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Antiplatelet Activity of [5-(2-Methoxy-5-chlorophenyl)furan-2-ylcarbonyl]guanidine (KR-32570), a Novel Sodium/hydrogen Exchanger-1 and Its Mechanism of Action

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The antiplatelet effects of a novel guanidine derivative, KR-32570 ([5-(2-methoxy-5-chlorophenyl) furan-2-ylcarbonyl]guanidine), were investigated with an emphasis on the mechanisms underlying its inhibition of collagen-induced platelet aggregation. KR-32570 significantly inhibited the aggregation of washed rabbit platelets induced by collagen (10 μg/mL), thrombin (0.05 U/mL), arachidonic acid (100 μM), a thromboxane (TX) A_2 mimetic agent U46619 (9,11-dideoxy-9,11-methanoepoxy-prostaglandin F_2, 1 μM) and a Ca^{2+} ATPase inhibitor thapsigargin (0.5 μM) (IC_{50} values: 13.8 ± 1.8, 26.3 ± 1.2, 8.5 ± 0.9, 4.3 ± 1.7 and 49.8 ± 1.4 μM, respectively). KR-32570 inhibited the collagen-induced liberation of [^{3}H]arachidonic acid from the platelets in a concentration dependent manner with complete inhibition being observed at 50 μM. The TXA_2 synthase assay showed that KR-32570 also inhibited the conversion of the substrate PGH_2 to TXB_2 at all concentrations. Furthermore, KR-32570 significantly inhibited the $[Ca^{2+}]_i$ mobilization induced by collagen at 50 μM, which is the concentration that completely inhibits platelet aggregation. KR-32570 also decreased the level of collagen (10 μg/mL)-induced secretion of serotonin from the dense-granule contents of platelets, and inhibited the NHE-1-mediated rabbit platelet swelling induced by intracellular acidification. These results suggest that the antiplatelet activity of KR-32570 against collagen-induced platelet aggregation is mediated mainly by inhibiting the release of arachidonic acid, TXA_2 synthase, the mobilization of cytosolic Ca^{2+} and NHE-1.

Key words: KR-32570, Platelet, Platelet swelling test, Washed rabbit platelet, NHE-1

INTRODUCTION

Sodium/hydrogen exchanger isoform-1 (NHE-1) plays an important role in regulating the function of the cardiovascular system such as the heart, platelets, blood vessels and the endothelium (Mentzer *et al.*, 2003; Rosskopf, 1999). Therefore, it has been repeatedly reported that NHE-1 is activated during cardiac ischemia, and contributes to the pathogenesis of ischemia/reperfusion-induced heart injury *via* multiple mechanisms of action (Karmazyn, 1999). NHE-1 inhibitors such as cariporide, eniporide, and sabiporide have been reported to have cardioprotective effects in various experimental models of ischemia/reperfusion heart injury either by inhibiting the electro-neutral exchange of intracellular H^+ for extracellular Na^+ and the resulting accumulation of intracellular Ca^{2+} *via* the reverse mode of the sodium/calcium exchanger (NCX) (Mentzer *et al.*, 2003; Karmazyn, 2001) or by delaying the acidification of the mitochondrial matrix and the exhaustion of ATP during ischemia (Ruiz-Meana *et al.*, 2003). It has been also shown that the

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platelet activation and aggregation evoked by various agonists such as thrombin, ADP, serotonin, AVP, epinephrine, PAF and collagen were inhibited to various degrees when extracellular Na^+ was replaced, the extracellular pH was decreased, or amiloride and its derivatives and NHE-1 inhibitors with less specificity were used. This indicates that NHE-1 plays a significant role of in regulating the function of platelets (Rosskopf, 1999; Roberts *et al.*; 2004).

Once a vascular injury occurs, the platelets are activated and adhere to the exposed subendothelium at the site of an injury (primary adhesion), which is followed by platelet-platelet adhesion (aggregation or cohesion) for thrombus growth *via* a complex multi-step process involving a wide variety of adhesive ligands and receptors on the platelet surface (Jackson *et al.,* 2003; Corti *et al.,* 2002, 2003). Of the many signaling processes that lead to platelet activation, the release of arachidonic acid, which leads to the formation of thromboxane (TX) A_2 *via* the cyclooxygenase (COX)-TXA_2 synthase pathway, is believed to be one of key components in the platelet response to different platelet agonists. This pathway is believed to be responsible for the change in the platelet shape and pseudopod formation as well as the platelet adhesion on the damaged vessel surface (Offermanns *et al.,* 1994; Klages *et al.,* 1999; Raychowdhury *et al.,* 1994). This suggests that the inhibition of TXA_2 synthesis or its action is theoretically an effective means for treating atherothrombotic disorders, as clinical evidence clearly shows that drugs such as aspirin, picotamide and ridogrel benefit those patients with acute coronary syndrome and myocardial infarction (The RAPT Investigators, 1994; Jneid *et al.,* 2003). cytosolic Ca^{2+} mobilization also plays an important role in platelet activation and aggregation. During platelet activation, the $[Ca^{2+}]_i$ assumes transient spike mobilization or elevated oscillatory flux as a result of either Ca^{2+} influx or its release from the intracellular stores and plays a key role in regulating the platelet responses to various agonists (Jackson *et al.,* 2003).

