in an ice bath. Imidazole (1 mM, 200 μl) was added to each tube. GTP and cyclic GMP were separated on neutral alumina and the radioactivity of [³²P]-cyclic GMP was determined in a liquid scintillation counter.

Materials

YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole; Figure 1] was chemically synthesized as described previously (Yoshina & Kuo, 1978), and dissolved in DMSO. Bovine thrombin (Parke Davis Co.) was dissolved in glycerol (50% v/v) for a stock solution of 100 NIH units ml⁻¹. Collagen (type I, bovine achilles tendon, Sigma Chem. Co.) was homogenized in acetic acid (25 mM) and stored (1 mg ml) at 4°C. U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy PGF_{2 α} , Sigma Chem. Co.) was dissolved in DMSO. Sodium nitroprusside was purchased from Sigma Chem. Co. Haemoglobin (oxyhaemoglobin) was prepared by adding a 10 fold molar excess of sodium dithionite to 1 mM solution of commercial haemoglobin (Sigma Chem. Co.). Sodium dithionite was then removed by dialysis against 100 volumes of distilled water for 2 h at 4°C. [³²P]-GTP was purchased from Amersham, U.K., methylene blue from Fluka Chem. Co., MY-5445 [1-(3-chlorophenylamino)-4-phenylphthalazine], HL-725 [9,10-dimethoxy-2-mesityl-imino-3-methyl-3,4,6,7-tetrahydro-2H-pyrimido(6,1-A)-isoquinoline-4-one-hydrochloride] and 2',5'-dideoxyadenosine from Biomol, U.S.A., cyclic AMP and cyclic GMP enzyme immunoassay

kits from Cayman Chem. Co. U.S.A., and protein assay kit from Bio-Rad. All other chemicals were purchased from Sigma Chem. Co.

Statistics

Results are expressed as the mean \pm standard error of the mean (s.e.mean) and comparisons were made with Student's *t* test. A probability of 0.05 or less was considered significant.

Results

Effect of YC-1 on the aggregation and ATP release of washed human platelets

In human washed platelets, U46619 (2 μM, a stable thromboxane A₂-mimetic), collagen (10 μg ml⁻¹) and thrombin (0.1 u ml⁻¹) all caused about 90% aggregation. YC-1 inhibited U46619-, collagen- and thrombin-induced platelet aggregation in a concentration-dependent manner with IC₅₀ values of 2.1 \pm 0.3, 11.7 \pm 2.1 and 59.3 \pm 7.1, respectively (Figure 2). YC-1 also inhibited ATP release from platelet dense-granules induced by these agents, in parallel with its inhibitory effect on the aggregation (Figure 3).

Effects of YC-1 on platelet guanylate cyclase activity and cyclic nucleotide levels

Using 30,000 g supernatants from human platelet homogenates, we found a basal activity of soluble guanylate cyclase (sGC) of 1.63 \pm 0.61 pmol min⁻¹ mg⁻¹ of protein. YC-1 (5–100 μM) increased the activity of sGC in a concentration-dependent manner (100 μM YC-1 raised the basal activity to 11.29 \pm 1.18 pmol min⁻¹ mg⁻¹ protein; Figure 4). In contrast,

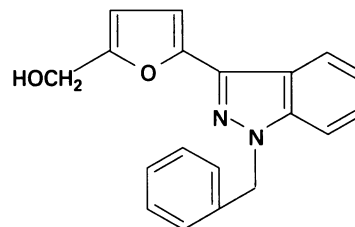


Figure 1 Chemical structure of YC-1.

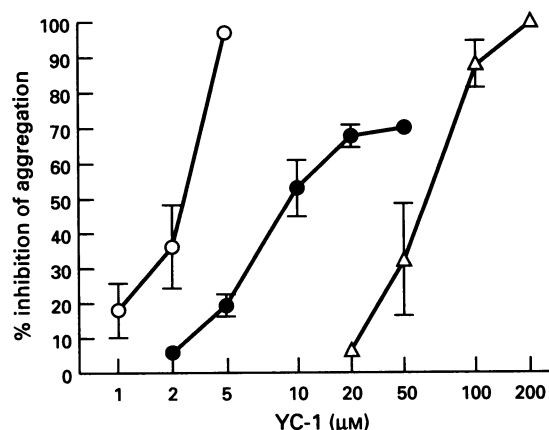


Figure 2 Concentration-inhibition curve of YC-1 on U46619- (2 μM, ○), collagen- (10 μg ml⁻¹, ●) and thrombin- (0.1 u ml⁻¹, △)-induced aggregation of human washed platelets. Percentages of inhibition are presented as means \pm s.e.mean (*n* = 5).

