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Mechanism of inhibition of platelet aggregation by HCL-31D

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Abstract

The antiplatelet effect of the pyridazinone analogue, 4,5-dihydro-6-[4-[2-hydroxy-3-(3,4 dimethoxybenzylamino)propoxy]naphth-1-yl]-3(2H)-pyridazinone (HCL-31D), was investigated in vitro with rabbit platelets. HCL-31D dose-dependently inhibited the platelet aggregation and ATP release induced by collagen (10 μg/ml), arachidonic acid (100 μM) or thrombin (0.1 U/ml) with an IC_{50} of about 0.95–5.41 μM. HCL-31D (0.5–5 μM) increased the platelet cyclic AMP level in a dose-dependent manner. Furthermore, HCL-31D potentiated cyclic AMP formation caused by prostaglandin E_1 but not that caused by 3-isobutyl-1-methylxanthine (IBMX). HCL-31D also attenuated phosphoinositide breakdown and intracellular Ca^{2+} elevation induced by collagen, arachidonic acid or thrombin. HCL-31D inhibited the formation of thromboxane B_2 induced by collagen or thrombin but not by arachidonic acid. In addition, HCL-31D did not affect platelet cylooxygenase and thromboxane synthase activity. These data indicate that HCL-31D is an inhibitor of phosphodiesterase and that its antiplatelet effect is mainly mediated by elevation of cyclic AMP levels.

Keywords: HCL-31D, platelet aggregation; cAMP; Thromboxane B_2; Phosphoinositide breakdown

1. Introduction

There is increasing evidence that platelets play important roles in the initiation and development of thrombosis and certain occlusive diseases (Mustard, 1976; Fitzgerald, 1991; Marcus and Safier, 1993). Advanced atherosclerotic blood vessels, ulcers or fissures in the fibrous cap of the atheroma are thought to recruit platelets and transform them into major prothrombotic offenders. Recently, antiplatelet therapy has become a useful means for preventing or treating thrombotic events in cardiovascular, cerebrovascular, and peripheral vascular diseases (Antiplatelet Trialists' Collaboration, 1994).

Platelet activation is a result of a complex signal transduction cascade reaction mediated by various stimulants. One important mediator in regulating platelet activation is the level of second messengers such as adenosine 3′, 5′-cyclic monophosphate (cyclic AMP). It is well-known that an increased intracellular cyclic AMP level results in inhibition of platelet activation, adhesion, and release of granule contents (Feinstein et al., 1985). In addition, cyclic AMP-elevating drugs have been shown to mediate the functions of some signaling molecules in platelets, through protein kinase A-dependent phosphorylation (Siess and Lapetina, 1990). The steady state level of cyclic AMP is maintained by a balance between the rate of synthesis by adenylate cyclase and the rate of degradation by cyclic AMP phosphodiesterase. Thus, stimulation of adenylate cyclase activity or inhibition of cyclic AMP phosphodiesterase activity resulting in elevation of the intracellular cyclic AMP level may lead to suppression of platelet function. However, adenylate cyclase activators, such as prostaglandin I_2, have had limited clinical utility due to their potent vasodilator activity. In recent years, phosphodiesterase inhibitors have been developed and evaluated in clinical studies for their efficacy in patients with conges-

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tive heart failure. Fortunately, phosphodiesterase type III inhibitors like amrinone and milrinone have been shown to have a beneficial effect on cardiac and renal function in patients as well as an antiplatelet effect in vivo and in vitro (Lejemtel et al., 1980; Maskin et al., 1982; Buerke et al., 1997). Therefore, the development of effective phosphodiesterase inhibitors may be a potential approach for future clinical therapy.

Here, we demonstrated that the new pyridazinone analogue, 4,5-dihydro-6-[4-[2-hydroxy-3-(3,4-dimethoxybenzylamino)propoxy]naphth-1-yl]-3(2H)-pyridazinone (HCL-31D), is a potent platelet function inhibitor. The antiplatelet activity of HCL-31D might be mediated by an elevation of cyclic AMP level as a result of inhibition of phosphodiesterase activity and suppressed agonist-induced phosphoinositide breakdown, thromboxane A_2 formation as well as increase in cytosolic Ca^{2+} level.

