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Antiplatelet effects of KW-7, a new inhibitor of cyclic nucleotide phosphodiesterases

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Abstract

The antiplatelet effect of a new synthetic compound, 8,9-dimethoxyl-1-(4-methoxy-phenyl)-5,6-dihydro-pyrrolo[2,1-*a*]isoquinoline-2,3-dione (KW-7), was determined in rabbit platelets. KW-7 concentration-dependently prevented platelet aggregation caused by arachidonic acid, collagen, platelet-activating factor, and thrombin. KW-7 induced a substantial increase in cyclic AMP levels and a smaller increase in cyclic GMP levels in platelets. In platelet homogenates, KW-7 inhibited both cyclic AMP- and cyclic GMP-phosphodiesterase activities. The antiplatelet effect of KW-7 was reversed by SQ22536 (an inhibitor of adenylate cyclase) and H89 (an inhibitor of protein kinase A) but not by ODQ (an inhibitor of soluble guanylate cyclase). These data suggest that the antiplatelet effect of KW-7 is cyclic AMP-dependent, and is through inhibition of platelet phosphodiesterases. In addition, KW-7 inhibited arachidonic acid-stimulated thromboxane production; this effect was associated with an increase in prostaglandin D_2 levels indicating KW-7 is also an inhibitor of thromboxane synthase. The dual inhibition of KW-7 on phosphodiesterase and thromboxane synthase might provide an attractive target in developing antiplatelet drugs.

Keywords: Antiplatelet; Phosphodiesterase inhibitor; Thromboxane; KW-7

1. Introduction

Numerous evidence indicate that platelets contribute significantly to the pathogenesis of arterial thromboembotic diseases, such as acute coronary syndrome and ischemic stroke, which are the major causes of death in developed countries (Schwortz et al., 1990; Ross, 1993). Antiplatelet drugs, such as aspirin and ticlopidine, have been shown to be beneficial in the treatment of thromboembotic diseases (Coller, 1992; Antiplatelet Trialists' Collaboration, 1994a,b; Antithrombotic Trialist' Collaboration, 2002). However, current antiplatelet drugs still have considerable limitation in their mode of action and efficacy. Greater understanding of platelet function in molecular levels will probably lead to the development of new more potent drugs (Gresele and Agnelli, 2002; Bhatt and Topol, 2003).

Following endothelial damage or disruption of atherosclerotic plaque in blood vessels, platelets rapidly adhere to newly exposed extracellular matrix. Adhesion is followed by platelet activation and release proaggregatory substances, such as ADP and thromboxane A_2, which thereby recruiting more circulating platelets, resulting in the formation of a platelet-rich thrombus. On the other hand, blood platelets are under tight negative control, mainly through prostacyclin and nitric oxide released from vascular endothelial cells, which increase platelet cyclic AMP and cyclic GMP levels and lead to inhibition of platelet activation (Siess, 1989; Schwarz et al., 2001).

Benzylisoquinolines present a class of the most common alkaloids in higher plants that show various biological activities such as antiplatelet, vasorelaxing, analgesic, and anticancer effects. In the search of new antiplatelet agents, 40 newly synthetic benzylisoquinoline derivatives were tested for their antiplatelet activities. Among them, 8,9-dimethoxyl-1-(4-methoxy-phenyl)-5,6-dihydro-pyrrolo[2,1-*a*] isoquinoline-2,3-dione (KW-7, Fig. 1) showed the most potent inhibitory effect on platelet aggregation elicited by various platelet stimulators (Kuo et al., 2003). In the present

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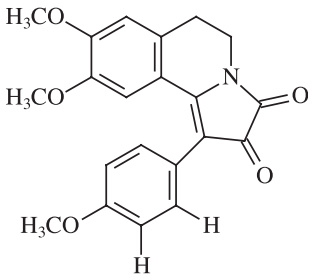

Fig. 1. Chemical structure of KW-7.

study, the mechanism of action of KW-7 was further investigated. Our data suggest that the antiplatelet effect of KW-7 is cyclic AMP-dependent, and is through inhibition of platelet phosphodiesterases.

2. Materials and methods

2.1. Preparation of washed rabbit platelets

Blood anticoagulated with ethylenediaminetetraacetic acid (EDTA) was collected from New Zealand rabbits. Rabbit platelet suspension was prepared according to the procedure previously described (Wu et al., 2000). The platelets, after washing, were finally suspended in Tyrode's solution containing Ca^{2+} (1 mM), glucose (11.1 mM) and bovine serum albumin (3.5 mg/ml) at a concentration of 3×10^8 platelets/ml.

2.2. Measurement of platelet aggregation

Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer (Chrono-Log, USA). The platelet suspension was incubated with dimethyl sulfoxide (DMSO, vehicle) or KW-7 at 37 °C for 3 min under a stirring condition (1200 rpm) prior to the addition of the platelet activators. The extent of platelet aggregation was measured as the maximal increase of light transmission within 5 min after the addition of inducers.

