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KF31327, a new potent and selective inhibitor of cyclic nucleotide phosphodiesterase 5

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Abstract

The effects of KF31327 (3-ethyl-8-[2-(4-hydroxymethylpiperidino)benzylamino]-2,3-dihydro-1*H*-imidazo[4,5-*g*]quinazoline-2-thione dihydrochloride) on phosphodiesterase 5 (cyclic GMP-specific phosphodiesterase) activity and platelet aggregation were investigated and compared with those of sildenafil, a well-known phosphodiesterase 5 inhibitor. KF31327 inhibited phosphodiesterase 5 from canine trachea (K_i = 0.16 nM) more potently than sildenafil (K_i = 7.2 nM). The kinetic analysis revealed that KF31327 was a non-competitive inhibitor. In the presence of nitroglycerin (nitric oxide generator), both compounds inhibited the collagen-induced aggregation of rabbit platelets at less than 0.1 μM, augmenting intracellular cyclic GMP level without affecting cyclic AMP. In contrast, in the absence of nitroglycerin, a higher concentration (10 μM) of KF31327 was required to inhibit platelet aggregation and increased both cyclic nucleotide levels. However, 10 μM sildenafil did not affect aggregation despite elevation of cyclic GMP comparable to that in the presence of nitroglycerin. These results indicate that in the presence of nitroglycerin, the inhibition of platelet aggregation by KF31327 is due to the elevation of cyclic GMP, whereas the mechanism underlying the inhibition without nitroglycerin might be related to a rise in intracellular cyclic AMP.

Keywords: KF31327 (3-ethyl-8-[2-(4-hydroxymethylpiperidino)benzylamino]-2,3-dihydro-1*H*-imidazo[4,5-*g*]quinazoline-2-thione dihydrochloride); Phosphodiesterase 5 (cyclic GMP-specific phosphodiesterase); cAMP; cGMP; Platelet; Sildenafil

1. Introduction

Cyclic nucleotides are major intracellular signaling molecules that play a crucial role in mammalian cells. Intracellular concentrations of cyclic nucleotides are regulated by two families of enzymes, adenylate and guanylate cyclases which synthesize cyclic AMP and cyclic GMP from corresponding nucleotide triphosphates, and cyclic nucleotide phosphodiesterases which catalyze the hydrolysis and inactivation of cyclic AMP and/or cyclic GMP. Cyclic nucleotide phosphodiesterases consist of at least 11 enzyme families in mammals each being encoded by a distinct gene or genes (Beavo, 1995; Francis et al., 2000). The phosphodiesterase isozymes can be distinguished by their kinetic properties, substrate preference, regulatory molecules and inhibitor sensitivity (Beavo, 1995; Torphy and Undem, 1991; Nicholson et al., 1991). Phosphodiesterases are attractive molecular targets for therapeutics. The focus of the present paper is phosphodiesterase 5, which, based on its substrate specificity, has been termed a cyclic GMP-specific phosphodiesterase. It is selectively inhibited by zaprinast (Beavo and Reifsnyder, 1990) and sildenafil (Boolell et al., 1996), and less selectively by dipyridamole (Weishaar et al., 1990). Phosphodiesterase 5 is mainly expressed in smooth muscles including vascular and tracheal smooth muscle (Francis et al., 1990), and also in platelets (Sheth and Colman, 1995). It has been recognized for 20 years that inhibitors of phosphodiesterase 5 are potent inhibitors of platelet aggregation, weak cardiac inotropic agents and vascular relaxants (Hidaka and Endo, 1984). Sildenafil enhances relaxation of smooth muscle in the corpus cavernosum and is thereby effective in the treatment of male erectile dysfunction (Boolell et al., 1996).

We have discovered a new compound KF31327 (3-ethyl-8-[-2(4 - hydroxymethylpiperidino)benzylamino] -2,3-dihydro-1*H*-imidazo[4,5-*g*]quinazoline-2thione dihydrochloride; Fig. 1), which potently, selectively and in a

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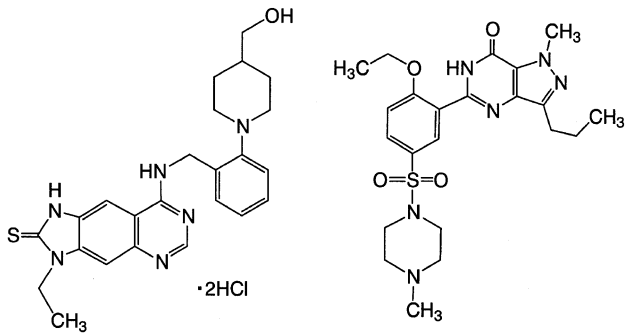

Fig. 1. Structure of KF31327 and sildenafil.

non-competitive manner, inhibits phosphodiesterase 5. We have compared the ability of KF31327 and sildenafil to inhibit collagen-induced platelet aggregation, and correlated this with their effects on intracellular cyclic GMP and cyclic AMP levels in platelets.