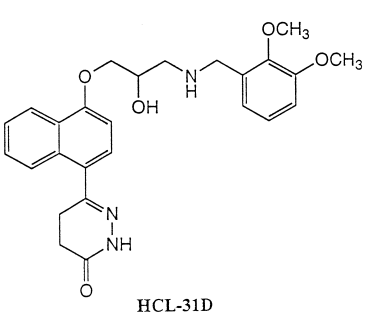
2. Materials and methods

2.1. Materials

Thrombin, collagen (type 1, equine tendon), arachidonic acid, bovine serum albumin, indomethacin, ethylenediaminetetraacetic acid, disodium salt (EDTA), luciferase–luciferin, imidazole, 3-isobutyl-1-methylxanthine (IBMX) and 1-[2-(5-carboxyazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*-*N*′-*N*′-tetraacetoxymethylester (fura-2/AM) were purchased from Sigma, USA. Prostaglandin H_2 and thromboxane B_2, prostaglandin E_2, cyclic AMP as well as cyclic-GMP enzyme immunoassay (EIA) kits were purchased from Cayman Chemical, Ann Arbor, USA. A cyclooxygenase activity assay kit was obtained from Biomol, Campus Drive, Plymouth Meeting, USA. HCL-31D, synthesized by Professor An-Rong Lee, was dissolved in dimethylsulfoxide (DMSO); its chemical structure is shown in Fig. 1.

2.2. Preparation of platelet suspension

Blood was withdrawn from rabbit marginal vein, mixed with anticoagulant, EDTA (100 mM, 14:1 v/v) and centrifuged at $160 \times g$, 25°C for 10 min to obtain platelet rich plasma. The platelet suspension was prepared from the platelet-rich plasma according to washing procedures described previously (Chou et al., 1996). Platelet pellets were then suspended in Tyrode's solution of the following composition (mM): $CaCl_2$ (1), NaCl (136.8), KCl (2.7), $NaHCO_3$ (11.9), $MgCl_2$ (2.1), NaH_2PO_4 (0.4), and glucose (10) containing bovine serum albumin (0.35%). The platelet concentration was adjusted to 3.0×10^8 platelets/ml.



HCL-31D

Fig. 1. Structure of HCL-31D.

2.3. Platelet aggregation and ATP release reaction

Aggregation was measured turbidimetrically at 37°C with constant stirring at 1000 rpm. The absorbance of Tyrode's solution was assigned as 100% aggregation and the absorbance of platelet suspension as 0% aggregation. Following a 3-min equilibration at 37°C, the platelet suspension was incubated with HCL-31D (0.5–10 μM) for 3 min before the addition of aggregation inducer. The extent of platelet aggregation was evaluated by measuring the maximum height reached by the aggregation curves. Data are expressed as percentages of maximal aggregation. The ATP released from platelets was detected by the bioluminescence method (DeLuca and McElory, 1978). Both the aggregation and release of ATP were simultaneously measured by a Lumi-aggregometer (Model 560, Chrono-Log, USA) connected to two dual-channel recorders. In order to eliminate the effect of the solvent on the aggregation and release reaction of platelets, the final concentration of DMSO was fixed at 0.5% (v/v).

2.4. Platelet cyclic AMP and cyclic GMP determination

Rabbit platelet suspension (3.0×10^8 platelets/ml) was incubated with prostaglandin E_1 (1 μM) or various concentrations of HCL-31D for 3 min at 37°C with stirring. In some experiments, platelets were pretreated with IBMX (50 μM), a known cyclic AMP phosphodiesterase inhibitor, or prostaglandin E_1, an adenyl cyclase activator, for 3 min followed by addition of HCL-31D. The incubation was stopped by adding 10 mM EDTA followed immediately by boiling for 5 min. After cooling to 4°C, the precipitated protein was sedimented by centrifugation. The cyclic AMP and cyclic GMP contents in the supernatant were measured with appropriate EIA kits, following acetylation of the samples as described by the manufacturer.