[5-(2-methoxy-5-chlorophenyl)furan-2-ylcarbonyl]guanidine (KR-32570), which is a newly synthesized guanidine derivative, was reported to exert a potent cardioprotective effect in ischemic rat and dog heart models *in vitro* and *in vivo* (personal communication). Therefore, this study examined the antiplatelet effects of KR-32570 on washed rabbit platelets activated by various agonists with special emphasis on the mechanisms of action underlying its inhibition of collagen-induced platelet aggregation.

MATERIALS AND METHODS

Chemicals

KR-32570 was synthesized at the Medicinal Science Division, Korea Research Institute of Chemical Technology (Daejeon, Korea). The bovine serum albumin (BSA), collagen and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). The calcium green-1/AM was purchased from Molecular Probes (Eugen, OR, U.S.A.). The thrombin and arachidonic acid were purchased from Chrono-Log Co. (Havertown, PA, U.S.A.). The TXB_2, PGD_2 and U46619 were purchased from Cayman Chemical Co. (Ann Arbor, MI, U.S.A.). The [^{3}H]Arachidonic acid (250 μCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). All other chemicals were of analytical grade.

Animals

New Zealand white rabbits were purchased from Sam-Tako Animal Co. (Osan, Korea) and acclimatized for 1 week at 24°C and 55% humidity, with access to a commercial pellet diet obtained from Samyang Co. (Wonju, Korea) and drinking water before the experiments *ad libitum*. The animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Chungbuk National University, Korea.

Rabbit washed platelet preparation

Blood was withdrawn from the ear arteries of the male New Zealand white rabbits and collected directly into 0.15 (v/v) of and anticoagulant citrate dextrose (ACD) solution containing 0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v). The washed platelets were prepared as described elsewhere (Jin *et al.,* 2005). Briefly, the platelet rich plasma (PRP) was obtained by centrifuging rabbit blood at 230 × g for 10 min. The platelets were sedimented by centrifuging the PRP at 800 × g for 15 min followed by washing with Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM Hepes, pH 6.5) containing 0.35% BSA and 0.4 mM EGTA (ethylene glycol bis(β-aminoethyl ether) N,N,N'N'-tetraacetic acid). The washed platelets were then resuspended in Hepes buffer (pH 7.4) and adjusted to 4×10^8 cells/mL.

Measurement of platelet aggregation *in vitro*

The level of platelet aggregation was measured using an aggregometer (Chrono-Log Co., Havertown, PA, U.S.A.) according to the turbidimetry method reported by Born and Cross (1963). Briefly, a washed suspension of rabbit platelets was incubated at 37°C for 4 min in an aggregometer with constant stirring at 1000 rpm before aggregation *via* the addition of collagen (10 μg/mL), thrombin (0.05 U/mL), arachidonic acid (100 μM), U46619 (1 μM) and thapsigargin (0.5 μM), respectively. The resulting aggregation, which was measured as the change in light transmission, was recorded for 10 min. The extent of the inhibition of platelet aggregation is

expressed as % of the control.

Measurement of arachidonic acid liberation

The level of arachidonic acid liberation was measured under the experimental condition where radio-labeled arachidonic acid was incorporated into phospholipids as previously described (Jin *et al.,* 2005). Briefly, the PRP was preincubated with [^{3}H]arachidonic acid (1 μCi/mL) at 37°C for 1.5 hr, and washed as described above. The [^{3}H]arachidonic acid pre-labeled platelets (4×10^8 cells/mL) were pretreated with 50 μM BW755C, which is a COX and lipoxygenase inhibitor, various concentrations of KR-32570 (10, 25 and 50 μM) and 50 μM $AACOCF_3$ at 37°C for 3 min in the presence of 1 mM $CaCl_2$ the platelets were then stimulated with collagen (50 μg/mL). The reaction was terminated by adding chloroform/methanol/HCl (200:200:1, v/v/v). The lipids were extracted and separated by TLC on silica gel G plates using the following development system: petroleum ether/diethyl ether/acetic acid (40/40/1, v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was measured by liquid scintillation counting.

TXA_2 synthase activity assay

The TXA_2 synthase activity was measured using PGH_2 as a substrate for TXA_2 synthase, as previously described (Jin *et al.,* 2005). Briefly, aliquots of PGH_2, in anhydrous acetone, were pipetted into glass tubes, and the acetone was then evaporated under a gentle stream of nitrogen. The PGH_2 was then re-dissolved immediately in ethanol. The platelet suspensions (4×10^8 platelets/mL) were preincubated in Hepes buffer at 37°C with 50 μM indomethacin for 3 min, and further incubated with the KR-32570 or imidazole (5 mM), which is a typical TXA_2 synthase inhibitor for 3 min prior to adding 5 μM PGH_2. The final ethanol concentration was 0.1% (v/v). Five min after PGH_2 addition, the incubations were terminated by adding cooled EGTA (2 mM) and centrifuged at 13000 × g at 4°C for 4 min. The amount of TXB_2 in the supernatants was measured using a commercial enzyme immunoassay kit according to the manufacturer's instructions (Amersham biosciences, Ltd., Little Chalfont, Buckinghamshire, UK). The TXA_2 synthase activity is reflected by the production of TXB_2.

