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Stretch-induced contraction of rabbit isolated pulmonary artery and the involvement of endothelium-derived thromboxane A_2

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1 The mechanism of stretch-induced contraction of the intrapulmonary artery of rabbit was studied with special regard to the endothelium-dependence and production of prostanoids.

2 Isolated intrapulmonary artery of rabbits in ring form produced contraction when stretched slowly up to 180% of its initial muscle length (=100%) at a rate of 0.44 mm s^{-1}, with a stimulus period of 5 min.

3 The stretch-induced contraction was attenuated by the mechanical removal of the endothelium, inhibitors of cyclo-oxygenase such as aspirin and indomethacin, [1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino) carbonyl] hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ 29,548), which is a thromboxane A_2/prostaglandin H_2 receptor antagonist, or by ozagrel, an inhibitor of thromboxane A_2 synthase.

4 Biochemical assay indicated that the production of thromboxane B_2, a stable metabolite of thromboxane A_2, was increased 17 times in response to stretch only when the endothelium was intact. The production of thromboxane B_2 was also inhibited by aspirin or ozagrel.

5 The production of 6-keto prostaglandin $F_{1\alpha}$, a stable metabolite of prostacyclin, was also increased in response to stretch in the preparation with intact endothelium. However, ozagrel showed no apparent effect on the production of 6-keto prostaglandin $F_{1\alpha}$.

6 These results suggest that a mechanical stimulus like stretch can act on endothelial cells of rabbit pulmonary artery to cause contraction by activation of arachidonic acid metabolism via the cyclo-oxygenase pathway and subsequent release of thromboxane A_2 and/or an increase in the ratio of thromboxane A_2/prostacyclin.

Keywords: Rabbit intrapulmonary artery; stretch-induced contraction; endothelium-derived thromboxane A_2; ozagrel; pulmonary circulation

Introduction

In addition to pharmacological receptor stimulation, mechanical stimulation, including stretch and pressure, have been considered as a regulatory factor for vascular contraction and relaxation (Bayliss, 1902). Mechanical factors have been also implicated as a stimulus for the induction of hypertrophy and/or hyperplasia in the cardiovascular system under physiological and pathological conditions (Morgan *et al*., 1987). The contractile reaction of vascular tissue to mechanical force such as stretch is a kind of physical response in the circulatory system and thus requires cellular signal transduction including Ca^{2+} mobilization. As to such a mechano-sensitivity, a rate-dependent excitation of the vascular tissue has been described (Johansson & Mellander, 1975), i.e., a dynamic/quick stretch stimulus, operating during the period of increasing muscle length, was more effective in causing excitatory responses than those evoked by a static/slow stretch stimulus (a slow elongation). We previously demonstrated that the contraction of cerebral and coronary arteries of various animal species, including rabbits, dogs, cats and pigs, produced in response to quick stretch (10 cm s^{-1}), was myogenic in nature (Nakayama, 1982; Nakayama *et al*., 1989; Tanaka & Nakayama, 1991) and that the contraction could be mediated through transmembrane influx of Ca^{2+} and the release of Ca^{2+} from intracellular storage sites (Nakayama *et al*., 1986; Tanaka *et al*., 1994a,b).

The interplay between endothelium and medial smooth muscle is considered to be important for the regulation of vascular contraction and relaxation under physiological and pathological conditions. In the pulmonary circulation, acetylcholine (ACh), a well-known endothelium-dependent vasodilator in the systemic circulation, functions as a vasoconstrictor and vasodilator, depending on the level of pre-existing vascular tone in the pulmonary circulation (see Barnes & Liu, 1995). The vasoconstrictor action of ACh was shown to be mediated by endothelium-derived thromboxane A_2 (TXA_2) in the rabbit pulmonary artery (Buzzard & Pfister, 1993; Altiere *et al*., 1986, 1994).