2. Materials and methods

2.1. Materials

[2, 8-^{3}H]-Adenosine 3′, 5′-cyclic phosphate ([^{3}H]cyclic AMP; 925 GBq/mmol) and [8-^{3}H]-guanosine 3′, 5′-cyclic phosphate ([^{3}H]cyclic GMP; 344.1 GBq/mmol) were obtained from NEN™ Life Science Products (Boston, MA). Diethylaminoethyl (DEAE)-Sephacel and Calmodulin-Sepharose 4B were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Cyclic AMP, cyclic GMP, 5′-nucleotidase, phosphodiesterase 3′:5′-cyclic nucleotide-activator (calmodulin) and L-nitro-arginine methyl ester (L-NAME) were from Sigma (St. Louis, MO). Collagen reagent was from Moriya (Tokyo, Japan). Nitroglycerin was from Midori-juji (Osaka, Japan). Zaprinast (M & B22948: 2-*o*-propoxyphenyl-8-azapurin-6-one) was from Research Biochemical International (Natick, MA). KF31327, sildenafil (1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-*oxo*-3-propyl-1H-pyrazolo[4,3-*d*]pyrimidin-5-yl) phenylsulphonyl]-4-methyl-piperazine), milrinone (2-methyl-5-cyano-(3,4′-bipyridin)-6(1*H*)-one) and rolipram (*R*,*S*-(±)-4-(3-cyclopentyloxy-4-methoxyphenyl)-2-pyrrolidine) were synthesized at the Department of Medicinal Chemistry at Kyowa Hakko Laboratories.

2.2. Animals

Male beagles (12–13 month old weighing 11.6–12.0 kg) were obtained from Nihon Nosan Kogyo K.K. (Yokohama, Japan). Male Japanese white rabbits (3.0–3.5 kg) were from Ichikawaya (Tokyo, Japan). The animals were kept in temperature-controlled rooms (22–24 °C) with a 12-h light–dark cycle. The animals received commercial standard diets and water. All animals were used in accordance with the guiding principles for the care and use of laboratory animals approved by the Japanese Pharmacological Society, and the experimental protocols were reviewed and approved by the ethical committee of Pharmaceutical Research Institute, Kyowa Hakko Kogyo.

2.3. Phosphodiesterase isozyme preparation

Ca^{2+}/calmodulin-stimulated phosphodiesterase (phosphodiesterase 1), cyclic GMP-stimulated phosphodiesterase (phosphodiesterase 2), cyclic GMP-inhibited phosphodiesterase (phosphodiesterase 3), cyclic AMP-specific phosphodiesterase (phosphodiesterase 4) and cyclic GMP-specific phosphodiesterase (phosphodiesterase 5) were separated from canine tracheal tissue as previously described (Torphy and Cieslinski, 1990) with slight modifications. Briefly, male beagles were killed with an i.v. injection of sodium pentobarbital (65 mg/kg). The tracheas were rapidly removed and stored at −80 °C until use. The tracheas were minced with fine scissors and homogenized with a Polytron (five bursts, 15 s/burst) (Kinematica, Luzerne, Switzerland). The homogenate of the trachea was centrifuged at 100,000 × *g* for 1 h at 4 °C. The supernatant was applied to a DEAE-Sephacel anion exchange column. The phosphodiesterase isozymes were eluted with 0 to 1 M sodium acetate as a linear gradient. Fractions were collected and assayed for phosphodiesterase activity. All preparation procedures were performed at 4 °C. Phosphodiesterase isozymes were stored at −80 °C in buffers containing 2000 U/ml aprotinin (Bayer, Leverkusen, Germany), and the characteristics of the enzymes were verified by assessing the effectiveness of regulatory factors or the effect of selective inhibitors.

2.4. Measurement of phosphodiesterase activity and kinetic analysis

Phosphodiesterase activity was measured by the method of Kincaid and Manganiello (1988). The reaction mixture contained: 50 mM *N*,*N*-bis(2-hydroxyethyl)-2-amino ethanesulfonic acid (BES)/sodium hydroxide, 1 mM $MgCl_2$, 0.1 mM EGTA and 0.1 mg/ml soybean trypsin inhibitor (pH 7.2). Unless otherwise stated, 1 μM [^{3}H]cyclic AMP or [^{3}H]cyclic GMP was used as a substrate. The reaction mixture was incubated for 10 to 30 min at 30 °C depending on the enzyme activity. The reaction was terminated by addition of 0.25 M hydrochloric acid and neutralized by 0.25 M NaOH buffered with 0.1 M Tris/HCl (pH 8.0). After conversion of the 5′-nucleotide to its corresponding nucleoside by 5′-nucleotidase, the samples were applied to a DEAE-Sephadex A-25 column (Amersham Pharmacia Biotech), and the radioactivities of the [^{3}H]nucleoside eluted with water was measured by scintillation counter LS6500 (Beckman Instruments, Fullerton, CA). The concentration of each drug

required to produce 50% inhibition (IC_{50}) was calculated by linear regression analysis of the percent inhibition data. In the kinetic analyses, phosphodiesterase 5 activity was assayed with [^{3}H]cyclic GMP (4, 8, 16 and 32 μM) as substrate in the presence of various concentration of KF31327 or sildenafil. K_i values for the compounds were calculated by drug concentration vs. slope replot from each of Lineweaver–Burk plots.