2.3. Thromboxane B_2, prostaglandin D_2, and cyclooxygenase assay

Because thromboxane A_2 is very unstable and rapidly converted to more stable metabolite thromboxane B_2, we thus measured the later instead of thromboxane A_2. After challenge of platelets with the aggregation inducer for 5 min, EDTA (2 mM) and indomethacin (50 μM) were added. The platelet suspensions were centrifuged for 3 min at 13,000 rpm, the thromboxane B_2 and prostaglandin D_2 in the supernatants were assayed using enzyme immunoassay kits according to the procedure described by the manufacturer.

The effect of KW-7 on purified ovine cyclooxygenase-1 was tested using a commercial kit according to the procedure described by the manufacturer. In this assay, cyclooxygenase catalyzed the metabolism of arachidonic acid to PGH_2. Next, $PGF_{2\alpha}$ produced by reduction of PGH_2 by $SnCl_2$ was measured by enzyme immunoassay.

2.4. Measurement of intracellular Ca^{2+} mobilization

Platelets pelleted from platelet-rich plasma were resuspended in Ca^{2+}-free Tyrode's solution, then incubated with fluo-3/AM (2 μM) at 37 °C for 30 min. In order to prevent leakage of dye, probenecid (2.5 mM) was added to the buffers throughout the experiments (Merritt et al., 1990). After washing twice, the fluo-3-loaded platelets were finally suspended in Ca^{2+}-free Tyrode's solution at a concentration of 5×10^7 platelets/ml. The fluo-3-loaded platelets were preincubated with KW-7 in the presence or absence of 1 mM extracellular calcium at 37 °C for 3 min prior to the addition of the platelet activators. Fluorescence (Ex 505 nm, Em 530 nm) was measured with a fluorescence spectrophotometer (Model F4000; Hitachi, Tokyo, Japan).

2.5. Estimation of platelet cyclic nucleotide contents

The method of Karniguian et al. (1982) was used. The platelet suspension was incubated with KW-7 or other agents at 37 °C for various length of time under a stirring condition (1200 rpm) in a light-transmission aggregometer. The reaction was stopped by adding EDTA (10 mM) followed immediately by boiling for 2 min. Upon cooling to 4 °C, cell debris was removed by centrifugation at $10{,}000 \times g$ for 5 min. The supernatant was then used to assay for cyclic AMP and cyclic GMP using enzyme immunoassay kits.

2.6. Measurement of activity of cyclic AMP and cyclic GMP phosphodiesterases

The cyclic nucleotide phosphodiesterase activity was measured according to the procedure previously described (Ko et al., 1994). In brief, washed rabbit platelets prepared as described above were resuspended in 50 mM Tris–HCl (pH 7.4, containing 0.4 mM EGTA). Platelets were disrupted by sonication at 4 °C and centrifuged at $1500 \times g$ at 4 °C for 20 min to remove cell debris. The supernatant was used as a source of crude phosphodiesterases. The enzyme preparation (1 mg/ml; 0.1 ml) was incubated with Tris–HCl (0.2 ml, containing 4 mM $MgCl_2$ and 0.4 mM EGTA) at 30 °C for 5 min; 0.1 ml cyclic AMP (final concentration, 0.5 μM containing 0.1 μCi [^{3}H] cyclic AMP) or cyclic GMP (final concentration, 0.5 μM containing 0.1 μCi [^{3}H] cyclic GMP) was then added. After 30 min at 30 °C, the reaction was terminated by addition of 0.25 M HCl and neutralized by 0.25 M NaOH. 5′-

nucleotidase snake venom (10 U/ml; 0.1 ml) was then added and incubated at 30 °C for 60 min to convert the 5′-AMP or 5′-GMP to the uncharged nucleosides, adenosine, or guanosine. The samples were applied to a Dowex-1 column (Sigma), and the radioactivity of the [^{3}H]nucleoside eluted with water was measured by a liquid scintillation counter.

2.7. Drugs

KW-7 was synthesized base on the methods described previously (Kuo et al., 2003). Bovine α-thrombin, arachidonic acid, collagen (type I, bovine Achilles tendon), platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine), prostaglandin E_1 (PGE_1), sodium nitroprusside (SNP), 3-isobutyl-1-methylxanthine (IBMX), SQ22536, H89, ODQ, 5′-nucleotidase, Dowex-1 (100–200 mesh: X8, chloride), and fluo-3/AM were obtained from Sigma, USA. Thromboxane B_2, cyclic AMP, and cyclic GMP enzyme immunoassay kits and the radioactive materials [^{3}H] cyclic AMP and [^{3}H] cyclic GMP were obtained from Amersham, USA. The cyclooxygenase assay kit and the prostaglandin D_2 enzyme immunoassay kit were purchased from Cayman, USA. All other chemicals were purchased from Sigma.

2.8. Statistics

Results are expressed as the mean ± standard error of the mean (S.E.M.) and comparisons were made using Student's *t*-test. A probability of 0.05 or less was considered significant.