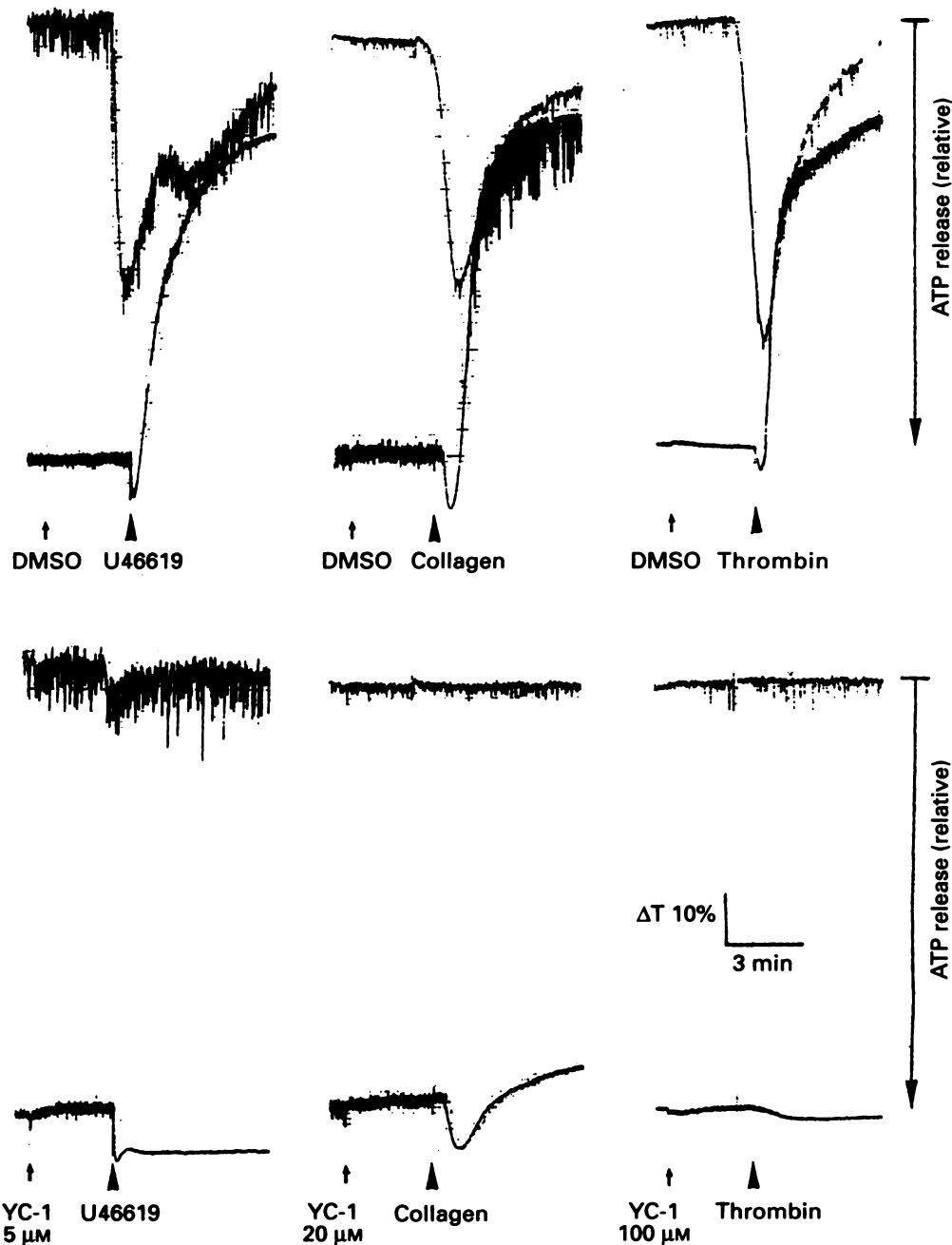


Figure 3 Typical patterns of inhibitory effect of YC-1 on U46619- ($2\ \mu\text{M}$), collagen- ($10\ \mu\text{g ml}^{-1}$) and thrombin- ($0.1\ \text{u ml}^{-1}$)-induced aggregation (upward tracing) and ATP release (downward tracing) of human washed platelets.