2.5. Preparation of rabbit washed platelets

Platelet-rich plasma was prepared as previously described by Simpson et al. (1988) with slight modifications. Peripheral blood of male healthy white rabbits was anticoagulated with 0.38 w/v% sodium citrate. The citrate-treated blood was centrifuged ($250 \times g$ for 10 min at 25 °C), and the platelet-rich plasma was collected. Washed platelets were prepared from the platelet-rich plasma centrifuged ($2000 \times g$ for 10 min at 25 °C). The pellet was resuspended in Ca^{2+}, Mg^{2+}-free HEPES-Tyrode buffer containing: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 5 mM HEPES, 5 mM glucose and 0.5 w/v% bovine albumin (pH 7.4), and centrifuged ($2000 \times g$ for 10 min at 25 °C). After repeating this procedure four times, the pellet was resuspended in HEPES-Tyrode buffer containing: 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.36 mM NaH_2PO_4, 5 mM HEPES, 5 mM glucose, 0.5 w/v% bovine albumin, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.4). The afforded washed platelets were adjusted at 3.6×10^8 cells/ml.

2.6. Platelet aggregation assay

Platelet aggregation was measured by the turbidimetric method (Born and Cross, 1963) in a TE-500 aggregometer (Erma Optical Works, Tokyo, Japan). Various concentrations of drugs were added to 8×10^7 cells of rabbit washed platelets in a final volume of 240 μl and pre-incubated for 5 min at 37 °C. After the addition of 10 μl collagen solution (final 2 μg/ml), aggregation was determined by the change in absorbance monitored for 10 min. Aggregation was expressed as a percentage of control sample. Aggregation in the presence of nitroglycerin and L-NAME was determined by the addition of nitroglycerin (10 μM) and L-NAME (100 μM) to each of platelet aliquots at the same time as the addition of the drugs.

2.7. Assay of intracellular cyclic nucleotides levels in washed platelets

The washed platelets were suspended in the same buffer (8×10^7 cells) as described in Section 2.6, supplemented with various drugs, and then incubated at 37 °C. The experimental schedule of pre-incubation and collagen-stimulation was identical to that used for the aggregation assays. Incubation was terminated by addition of trichloroacetic acid (final 5 w/v%) at the following incubation times; 0, 2 and 5 min pre-incubation and after 10 min incubation with collagen. Effect of L-NAME on platelet cyclic nucleotide was assessed after 10 min incubation with collagen. After centrifugation ($2000 \times g$ for 10 min at 25 °C), trichloroacetic acid in the supernatant was removed by four times of extraction with 2.5 volumes of water-saturated diethylether. The aqueous phase was lyophilized and the cyclic nucleotide contents were determined using commercially available enzyme immunoassay kits (Amersham Pharmacia Biotech). The data were analyzed using DeltaSoft3 ver.1.3 (BioMetallics, Princeton, NJ), and expressed as pmol cyclic AMP or cyclic GMP per 10^8 platelets.

2.8. Statistical analysis

All results were expressed as the means ± S.E.M. IC_{50} values of enzyme inhibition for the compounds were determined on the basis of concentration–response curves in which the concentrations ranged from 0.001 nM to 10 μM using the Probit (logistic model) method in SAS System for Windows release.6.12 (SAS Institute, Cary, NC). Comparison of platelet aggregation and cyclic nucleotide content in vehicle and drug-treated platelets was made with one-way analysis of variance (ANOVA) followed by Dunnett's test. Statistical differences between the two groups were analyzed by Wilcoxson's test and Student's unpaired *t*-test for platelet aggregation and cyclic nucleotide contents, respectively. Concentration dependency of phosphodiesterase 5 inhibitors was analyzed by max-*t*-test. A probability (P) of less than 0.05 was considered as significant.

3. Results

3.1. Potent and selective inhibition of phosphodiesterase 5 by KF31327

Five phosphodiesterase isozymes were separated from canine trachea as Torphy and Cieslinski (1990) previously reported. Phosphodiesterase 1 to 4 were assayed with [^{3}H]cyclic AMP as substrate and phosphodiesterase 5 with [^{3}H]cyclic GMP. Phosphodiesterase 1 was activated 2-fold by the addition of 20 U/ml calmodulin and 0.45 mM $CaCl_2$. Phosphodiesterase 2 was stimulated 20-fold by 10 μM cyclic GMP. Phosphodiesterase 3, 4 and 5 were identified by their substrate specificity and the effect of selective inhibitors. Phosphodiesterase 3 was inhibited by milrinone ($IC_{50} = 1.8$ μM), phosphodiesterase 4 by rolipram ($IC_{50} = 3.0$ μM) and phosphodiesterase 5 by zaprinast ($IC_{50} = 0.53$ μM). Each isozyme displayed Michaelis–Menten type kinetics and each K_m value was similar to the previously reported value (Torphy and Cieslinski, 1990). KF31327 showed considerable degree of selectivity for phosphodiesterase 5; it potently inhibited