2.5. Phosphoinositide breakdown assay

Rabbit EDTA-platelet-rich plasma was centrifuged at $500 \times g$ for 10 min at room temperature. The platelet pellets were suspended in 1 ml of Ca^{2+}-free and bovine serum albumin-free Tyrode's solution containing 75 μCi/ml of myo-[2-^{3}H]inositol. After incubation at 37°C for 2 h, the platelets were collected by centrifugation at $500 \times g$ for 5 min and resuspended in Ca^{2+}-free Tyrode's

solution and the platelet count was adjusted to 3×10^8 platelets/ml. One-milliliter aliquots of platelet suspension were prewarmed at 37°C with 5 mM LiCl, an inhibitor of inositol-monophosphate phosphatase, in a 3.5-ml cuvette with stirring at 1000 rpm. HCL-31D was preincubated with loaded platelets at room temperature for 3 min, and then collagen (10 μg/ml), arachidonic acid (100 μM) or thrombin (0.1 U/ml) was added to trigger aggregation for 6 min. The reaction was stopped by adding ice-cold trichloroacetic acid (10% w/v), followed by centrifugation at $1000 \times g$ for 5 min. One-milliliter of supernatant was collected, and trichloroacetic acid was extracted by washing with 10 ml of diethyl ether three times. The mixture was then incubated at 80°C, in hot water, to remove the residual diethyl ether. The aqueous phase containing the inositol phosphates was adjusted to pH 7–8 and loaded onto a Dowex-1 anion exchange column (50% w/v, 1 ml) for separation of the inositol phosphates as described previously (Neylon and Summers, 1987). In this study, only [^{3}H]inositol monophosphate (IP_1) was measured as an index of total inositol phosphate formation, because the levels of inositol biphosphate and inositol triphosphate were very low.

2.6. Measurement of intracellular Ca^{2+}

According to the method described by Pollock and Rink (1986), platelets (3×10^8 platelets/ml) were incubated with fura-2/AM (5 μM) for 50 min at 37°C and then centrifuged and washed twice and finally suspended in the Tyrode's solution containing 1 mM Ca^{2+}. The fluorescence (excitation 340 nm, emission 500 nm) was measured with a fluorescence spectrophotometer (CAF-100, Jasco, Japan). At the end of the experiment, the cells were treated with 0.1% Triton X-100 followed by the addition of 10 mM ethyleneglycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid to obtain the maximal and minimal fluorescence, respectively. The intracellular free calcium concentration ($[Ca^{2+}]_i$) was calculated as described for fura-2, using a Ca^{2+}-dye dissociation constant of 224 nM (Grynkiewicz et al., 1985).

2.7. Measurement of thromboxane B_2 formation

After incubation with HCL-31D for 3 min followed by incubation with collagen (10 μg/ml), arachidonic acid (100 μM) or thrombin (0.1 U/ml) for 6 min, 2 mM EDTA and 50 μM indomethacin, a cyclooxygenase inhibitor, were added to halt thromboxane B_2 formation. After centrifugation at $10{,}000 \times g$ for 5 min, thromboxane B_2 in the supernatant was assayed with the EIA kit.

2.8. Cyclooxygenase activity determination

The effect of HCL-31D on cyclooxygenase activity was determined using a cyclooxygenase activity assay kit (Chou et al., 1996). In brief, sheep vesicular gland microsomes (0.2 mg/ml; 100 μl) were incubated with DMSO (2 μl), indomethacin (0.01 mg/2 μl) or various concentrations of HCL-31D (2 μl) at 4°C for 3 min. Reaction cofactor (epinephrine, tryptophan, hydroqinone and GSH; 10 μl) was then added and the mixture was incubated at 25°C for 3 min with shaking. After that, arachidonic acid (2 μg/2 μl) was added and the mixture was incubated for another 3 min. $FeCl_2$ (25 mM, 10 μl) was then added to halt the reaction, and the reaction mixture was allowed to stand at room temperature for 15 min. After centrifugation at 3000 $\times g$ and 4°C for 10 min, prostaglandin E_2 in the supernatant was assayed using the EIA kit. Cyclooxygenase activity was reflected by the production of prostaglandin E_2.