$10\ \mu\text{M}$ and $100\ \mu\text{M}$ sodium nitroprusside (SNP) raised the activity of sGC to 10.98 ± 0.97 and $43.27 \pm 15.24\ \text{pmol min}^{-1}\ \text{mg}^{-1}$ of protein, respectively.

When DTT ($10\ \text{mM}$) was absent from the reaction mixture, the basal activity of sGC increased to $3.08 \pm 0.24\ \text{pmol min}^{-1}\ \text{mg}^{-1}$ of protein, and the action of SNP on sGC was attenuated ($10\ \mu\text{M}$ and $100\ \mu\text{M}$ SNP increased the activity of sGC to 3.22 ± 0.14 and $13.75 \pm 3.83\ \text{pmol min}^{-1}\ \text{mg}^{-1}$ of protein, respectively). In contrast, $100\ \mu\text{M}$ YC-1 raised the activity of sGC to $10.21 \pm 1.73\ \text{pmol min}^{-1}\ \text{mg}^{-1}$ of protein in the absence of DTT.

In parallel with the increase of activity of sGC, YC-1 (5 – $100\ \mu\text{M}$) markedly elevated cyclic GMP levels in intact platelets. At higher concentrations (20 – $100\ \mu\text{M}$), YC-1 also caused small but significant increases in platelet cyclic AMP levels (Figure 5). SNP ($50\ \mu\text{M}$) had effects similar to those obtained with YC-1, causing a substantial increase in platelet cyclic GMP (from 1.75 ± 0.03 to $11.7 \pm 0.3\ \text{pmol}/10^9$ platelets) and a small increase in cyclic AMP (from 17.3 ± 1.1 to $23.8 \pm 3\ \text{pmol}/10^9$ platelets).

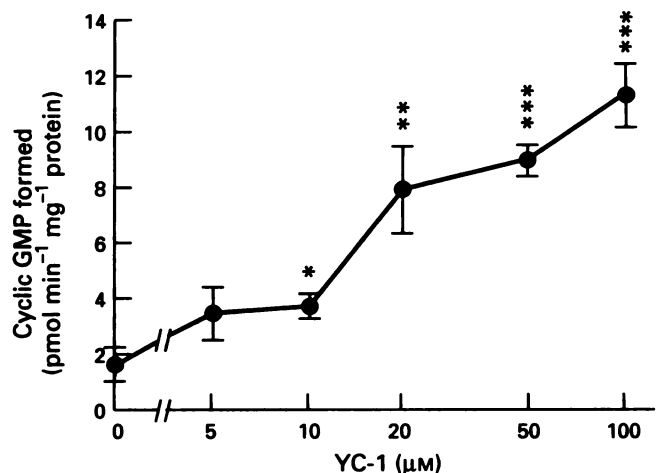


Figure 4 Effects of YC-1 on human platelet guanylate cyclase. Guanylate cyclase activity in the $30,000g$ supernatant fraction was assayed in the presence of $10\ \text{mM}$ dithiothreitol for $10\ \text{min}$ at 37°C as described under 'Methods' with the indicated concentrations of YC-1. Values are presented as means \pm s.e. mean ($n=3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ as compared with the basal activity.