Table 1
Phosphodiesterase inhibition profiles of KF31327 and sildenafil in each phosphodiesterase isozyme fraction prepared from canine trachea

	KF31327	Sildenafil
	IC_{50} (nM)	
Phosphodiesterase 1	380 ± 30	780 ± 110
Phosphodiesterase 2	670 ± 120	80,000 ± 5,000
Phosphodiesterase 3	38 ± 3	35,000 ± 2,000
Phosphodiesterase 4	800 ± 50	22,000 ± 4,000
Phosphodiesterase 5	0.074 ± 0.005	2.7 ± 0.2

IC_{50} values were determined using 1 μM [^{3}H]cyclic AMP as substrate for phosphodiesterases 1 to 4 and 1 μM [^{3}H]cyclic GMP as substrate for phosphodiesterases 5. Each value represents the mean ± S.E.M. of three experiments.

phosphodiesterase 5 with an IC_{50} value of 0.074 nM but the IC_{50} values against phosphodiesterase 1, 2, 3 and 4 were 380, 670, 38 and 800 nM, respectively (Table 1). Thus, KF31327 was at least 500-fold selective for inhibition of phosphodiesterase 5. Sildenafil, which is a well-known inhibitor of phosphodiesterase 5, potently inhibited the isozyme (IC_{50} = 2.7 nM). Sildenafil possessed similar inhibitory selectivity for phosphodiesterase 5 to that exhibited by KF31327. KF31327 inhibited phosphodiesterase 5 reversibly (data not shown).

3.2. Kinetic analysis of phosphodiesterase 5 inhibition by KF31327 and sildenafil

To compare the mode of inhibition of phosphodiesterase 5 by KF31327 and sildenafil, the enzyme activity was measured in the presence of different concentrations of the compounds together with varying substrate cyclic GMP concentrations. Fig. 2 shows the kinetic analyses by means of Lineweaver–Burk plots. The analysis clearly indicates that KF31327 inhibits phosphodiesterase 5 in a non-competitive manner, in contrast, sildenafil acted as a competitive inhibitor. The K_i values for KF31327 and sildenafil, calculated by drug concentration vs. slope replots (Royer, 1986), were 0.16 ± 0.02 and 7.2 ± 0.7 nM,

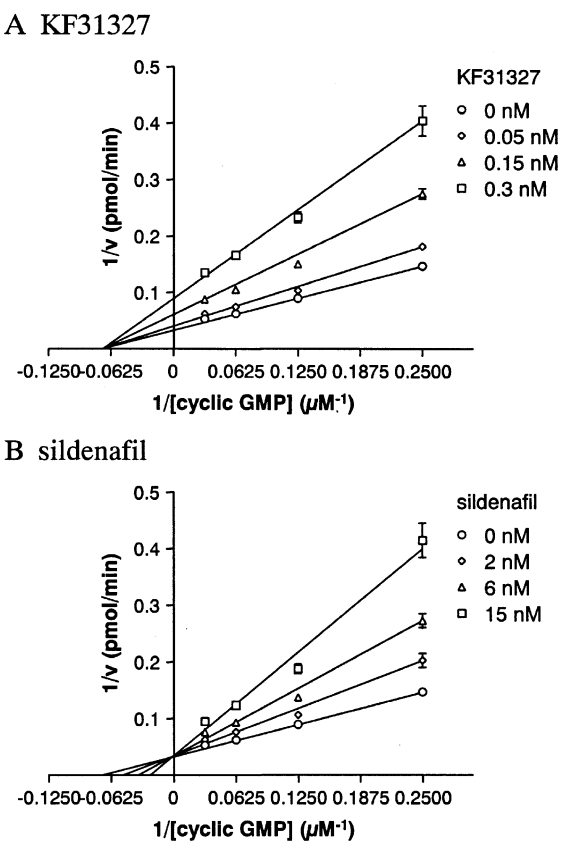

Fig. 2. Lineweaver–Burk plots of KF31327 (A) and sildenafil (B) in inhibition of phosphodiesterase 5 (n = 3). Phosphodiesterase 5 activity was assayed with [^{3}H]cyclic GMP (4, 8, 16 and 32 μM) as substrate. Concentrations of the inhibitors were 0.05, 0.15 and 0.3 nM for KF31327 and 2, 6 and 15 nM for sildenafil, respectively. Lines were fitted according to non-competitive and competitive inhibition modes for KF31327 and sildenafil, respectively.