However, the role of endothelium in the ontogeny of the contractile response of vascular tissues in response to a mechanical stimulation such as stretch or pressure is still controversial. For instance, a quick/rapid stretch applied to pulmonary arteries of the guinea-pig (Belik, 1994), intrapulmonary arteries of cats (Kulik *et al*., 1988) and cerebral arteries of various animal species, including rabbits (Nakayama, 1982; Nakayama *et al*., 1986, 1989; Tanaka & Nakayama, 1991; Nakayama & Tanaka, 1993), produced myogenic contractions, whereas a slow/static stretch at about 0.3 mm s^{-1} or slow pressurization produced endothelium-dependent contraction in canine basilar (Katsusic *et al*., 1987), carotid (Rubanyi, 1988), and feline cerebral (Harder, 1987) arteries. In contrast, the contraction in response to slow stretch or pressure in rabbit ear arterioles (Hwa & Bevan, 1986) and rat posterior cerebral arteries (McCarron *et al*., 1989) was not dependent on the endothelium.

In the present study, we compared the effects of mechanical stretch on the intrapulmonary artery of the rabbit with those of ACh with special regard to the contractile activity and production of prostanoids, such as TXA_2 and

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prostacyclin. Our experimental results showed that a slow stretch (at a rate of less than 1 mm s^{-1}) can lead to augmentation of arachidonic acid metabolism and generation of contraction in an endothelium-dependent manner. A part of the present work has been published in a preliminary form (Ueta *et al*., 1995).

Methods

Experiments were performed on blood vessels isolated from Japanese White rabbits of either sex, weighing 2.0–3.5 kg. The animals were treated as approved by the Institutional Animal Care and Use Committee and the Guideline of Animal Experiments established by the Japanese Pharmacological Society. Animals were given heparin (200 u kg^{-1}) via an ear vein and lightly anaesthetized with pentobarbitone sodium (25 mg kg^{-1}, i.v.). The rabbits were killed by rapid exsanguination from the common carotid arteries; and after the chest was opened, the lungs and heart were removed as a whole and immersed in Tyrode solution consisting of the following (in mM): NaCl 158.3, KCl 4.0, $CaCl_2$ 2.0, $MgCl_2$ 1.05, $NaHCO_3$, 10.0, NaH_2PO_4 0.42 and glucose 5.6. The solution was bubbled with 97% O_2 and 3% CO_2 and kept at pH 7.35 at 37°C. The first branch of the intrapulmonary lobular artery having an outer diameter of 2 mm, i.e., about 1.5 cm distant from the hilum of right and left lungs, was carefully isolated. Each artery was cleared of fat and connective tissue under a dissection microscope and cut into a pair of ring segments about 2 mm wide for functional experiments, or about 4 mm wide for biochemical experiments. In experiments designed to examine the effect of removal of endothelium, the intimal layer of the artery was rubbed with a moist cotton pledget.

Repeated histological examinations of the intimal surface of the arteries with and without endothelium were carried out by scanning electron microscopy (S-500, Hitachi, Tokyo, Japan) (Nakayama, 1988). The microscopic observation indicated the absence of not only endothelial cells but also platelet or macrophages that may have adhered to the luminal surface. The effectiveness of the endothelial removal was also established functionally by the absence of ACh (30 nM)-induced relaxation of the artery preconstricted with 1 μM prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).

Recording of isometric tension

Two L-shaped tungsten wires (150 μm diameter) were inserted through the lumen of the rings. The lower wire was attached to a supporting hook, and the upper one was connected to the lever of a mechanoelectric transducer (T7-30-240, Orientec, Tokyo, Japan). Each artery was mounted in an organ bath containing 10 ml of Tyrode solution. Isotonic high K^+ solution was prepared by replacing the NaCl with an equimolar amount of KCl. Ca^{2+}-free Tyrode solution was prepared by omitting $CaCl_2$ and adding 0.2 mM EGTA. The Tyrode solution was bubbled with 97% O_2 and 3% CO_2 and kept at pH 7.35 and at 37°C. The artery in the organ bath was allowed to equilibrate for at least 60 min under an arbitrarily chosen passive load of 24.5 mN, at which the contractile responsiveness was intermittently ascertained by 80 mM KCl. During the equilibration period the bath fluid was exchanged at 20 min intervals with fresh solution. All agonistic and depolarizing stimuli were given to the artery segment at the optimally-stretched condition (1.8 Li, see later section).

All signals from the mechanotransducer were amplified with a biological amplifier (6M82, NEC-San-ei, Tokyo, Japan) and recorded on a pen-writing recorder (8K-1-S, NEC-San-ei, Tokyo, Japan). Contractions of pulmonary arteries in response to agonistic or depolarizing stimuli were expressed as tension developed (mN) per circumferential length of the ring segment (mm).