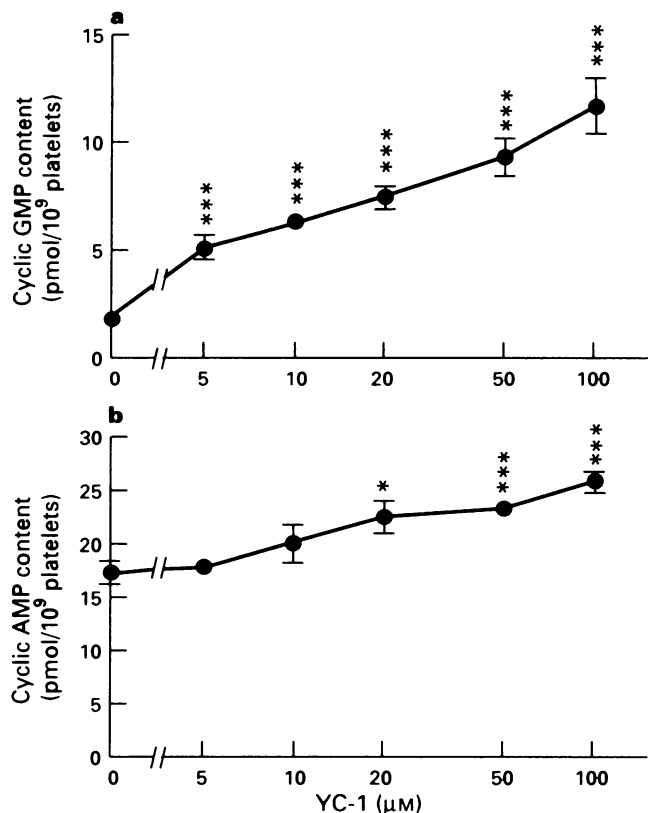


Figure 5 Effects of YC-1 on platelet cyclic GMP (a) and cyclic AMP (b). Washed human platelets (4×10^8 platelets) were incubated for 2 min at 37°C with various concentrations of YC-1. Reactions were then terminated by addition of trichloroacetic acid. The cyclic GMP and cyclic AMP contents were determined by enzyme immunoassay. Values are presented as means \pm s.e. mean ($n=4$). * $P < 0.05$; *** $P < 0.001$ as compared with the respective control.

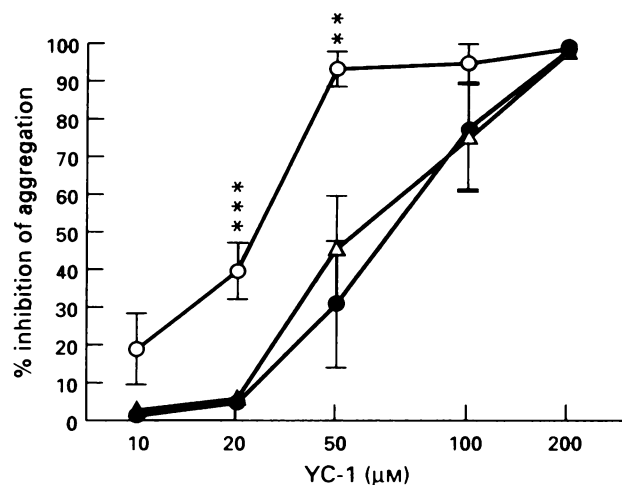


Figure 6 Potentiation of the anti-aggregatory effect of YC-1 by MY-5445, but not by HL-725. Washed human platelets were preincubated with DMSO (control, ●), MY-5445 (20 μM, ○) or HL-725 (1 pM, △) for 1 min at 37°C, the YC-1 (as indicated concentration) was added and the incubations were continued for an additional 3 min. Platelet aggregation was then induced by the addition of thrombin (0.1 u ml^{-1}). Percentage inhibition is presented as mean \pm s.e. mean ($n=4$). ** $P < 0.01$; *** $P < 0.001$ as compared with the respective control.

Synergistic effects of MY-5445 and YC-1

MY-5445 (20 μM), a selective inhibitor of cyclic GMP phosphodiesterase (Hagiwara *et al.*, 1984), potentiated the inhibitory effect of SNP on thrombin-induced platelet

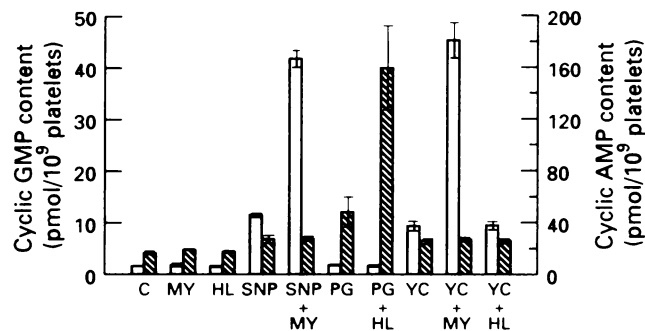


Figure 7 Effects of MY-5445 and HL-725 on the increases in platelet cyclic GMP (open columns) and cyclic AMP (hatched columns) caused by YC-1, sodium nitroprusside (SNP) or prostaglandin E₁ (PGE₁). Washed human platelets were incubated at 37°C with DMSO (0.5%, C), SNP (50 μM), PGE₁ (0.1 μM, PG) or YC-1 (50 μM, YC) in the absence or presence of MY-5445 (20 μM, MY) or HL-725 (1 pM, HL). MY-5445 or HL-725 was added 1 min before YC-1, SNP or PGE₁ and the incubations were continued for additional 2 min. The cyclic GMP and cyclic AMP contents were determined by enzyme immunoassay. Values are presented as means \pm s.e. mean ($n=4$).

aggregation (from 1.6 ± 0.3 to $95.9 \pm 3.6\%$ inhibition of aggregation, $n=4$). Similarly, MY-5445 enhanced the anti-aggregatory effect of YC-1 and shifted the concentration-response curve to the left (Figure 6). This effect was associated with a marked increase in cyclic GMP accumulation (Figure 7).