3. Results

3.1. Effect of KW-7 on the aggregation of washed rabbit platelets

In rabbit washed platelets, KW-7 completely inhibited arachidonic acid (AA, 100 μM)-, collagen (10 μg/ml)-, platelet activating factor (PAF, 1 ng/ml)-, and thrombin (0.1 U/ml)-induced platelet aggregation in a concentration-dependent manner with IC_{50} values of 10.4 ± 0.1, 10.9 ± 2.0, 32.9 ± 1.8, and 32.7 ± 0.4, respectively (n = 4). In contrast, the IC_{50} values of the non-selective phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) on platelet aggregation caused by these four stimulators are 27.6 ± 4.1, 29.5 ± 3.4, 180.8 ± 25.7, and 69.0 ± 8.2, respectively (n = 4).

Moreover, addition of KW-7 (5–20 μM) to rabbit platelets that had been aggregated by the above inducers caused rapid disaggregations in a concentration-dependent manner. The maximal effect of KW-7 on platelet disaggregation occurs at 20 μM KW-7. Similarly, IBMX (300 μM) also could disperse formed platelet aggregates (data not shown).

3.2. Effect of KW-7 on thromboxane formation in platelets

As shown in Table 1, KW-7 inhibited thromboxane B_2 formation caused by arachidonic acid, collagen, PAF or thrombin in similar concentration ranges. In contrast, IBMX had no effect on arachidonic acid-induced thromboxane formation, although it could inhibit those induced by collagen, PAF and thrombin.

To explore the mechanism of KW-7 on thromboxane formation in platelets, the effect of KW-7 on cyclooxygenase activity and on prostaglandin D_2 production were tested. In the purified enzyme system, KW-7, up to 50 μM, did not affect the activity of ovine cyclooxygenase-1 (data not shown). In the prostaglandin D_2 assay, arachidonic acid (100 μM) increased the prostaglandin D_2 levels in platelets from 0.06 ± 0.004 ng/3 × 10^8 platelets to 3.7 ± 0.4 ng/3 × 10^8 platelets (n = 4). Pretreatment of platelets with 50 and 20 μM of KW-7 further enhanced arachidonic acid-stimulated prostaglandin D_2 to 5.0 ± 0.4 ng/3 × 10^8 platelets (n = 4, P < 0.05) and 7.0 ± 0.5 ng/3 × 10^8 platelets (n = 4, P < 0.001), respectively. In contrast, indomethacin (10 μM) almost completely prevented arachidonic acid-stimulated prostaglandin D_2 (0.3 ± 0.04 ng/3 × 10^8 platelets, n = 4, P < 0.001).

3.3. Effect of KW-7 on the intracellular free calcium of platelets

In fluo-3-loaded platelets, thrombin, PAF or arachidonic acid caused an increase of intracellular free calcium. As shown in Fig. 2, in the presence of 1 mM of extracellular calcium, pretreatment of platelets with KW-7 prevented calcium signal elicited by these inducers. Furthermore, when added after thrombin-induced calcium signal had reached the maximal level, KW-7 (20 μM) induced a rapid

Table 1
Effects of KW-7 and IBMX on the thromboxane B_2 formation in washed rabbit platelets caused by arachidonic acid, collagen, thrombin, and PAF

Treatment	Thromboxane B_2 (ng/3 × 10^8 platelets)			
	AA	Collagen	Thrombin	PAF
DMSO (control)	445.6 ± 40.0	177.2 ± 23.9	6.7 ± 0.4	28.0 ± 3.3
KW-7 50 μM	15.7 ± 4.6***	9.4 ± 3.5***	1.4 ± 0.4***	4.5 ± 0.6***
20 μM	188.7 ± 29.3***	41.9 ± 6.2***	2.1 ± 0.2***	11.2 ± 2.1**
10 μM	336.7 ± 52.8	78.5 ± 10.6*	2.7 ± 0.2***	14.9 ± 0.6*
5 μM	462.9 ± 53.4	120.6 ± 16.7	4.2 ± 0.2***	17.1 ± 1.1*
IBMX 300 μM	331.3 ± 95.5	6.6 ± 0.4***	1.0 ± 0.4**	2.1 ± 0.3**

Washed rabbit platelets were preincubated with DMSO, KW-7 or IBMX at 37 °C for 3 min and then arachidonic acid (AA, 100 μM), collagen (10 μg/ml), thrombin (0.1 U/ml) or PAF (1 ng/ml) was added. Reactions were terminated by EDTA (2 mM) and indomethacin (50 μM) 5 min after the addition of the inducers. The thromboxane B_2 level of resting platelets was 1.6 ± 0.2 ng/3 × 10^8 platelets. Values are presented as mean ± S.E.M. (n = 4–8). *P < 0.05, **P < 0.01, ***P < 0.001 as compared with the respective control.