2.9. Thromboxane synthase determination

After incubation of platelets with HCL-31D (5 μM) or imidazole (1 mM) for 3 min, prostaglandin H_2 5 μM was added for a further 6 min, and then 2 mM EDTA and 50 μM indomethacin were added. After centrifugation at $10{,}000 \times g$ for 5 min, the amount of thromboxane B_2, reflecting thromboxane synthase activity, in the supernatant was measured with the EIA kit.

2.10. Statistical analysis

The experimental results are expressed as means ± SEM. Statistical analysis was performed with analysis of variance (ANOVA). If the analysis showed significant differences among the group means, then each group was compared by the Newman–Keuls method. Results were considered significantly different at a value of $P < 0.05$.

3. Results

3.1. Effect of HCL-31D on platelet aggregation and ATP release

HCL-31D dose-dependently inhibited rabbit platelet aggregation induced by collagen (10 μg/ml), arachidonic acid (100 μM) and thrombin (0.1 U/ml) with an IC_{50} of 0.95 ± 0.05, 0.75 ± 0.02 and 5.41 ± 0.23 (μM), respectively (Fig. 2). Furthermore, HCL-31D also dose-dependently inhibited the ATP release induced by collagen (Fig. 3). Similar results were also observed for arachidonic acid- and thrombin-induced ATP release (data not shown). The inhibition of ATP release was parallel to the inhibition of platelet aggregation. In addition, HCL-31D also inhibited human platelet aggregation induced by the above inducers with an IC_{50} of about 2–8 μM.

3.2. Effect of HCL-31D on platelet cyclic AMP and cyclic GMP content

The level of cyclic AMP in unstimulated platelets was very low (0.40 ± 0.12 pmol/10^8 platelets). Prostaglandin

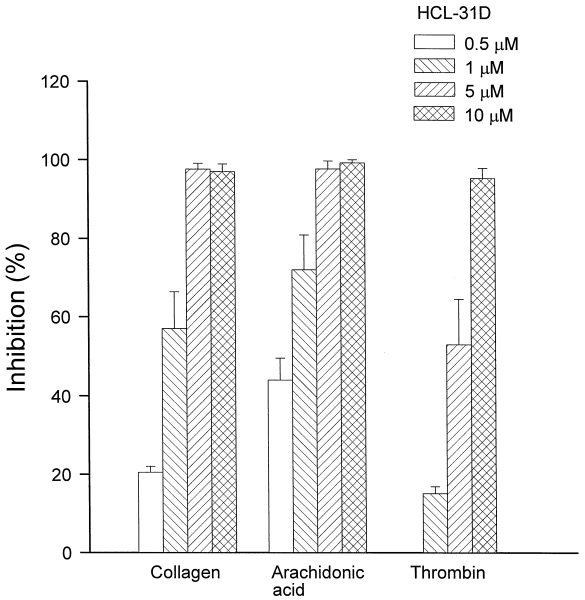


Fig. 2. The inhibition by HCL-31D of platelet aggregation induced by collagen, arachidonic acid or thrombin. Washed rabbit platelets were preincubated with HCL-31D (0.5–10 μM) for 3 min, and then collagen (10 μg/ml), arachidonic acid (100 μM) or thrombin (0.1 U/ml) was added to trigger platelet aggregation. Percent inhibition is presented as the mean ± SEM ($n = 6$).