HL-725 (1 pM), a selective inhibitor of cyclic AMP phosphodiesterase (Ruppert & Weithmann, 1982), had synergistic effects on both the increase in cyclic AMP levels (Figure 7) and the anti-aggregatory effect caused by low concentration (0.1 μM) of PGE₁ (from 1.2 ± 0.4 to $93.2 \pm 6.1\%$ inhibition of aggregation, $n=5$). In the presence of 1 pM HL-725, however, the anti-aggregatory effect of YC-1 remained unchanged (Figure 6), and there was no additional effect on platelet cyclic AMP levels (Figure 7).

Neither MY-5445 nor HL-725 had any effect on thrombin-induced platelet aggregation at the concentrations used (data not shown).

Blockade of the actions of YC-1 by methylene blue

Methylene blue, an inhibitor of sGC (Gruetter *et al.*, 1979), was used for further investigation of the relationship between guanylate cyclase activation and antiplatelet effects caused by YC-1. Methylene blue (10 μM) alone did not affect the basal level of platelet cyclic GMP ($2.1 \pm 0.1 \text{ pmol/10}^9$ platelets, $n=4$) and the platelet aggregation induced by thrombin ($91.3 \pm 1.1\%$ aggregation, $n=5$). When platelets were preincubated with methylene blue for 4 min, the increase in platelet cyclic GMP caused by SNP or YC-1 was almost completely abolished. Furthermore, the inhibitory effect of YC-1 on thrombin-induced platelet aggregation was markedly attenuated (Figure 8).

DDA (300 μM), an inhibitor of adenylate cyclase (Haslam *et al.*, 1978), prevented both the inhibitory effect of PGE₁ (10 μM) on platelet aggregation and the increases of platelet cyclic AMP. In contrast, DDA significantly inhibited the increases of platelet cyclic AMP, but did not affect the anti-aggregatory effect caused by YC-1 (Figure 8).

Effects of haemoglobin on actions of YC-1

In the platelet suspension, haemoglobin (5 μM) completely prevented the inhibitory effect of SNP on collagen-induced platelet aggregation. In the soluble fraction from platelet homogenate, haemoglobin (5 μM) also completely blocked the increase in platelet cGMP caused by SNP. In contrast, haemoglobin did not affect these actions of YC-1 (Figure 9). These results suggest that YC-1 activated sGC and inhibited platelet aggregation via a NO-independent mechanism.