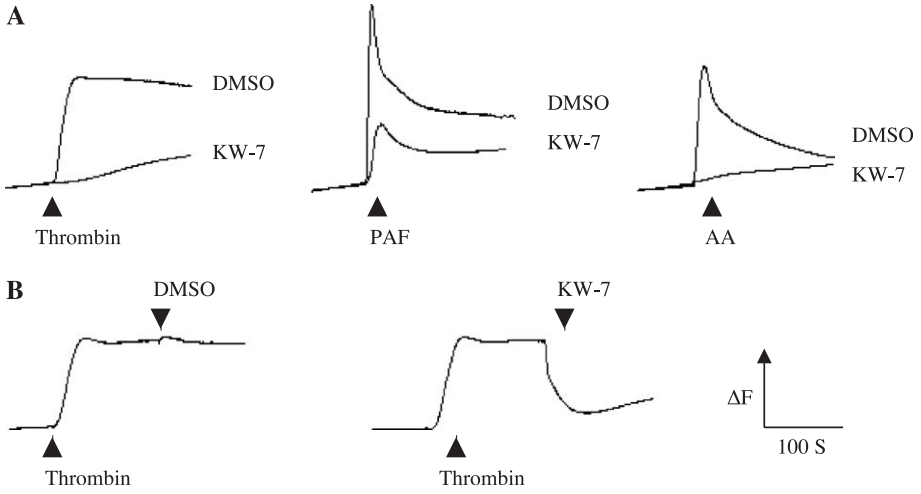

Fig. 2. Effects of KW-7 on intracellular calcium mobilization in rabbit platelets. (A) Fluo-3-loaded rabbit platelets were incubated with DMSO (0.5%, control) or KW-7 (20 μM) at 37 °C for 3 min, thrombin (0.1 U/ml), PAF (1 ng/ml) or arachidonic acid (AA, 100 μM) was then added to trigger the increase of $[Ca^{2+}]_i$ (fluorescence, F). (B) Fluo-3-loaded rabbit platelets were pre-warmed at 37 °C for 3 min before addition of thrombin (0.1 U/ml) for the induction of the increase of $[Ca^{2+}]_i$. KW-7 (20 μM) was added when thrombin-evoked calcium signal had reached the maximal level. Similar results were obtained in three separate experiments.

and complete decline of calcium signal toward the baseline. In the absence of extracellular calcium, pretreatment of platelets with KW-7 also blunted the increase in intracellular calcium levels by these platelet stimulators (data not shown).

3.4. Effect of KW-7 on cyclic nucleotide levels in platelets

The basal levels of cyclic AMP and cyclic GMP in washed rabbit platelets were 3.1 ± 0.3 and 1.5 ± 0.1 pmol/10^9 platelets, respectively. After incubated with platelets for 2 min, KW-7 (5–50 μM) concentration-dependently elicited substantial increases in cyclic AMP levels and smaller increases in cyclic GMP levels (Fig. 3).

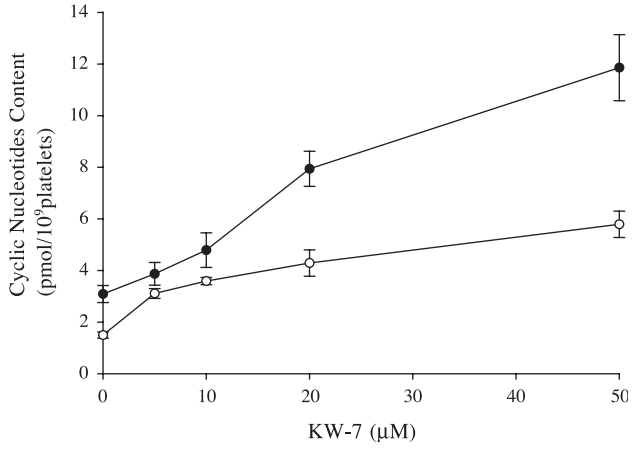

Fig. 3. Effects of KW-7 on platelet cyclic AMP (●) and cyclic GMP (○) levels. Washed rabbit platelets (1×10^9 platelets/ml) were incubated at 37 °C for 2 min with DMSO (0.5%, control) or various concentrations of KW-7. The reaction was stopped, platelets were then pelleted and the supernatants were assayed for cyclic AMP and cyclic GMP by enzyme immunoassay. Values are presented as mean ± S.E.M. ($n = 4$).

In the presence of either KW-7 (10 μM) or IBMX (100 μM), both PGE_1 (1 μM)-elicited elevation of cyclic AMP levels and sodium nitroprusside (SNP, 1 μM)-elicited elevation of cyclic GMP levels were significantly enhanced (Table 2). On the other hand, the combination of KW-7 and IBMX had no additional effect on platelet cyclic nucleotide levels (Table 2).

