Inhibition of Thromboxane A₂-Induced Vasocontraction by KF4939, a New Anti-Platelet Agent, in Rabbit Mesenteric and Dog Coronary Arteries

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Abstract—The effect of a new anti-platelet agent, KF4939, on thromboxane A₂ (TXA₂)-induced vasocontraction was studied in superfused rabbit mesenteric and dog coronary arteries, in comparison with the effects on the contractions evoked by KCl, noradrenaline, serotonin, angiotensin II and histamine. The calcium sources involved in the TXA₂-induced vasocontraction were also examined. The TXA₂-induced contraction of the rabbit mesenteric artery was partly attenuated after exposure to the calcium-free medium, but was not attenuated by nifedipine. The TXA₂-induced contraction of the dog coronary artery was markedly attenuated by nifedipine. These results indicate that TXA₂ utilizes both intracellularly stored calcium and an extracellular source of calcium for its vasocontraction, and the voltage-dependent calcium channel plays an important role in the dog coronary artery, but in the rabbit mesenteric artery. KF4939 inhibited the TXA₂-induced contraction in both arteries. In the rabbit mesenteric artery, three times and more higher concentration than that to inhibit TXA₂-induced one were required to inhibit other agonist induced contractions. KF4939 caused no alteration in the KCl-induced contraction of both arteries. Thus, KF4939 seems to be a selective inhibitor of TXA₂-induced vasocontraction, and the receptor-linked mechanism may be a possible site of the TXA₂ antagonistic action of KF4939.
other agonists and sources of calcium involved in the vascular action of TXA₂ were also examined in order to clarify the mechanism of vascular action of KF4939.

A reaction mixture of blood platelets with arachidonic acid was used as the source of TXA₂. Since cumulative addition of the biosynthetic TXA₂ into the organ bath is impossible due to its instability, a superfusion technique which we have been using for the bioassay of TXA₂ was employed.

Materials and Methods

Isolated superfused arteries: Rabbit mesenteric artery or dog coronary artery was used for separate experiments. Male rabbits weighing 2-3 kg were allowed to bleed. A section of the superior mesenteric artery was removed and helically cut into strips with a width of 2-3 mm and a length of approx. 1.5 cm. Mongrel dogs of either sex weighing 7-12 kg were killed by intravenous injection of an over-dose of sodium pentobarbital, and the heart was rapidly removed. The circumflex branch of the left coronary artery was isolated from the heart and helically cut into strips with a width of 3-4 mm and a length of 1-1.5 cm. Both ends of the strips were tied with silk thread. Then the strips were mounted between a fixing rod and a force displacement transducer, SB-1T (Nihon Kohden), at a resting tension of 1.5 g and superfused with Krebs-Henseleit (K-H) solution at a constant speed, 10 ml/min, using a Harvard peristaltic pump (1210). The perfusing medium was continuously oxygenated with a 95% O₂+5% CO₂ gas mixture and maintained at a constant temperature of 37°C. The composition of the medium was (in mM): NaCl, 118; KCl, 4.75; CaCl₂, 2.54; KH₂PO₄, 1.19; MgSO₄ 7H₂O, 1.19; NaHCO₃, 12.5; and glucose, 10.0. Before the start of experiments, the arterial strips were allowed to equilibrate for 60-90 min. Isometric contractions were recorded on a polygraph RM-45 (Nihon Kohden). Vasocontracting substances were applied into the perfusion medium through a side cannula placed near the upper end of the strip. Test drugs were dissolved in the perfusing medium at stated concentrations and infused from 10 min before the first application of vasocontracting substance to the end of the last contractile response. When the contractile responses to TXA₂ were measured, K-H solution containing the following antagonists was used in order to determine their specificity for TXA₂. The antagonists were phenoxybenzamine hydrochloride (10⁻⁷ g/ml), diphenhydramine hydrochloride (10⁻⁵ g/ml), methysergide maleate (10⁻⁶ g/ml) and indomethacin (10⁻⁶ g/ml).

Some of the rabbit mesenteric arterial strips were submitted for the study on the effect of the removal of extracellular calcium on the contractions induced by TXA₂, KCl or NA. After the contractile responses to 0.6 mmoles KCl, 10 μg NA or TXA₂ generated by 5×10⁸ blood platelets were obtained in normal K-H solution, the perfusion medium was changed to calcium-free K-H solution containing 0.2 mM EGTA, and the contractile responses to each vasoconstrictor were obtained again.