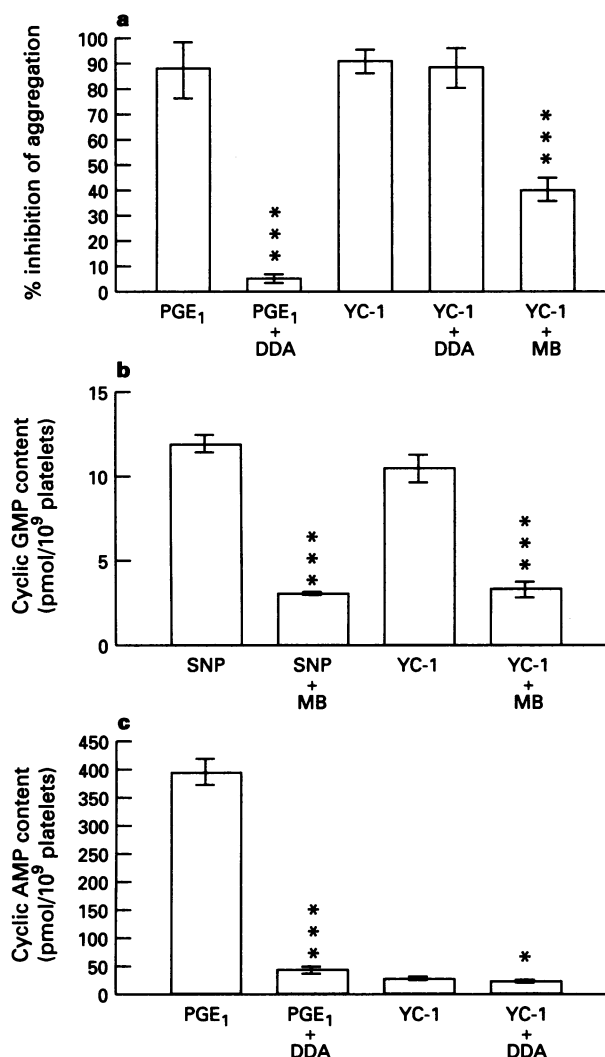


Figure 8 Effects of methylene blue and 2',5'-dideoxyadenosine (DDA) on the inhibition of platelet aggregation (a) and the increases of platelet cyclic GMP (b) and cyclic AMP (c) caused by YC-1. Washed human platelets were incubated with YC-1 (100 μ M), prostaglandin E₁ (PGE₁, 10 μ M) or sodium nitroprusside (SNP, 50 μ M) at 37°C for 3 min (a) or 2 min (b, c) in the absence or presence of methylene blue (MB, 10 μ M) or DDA (300 μ M). Platelet aggregation was induced by thrombin (0.1 u ml⁻¹). The cyclic GMP and cyclic AMP contents were determined by enzyme immunoassay. Values are presented as means \pm s.e. mean ($n=5$ in a, $n=4$ in b and c) * $P<0.05$; *** $P<0.001$ as compared with the respective control.

Discussion

This study has demonstrated that YC-1 activated sGC and inhibited aggregation of human platelets. These results are consistent with those found in rabbit platelets (Ko *et al.*, 1994). By using specific inhibitors of enzymes, the relationship between sGC/cyclic GMP and the antiplatelet effects of YC-1 was further investigated in this study. MY-5445, an inhibitor of the breakdown of platelet cyclic GMP, dramatically potentiated the increases in cyclic GMP and the anti-aggregatory effect caused by YC-1. Furthermore, blockade of sGC by methylene blue almost completely prevented the YC-1-induced rise in platelet cyclic GMP and markedly attenuated the anti-aggregatory effect of YC-1. These results imply that the inhibitory effect of YC-1 on platelet aggregation was mediated by activation of the sGC/cyclic GMP pathway.

YC-1 not only increased platelet cyclic GMP but also caused slight increases in platelet cyclic AMP. This effect was similar to that produced by SNP. Maurice & Haslam (1990) reported that the increases in platelet cyclic AMP by SNP were

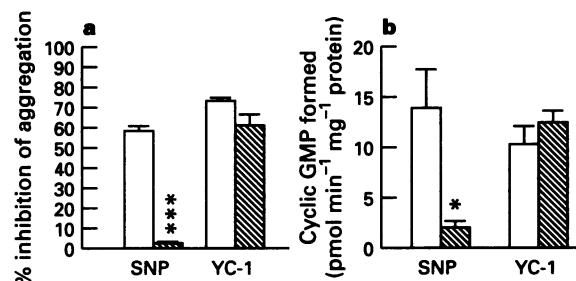


Figure 9 Effects of haemoglobin on the inhibition of platelet aggregation (a) and activation of platelet guanylate cyclase (b) caused by YC-1 and sodium nitroprusside (SNP). (a) Washed human platelets were incubated with YC-1 (20 μ M) or SNP (20 μ M) at 37°C for 3 min, in the absence (open column) or presence (hatched column) of haemoglobin (5 μ M). Platelet aggregation was then induced by collagen (10 μ g ml⁻¹). (b) The 30,000 g supernatant fraction of human platelets was incubated with YC-1 (100 μ M) or SNP (100 μ M) at 37°C for 10 min. The guanylate cyclase activity was assayed as described under 'Methods' with the exception that DTT was omitted from the reaction mixture. Values are presented as means \pm s.e. mean. ($n=4$ in a and b) * $P<0.05$; *** $P<0.001$ as compared with the respective control.

mediated by the inhibition by cyclic GMP of cyclic AMP breakdown. Because YC-1 and SNP greatly enhanced the accumulation of cyclic AMP caused by PGE₁ (Ko *et al.*, 1994), and HL-725 did not further increase platelet cyclic AMP caused by YC-1 or SNP, they must act by inhibiting cyclic AMP breakdown and not by promoting cyclic AMP formation. The common target of YC-1, SNP and HL-725 is most likely cyclic GMP-inhibited low K_m cyclic AMP phosphodiesterase, an enzyme known to be present in many cells including platelets. However, the attenuation of the increases in cyclic AMP by DDA, without a corresponding effect on platelet aggregation, indicates that cyclic AMP does not play a significant role in the inhibition of platelet aggregation by YC-1.