E_1 (1 μM) increased the cyclic AMP level to 6.24 ± 1.82 pmol/10^8 platelets. Incubation with HCL-31D (0.5–5 μM) for 3 min resulted in a significant elevation of the platelet cyclic AMP level to about 2–8.5-fold that of control. Addition of IBMX (50 μM) had little effect on cyclic AMP formation by HCL-31D (Table 1). In addition, HCL-31D or IBMX further potentiated the ability of prostaglandin E_1 to increase cyclic AMP. Similarly, HCL-31D (1–5 μM) also increased cyclic AMP formation in human platelets (data not shown). However, HCL-31D (0.5–5 μM) had no significant effect on the platelet level of cyclic GMP.

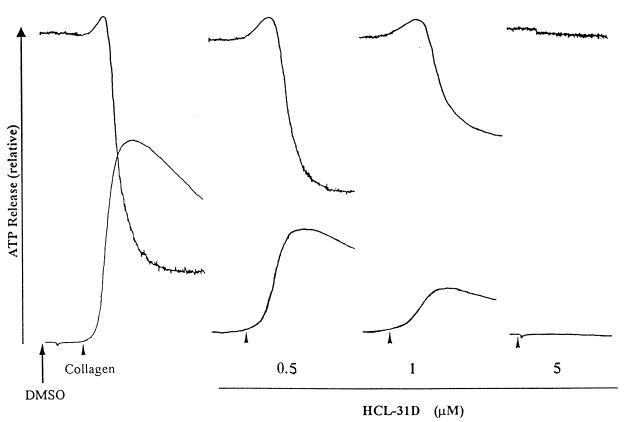


Fig. 3. Effect of various concentrations of HCL-31D on the platelet aggregation and ATP release induced by collagen. Washed rabbit platelets were preincubated with HCL-31D (0.5–5 μM) for 3 min, and then collagen (10 μg/ml) was added to trigger platelet aggregation (downward tracings) and ATP release (upward tracings). The traces are representative of five similar experiments.

Table 1
Effect of prostaglandin E_1 and HCL-31D on the cyclic AMP level of washed rabbit platelets
DMSO (0.5%, resting), HCL-31D, prostaglandin E_1 (PGE_1), the combination of PGE_1 with 3-isobutyl-1-methylxanthine (IBMX) or HCL-31D, or the combination of IBMX with HCL-31D was preincubated with platelets at 37°C for 3 min. Cyclic AMP formation was stopped by adding 10 mM EDTA and immediate boiling for 5 min. Values are presented as means ± SEM ($n = 6$).

Treatment	Cyclic AMP (pmol/10^8 platelets)
Resting	0.40 ± 0.12
HCL-31D (μM)	
0.5	0.94 ± 0.45
1	1.98 ± 0.31[a]
5	3.42 ± 0.42[b]
PGE_1 (1 μM)	6.24 ± 1.82[b]
PGE_1 (1 μM) + IBMX (50 μM)	10.35 ± 2.22[b]
PGE_1 (1 μM) + HCL-31D (1 μM)	9.42 ± 1.90[b]
IBMX (50 μM) + HCL-31D (1 μM)	2.11 ± 0.44[a]
IBMX (50 μM) + HCL-31D (5 μM)	3.67 ± 0.52[b]

[a] $P < 0.05$ as compared with that of resting state.
[b] $P < 0.01$ as compared with that of resting state.

3.3. Effect of HCL-31D on phosphoinositide breakdown

Phosphoinositide breakdown in platelets was activated by many agonists. As shown in Fig. 4, collagen (10 μg/ml), arachidonic acid (100 μM) and thrombin (0.1 U/ml) increased IP_1 formation 3.3 ± 0.7-, 2.6 ± 0.7- and 4.3 ± 0.9-fold, respectively, as compared to that of resting platelets. HCL-31D (5 μM) significantly inhibited the IP_1 formation induced by the above inducers. HCL-31D alone did not significantly affect IP_1 formation in resting platelets.

3.4. Effect of HCL-31D on $[Ca^{2+}]_i$ mobilization

In fura-2-loaded platelets, arachidonic acid (100 μM) collagen (10 μg/ml) or thrombin (0.1 U/ml) markedly increased $[Ca^{2+}]_i$ in platelets in the presence of external Ca^{2+} (1 mM). As shown in Fig. 5, incubation with HCL-31D (5 or 10 μM) for 3 min significantly attenuated the rise in $[Ca^{2+}]_i$ in platelets induced by arachidonic acid, collagen or thrombin.