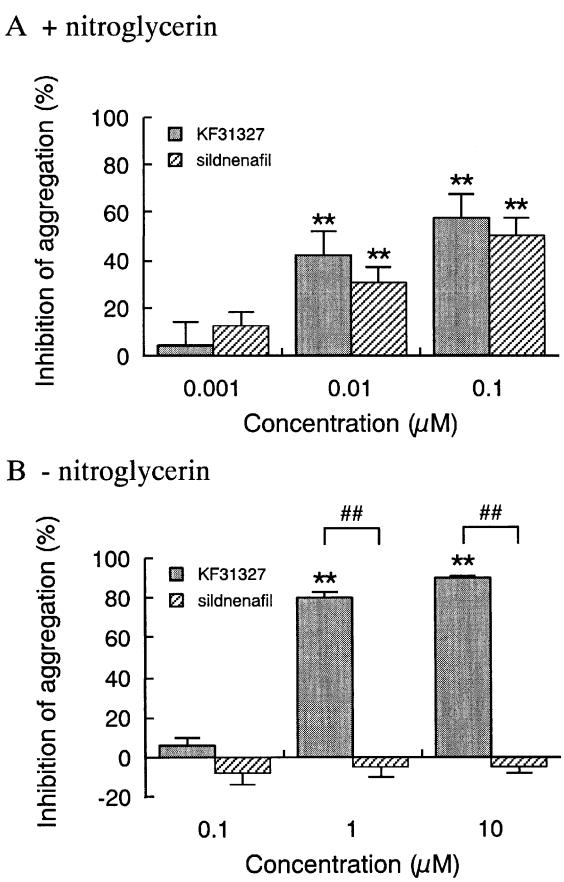

Fig. 3. Inhibitory effects of KF31327 (gray bars) and sildenafil (hatched bars) on collagen-induced platelet aggregation in rabbit washed platelets in the presence (A) and absence (B) of nitroglycerin. Platelets were pre-incubated for 5 min with 10 μM nitroglycerin and each of drugs at indicated concentration, and then challenged with 2 μg/ml of collagen for 10 min. Values represent mean ± S.E.M. (n = 4). $^{**}P < 0.01$ compared with vehicle. $\#\#P < 0.01$ KF31327 vs. sildenafil. KF31327 (A and B) and sildenafil (A) concentration dependently inhibited platelet aggregation verified by max-t-test ($P < 0.01$).

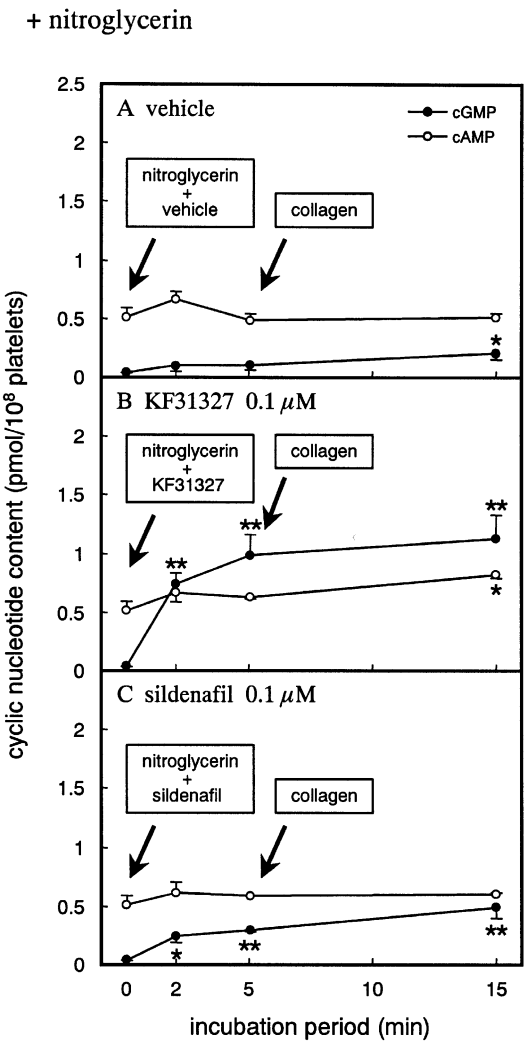

Fig. 4. Time course of change on cyclic nucleotide levels in rabbit washed platelets in the presence of 10 μM nitroglycerin. Control (A), 0.1 μM KF31327-treated (B) and 0.1 μM sildenafil-treated (C) platelets were pre-incubated for 5 min then stimulated by collagen for 10 min. The cyclic AMP and cyclic GMP contents were determined by enzyme immunoassay. Values are presented as means ± S.E.M. ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with content at time zero.

respectively. Thus, KF31327 was 45-fold more potent than sildenafil.