Measurement of TXB_2 and PGD_2 generation

The level of TXB_2 and PGD_2 generation was measured under the experimental conditions where radiolabeled arachidonic acid was loaded as a substrate for the metabolic cascade in platelets, as previously described (Jin *et al.,* 2005). Briefly, the washed platelets (4×10^8 cells/mL) were preincubated with various concentrations of KR-32570 at 37°C for 3 min, and further incubated with a mixture of [^{3}H]arachidonic acid and the unlabeled arachidonic acid (2 μM, 1 μCi/mL) for 5 min. The reaction was quenched by adding a stop solution containing 2.6 mM EGTA and 130 μM BW755C (1-Phenyl-3-pyrazolidone (phenidone) and 3-amino-1-(m-(trifluoromethyl)-phenyl)-2-pyrazoline), a COX and lipoxygenase (LOX) inhibitor. The lipids were extracted and separated by thin layer chromatography (TLC) on silica gel G plates (Analtech, Delaware, U.S.A.) using the following development system: ethyl acetate/isooctane/acetic acid/H_2O (9:5:2:10, v/v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting (model LS 3801, Beckman, Buckinghamshire, UK).

Measurement of $[Ca^{2+}]_i$

The cytosolic Ca^{2+} measurements used fluorescent dye calcium green-1, which involved incubating the platelets with cell permeant acetoxymethyl ester. The rabbit platelets (isolated as described above) were incubated with 2 μM calcium green-1/AM at room temperature for 1 hr (on a rocking platform) in a loading buffer (137 mM NaCl, 27 mM KCl, 0.4 mM NaH_2PO_4, 10 mM HEPES, 12 mM $NaHCO_3$, 5.5 mM dextrose, 0.35% BSA, pH 7.4). The excess calcium green-1/AM was removed by centrifugation (500 × g for 10 min) and the platelets were suspended in fresh buffer, without added EGTA. Aliquots of the platelet suspension (2.5 mL) were added to 4 mL cuvettes containing a Teflon coated stirrer bar (Chrono-log, Havertown, PA, U.S.A.). Immediately before measuring the $[Ca^{2+}]_i$, EGTA was added back to the buffer to a final concentration of 4 mM. The $[Ca^{2+}]_i$ measurements were carried out at room temperature in a MSIII fluorimeter (Photon Technology International, S. Brunswick, NJ, U.S.A.) using excitation and emission wavelengths of 506 nm and 533 nm, respectively. $[Ca^{2+}]_i$ was calculated using the SPEX dM3000 software package.

Measurement of serotonin secretion

The serotonin concentration was determined using the fluorimetric method reported by Holmsen and Dangelmaier (Holmsen and Dangelmarier, 1989). The washed platelet suspension was pretreated with KR-32570 at 37°C for 3 min, and then with imipramine (5 μM) to prevent the reuptake of serotonin. Collagen (10 μg/mL) was then added. After 5 min, the reaction was quenched by adding 5 mM EDTA on ice and the supernatant was centrifuged at 12,000 × g for 2 min. The supernatant was mixed with 6 M trichloroacetic acid (TCA) and centrifuged at 12,000 × g for 2 min. A 0.6 mL aliquot of the TCA supernatant was mixed with 2.4 mL of the solution (0.5% *o*-phthalaldehyde in ethanol diluted 1:10 with 8 N HCl), placed in a boiling water bath for 10 min, and then cooled in ice. The excess

TCA was extracted with chloroform and the fluorophore was measured at excitation and emission wavelengths of 360 nm and 475 nm, respectively. Serotonin creatinine sulfate was used as a standard solution to calculate the level of serotonin release. 100% release of serotonin was determined by treating the platelets with Triton X-100.