Experimental protocol for slow stretch activation of pulmonary artery

After an accommodation period of about 1 h in control medium, the transducer support attached to a micromanipulator was lowered once and then raised until any further increase in length of the artery caused a measurable increase in tension, as assessed by the procedure previously described for determination of the length-tension relationship (Nakayama, 1988). The length of the ring segment at this stage, i.e., the unloaded passive condition, was determined from the initial muscle length (Li = 1.0 or 100%), which corresponds to the following general formula: $Li = 2a + 2b(\pi + 2)$, where a = distance (in mm) between the inner edges of the upper and lower tungsten wires that were parallel to each another; b = diameter of tungsten wire (0.15 mm in the present case). For the ring segments, Li is the distance around the inside edge of the ring, i.e., the inside circumference assuming the segment to be supposedly cylindrical in shape. After determination of Li, the artery was equilibrated once more for about 1 h and the actual experiments were then started. The amount of stretch was adjusted with the micromanipulator and the rate of static stretch was kept constant at 0.44 ± 0.01 mm s^{-1}, which was determined in a selected number of preparations ($n = 14$). In usual experiments, an amount of stretch equal to 1.8 Li, i.e., 180% of the initial muscle length (= 100%), given to the artery segment evoked a contraction that became stable within 5 min (see Figure 1), such that the stretches could be repeated at 65 min intervals with a stimulus period of 5 min to yield reproducible responses. For assessment of drug actions, enzyme inhibitors, such as aspirin, indomethacin or ozagrel, and a thromboxane A_2/prostaglandin H_2 receptor antagonist (SQ 29,548), were added at 40 min and 5 min, respectively, before stretch. For quantitative analysis of the stretch-induced contraction (Nakayama, 1982), the area under the tension curve above the control resting level for the 5 min period was measured on the recording chart with a planimeter. The passive increase in tension elicited by stretch was defined as the state of response to stretch after administration of 10 μM papaverine, which could totally eliminate the active contractile response. The active mechanical response during stretch was obtained by subtracting the area corresponding to the increase in passive tension from the total circumscribed area, divided by the wet weight of the tissue, and was expressed as the contractile activity. The dimension of unit of the activity was the product of force and time.

Measurement of thromboxane and prostacyclin released from the artery

TXA_2 and prostacyclin were measured in the bathing fluid as their stable metabolites, thromboxane B_2 (TXB_2) (Code No. RPN 220) and 6-keto-$PGF_{1\alpha}$ (Code No. RPN 221), respectively, by use of enzyme immunoassay kits (Amersham Life Science, Buckinghamshire, U.K.). The lower limit of detection for TXB_2 was 3.6 pg ml^{-1} and that for 6-keto $PGF_{1\alpha}$ was 3.0 pg ml^{-1}. All control media containing various agents used in the present study had no apparent effect on the measurement. In order to avoid the effect of mechanical stretch, the artery segment was relaxed to the initial muscle length when measurements of thromboxane and prostacyclin in response to ACh were performed. The enzyme inhibitor or receptor antagonist was added in a similar manner as in the tension-recording experiments. For measurement of TXB_2 and 6-keto-$PGF_{1\alpha}$ released from the artery, a 150 μl volume of bathing fluid (total 10 ml) was collected 30 s before agonistic or stretch stimulation and the concentrations were determined and designated as basal values. Another 150 μl of bathing fluid was also collected 5 min after the start of the agonistic or stretch stimulation. The volume of bathing fluid was kept constant (total 10 ml) by addition of the same volume of control media each time after the collection of a sample. All the collected samples were stored at −20°C until assayed. The

TXB_2 and 6-keto-$PGF_{1\alpha}$ values, determined in duplicate, were normalized to the tissue wet weight, i.e., pg min^{-1} mg^{-1} tissue wet weight. In some pilot studies, prostaglandin E_2 (RPN 22) and prostaglandin $F_{2\alpha}$ (TRK 900) were also measured in the bathing fluid by Amersham assay systems.