3.3. Effects of KF31327 on platelet aggregation

In the presence of 10 μM nitroglycerin, both KF31327 and sildenafil prevented aggregation of rabbit washed platelets in concentration-dependent manners (Fig. 3A). KF31327 and sildenafil at 0.1 μM inhibited platelet aggregation by 58 ± 10% and 50 ± 8%, respectively. In the absence of nitroglycerin, higher concentrations (1 and 10 μM) of KF31327 were required to inhibit platelet aggregation (Fig. 3B). KF31327 inhibited washed platelet aggregation by 90 ± 1% at 10 μM. In contrast, sildenafil, at concentrations up to 10 μM, had no effect on platelet aggregation in the absence of nitroglycerin. Platelet aggregation inhibited by 10 μM KF31327 in the absence of nitroglycerin was not affected by the addition of 100 μM L-NAME, an inhibitor of nitric oxide synthase (% inhibition of aggregation; 85 ± 2% without L-NAME vs. 84 ± 1% with L-NAME, $n = 3$).

3.4. Effect of KF31327 on cyclic nucleotide levels in platelets

Cyclic nucleotide levels in washed platelets were measured in order to investigate the relationship between the inhibitory activity for platelet aggregation and the alteration of cyclic nucleotides by KF31327 and sildenafil. Naive washed platelets contain 0.04 ± 0.01 and 0.51 ± 0.08

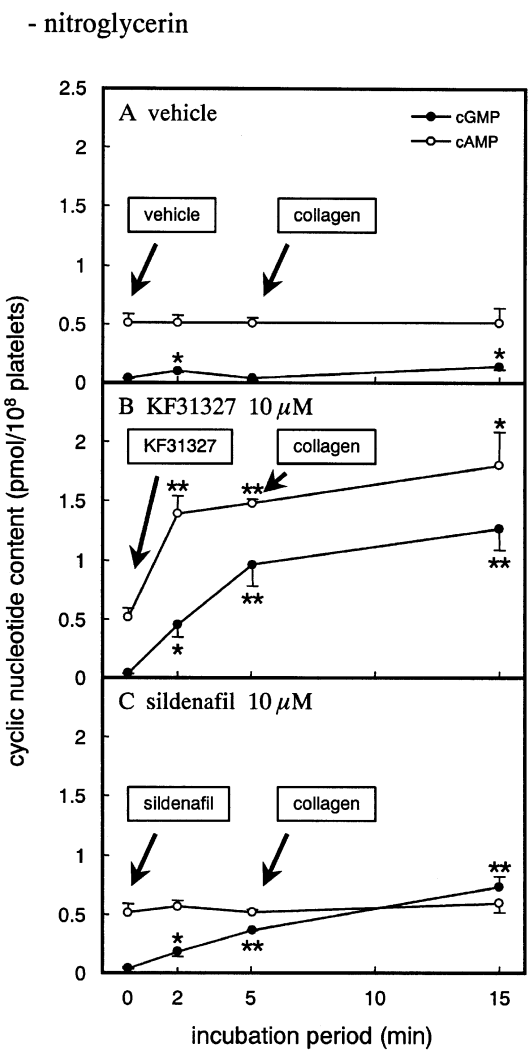

Fig. 5. Time course of change on cyclic nucleotide levels in rabbit washed platelets in the absence of nitroglycerin. Control (A), 10 μM KF31327-treated (B) and 10 μM sildenafil-treated (C) platelets were pre-incubated for 5 min then stimulated by collagen for 10 min. Values are presented as means ± S.E.M. ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with content at time zero.

pmol/10^8 cells of cyclic GMP and cyclic AMP, respectively (at time zero in Figs. 4 and 5). After 5 min pre-incubation, 10 μM nitroglycerin marginally raised intracellular cyclic GMP levels to 0.10 ± 0.04 pmol/10^8 cells but had little effect on the level of cyclic AMP (Fig. 4A). Stimulation of cells by collagen for 10 min resulted in a slight increase of cyclic GMP level (Fig. 5A). KF31327 and sildenafil at 0.1 μM increased the level of cyclic GMP in the presence of 10 μM nitroglycerin without affecting cyclic AMP (Fig. 4B and C). After 5 min pre-incubation, cyclic GMP levels increased to 0.98 ± 0.17 and 0.29 ± 0.02 pmol/10^8 cells for KF31327 and sildenafil, respectively. The accumulation of cyclic GMP by KF31327 during 5 min incubation with nitroglycerin was concentration-dependent and greater than that by sildenafil (Fig. 6A).

In the absence of nitroglycerin, KF31327 (10 μM) inhibited platelet aggregation, whereas sildenafil alone at 10 μM had no effect (Fig. 3B). Therefore, the changes of cyclic nucleotide levels caused by KF31327 and sildenafil without addition of nitroglycerin were investigated. Both KF31327 and sildenafil showed significant increase in cyclic GMP at 10 μM. After 5 min incubation, the mean cyclic GMP levels of KF31327 and sildenafil-treated cells were 0.95 ± 0.17 and 0.36 ± 0.01 pmol/10^8 cells, respectively (Fig. 5B and C). The cyclic AMP level was also significantly raised by KF31327 to 1.48 ± 0.04 pmol/10^8 cells, but not by sildenafil. Augmentation of both cyclic GMP and cyclic AMP by KF31327 was concentration-dependent (Fig. 6B) and was not inhibited by 100 μM L-NAME (data not shown).