Platelet swelling assay

Blood samples (9/1 blood/ACD solution, vol/vol %) were withdrawn from the rabbits by venipuncture of the ear vein into tubes containing an ACD solution (65 mM citric acid, 85 mM trisodium citrate, 2% dextrose). Each sample was centrifuged at 1300 rpm for 10 min at room temperature, and the platelet-rich plasma (PRP) was obtained from the upper two-thirds of the supernatant. The remainder of the blood sample was then centrifuged at 3000 rpm for 10 min to obtain the platelet-poor plasma (PPP). The platelets in PRP were counted in a hemocytometer using optical microscopy, and adjusted to 1×10^8 cells/mL (final counts). The platelet NHE-1 activity was measured using a minor modification of the method reported by Rosskopf *et al.* (1991). Briefly, the increase in light transmission associated with cell swelling was measured with an aggregometer (Chrono-Log 490 4D, Havertown, PA, U.S.A.). A Na propionate solution (250 μL, in mmol L^{-1}: Na propionate 135, HEPES 20, $CaCl_2$ 1, $MgCl_2$ 1, glucose 10, pH 6.7) in a cuvette was stirred at 1000 rpm and prewarmed to 37 for 5 min. An increase in the light transmission of PRP induced by platelet swelling was observed after applying PRP (50 μL). The PRP and Na propionate solution were mixed, which resulted in the production of an acidic intracellular pH in which the platelet NHE is activated. The increase in Na^+ influx associated with the excretion of cytosolic H^+ *via* NHE causes cellular swelling as a result of water accumulation in the cytoplasm (Rosskopf *et al.*, 1991). Light transmission through PRP was increased because the density of the cellular component decreases with swelling (Rosskopf *et al.*, 1991). This simple assay system was used to evaluate the effects of drugs on NHE-1, because NHE-1 is believed to be the dominant subtype of NHE in platelets. KR-32568 was added to the cuvette 3 min before adding PRP. A solution containing an equivalent mixture of PPP and Na propionate was used to correct for light transmission through the non-platelet portion of PRP. The changes in light transmission were recorded continuously at 37°C for 5 min, and rate constants were calculated from the slopes generated during the first 42 s, as described by Rosskopf *et al.* (1991). The inhibitory effect of KR-32560 at various concentrations is expressed as the rate constant percentage relative to the value obtained in the presence of the vehicle. The half-maximum inhibitory concentration (IC_{50}) value of KR-32560 was obtained from the slope of the linear part of the relationship between the log concentration and the NHE activity using linear regression analysis.

Statistical analysis

The results are expressed as a mean ± S.E.M. A one-way analysis of variance (ANOVA) was used for multiple comparisons (Sigma Stat®, Jandel Co., San Rafael, CA, U.S.A.). A Dunnett's test was applied if there was a significant variation between the treated-groups. A P value < 0.05 were considered significant.

RESULTS

Effect of KR-32570 on rabbit platelet aggregation *in vitro*

As shown in Fig. 1, KR-32570 inhibited the aggregation of rabbit platelets induced by collagen (10 μg/mL), thrombin (0.05 U/mL), arachidonic acid (100 μM), U46619 (1 μM), a TXA_2 mimic, and thapsigargin (0.5 μM), a Ca^{2+} ATPase inhibitor, in a concentration dependent manner with IC_{50} values of 13.8 ± 1.8, 26.3 ± 1.2, 8.5 ± 0.9, 4.3 ± 1.7 and 49.8 ± 1.4 μM, respectively. However, cariporide and sabiporide, which are two selective NHE-1 inhibitors with respective rapid and slow dissociation kinetics, showed very weak inhibition of collagen-induced platelet aggregation under the same experimental conditions (inhibition %: 4.6 ± 1.8 and 10.8 ± 0.8% at 100 μM, respectively).

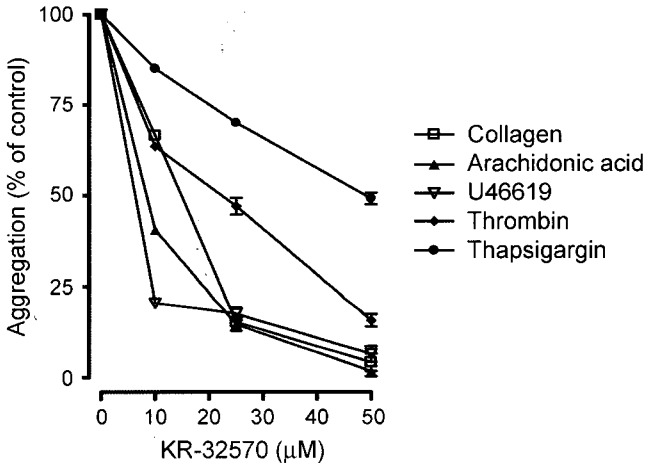

Fig. 1. Effect of KR-32570 on the aggregation of washed rabbit platelets. A washed rabbit platelet suspension was incubated at 37°C in an aggregometer with constant stirring at 1,000 rpm, followed by the addition of KR-32570. After 3 min preincubation, platelet aggregation was induced by the addition of thrombin (0.05 U/mL), arachidonic acid (100 μM), collagen (10 μg/mL), U46619 (1 μM) or thapsigargin (0.5 μM). The percentage aggregation is expressed as the % of the maximum aggregation induced by the respective inducers. The data is expressed as mean ± S.E.M. (n = 4).