3.5. Effect of HCL-31D on platelet thromboxane B_2 production

The thromboxane B_2 level of resting platelets was 1.3 ± 0.2 ng/ml. In rabbit washed platelets, collagen (10 μg/ml), arachidonic acid (100 μM) and thrombin (0.1 U/ml) caused marked thromboxane B_2 formation. HCL-31D (0.5–5 μM) significantly inhibited the collagen- and thrombin-induced thromboxane B_2 formation but had no

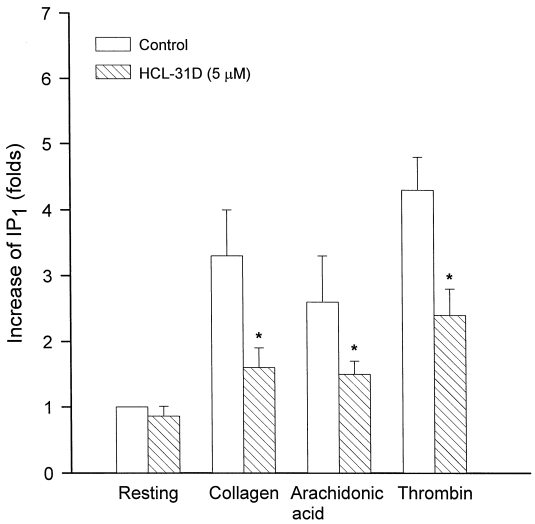


Fig. 4. Inhibitory effect of HCL-31D on the formation of inositol monophosphate (IP_1) induced by some agonists in washed rabbit platelets. [^{3}H] Inositol-labeled platelets were incubated with DMSO (0.5%, control) or HCL-31D (5 μM) at 25°C for 3 min; then collagen (10 μg/ml), arachidonic acid (100 μM) or thrombin (0.1 U/ml) was added for another 6 min. The [^{3}H] IP_1 level of resting platelets was 620 ± 95 cpm/3×10^8 platelets. The increase (fold) in IP_1 is presented as the mean ± SEM ($n = 5$–6). $^*P < 0.05$ as compared with the respective control.

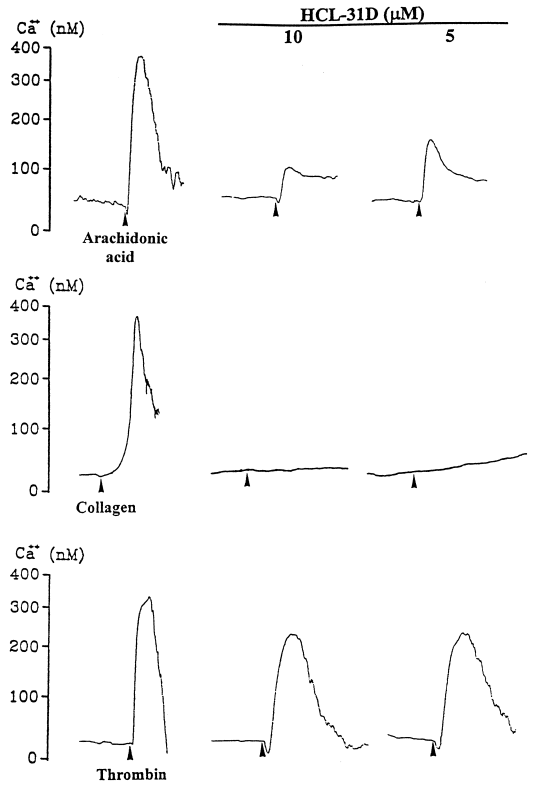


Fig. 5. Effect of HCL-31D on the increase in $[Ca^{2+}]_i$ in rabbit platelets caused by various inducers. Fura-2-loaded platelets were preincubated with DMSO (0.5%, control) or HCL-31D (5 or 10 μM) at 37°C for 3 min and then arachidonic acid (100 μM), collagen (10 μg/ml) or thrombin (0.1U/ml) was added. The traces are representative of five similar experiments.