3.5. Effect of KW-7 on platelet phosphodiesterases (PDEs)

It has been demonstrated that rabbit platelets exclusively express PDE 3 and 5 that is responsible for hydrolysis of cyclic AMP and cyclic GMP in platelets, respectively (Hidaka et al., 1979; Liao et al., 1998). To test if the cyclic nucleotides-elevating effect of KW-7 is due to the inhibition of phosphodiesterases, [^{3}H] cyclic AMP and [^{3}H] cyclic

Table 2
Interactions of KW-7, IBMX, PGE_1 and SNP on platelet cyclic AMP and cyclic GMP levels

	Cyclic AMP (pmol/ml)	Cyclic GMP (pmol/ml)
DMSO	3.1 ± 0.3	1.5 ± 0.1
PGE_1	19.9 ± 4.1	–
SNP	–	18.0 ± 1.7
KW-7	4.8 ± 0.7	3.6 ± 0.1
KW-7 + PGE_1	146.0 ± 15.6	–
KW-7 + SNP	–	33.9 ± 3.8
IBMX	5.6 ± 0.6	4.8 ± 0.4
IBMX + KW-7	5.6 ± 0.7	6.1 ± 0.2

Washed rabbit platelets (1×10^9 platelets/ml) were incubated with PGE_1 (1 μM) or SNP (1 μM) at 37 °C for 1 min in the presence of DMSO, IBMX (100 μM) or KW-7 (10 μM). The reaction was stopped, platelets were then pelleted and supernatants were used to assay for cyclic AMP and cyclic GMP using enzyme immunoassay kits. Values are presented as mean ± S.E.M. ($n = 4$).

GMP were used as substrates in rabbit platelet homogenates. As shown in Fig. 4, KW-7 concentration-dependently inhibited PDE3 and PDE5 activities with IC_{50} values of 7.8 ± 0.8 and 14.5 ± 5.1 μM, respectively. IBMX also inhibited both enzymes with IC_{50} values of 28.8 ± 2.0 and 42.4 ± 12.2 μM, respectively.

3.6. The antiplatelet effect of KW-7 is related with cyclic nucleotides

To demonstrate if the antiplatelet effect of KW-7 is due to elevation of cyclic nucleotide levels in platelets, the adenylate cyclase inhibitor SQ22536 (Daniel et al., 1999), the protein kinase A inhibitor H89 (Chijiwa et al., 1990), and the guanylate cyclase inhibitor ODQ (Garthwaite et al., 1995) were used in the experiments. As shown in Fig. 5, pretreatment of platelets with SQ22536 almost completely prevented the increase in platelet cyclic AMP caused by KW-7 and markedly attenuated the capacity of KW-7 to inhibit platelet aggregation. H89 even had more pronounced effect than SQ22536 on prevention of KW-7's antiaggregatory activity. In contrast, ODQ totally abolished the increases of platelet

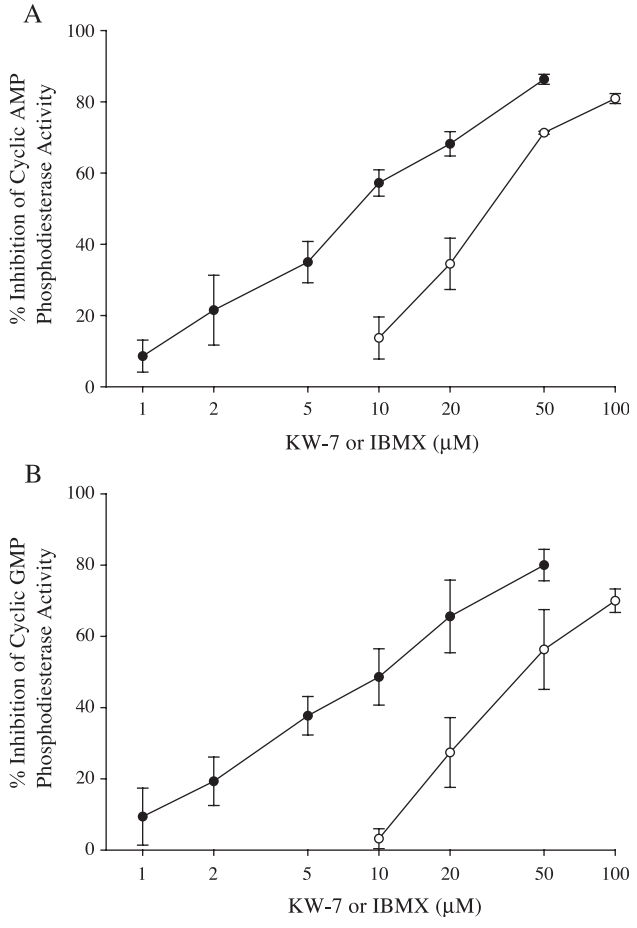

Fig. 4. Effects of KW-7 and IBMX on platelet cyclic AMP (A) and cyclic GMP (B) phosphodiesterase activities. Platelet homogenates were incubated with various concentrations of KW-7 (●) or IBMX (○) at 30 °C for 5 min, [^{3}H] cyclic AMP or [^{3}H] cyclic GMP was then added to the reaction mixture for another 30 min. Phosphodiesterase activity was measured as described in Materials and Methods. Percentages of inhibition are presented as mean ± S.E.M. ($n = 3$).