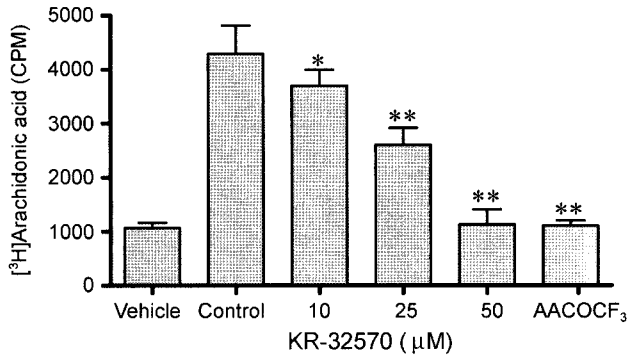

Fig. 2. Effect of KR-32570 on the collagen-induced liberation of arachidonic acid in rabbit platelets. [^{3}H]Arachidonic acid-prelabelled platelets were incubated with various concentrations of KR-32570 or $AACOCF_3$ for 2 min at 37°C in the presence of 50 μM BW755C, and then stimulated with 50 μg/mL collagen for 2 min. The [^{3}H]arachidonic acid liberated was determined as described in Materials and Methods. Each point is expressed as a mean ± S.E.M. (n = 3). *P < 0.05 and **P < 0.01 vs. corresponding stimulus control.

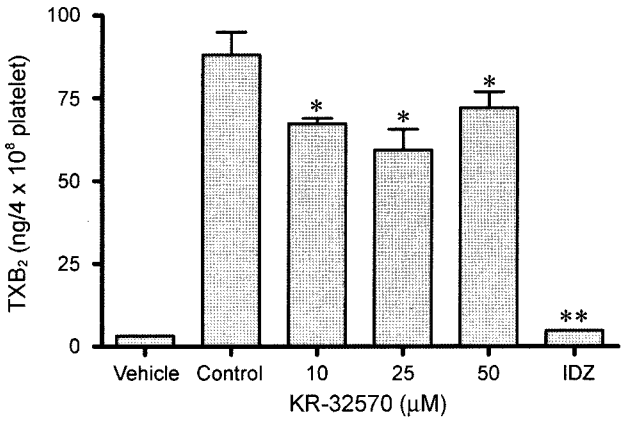

Fig. 3. Effect of KR-32570 on the TXA_2 synthase activity. After preincubating the platelets with indomethacin (50 μM) at 37°C for 2 min, a platelet suspension containing DMSO (0.1%), KR-32570 or imidazole (IDZ, 5 mM) was further incubated for 3 min, which was followed by the addition of 5 μM PGH_2. Five min after the addition of PGH_2, the incubations were quenched by adding cooled EGTA (2 mM) and centrifuging the mixture 13000 × g at 4°C for 4 min. The level of TXB_2 formation in the supernatants was determined by an enzymeimmunoassay. The TXA_2 synthase activity is reflected by the production of TXB_2, which is presented as a mean ± S.E.M. (n = 3). *P < 0.05 and **P < 0.01 vs. corresponding stimulus control.

Effect of KR-32570 on collagen-induced arachidonic acid liberation

As shown in Fig. 2, a pretreatment with KR-32570 at concentrations of 10, 25 and 50 μM inhibited the collagen-induced arachidonic acid liberation in the [^{3}H]arachidonic acid pre-labeled rabbit platelets by 18.2, 52.2 and 97.9%, respectively. $AACOCF_3$, which is a phospholipase A_2 inhibitor used as a positive control, almost completely (by 89.4%) blocked the liberation of arachidonic acid at a concentration of 50 μM.

Effect of KR-32570 on TXA_2 synthase activity

The conversion of arachidonic acid to TXA_2 in platelets requires the action of two enzymes, COX and TXA_2 synthase. TXA_2 synthase catalyzes the conversion of PGH_2 to TXA_2 in platelets. However, the use of PGH_2 allows the COX step to be circumvented during the arachidonic acid metabolism. The addition of increasing concentrations of PGH_2 to the washed rabbit platelet suspensions produced a concentration-dependent increase in TXB_2 (data not shown). Therefore, the washed rabbit platelet suspensions containing PGH_2 can be used for a direct evaluation of the TXA_2 synthase inhibitor. In the washed rabbit platelet suspensions, the level of TXB_2 in the unstimulated platelets was approximately 3 ng/4 × 10^8 platelets. After incubating the washed platelet suspensions with PGH_2 (5 μM) at 37°C for 5 min, the level of TXB_2 formation was increased to 92.2 ng/4 × 10^8 platelets. As shown in Fig. 3, KR-32570 at concentrations of 10, 25 and 50 μM inhibited the conversion of PGH_2 to TXB_2 by 27.9, 36.9 and 22.5 %in the washed rabbit platelet suspensions, respectively. Imidazole (50 mM), a typical TXA_2 synthase inhibitor, completely (by 98.9%) inhibited the conversion of PGH_2 to TXB_2.