Drugs

The following pharmacological agents were used: atropine sulphate, EGTA (ethylene glycol-bis(β-aminoethylether)N,N,N′,N′-tetraacetic acid), indomethacin, nicardipine hydrochloride, propranolol hydrochloride, (−)-isoprenaline hydrochloride, tetrodotoxin (Sigma, St. Louis, MO); acetylcholine chloride (Daiichi, Tokyo, Japan); acetylsalicylic acid (aspirin), prostaglandin $F_{2\alpha}$, papaverine hydrochloride (Wako, Osaka, Japan); phentolamine mesylate (CIBA-Geigy); chlorpheniramine maleate (Schering); ketanserin tartrate (Kyowa Hakko, Tokyo, Japan); the thromboxane A_2 receptor antagonist [1S-[1α,2α(Z),3α,4α]]-7-[3-[[2-[(phenylamino) carbonyl]hydrazino]methyl-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ 29,548) (Research Biochemicals International, Natick, MA), and ozagrel ((E)-3-[4-(1-imidazoylmethyl)phenyl]-2-propenate) (OKY-046); Kissei, Matsumoto, Japan). Aspirin and indomethacin were dissolved in ethanol and 0.1 M sodium bicarbonate, respectively. SQ 29,548 was dissolved in ethanol. All drugs were further diluted with distilled water. Assay systems for prostanoids were obtained from Amersham Life Science, Tokyo, Japan. The molar concentration (M) in the bathing solution is given for all drugs used.

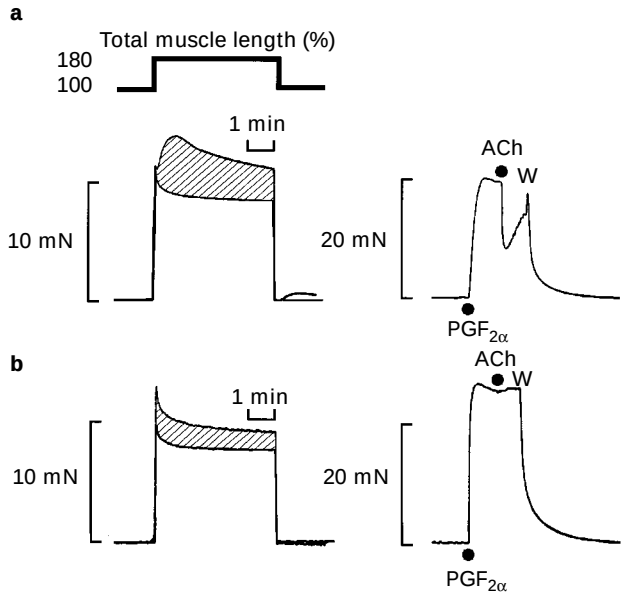


Figure 1 Typical mechanical responses of rabbit intrapulmonary arteries with and without endothelium to stretch and ACh. (a) Tracing show the effects of stretch (left panel) and ACh (right panel) on mechanical responses of the pulmonary arteries with intact endothelium. Active tension is shown as the hatched area of the mechanogram in response to stretch to 1.8 Li (180% of the initial muscle length = 100%) with a stimulus period of 5 min, in which tension was superimposed on the passive increase in tension after administration of 0.1 mM papaverine. The artery preconstricted with $PGF_{2\alpha}$ (1 μM) was relaxed with 30 nM ACh in the presence of endothelium. (b) Tracings show a reduced active mechanical activity in response to stretch (left panel) and no apparent relaxation produced by 30 nM ACh in the arteries without endothelium. Procedures for stretch and agonistic stimuli were the same as in (a). In (a) and (b), two pairs of cylindrical artery segments obtained from the same rabbit were used. W, washout.

Statistical analysis

The data are expressed as the mean ± s.e.mean. EC_{50} (concentration of agonist required to induce half the maximum response) was determined by probit analysis (Finney, 1952). Statistical analyses were done by paired or unpaired Student's *t* test. To determine whether responses differed significantly between groups, we performed Tukey's multiple range tests after analysis of variance (ANOVA). Differences were considered significant when the *P* value was less than 0.05.