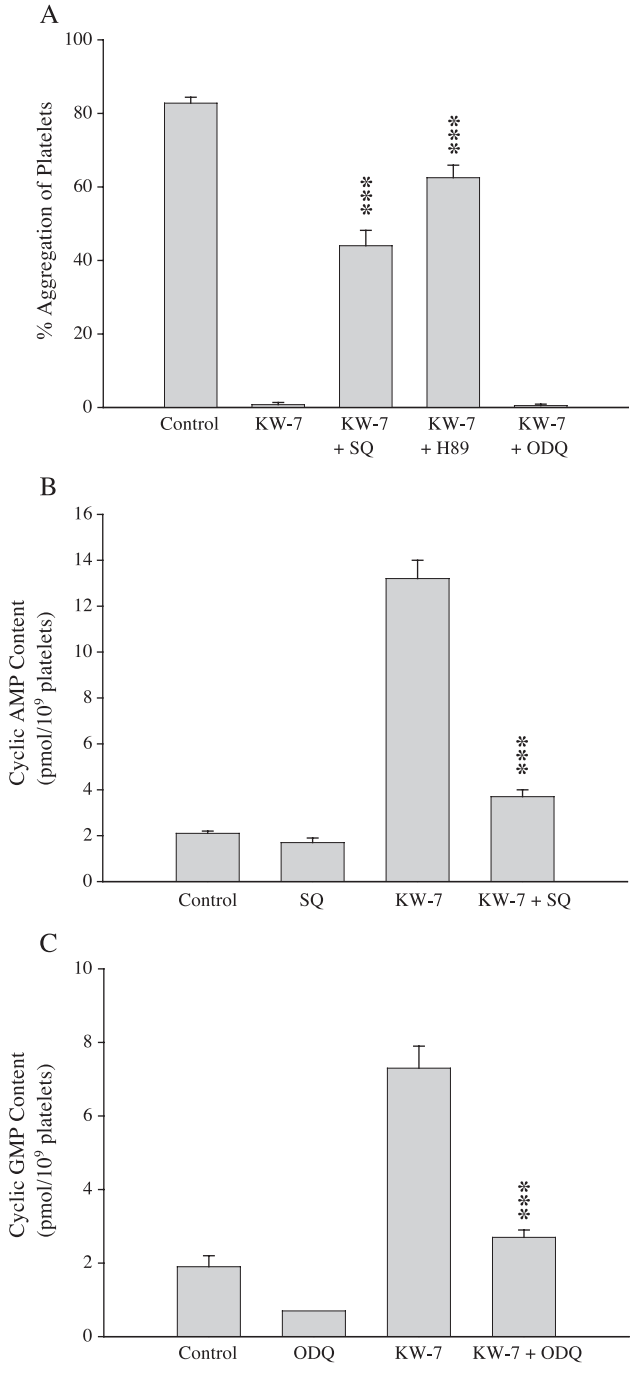

Fig. 5. Effects of SQ22536, H89 and ODQ on the inhibition of platelet aggregation (A) and the increases of platelet cyclic AMP (B) and cyclic GMP (C) levels by KW-7. Washed rabbit platelets were incubated with KW-7 (50 μM) at 37 °C for 3 min in the presence of SQ22536 (80 μM), H89 (20 μM) or ODQ (2 μM). Platelet aggregation was induced by thrombin (0.1 U/ml). The cyclic nucleotide contents were determined by enzyme immunoassay. Values are presented as mean ± S.E.M. ($n = 4$). ***$P < 0.001$ as compared with KW-7 alone.

cyclic GMP, but did not affect the antiaggregatory effect caused by KW-7.

3.7. Synergistic effect of KW-7 on the antiplatelet activity of PGE1 and SNP

By using KW-7 at a concentration (5 μM) which does not affect thrombin-induced platelet aggregation, the interaction between KW-7 and cyclic nucleotide-elevating agents was investigated. In the absence of KW-7, PGE_1 inhibited thrombin-induced platelet aggregation with a IC_{50} value of 43.0 ± 6.5 nM. When platelets were pretreated with KW-7 for 2 min before addition of PGE_1, the concentration-response curve of PGE_1 was significantly shifted to the left with a IC_{50} value of 7.6 ± 1.5 nM (Fig. 6A). In a similar but much more pronounced manner, the antiaggregatory activity of SNP was enhanced nearly 5000-fold by KW-7 (121.3 ± 28.4 μM vs. 24.4 ± 4.0 nM, Fig. 6B).