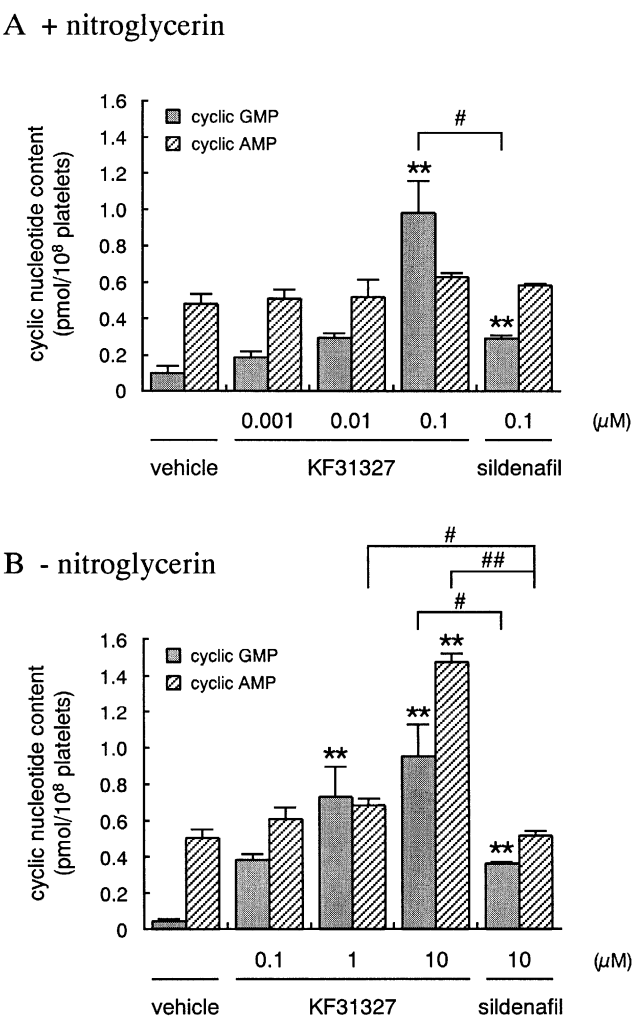

Fig. 6. Cyclic nucleotide levels in rabbit washed platelets after 5 min incubation with drugs in the presence (A) or the absence (B) of 10 μM nitroglycerin just before the induction of aggregation by collagen. Values are presented as means ± S.E.M. ($n = 3$). $^{**}P < 0.01$ compared with vehicle. $\#P < 0.05$ and $\#\#P < 0.01$ KF31327 vs. sildenafil. Cyclic GMP level (A and B) and cyclic AMP level (B) were concentration dependently increased by treatment of KF31327 verified by max-*t*-test ($P < 0.05$).

4. Discussion

KF31327 inhibited phosphodiesterase 5 from canine trachea with a high potency (45 times than that of sildenafil) and a high selectivity (at least 500-fold) over phosphodiesterase 1 to 4 (Table 1). Kinetic analysis revealed that the inhibition of phosphodiesterase 5 by KF31327 was non-competitive, whereas sildenafil was competitive as Ballard et al. (1998) reported (Fig. 2). To date, several phosphodiesterase 5 selective inhibitors have been reported, including zaprinast, E4021, sildenafil and T-1032 (Ballard et al., 1998; Kotera et al., 2000; Miyahara et al., 1995; Turko et al., 1998). All these inhibitors are competitive; KF31327 is the first non-competitive inhibitor of this isozyme to be reported. Non-competitive inhibitors could achieve enzyme inhibition independent of substrate concentration. Hence the elevation of cyclic GMP in a specific intracellular region via phosphodiesterase 5 inhibition by KF31327 might not diminish the efficacy of the inhibitor, which is distinct from the actions of competitive phosphodiesterase 5 inhibitors, and lead to preferred pharmacological effects.

Elevation of intracellular cyclic GMP and/or cyclic AMP suppresses platelet aggregation (Sheth and Colman, 1995; Haslam et al., 1999), and inhibition of platelet aggregation by selective phosphodiesterase 5 inhibitors is due to an augmentation of cyclic GMP level (Bowen and Haslam, 1991; Chiu et al., 1997; Hagiwara et al., 1984). We compared the efficacy of KF31327 and sildenafil in rabbit platelet aggregation induced by collagen. Rabbit platelets exclusively express phosphodiesterase 3 (cyclic GMP-inhibited phosphodiesterase) and phosphodiesterase 5 (Hidaka et al., 1979; Liao et al., 1998). In human platelets, these two isozymes are also the major phosphodiesterases (Ito et al., 1996).