Table 2
Effect of HCL-31D on the thromboxane B_2 formation of washed rabbit platelets induced by collagen, arachidonic acid and thrombin
HCL-31D or DMSO (0.5%; control) was preincubated with platelets at 37°C for 3 min, and then inducer was added. Aggregation and thromboxane B_2 formation were terminated by the addition of EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of inducer. Values are presented as means ± SEM ($n = 5$). The thromboxane B_2 level of resting platelets was 1.3 ± 0.2 ng/ml.

	Thromboxane B_2 (ng/ml) formation induced by		
	Collagen (10 μg/ml)	Arachidonic acid (100 mM)	Thrombin (0.1 U/ml)
Control	487.6 ± 19.8	560.9 ± 26.2	131.5 ± 6.5
HCL-31D (μM)			
0.5	157.3 ± 22.1[a]	570.1 ± 24.6	55.7 ± 5.8[a]
1	101.5 ± 18.7[a]	558.6 ± 9.5	15.7 ± 3.1[a]
5	17.4 ± 2.1[a]	583.5 ± 36.4	8.4 ± 1.5[a]

[a] $P < 0.001$ as compared with the control value.

effect on arachidonic acid-induced thromboxane B_2 production (Table 2). In addition, HCL-31D alone did not affect the thromboxane B_2 level in resting platelets.

3.6. Effect of HCL-31D on cyclooxygenase activity

Cyclooxygenase activity was reflected by the formation of prostaglandin E_2. After incubation of sheep vesicular gland microsomes with arachidonic acid (2 μg) at 25°C for 3 min, the prostaglandin E_2 level was 36.8 ± 9.5 ng/ml. HCL-31D (5, 10 μM) had no significant effect on prostaglandin E_2 levels (35.4 ± 2.6 and 33.8 ± 2.8 ng/ml, respectively). However, indomethacin (0.01 mg) significantly reduced prostaglandin E_2 levels to 5.2 ± 1.3 ng/ml.

3.7. Effect of HCL-31D on thromboxane synthase activity

Prostaglandin H_2-induced thromboxane B_2 formation in washed rabbit platelets was 5.68 ± 0.64 ng/ml. After incubation with HCL-31D (5, 10 μM) for 3 min followed by addition of prostaglandin H_2 (5 μM), the thromboxane B_2 levels were 5.55 ± 0.71 and 5.38 ± 0.59 ng/ml, respectively, with no significant difference compared with control. However, imidazole (1 mM), a thromboxane synthase inhibitor, strongly inhibited thromboxane B_2 formation (0.98 ± 0.06 ng/ml).

4. Discussion

The present study demonstrates that HCL-31D is a potent inhibitor of platelet aggregation and ATP release induced by collagen, arachidonic acid and thrombin. Its mechanisms of action may include an increase in the cyclic AMP level through inhibition of cyclic AMP phosphodiesterase activity and subsequent inhibition of phos-

phoinositide breakdown, thromboxane A_2 formation and intracellular Ca^{2+} mobilization.

It is known that elevation of the cyclic AMP level is the most inhibitory pathway to regulate platelet responses, including aggregation, ATP release, phosphoinositide breakdown and rise in $[Ca^{2+}]_i$ (Siess, 1989; Geiger et al., 1992; Rhee et al., 1993). In our study, HCL-31D significantly increased the cyclic AMP content of platelets in a concentration-dependent manner that paralleled its antiplatelet effect. In the presence of IBMX, the increased cyclic AMP level caused by HCL-31D was not further enhanced. In contrast, the elevating effect on cyclic AMP of prostaglandin E_1 was markedly potentiated by HCL-31D. These results imply that the increase in cyclic AMP level caused by HCL-31D is mainly mediated by inhibition of cyclic AMP phosphodiesterase activity. However, HCL-31D did not increase cyclic GMP formation, which may indicate that HCL-31D is a more specific inhibitor of cyclic AMP phosphodiesterase in rabbit platelets.