NO has been shown to be an endogenous activator of sGC. Additionally, nitrovasodilators, such as SNP and nitroglycerin, are believed to exert their effects via the release of NO. Therefore, investigating whether the actions of YC-1 are dependent or independent on NO is of primary concern here. In this study, we used haemoglobin as a scavenger of NO. NO binds to the iron-haeme group of haemoglobin with a very high affinity, and loses its activity (Keilin & Hartree, 1937; Murad *et al.*, 1977). Unlike the NO-donor SNP, the inhibitory effect of YC-1 on platelet aggregation remained unaffected by co-incubation with haemoglobin. These data imply that YC-1 inhibits platelet aggregation through a NO-independent mechanism. Nevertheless, the possibility that YC-1 activates the platelet L-arginine/NO pathway cannot be totally ruled out. It has been proposed that platelets contain the L-arginine/NO pathway (Radomski *et al.*, 1990), and a NO synthase has been purified from human platelet (Muruganandam & Mutus, 1994). Haemoglobin did not penetrate platelet membrane effectively, thus it cannot block the effect of NO produced by NO synthase within the cell. However, with YC-1 this is not the case. That is, in a cell-free system, haemoglobin still would not inhibit the action of YC-1 on sGC.

Two additional pieces of evidence imply that YC-1 activates sGC through a mechanism which is distinct from NO. First, unlike SNP, the activation of sGC caused by YC-1 was much less sensitive to the reducing agent DTT. It has been recognized that regulation of sGC involves oxidation-reduction reactions; reducing agents like DTT promoted, whereas oxidizing agents inhibited activation of sGC by NO and nitrovasodilators (Waldman & Murad, 1987). DTT might be a potentiating activator by preventing both oxidation of NO to inactive metabolites, and formation of oxidized sGC which lost most of its ability to be modulated by NO. Second, the chemical structure of YC-1 implies it possesses no functional group which can be converted to NO.

Apart from NO, carbon monoxide (CO) and hydroxyl radical have been shown to activate sGC (Schmidt, 1992). However, it is unlikely that YC-1 activated sGC through CO or hydroxyl radical since the activation of sGC caused by YC-1 was affected by neither haemoglobin which also bound CO, nor mannitol (50 mM), a scavenger of the hydroxyl radical (data not shown).

The above results suggest that YC-1 activates sGC of human platelets in a NO-independent manner, and exerts its antiplatelet effects through the sGC/cyclic GMP pathway. It is interesting that YC-1 can bypass NO to activate sGC, because almost all known activators of sGC, including nitrovasodilators, exert their action via the release of NO. NO binds to the iron-containing haem group of sGC, and causes a three-dimensional change that increases the production of cyclic GMP (Ignarro *et al.*, 1984; Traylor & Sharma, 1992).