Results

Characteristics of mechanical responses to slow stretch and acetylcholine and their dependence on endothelium

Figure 1 shows the typical mechanical response of a pair of ring segments of rabbit pulmonary artery in the presence (a) and absence (b) of endothelium to stretch by the standard procedure described in the Methods section, i.e., a rate of stretch of 0.44 mm s^{-1}, an amount of stretch of 1.8 Li, i.e., 180% of the initial muscle length (= 100%), and a stimulus period of 5 min given at 65 min intervals. An initial rise in tension occurred with the stretch and the subsequent fall at the completion of stretch was followed within about 15 s by a delayed contraction that reached a maximum about a minute after application of the stretch. The hatched area of the mechanogram shows the active tension that was superimposed on the passive increase in tension after repeated stretches in a medium containing 100 μM papaverine (Figure 1a, left). In contrast, the contraction in response to stretch was strongly attenuated when the endothelium was mechanically removed (Figure 1b, left). In accordance with the endothelium-dependent response to stretch, a small amount of acetylcholine (ACh), i.e., 30 nM, produced relaxation of the artery preconstricted with 1 μM $PGF_{2\alpha}$ only when the endothelium was intact (Figure 1a, b, right).

Table 1 summarizes the mechanical responses to various stimuli of ring segments of rabbit pulmonary arteries with and without endothelium. The stretch-induced contraction and the relaxation of $PGF_{2\alpha}$-induced contraction upon addition of ACh (30 nM) were significantly reduced in arteries without endothelium ($P < 0.05$ for stretch-induced contraction and

Table 1 Contractile responses to stretch, 80 mM KCl and 1 μM $PGF_{2\alpha}$ and relaxation produced by 30 nM ACh, in ring segments of rabbit pulmonary arteries with and without endothelium

	Contractile activity [1] (unit mg^{-1})	*KCl 80 mM* [2] (mN mm^{-1})	*$PGF_{2\alpha}$* [2] (mN mm^{-1})	*ACh* [3] (%)
Endothelium (+)	0.87 ± 0.15	11.67 ± 1.96	7.26 ± 1.86	57.38 ± 4.89
Endothelium (−)	0.31 ± 0.06*	11.87 ± 1.57	11.08 ± 1.47*	2.27 ± 1.01**

[1]Contractile responses to stretch (amount of stretch: 180% of the initial muscle length = 100%, stimulus period of 5 min with 65 min intervals) are expressed as contractile activity (unit mg^{-1} tissue wet weight). [2]Contractions in response to 80 mM KCl and 1 μM $PGF_{2\alpha}$ are expressed as contraction amplitude (mN) per circumferential muscle length (mm). [3]Relaxation produced by 30 nM ACh is expressed as a % of relaxation from steady-state level of contraction in response to a submaximal concentration of 1 μM $PGF_{2\alpha}$. Each value represents the mean ± s.e.mean for 5 experiments. *$P < 0.05$, **$P < 0.01$ vs corresponding values obtained for the artery segments with endothelium intact.

$P<0.01$ for ACh, each vs corresponding value for endothelium-intact artery), whereas the contraction in response to $PGF_{2\alpha}$ (1 μM) was augmented in the endothelium-deprived artery ($P<0.01$ vs corresponding value for endothelium). However, the 80 mM KCl-induced contraction was independent of the presence or absence of endothelium. Therefore, the following experiments were performed in the artery with intact endothelium.

The magnitude of stretch was found to have a profound effect on the contraction of the rabbit pulmonary artery in response to stretch: As shown in Figure 2a, the contractile activity of the pulmonary artery produced by stretch was increased, depending on the amount of stretch, and reached a maximum at 1.8 Li. As shown in Figure 2b, under the optimally-stretched condition (1.8 Li), ACh (3 nM – 0.1 mM) contracted the artery in a concentration-dependent manner only when the endothelium was present; whereas the agonist produced only a slight contraction in the artery without endothelium.