Effects of KR-32570 on conversion of arachidonic acid to TXB_2 and PGD_2

As shown in Fig. 4A, after adding [^{3}H]arachidonic acid to the intact rabbit platelet suspension (inhibition percentage: 46.8, 36.6 and 35.5% at concentrations of 10, 25 and 50 μM, respectively), KR-32570 suppressed the generation of TXB_2 reflecting the formation of TXA_2 at all concentrations used. However, KR-32570 had no effect on the generation of PGD_2 (Fig. 4B). Indomethacin (50 μM), a COX inhibitor, almost completely abolished the production of TXB_2 and PGD_2.

Effect of KR-32570 on $[Ca^{2+}]_i$

Fig. 5 shows a representative trace, in which collagen was added to induce $[Ca^{2+}]_i$ mobilization. The effect of KR-32570 on $[Ca^{2+}]_i$ mobilization was observed after 3 min incubation with the platelets before adding collagen (10 μg/mL). Collagen induced a gradual but transient increase in $[Ca^{2+}]_i$ which reached at 200 μM after 4-6 min. Treating the platelet suspension with KR-32570 (50 μM) significantly inhibited (by 52%) the increase in $[Ca^{2+}]_i$ in response to collagen. The right panel shown in Fig. 5 indicates the average of 3 separate experiments, which is similar to that shown in the left panel.

Effect of KR-32570 on serotonin release

As shown in Fig. 6, pretreating the rabbit platelets with

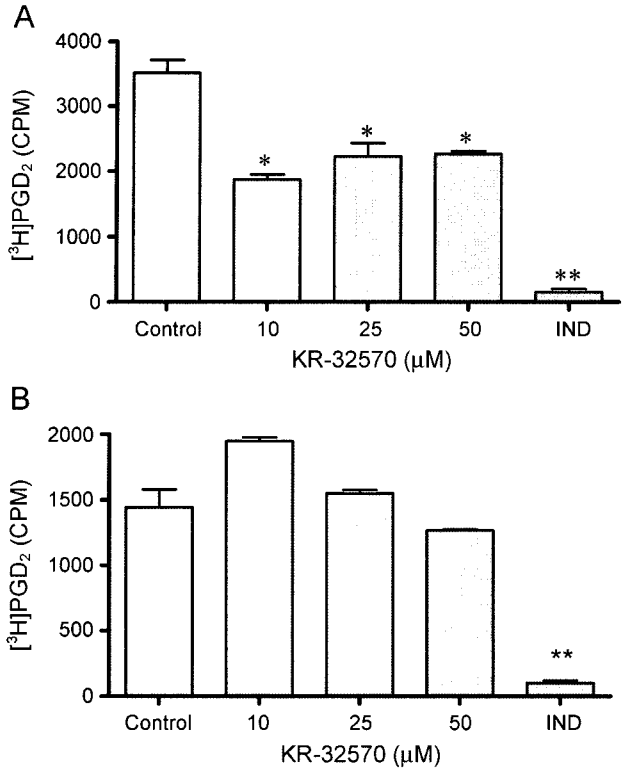

Fig. 4. Effects of KR-32570 on the conversion of arachidonic acid to TXB_2 and PGD_2. The washed rabbit platelets were preincubated with various concentrations of KR-32570 or indomethacin (IND, 50 μM) for 3 min without $CaCl_2$, and then further incubated with a mixture of [^{3}H]arachidonic acid and the unlabelled arachidonic acid (2 μM) for 5 min. The level of [^{3}H]thromboxane B_2 generation (A) and [^{3}H]prostaglandin D_2 generation (B) were measured as described in Materials and Methods. The data is expressed as mean ± S.E.M. (n = 3). *P < 0.05 and **P < 0.01 vs. the corresponding stimulus control.

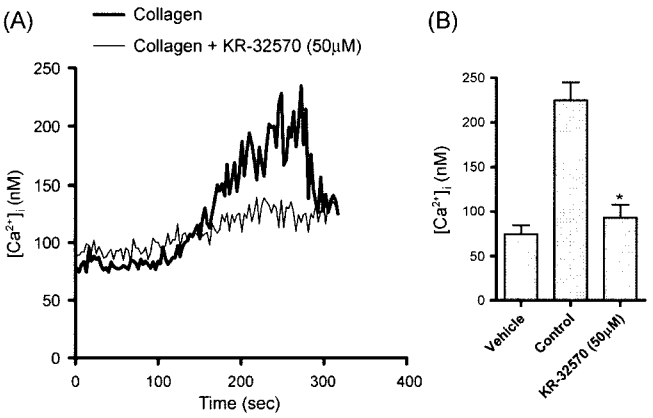

Fig. 5. Effects of KR-32570 on $[Ca^{2+}]_i$ in collagen (10 μg/mL)-stimulated rabbit platelet. The KR-32570 solution was added to yield a final concentration of 50 μM in the platelet suspension. Collagen (10 μg/mL) was added 3 min later. The traces (left panel) shown are from a representative experiment (A); similar results were obtained from three separate experiments and the average data are shown in the right panel (B). *P < 0.05 vs. corresponding stimulus control.