References

- BUECHLER, W.A., IVANOVA, K., WOLFRAM, G., DRUMMER, C., HEIM, J.M. & GERZER, R. (1994). Soluble guanylyl cyclase and platelet function. *Ann. N.Y. Acad. Sci.*, **714**, 151–157.
- DELUCA, M. & MCELORY, W.D. (1978). Purification and properties of firefly luciferase. *Methods Enzymol.*, **57**, 3–15.
- GERZER, R. (1983). Calcium-induced release from platelet membranes of fatty acids that modulate soluble guanylate cyclase. *J. Pharmacol. Exp. Ther.*, **266**, 180–186.
- GRUETTER, C.A., BARRY, B.K., MCNAMARA, D.B., GRUETTER, D.Y., KADOWITZ, P.J. & IGNARRO, L.J. (1979). Relaxation of bovine coronary artery and activation of coronary arterial guanylate cyclase by nitric oxide, nitroprusside and a carcinogenic nitrososamine. *J. Cyclic Nucleotide Res.*, **5**, 211–224.
- HAGIWARA, M., ENDO, T., KANAYAMA, T. & HIDAKA, H. (1984). Effect of 1-(3-chloroanilino)-4-phenylphthalazine (MY-5445), a specific inhibitor of cyclic GMP phosphodiesterase, on human platelet aggregation. *J. Pharmacol. Exp. Ther.*, **228**, 467–471.
- HASLAM, R.J., DAVIDSON, M.M.L. & DESJARDINS, J.V. (1978). Inhibition of adenylate cyclase by adenosine analogues in preparations of broken and intact human platelets: evidence for the unidirectional control of platelet function by cAMP. *Biochem. J.*, **176**, 83–95.
- HUANG, T.F., SHEU, J.R. & TENG, C.M. (1991). A potent antiplatelet peptide, triflavin, from *Trimeresurus flavoviridis* snake venom. *Biochem. J.*, **277**, 351–357.
- IGNARRO, L.J., WOOD, K.S. & WOLIN, M.S. (1984). Regulation of purified soluble guanylate cyclase by porphyrins and metalloporphyrins: a unifying concept. *Adv. Cyclic Nucleotide Res.*, **17**, 267–274.
- KEILIN, D. & HARTREE (1937). Reaction of nitric oxide with haemoglobin and methaemoglobin. *Nature*, **139**, 548–551.
- KO, F.N., WU, C.C., KUO, S.C., LEE, F.Y. & TENG, C.M. (1994). YC-1, a novel activator of platelet guanylate cyclase. *Blood*, **84**, 4226–4233.
- MAURICE, D.H. & HASLAM, R.J. (1990). Molecular basis of the synergistic inhibition of platelet function by nitrovasodilators and activators of adenylate cyclase: inhibition of cyclic AMP breakdown by cyclic GMP. *Mol. Pharmacol.*, **37**, 671–681.
- MURAD, F., MITTAL, C.K., ARNOLD, W.P., KATSUSKI, S. & KIMURA, H. (1978). Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv. Cyclic Nucleotide Res.*, **9**, 145–158.
- MURUGANANDAM, A. & MUTUS, B. (1994). Isolation of nitric oxide synthase from human platelets. *Biochim. Biophys. Acta*, **1200**, 1–6.
- O'BRIEN, J.R. (1962). Platelet aggregation II, some results from a new method of study. *J. Clin. Pathol.*, **15**, 452–455.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1987). The antiaggregating properties of vascular endothelium: interactions between prostacyclin and nitric oxide. *Br. J. Pharmacol.*, **92**, 639–646.
- RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). An L-arginine: nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5193–5197.
- RUPPERT, D. & WEITHMANN, K.U. (1982). HL 725, an extremely potent inhibitor of platelet phosphodiesterase and induced platelet aggregation *in vitro*. *Life Sci.*, **31**, 2037–2043.
- SCHMIDT, H.H.H.W. (1992). Endogenous soluble guanylyl cyclase-activating factors. *FEBS Lett.*, **307**, 102–107.
- SCHMIDT, H.H.H.W., LOHMANN, S.M. & WALTER, U. (1993). The nitric oxide and cGMP signal transduction system: regulation and mechanism of action. *Biochim. Biophys. Acta*, **1178**, 153–175.
- SCHMIDT, H.H.H.W. & WALTER, U. (1994). NO at work. *Cell*, **78**, 919–925.
- TRAYLOR, T.G. & SHARMA, V.S. (1992). Why NO? *Biochemistry*, **31**, 2847–2849.
- WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.
- WALTER, U. (1989). Physiological role of cGMP and cGMP-dependent protein kinase in the cardiovascular system. *Rev. Physiol. Biochem. Pharmacol.*, **113**, 41–88.
- WALTER, U., NOLTE, C., GEIGER, J., SCHANZENBACHER, P. & KOCHSIEK, K. (1991). Inhibition of platelet function by cyclic nucleotides and cyclic nucleotide-dependent protein kinases. In *Antithrombotics*, ed. Herman, A.G. pp. 121–138. Netherlands: Kluwer.
- YOSHINA, S. & KUO, S.C. (1978). Studies on heterocyclic compounds XXXV. Synthesis of furo[3,2-C]pyrazole derivatives. (2) electrophilic substitution of 1,3-diphenylfuro[3,2-C]pyrazole. *Yakugaku Zasshi*, **98**, 204.

(Received February 14, 1995
Revised May 10, 1995
Accepted June 16, 1995)