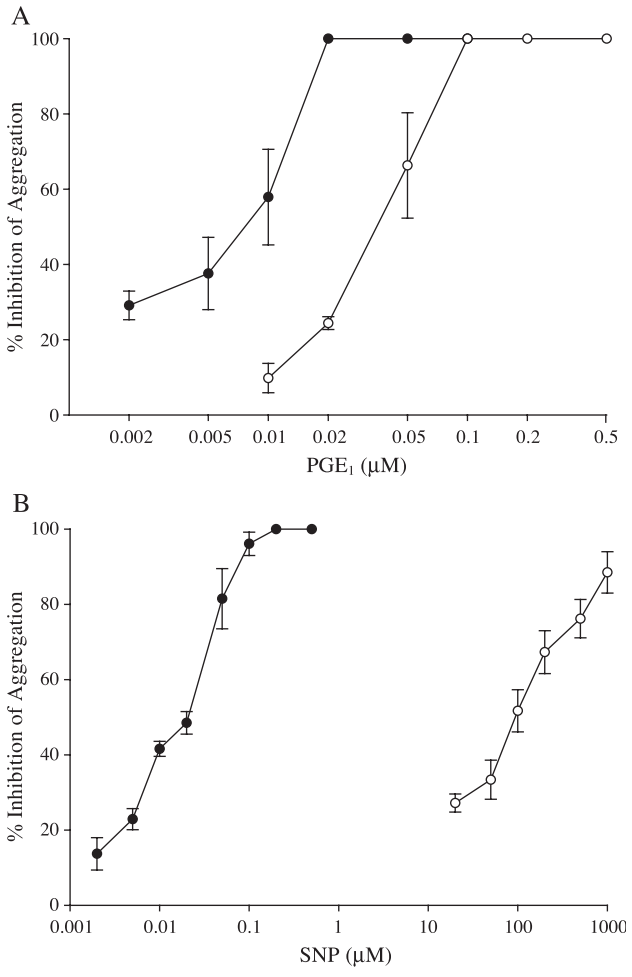

Fig. 6. Potentiation of the antiaggregatory effects of PGE_1 and SNP by KW-7. Washed rabbit platelets were incubated with various concentrations of PGE_1 (A) or SNP (B) at 37 °C for 1 min in the presence (○) or absence (●) of KW-7 (5 μM). Platelet aggregation was then induced by thrombin (0.1 U/ml). Values are presented as mean ± S.E.M. (n = 4).

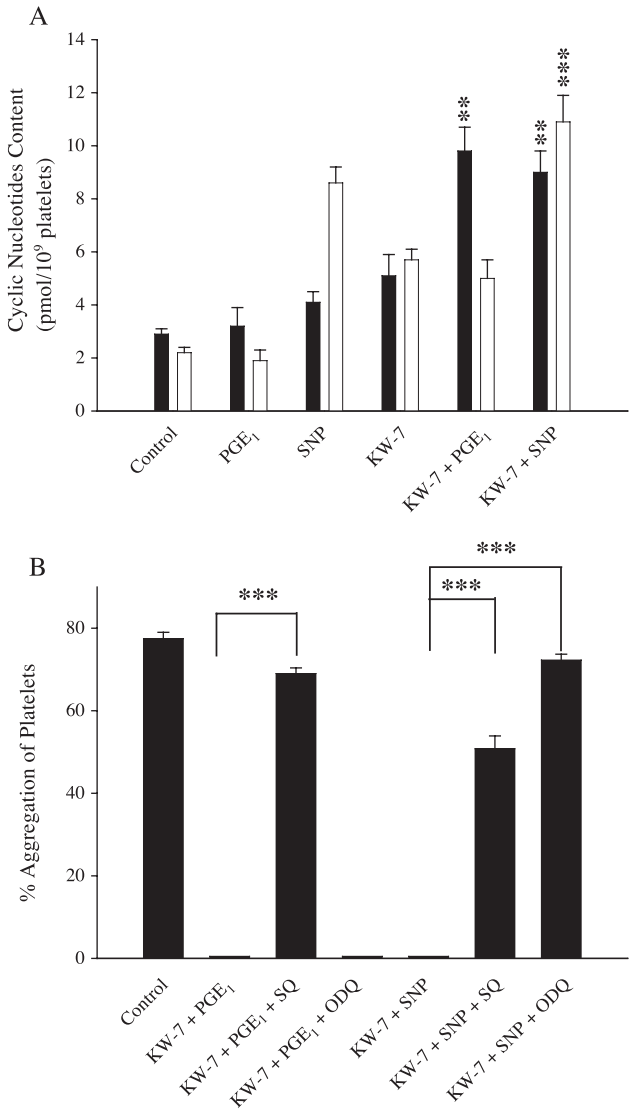

Fig. 7. (A) Potentiation of the cyclic nucleotide-elevating activities of PGE_1 and SNP by KW-7. Washed rabbit platelets were incubated with PGE_1 (0.02 μM) or SNP (0.2 μM) at 37 °C for 1 min in the presence of DMSO (control) or KW-7 (5 μM). The cyclic nucleotide contents were determined by enzyme immunoassay. Values are presented as mean ± S.E.M. (n = 4). $^{**}P < 0.01$, $^{***}P < 0.001$ as compared with KW-7 alone. (B) Effects of SQ22536 and ODQ on the inhibition of platelet aggregation by KW-7 with PGE_1 or SNP. Washed rabbit platelets were incubated with PGE_1 (0.02 μM) or SNP (0.2 μM) at 37 °C for 1 min in the presence of DMSO (control) or KW-7 (5 μM), platelet aggregation was then induced by thrombin (0.1 U/ml). In some experiments, platelets were preincubated with SQ22536 (80 μM) or ODQ (2 μM) before the addition of KW-7, PGE_1 or SNP. Values are presented as mean ± S.E.M. (n = 4). $^{***}P < 0.001$.