Phosphoinositide breakdown is an important pathway in the signal transduction of agonist-induced platelet activation. Many aggregation inducers like collagen and thrombin can stimulate the phospholipase C-catalyzed hydrolysis of the plasma membrane phospholipid phosphatidylinositol 4,5-biphosphate (Berridge, 1984). This process generates two active products, diacylglycerol and inositol trisphosphate. Diacylglycerol activates protein kinase C, leading to protein phosphorylation and a release reaction (Somlyo and Somlyo, 1994). The inositol trisphosphate triggers calcium mobilization from intracellular calcium stores (O'Rourke et al., 1985). It has been reported that the elevation of cyclic AMP leads to activation of protein kinase A, which phosphorylates phospholipase C-β2, and that phosphorylation inhibits activation of phospholipase C-β2 by G-protein βγ subunits (Liu and Simon, 1996). Recently, other studies have suggested that cyclic AMP may suppress the resynthesis of phosphatidylinositol 4,5-biphosphate as well as phosphatidylinositol and lead to inhibition of phosphoinositide turnover (Ryningen et al., 1998). Increased cyclic AMP levels also lower platelet $[Ca^{2+}]_i$ both by stimulating Ca^{2+} extrusion from the cells and by stimulating uptake of cytosolic Ca^{2+} into the dense tubular system through the phosphorylation of a 24-kDa Ca^{2+}-ATPase by protein kinase A (Zavoico and Feinstein, 1984; Enouff et al., 1987). Our results showed that HCL-31D inhibited phosphoinositide breakdown and $[Ca^{2+}]_i$ mobilization caused by platelet stimulators in rabbit platelets, which supports the results of previous studies (Benjamin et al., 1992). The reason why the attenuation of $[Ca^{2+}]_i$ mobilization in response to thrombin was much weaker than that in response to collagen is unclear. However, the mobilization caused by thrombin was almost completely inhibited by a higher concentration of HCL-31D (30 μM, data not shown). In addition, cyclic AMP exerts multiple inhibitory actions on thrombin-activated platelets, such as reduced ability of thrombin to bind to its receptors and inhibition of several thrombin-induced responses like phosphatidic acid formation (Lapetina, 1986; Lerea et al., 1987). Thus, the suppression by HCL-31D of platelet aggregation induced by thrombin is unlikely to be mediated by inhibition of Ca^{2+} mobilization alone.

It is well-known that thromboxane A_2 is an important mediator of the release reaction and aggregation of platelets (FitzGerald, 1991). When platelets are activated by collagen or thrombin, arachidonic acid can be liberated from membrane phospholipids by Ca^{2+}-dependent phospholipase A_2, diglyceride lipase or phosphatidic acid-specific phospholipase A_2 (Bell et al., 1979; Billah et al., 1981). Recently, it was reported that cyclic AMP could inhibit phospholipase A_2-mediated arachidonic acid release (Xing et al., 1999). Our results showed that HCL-31D markedly inhibited the formation of thromboxane B_2, a stable metabolite of thromboxane A_2, induced by collagen or thrombin but not by arachidonic acid (Table 2). The aggregation of and ATP release by platelets induced by arachidonic acid are due to thromboxane A_2 formation (Hamberg et al., 1975). Furthermore, our results also demonstrated that HCL-31D did not affect the activity of cyclooxygenase and thromboxane synthase. Thus, the antiplatelet action of HCL-31D may not be due to direct inhibition of thromboxane A_2 formation. These data also support the previous finding that cyclic nucleotides do not affect the activity of cyclooxygenase (Minkes et al., 1977).

In conclusion, we demonstrated that HCL-31D is a potent inhibitor of platelet aggregation. The mechanisms by which HCL-31D suppressed platelet aggregation may involve increased cyclic AMP formation and subsequent inhibition of phosphoinositide breakdown, thromboxane A_2 production and intracellular Ca^{2+} mobilization.

Acknowledgements

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