When soluble guanylate cyclase in platelets was stimulated by a nitric oxide generator, nitroglycerin, low concentrations (less than 0.1 μM) of both KF31327 and

sildenafil inhibited aggregation (Fig. 3A). This effect was clearly associated with an increase in cyclic GMP, while cyclic AMP level did little change (Figs. 4B,C and 6A). KF31327 produced a much higher elevation of cyclic GMP level, possibly reflecting a different inhibitory mode from that of sildenafil described above. However, KF31327 showed only a slight potency on platelet aggregation over sildenafil, for which at present we have no appropriate explanation (Fig. 3A). Although KF31327 inhibited phosphodiesterase 5 with a K_i value of 0.16 ± 0.02 nM, anti-platelet activity and augmentation of cyclic GMP by KF31327 were observed at higher concentrations (Figs. 3A and 6A). This difference could be due to binding of KF31327 to bovine albumin in the suspension buffer or some proteins in platelets, or limited penetration of KF31327 into platelet cells.

In contrast, in the absence of nitroglycerin only high concentrations (1 and 10 μM) of KF31327 inhibited aggregation, sildenafil had no effect in this study (Fig. 3B) and the similar result of sildenafil was reported by Wallis et al. (1999). It is also reported that E4021, another phosphodiesterase 5 selective inhibitor, alone up to 10 μM had no anti-platelet activity induced by thromboxane A_2 receptor agonist, but that E4021 plus a nitric oxide generator, 3-morpholino-sydnonimine (SIN-1), inhibited aggregation at concentrations that had no effect individually (Ito et al., 1996). In spite of the absence of nitroglycerin, KF31327 increased cyclic GMP level in platelets at effective concentrations. KF31327 also caused accumulation of cyclic AMP in platelets in a concentration-dependent manner, although not statistically significant from control platelets at 1 μM (Fig. 6B). Sildenafil (10 μM) augmented cyclic GMP in the absence of nitroglycerin which was comparable to that observed in its presence, yet it failed to inhibit platelet aggregation. There is not so much difference in the time course of cyclic GMP accumulation between the platelets treated with both nitroglycerin and phosphodiesterase 5 inhibitors (0.1 μM) and those with phosphodiesterase 5 inhibitors alone (10 μM) (Figs. 4 and 5). In addition, the result that neither the inhibition of platelet aggregation nor the increase of cyclic GMP by KF31327 was affected by nitric oxide synthase inhibitor L-NAME confirmed no involvement of intrinsic nitric oxide for generation of cyclic GMP in platelets. Thus, the distinctive activity of the phosphodiesterase inhibitors on platelet aggregation between the presence and the absence of nitroglycerin suggests that the total level of intracellular cyclic GMP is not the only factor controlling platelet aggregation.

The inhibitory mechanism of platelet aggregation by KF31327 in the absence of nitroglycerin may underlie on other determinants. One possibility is that localization of cyclic GMP in cells is different between platelets treated with KF31327 and sildenafil. KF31327 produces much larger increase in cyclic GMP level than sildenafil at effective concentrations. The elevated cyclic GMP by KF31327, more than 20-fold of basal level, might reach the target site for cyclic GMP in the pharmacological effect. The other possibility is the effect of cyclic AMP. In the absence of nitroglycerin KF31327, but not sildenafil, increased cyclic AMP and inhibited platelet aggregation. The result that KF31327 produced higher inhibition rate of platelet aggregation in the absence of nitroglycerin than that in its presence (Fig. 3), though apparent cyclic GMP levels were similar, suggests that the inhibition of aggregation is at least in part mediated by cyclic AMP. Previous reports have shown that phosphodiesterase 3 inhibitors, milrinone and SK & F94120, alone suppress the aggregation of rabbit and human platelets through the elevation of cyclic AMP (Simpson et al., 1988; Sly et al., 1997). KF31327 is highly selective for phosphodiesterase 5 but it does also inhibit phosphodiesterase 3 ($IC_{50} = 38$ nM). Consequently, inhibition of phosphodiesterase 3 activity by KF31327 provides an explanation for the increase of cyclic AMP level that probably contributes to the inhibition of platelet aggregation.

In summary, KF31327 is a potent, selective and non-competitive inhibitor of phosphodiesterase 5, and it potently inhibits collagen-induced platelet aggregation. The elevation of both cyclic nucleotides, i.e. cyclic GMP and cyclic AMP, in platelets provides a dual inhibitory mechanism for the inhibition of platelet aggregation by KF31327. When activation of soluble guanylate cyclase by nitric oxide occurs, lower concentration of KF31327 inhibits platelet aggregation and cyclic GMP mediates its inhibitory effect. However, in the absence of nitric oxide, cyclic AMP, as well as cyclic GMP, accumulates due to inhibition of phosphodiesterase 3 by higher concentration of KF31327, and this raised level of cyclic AMP contributes to the anti-aggregatory effect. In most cardiovascular tissues, including vascular smooth muscle and platelets, both phosphodiesterase 3 and 5 are expressed, and cyclic AMP and cyclic GMP could act synergistically as vasodilating and anti-aggregating mediators. The characteristics of KF31327, a potent and non-competitive phosphodiesterase 5 inhibitor with relatively weak activity for phosphodiesterase 3, seem to have therapeutic potential in a series of disorders.

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