The combination of PGE_1 and KW-7 could lead to an increase in cyclic AMP accumulation but had no additional effect on cyclic GMP accumulation (Fig. 7A). Furthermore, SQ22536, but not ODQ, reversed the antiaggregatory effect of PGE_1 with KW-7 (Fig. 7B). In contrast, the combination of SNP and KW-7 caused additional increases in both platelet cyclic AMP and cyclic GMP levels and, either

SQ22536 or ODQ markedly attenuated the antiaggregatory effect of SNP with KW-7 (Fig. 7A and B).

4. Discussion

Elevation of intracellular cyclic AMP and cyclic GMP levels, either by stimulation of adenylate or guanylate cyclase or by inhibition of phosphodiesterases, is the most potent mechanism of platelet inhibition (Haslam et al., 1999; Schwarz et al., 2001). Similar to the cyclic nucleotide-elevating agents (Rao et al., 1980; Radomski et al., 1987; Sargeant and Sage, 1994), KW-7 also prevented platelet aggregation, dispersed formed platelet aggregates and blunted the increase of intracellular free calcium. These effects of KW-7 were closely associated with the increased levels of cyclic AMP and cyclic GMP in platelets. In addition, KW-7 markedly enhanced the cyclic nucleotide-elevating activity of PGE_1 and SNP, but not that of IBMX, suggesting that KW-7 inhibited phosphodiesterases rather than stimulated cyclases. By using rabbit platelet homogenates as the enzyme sources, we demonstrated that KW-7 is a non-selective inhibitor of PDE3 and PDE5. Furthermore, SQ22536 and H89, but not ODQ, greatly reversed the inhibitory effect of KW-7 on platelet aggregation indicating that cyclic AMP is the major mediator of the antiplatelet action of KW-7.

The synergistic inhibition of cyclic AMP and cyclic GMP on platelet activation has been well established (Radomski et al., 1987; Maurice and Haslam, 1990). This effect could be of physiological importance to the antithrombotic property of vascular endothelium, because endothelial cells secret both prostacyclin (a cyclic AMP-elevating agent) and NO (a cyclic GMP-elevating agent). In the present work, KW-7 enhanced the antiaggregatory potency of PGE_1 and SNP by 6- and 5000-fold, respectively. The dramatic enhancement of action of subthreshold concentrations of SNP by KW-7 was accompanied with additional increases of both cyclic AMP and cyclic GMP levels in platelets and may lead to synergistic inhibition of platelet responses. This suggestion was supported by the fact that either SQ22536 or ODQ markedly attenuated the antiaggregatory effect of SNP with KW-7. On the contrary, the combination of PGE_1 and KW-7 only led to an increase of cyclic AMP accumulation but had no additional effect on cyclic GMP accumulation. Moreover, SQ22536, but not ODQ, reversed the antiaggregatory effect of PGE_1 with KW-7. Taken together, these results suggest that KW-7 acts synergistically with SNP or, to a small extent, PGE_1 to inhibit platelet aggregation through enhanced elevation of cyclic AMP and/or cyclic GMP levels.

Released thromboxane A_2 from the activated platelets is an important factor in amplification of the original stimulus due to recruitment of additional platelets from the circulation to the site of aggregation (FitzGerald, 1991). By acting differently from IBMX and other cyclic nucleotide-elevating agents that have no effect on arachidonic acid metabolism (Minkes et al., 1977; Ko et al., 1994), KW-7 concentration-dependently inhibited exogenous arachidonic acid-induced thromboxane production in platelets. Furthermore, this inhibition was associated with an increase in prostaglandin D_2 levels. Because KW-7 does not affect cyclooxygenase-1 activity, these results indicate that KW-7 inhibited thromboxane synthase and produced a re-diversion of arachidonic acid metabolism toward prostaglandin D_2, which is the major prostaglandin in platelets. It is well known that prostaglandin D_2 inhibits platelet aggregation through the activation of adenylate cyclase (Armstrong, 1996); this effect may be enhanced by the inhibition of KW-7 on phosphodiesterases. Further study is needed to explore the precise role of PGD_2 in KW-7's antiplatelet effects.

In conclusion, the newly synthetic pyrrolo-benzylisoquinoline derivative, KW-7, is an effective antiplatelet agent and phosphodiesterase inhibitor. KW-7 does not show structural similarity to any known phosphodiesterase inhibitor and thus provides a new active skeleton in the development of phosphodiesterase inhibitors. Moreover, KW-7 with the additional inhibitory activity on platelet thromboxane synthesis may be of extra benefit for its antiplatelet effects.

Acknowledgements

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