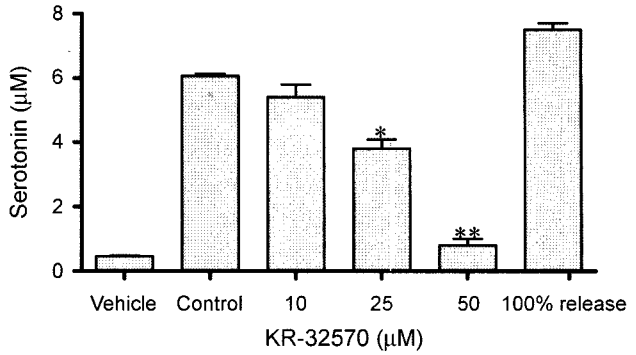

Fig. 6. Effect of KR-32570 on the collagen-induced secretion of serotonin in rabbit platelets. The washed platelet suspension was pretreated with various concentrations of KR-32570 at 37°C for 3 min in the presence of imipramine (5 μM) in order to prevent the reuptake of serotonin secreted from the dense-granule contents into the platelets. The suspension was then stimulated with 10 μg/mL collagen for 5 min. The amount of serotonin secreted was determined as described in Materials and Methods. Each point is expressed as a mean ± S.E.M. (n = 3). *P < 0.05 and **P < 0.01 vs. the corresponding stimulus control.

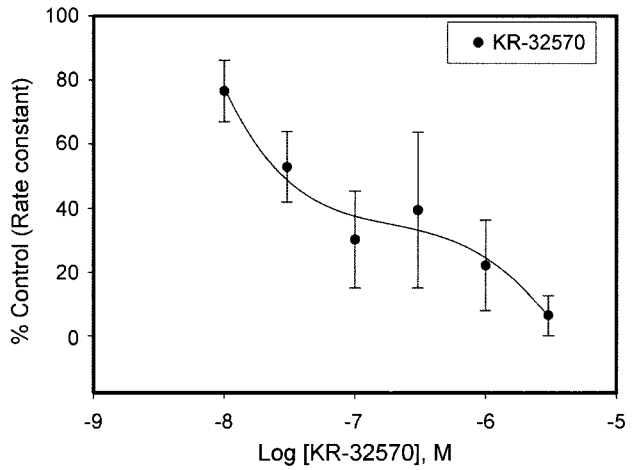

Fig. 7. Effects of KR-32560 on endogenous NHE-1-mediated rabbit platelet swelling induced by the application of Na propionate, which is expressed as the percentage of the swelling rate constant relative to the control rate constant. Values are represented as a mean ± SEM (n = 4).

KR-32570 at concentrations of 10, 25 and 50 μM inhibited the collagen (10 μg/mL)-induced release of serotonin from the dense-granule contents of platelets by 10, 36.7 and 88.3%, respectively.

Inhibition of NHE-1-mediated rabbit platelet swelling

KR-32570 inhibited the NHE-1-mediated platelet swelling induced by the intracellular acidification of the rabbit platelet rich plasma in a concentration-dependent manner (Fig. 7) The IC_{50} (KR-32570 concentration needed to decrease the rate of rabbit platelet swelling by 50%) was 0.036 μM.

DISCUSSION

This study evaluated the inhibitory effect of a novel NHE-1 inhibitor, KR-32570, on rabbit platelet aggregation along with its anti-platelet mechanisms of action. The platelet aggregation study (Fig. 1) showed that KR-32570 inhibited the aggregation of washed rabbit platelets induced by various platelet agonists in a concentration dependent manner, with potency in the order of U46619 > arachidonic acid > collagen > thrombin > thapsigargin. In addition, KR-32570 concentration-dependently inhibited the collagen-mediated liberation of arachidonic acid from the [^{3}H]arachidonic acid pre-labeled rabbit platelets, with complete inhibition being observed at 50 μM (Fig. 2). This shows the involvement of a blockade of arachidonic acid release and a metabolic cascade in the inhibitory effect of KR-32570 against collagen-induced platelet aggregation. KR-32570 was also found to significantly reduce the formation of TXB_2, which is a stable metabolite of TXA_2, from PGH_2. PGH_2 is a precursor of TXA_2, which was added to the collagen-unstimulated rabbit platelets that showed basal enzyme activity (Fig. 3). This indicates the inhibition of TXA_2 synthase by KR-32570. Therefore, it appears that the combined inhibition of TXA_2 synthase and the release of arachidonic acid by KR-32570 may contribute to the inhibitory effect on collagen-induced platelet aggregation. The finding that KR-32570 decreased the level of serotonin secretion from the granules in collagen-activated rabbit platelets in a concentration-dependent manner (Fig. 6), shows that the inhibitory effects of KR-32570 on collagen-induced platelet aggregation might in part be due to the inhibition of serotonin release or some other granular contents such as ADP and Ca^{2+} (Schlienger and Meier, 2003).