Production of thromboxane A₂: Washed rabbit platelets were prepared according to the method of Hamberg et al. (13). Briefly, the blood was collected in a plastic tube containing 0.75 vol. of 77 mM EDTA. Platelet-rich plasma (PRP) was obtained by centrifugation at 200×g for 15 min. Then, platelets were sedimented by further centrifugation at 650×g for 15 min and washed with a washing medium composed of 90 parts of 154 mM NaCl, 8 parts of 150 mM Tris-HCl buffer (pH 7.4) and 2 parts of 77 mM EDTA. The platelet pellet was resuspended in calcium-free K-H solution at a concentration of 10⁹ cells/ml. The suspension (0.5 ml) was incubated with arachidonic acid (final concentration 0.02 mM) for 20 sec at 37°C using an aggregometer (Rikadenki, RAM-31) to form thromboxane A₂. Immediately after the incubation, the total volume of the reaction mixture was taken into a syringe and applied to the arterial preparations. Since there was a fear that the activity of platelets to form TXA₂ by the incubation with arachidonic acid may have varied, as time passed, the stability of the contractile response to the incubation mixture (as TXA₂) was checked. As shown in Table 1, no significant change was observed in the contractile response until 4 hr after
the start of the experiment. Therefore, all experiments were performed within 4 hr.

**Chemicals:** KF4939 was synthesized at Permachem Asia Co. (Tokyo) and was used as an aqueous solution in ethanol. Nifedipine was synthesized at the Pharmaceuticals Res. Lab. of Kyowa Hakko Kogyo, Co., Ltd. Arachidonic acid, sodium salt (Sigma), noradrenaline (Noradrenalin®, Sankyo), potassium chloride (Kanto Chemical), serotonin creatinine sulfate (Tokyo Kasei), histamine dihydrochloride (Wako Pure Chemical) angiotensin II (Nakarai Chemicals) phenoxymethyl benzamine hydrochloride (Tokyo Kasei), diphenhydramine hydrochloride (Vena®, Tanabe), methysergide maleate (Sandoz) and indomethacin (Sigma) were used.

**Results**

**Contractile responses to thromboxane A₂ and several other agonists in superfused arteries:** In the rabbit mesenteric artery, the reaction mixture of rabbit platelets with arachidonic acid (as TXA₂) caused a fast and transient contractile response, which was dependent on the number of the platelets. Fast and transient contractions were also dose-dependently caused by 0.2–0.8 mmoles KCl, 0.3–10 µg noradrenaline (NA), 0.1–10 mmoles serotonin (5HT), 0.03–0.3 mmoles angiotensin II (Ang II) and 0.1–1 moles histamine (Hist) (Fig. 1). The amplitude of the response to TXA₂ (5×10⁸ cells) was 1.50±0.16 g (n=14). The amplitude of the responses to other agonists were as follows: 0.6 mmoles KCl (1.02±0.17 g, n=7), 10 µg NA (1.45±0.11 g, n=15), 10 mmoles 5HT (1.16±0.15 g, n=6), 0.3 mmoles Ang II (1.45±0.11, n=7) and 1 µmole Hist (1.26±0.07, n=4).

In dog coronary artery, TXA₂ caused a contraction which was dependent on the number of the platelets, similar to that in rabbit mesenteric artery. The amplitude of the response to TXA₂ (5×10⁸ cells) was 0.82±0.14 g (n=6). When KCl below 1 mmole was applied by a bolus injection, the contractile response obtained was very small.

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**Fig. 1.** Typical tracing of contractile responses of superfused rabbit mesenteric artery to thromboxane A₂, KCl, noradrenaline, serotonin, angiotensin II and histamine. Thromboxane A₂ was produced by the incubation of rabbit platelets with arachidonic acid for 20 sec at 37°C using an aggregometer.
or absent. On the other hand, when KCl was applied cumulatively with a continuous infusion, a dose-dependent contraction was obtained. A maximum contraction was achieved by 30 or 60 mM KCl. Therefore, 30 mM KCl was applied with continuous infusion for 10 min in the following experiments.