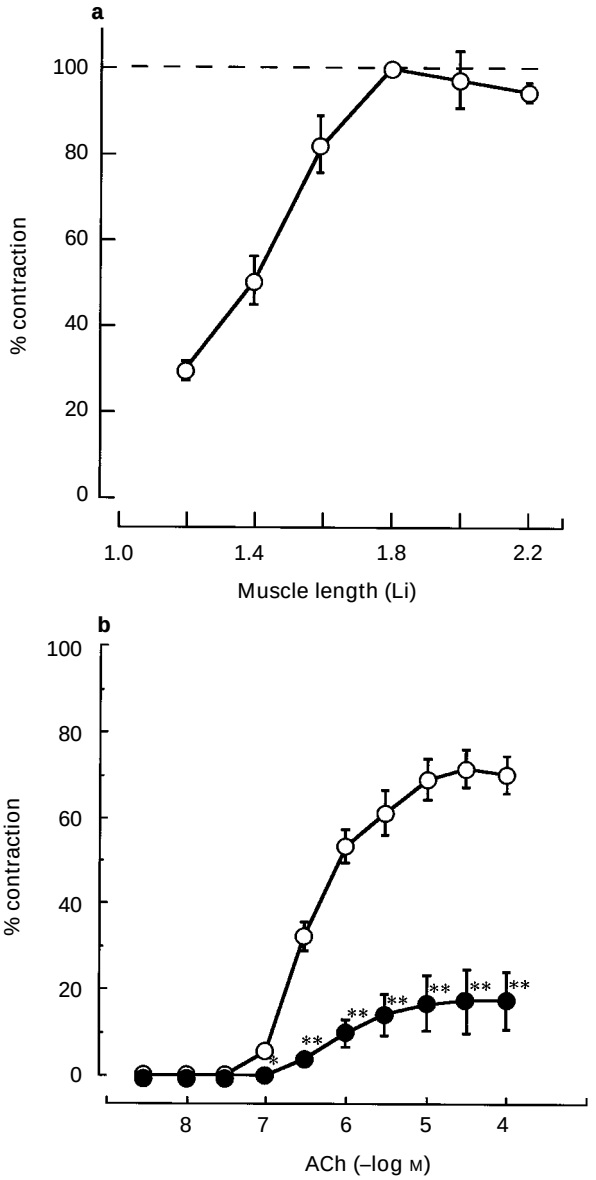


Figure 2 Mechanical responses of rabbit pulmonary arteries to stretch and ACh. (a) Effects of varying the amount of stretch on tension development of rabbit pulmonary artery. Relation between % change in contractile response to stretch (ordinate scale) and amount of stretch (abscissa scale) is shown. Active tension in response to stretch to 180% of initial muscle length is taken as 100%. Points and vertical lines indicate means ± s.e.mean for 5 experiments. Total of 15 segments of pulmonary arteries from the same number of rabbits was used. (b) Effects of ACh on the mechanical activity of rabbit pulmonary arteries in the presence (○) and absence (●) of endothelium. All contractions were normalized as a % of the contraction induced by 80 mM KCl (=100%). Separate artery segments were used for endothelium-intact and endothelium-denuded preparations. Points and vertical lines represent means ± s.e.mean for at least 5 experiments. *$P<0.05$, **$P<0.01$ vs corresponding value of the response to ACh in the preparations with intact endothelium.

Effects of autonomic blockers, tetrodotoxin, nicardipine, Ca^{2+}-withdrawal, aspirin, indomethacin, SQ 29,548 and ozagrel

Initially, the involvement of various nervous systems in the effects of stretch was examined. Blockade of α-adrenoceptors with 1 μM phentolamine, β-adrenoceptors with 1 μM propranolol, or of muscarinic effects with 30 nM atropine did not produce any significant change in the contractile response to stretch. Moreover, chlorpheniramine or ketanserin, each at 1 μM, was ineffective at inhibiting the stretch-induced contraction. In addition, hexamethonium at 1 μM or tetrodotoxin at 0.3 μM did not alter the mechanical response to stretch (data not shown). Hence, the mechanical response produced by stretch is unlikely to be attributable to stimulation of neuronal elements.

The concentration-response curves for ACh were shifted to the right, and the maximum response to 0.1 mM ACh ($78.5\pm4.5\%$ of 80 mM KCl-induced contraction = 100%, $n=4$) was reduced by $49.4\pm6.5\%$ ($P<0.05$, $n=4$), when the segment was pretreated with 30 nM atropine for 30 min. EC_{50} values (μM) for ACh before and after treatment with atropine were 0.62 ± 0.16 and 62.82 ± 1.16, respectively (each $n=4$, $P<0.01$). However, autonomic blockade with 1 μM phentolamine or 1 μM propranolol, as well as with 1 μM hexamethonium or 0.3 μM tetrodotoxin, did not alter the contractile response to ACh (data not shown).