In the platelet aggregation study (Fig. 1), KR-32570 significantly inhibited arachidonic acid- and U46619 (a TXA_2 mimetic)-induced platelet aggregation, indicating the involvement of several sites of action (e.g., enzymatic conversion of arachidonic acid to TXA_2, TXA_2 receptor and postreceptor signaling pathway) either alone or in combination. As shown in Fig. 7, KR-32570 inhibited the formation of [^{3}H]TXB_2, which is a stable metabolite derived from TXA_2, in the [^{3}H]arachidonic acid-loaded intact rabbit platelets. However, it had no significant effect on the production of [^{3}H]PGD_2, indicating the selective inhibition of TXA_2 synthase rather than COX. This finding is in line with the results from the TXA_2 synthase assay showing that KR-32570 significantly reduced the formation of TXB_2 from PGH_2, which is a common precursor of PGs and TXA_2 (Fig. 3). However, considering that the accumulation of PGH_2 could result in platelet activation *via* the same receptor (TXA_2/PGH_2 receptor) as TXA_2, it also is possible that KR-32570 may also interfere with some components of TXA_2/PGH_2 receptor signaling pathway leading to platelet aggregation.

The experiment using calcium green-1/AM-loaded platelets showed that KR-32570 inhibited the cytosolic Ca^{2+} mobilization induced by collagen (10 μg/mL) at 50 μM, a concentration that almost completely inhibited platelet aggregation (Fig. 5). This indicates that the blockade of cytosolic Ca^{2+} mobilization makes a partial contribution to the antiplatelet activity of KR-32570. The inhibitory effect of KR-32570 on cytosolic Ca^{2+} mobilization has significant meaning because strong platelet agonists such as thrombin and collagen induce platelet aggregation through different mechanisms (different types of phospholipase: Cβ and Cγ2, respectively) leading to the release of Ca^{2+} from the intracellular Ca^{2+} stores *via* the breakdown of phosphoinositide (Jackson *et al.,* 2003; Lapetina, 1990). Therefore, it appears that the inhibitory effect of KR-32570 on phosphoinositide breakdown and the resulting decrease in $[Ca^{2+}]_i$ mobilization could lead to a significant blockade of arachidonic acid liberation by PLC and $cPLA_2$, which are the two main types of $[Ca^{2+}]_i$-sensitive enzymes responsible for liberating arachidonic acid, as shown in Fig. 2 (Balsinde *et al.,* 2002; Drayer *et al.*, 1995).

It is well known that the swelling of platelets from humans, rats and rabbits in the presence of intracellular acidosis is mediated by the activation of NHE-1 (Kusumoto *et al.,* 2002; Marala *et al.,* 2002; Knight *et al.,* 2001). Hence, the results from the rabbit platelet-swelling assay suggest that KR-32570 has an inhibitory effect on NHE-1, with a similar potency to cariporide and eniporide reported for rat and human platelets. In this study, unlike KR-32570, cariporide and sabiporide, which are two known selective NHE-1 inhibitors with rapid and slow dissociation kinetics, respectively (Touret *et al.,* 2003), did not have any potent inhibitory effects against collagen-induced platelet aggregation. This indicates that these three compounds have different potencies and modes of action.

The results from the platelet swelling test suggests another explanation for KR-32570-induced decrease in $[Ca^{2+}]_i$, the inhibition of the Ca^{2+} influx through the NCX functioning in a reverse mode, which leads to the inhibition of both arachidonic acid-liberating enzymes (e.g., $cPLA_2$) as well as platelet aggregation induced by collagen or other NHE-1 activating platelet agonists, which was recently demonstrated in collagen-treated human platelets using Ca^{2+}, Na^+ and pH sensitive fluorescent dyes (Roberts *et al.,* 2004). This suggests that KR-32570 could serve as an effective antiplatelet agent because both the transient and robust cytosolic Ca^{2+} fluxes play a key role throughout the thrombogenic process regardless of the activating stimulus (soluble agonist or adhesive substrate) (Jackson *et al.,* 2003). Platelet agonists such as epinephrine,

ADP, thrombin, collagen and PMA induce part of the Na^+ influx *via* the activation of NHE-1, which contributes both to the platelet activation due to an increase in Ca^{2+} *via* a reverse mode of NCX and to the procoagulant activity due to the release of microvesicles that serve as catalytic sites for the assembly of tenase and prothrombinase complexes (Rosskopf, 1999; Roberts *et al.*, 2004; Siffert, 1995; Stelmach *et al.*, 2002). Accordingly, KR-32570 with an inhibitory effect on NHE-1 might be a promising agent for inhibiting platelet aggregation and blood coagulation.

In summary, these results demonstrate that KR-32570 has significant inhibitory effects on platelet aggregation induced by various platelet agonists. In addition, the anti-platelet activity of KR-32570 may be mediated *via* the inhibition of $[Ca^{2+}]_i$ mobilization, which also leads to the inhibition of arachidonic acid liberation and the formation of its active metabolites in rabbit platelets. These inhibitory effects on platelet aggregation might enhance the therapeutic potential of this new compound as a cardioprotective agent against ischemic heart disease.

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