Effects of KF4939 on vasoconstrictions induced by thromboxane A$_2$ and several other agonists: In the rabbit mesenteric artery, KF4939 at concentrations of 7 x 10$^{-6}$ M and more caused a significant inhibition in the contractile response to TXA$_2$ in a non-competitive fashion.

IC$_{50}$ (the concentration required to decrease by 50% the response to agonist) of KF4939 against TXA$_2$ which was formed by 5 x 10$^8$ cells was 4.76 x 10$^{-6}$ M. The effects of KF4939 on the dose-response curves for NA, Ang II, 5HT and Hist were also examined. KF4939 at a concentration of 7 x 10$^{-6}$ M caused no significant alteration in the dose-response curves for all agonists used, and at a concentration of 2.4 x 10$^{-5}$ M, it caused a significant inhibition only in the Hist-induced contraction. A significant inhibition in the contractile responses to other agonists was recognized in the presence of only 7 x 10$^{-5}$ M KF4939 (Fig. 2). The contractile response to 0.6 mmoles KCl was not altered by KF4939 even at 7 x 10$^{-5}$ M (Fig. 3).

In the dog coronary artery, KF4939 at concentrations of 10$^{-6}$ M and more caused a
significant inhibition in the TXA2-induced contraction, in a non-competitive fashion (Fig. 4). The IC50 of KF4939 against TXA2 (5×10^8 cells) was 2.1×10^-6 M. This value was lower than that obtained in the rabbit mesenteric artery. The contractile response evoked by 30 mM KCl was not altered by KF4939 even at 10^-5 M (Fig. 5).

Role of external calcium in TXA2-induced vasocontraction: The contractile response to TXA2 in the rabbit mesenteric artery was gradually reduced under perfusion with the calcium-free medium containing 0.2 mM EGTA. It, however, still remained after exposure to the calcium-free medium for 20 min. When the perfusing medium was returned to normal K-H solution, the response to TXA2 was readily recovered (data not shown). After exposure to the calcium-free medium for 20–25 min, the magnitude of the contraction induced by TXA2 was 43.7% of that seen in normal K-H solution. The contraction induced by NA also remained at 29.6% of that seen in normal K-H solution. The contraction evoked by KCl was nearly completely abolished. Nifedipine (3×10^-7 M) used in normal K-H solution almost completely inhibited the KCl-induced contraction, but caused no significant alteration in the both TXA2 and NA-induced contractions (Fig. 6).

In the dog coronary artery, both the TXA2- and KCl-induced contractions were markedly attenuated by nifedipine (Figs. 7 and 8).

Discussion

This study clarified that KF4939 has the...
ability to inhibit the action of TXA2 in arteries, like it does in platelets. There are few reports on the mode of action of TXA2 synthesized biologically. It, however, may be possible to consider that TXA2 contracts arteries through its receptor activation from the studies using its stable analogue cTXA2 (11). It is well known that NA, 5HT, Ang II and Hist also evoke the contractions through an activation of each specific receptor. We confirmed that the NA-, 5HT and Hist-induced contractions in the superfused rabbit mesenteric artery were antagonized by phentolamine, methysergide and diphenhydramine, respectively (data not shown). Among these receptor-mediated vasoconstrictions, the TXA2-induced one was selectively attenuated by KF4939. The threshold concentration of KF4939 to inhibit the TXA2-induced contraction was approximately one-third or less of that to inhibit other agonist-induced contractions. Thus, the TXA2 receptor activation-contraction coupling mechanism seems to be most susceptible to KF4939.

TXA2 is known as one of the most potent proaggregators of blood platelets and vasoconstrictors. Because calcium has a very significant role in platelet aggregation and vasocontraction, it may be important to clarify the movement of calcium in the two physiological actions of TXA2 in order to elucidate the mechanism of the drug effect on both of these actions.

It is generally accepted that the contractile machinery in vascular smooth muscle is activated by a rise of the cytoplasmic free calcium concentration (14). A transmembrane flux of calcium and a release from intracellular storage sites are considered as potential sources of the activating calcium. Calciums from both sources are mobilized in the NA-induced contraction. It seems that the initial phase of the NA-induced contraction (phasic contraction) is caused by calcium from the intracellular source and the maintained contraction (tonic contraction) by calcium from the extracellular source. On the other hand, the contractions elicited by high potassium depolarization are almost dependent on extracellular calcium (15, 16). Nifedipine is known as a calcium channel blocker. The contraction evoked by KCl was suppressed by nifedipine to a greater extent than that induced by NA. This agent blocks the calcium influx evoked by depolarization, but does not affect the receptor-operated calcium influx in rabbit mesenteric arteries (17). Most of all the experiments on vasocontraction in isolated arteries were performed using an organ bath method, and there are few reports using a superfusion technique. Therefore, we examined the calcium sources utilized for the contractile response to TXA2 in comparison with those to NA and KCl in superfused arteries using nifedipine and a calcium-free medium. Both NA and KCl caused fast and transient contractile responses in rabbit mesenteric artery in a dose-related manner. The contractile response to KCl was completely abolished after exposure to the calcium-free medium and almost complete inhibition was induced by nifedipine. The NA-induced contraction was partly di-

![Fig. 7. Effect of nifedipine on the contractile response of the dog coronary artery to thromboxane A2. Experimental procedures were the same as described in the legend for Fig. 4. Each point represents the mean±S.E. of 6 experiments. *P<0.05, **P<0.01: significantly different from the control.](image)

![Fig. 8. Effect of nifedipine on the KCl-induced contraction of dog coronary artery.](image)
minished after exposure to the calcium-free medium; however, it was not altered by nifedipine. These evidences indicate that the contractile response to KCl depends on the influx of calcium from the extracellular medium through the voltage-dependent calcium channel, and the response to NA depends on both the influx of calcium from the extracellular medium through the receptor-operated calcium channel and the release of calcium from the intracellular storage sites.

Toda (12) discussed that the increase in the transmembrane influx of calcium as well as the release of calcium from intracellular storage sites is involved in the cTXA2-induced contraction of dog mesenteric arteries, from the evidence that the contraction of arteries exposed to calcium-free medium was about half of the contraction obtained in normal medium. In this investigation, a similar attenuation of the TXA2-induced contraction after exposure to the calcium-free medium was observed in rabbit mesenteric artery. No significant inhibition, however, was caused by nifedipine. From these results, we may conclude that the TXA2-induced contraction of rabbit mesenteric artery depends on both the influx of calcium from the extracellular medium through the receptor-operated calcium channel and the release of calcium from intracellular storage sites, similar to the NA-induced contraction. The cTXA2-induced contraction of coronary artery is dependent on an influx of extracellular calcium through the voltage-dependent channel (11). Since the contraction induced by TXA2 in dog coronary artery was inhibited by nifedipine, it seems to be dependent on the voltage-dependent influx of extracellular calcium, like cTXA2.

KF4939 caused no significant inhibition in the KCl-induced contraction both in rabbit mesenteric and dog coronary arteries. Therefore, a block of the voltage-dependent calcium channel and non-specific inhibition of the final common mechanism of contraction after establishment of elevated cytoplasmic-free calcium level can be excluded from the mechanism of vascular action of KF4939. KF4939 inhibited both the TXA2-induced contractions which were dependent (in dog coronary arteries) and independent (in rabbit mesenteric arteries) of the voltage-dependent calcium influx. The sources of calcium involved in the TXA2-induced contraction of the rabbit mesenteric artery were similar to NA and may be similar to other receptor agonists (18). Nevertheless, KF4939 showed a selective inhibition in the TXA2 induced one. In addition, because the inhibition was in a non-competitive fashion, the competition on the receptor with TXA2 is improbable. Consequently, it can be presumed from these evidences that the TXA2 receptor-linked mechanism may be the most probable site of the TXA2 antagonistic action of KF4939. However, because high concentration of KF4939 inhibited the contractile responses not only to TXA2, but also to other receptor agonists in the mesenteric artery, non-specific inhibition of the receptor-operated calcium influx and/or the release of calcium stored in the cell may also be involved in the mechanism of vascular action of KF4939. TXA2 contracts the coronary arteries. The increase of plasma TXA2 levels in patients with ischemic heart disease has also been reported (19, 20). Thus, TXA2 has been suggested as a possible mediator of coronary vasospasm. Accordingly, the property of KF4939 to inhibit TXA2-induced vasoconstriction may afford another benefit for the therapy of this disease, in addition to its anti-platelet action.

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