Typical effects of nicardipine and Ca^{2+} withdrawal on the stretch-induced contraction are depicted in Figure 3a and b. Pretreatment with 0.1 μM nicardipine for 60 min, a concentration that abolished 80 mM KCl-induced contraction (data not shown); partially inhibited the stretch-induced contraction (only about 10%); whereas Ca^{2+}-withdrawal for 10 min before stretch significantly attenuated the stretch-induced contraction (Figure 3c). ACh in the concentration range 1 nM to 0.1 mM contracted the artery in a concentration-dependent manner with an EC_{50} value of 0.56 ± 0.14 μM ($n=4$), and the maximum contraction in response to 30 μM ACh was $72.5\pm4.8\%$ ($n=4$), when it was expressed as a % of the amplitude of the 80 mM KCl-induced contraction (=100%). After treatment with nicardipine, the maximum contractile response to 30 μM ACh was reduced to $82.4\pm7.1\%$ ($n=4$), and the EC_{50} value was not significantly changed (0.84 ± 0.15 μM, $n=4$). However, incubation of the artery segments in Ca^{2+}-free medium for 10 min inhibited almost completely the contractile response to ACh in the same concentration range (1 nM to 0.1 mM; data not shown).

Interestingly, the mechanical responses produced by both stretch and ACh were very susceptible to inhibitors of cyclooxygense and TXA_2 synthase or a PGH_2/TXA_2 receptor antagonist. Figure 4a shows the typical inhibitory effects of aspirin (10 μM) and indomethacin (10 μM), well-known cyclooxygenase inhibitors and ozagrel (10 μM), a TXA_2 synthase inhibitor, on the stretch-induced contraction. As summarized in Figure 4b, these three drugs significantly attenuated the stretch-induced contraction of rabbit pulmonary artery with intact endothelium.

Furthermore, the inhibition of the stretch-induced contraction by aspirin became very marked in the artery preparations with intact endothelium, when compared with the slight attenuation by aspirin in the endothelium denuded artery (Figure 5a). The remaining contractile activity in response to stretch in either type of segment was abolished by 0.1 mM papaverine (data not shown).

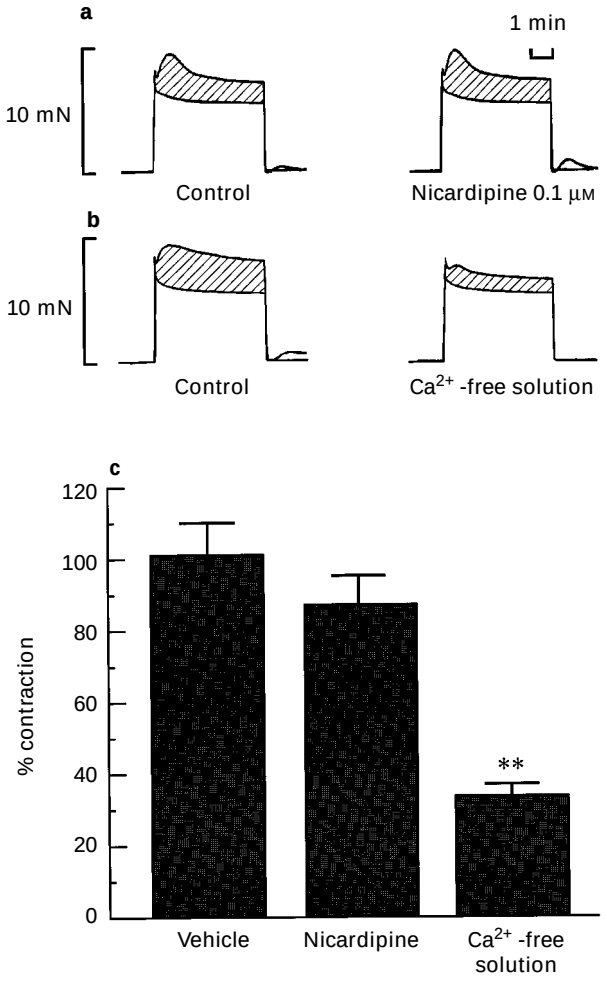


Figure 3 Effects of nicardipine and Ca^{2+}-free solution on the stretch-induced contraction of rabbit pulmonary artery. All mechanical activities were observed in endothelium-intact preparations. (a) Actual tracings showing the effects of 0.1 μM nicardipine on the contractile response to stretch. Nicardipine was added to the organ bath 30 min before the second stretch. Hatched area of the mechanogram shows the active tension as explained in the legend of Figure 1. (b) Actual tracings before and after incubation with Ca^{2+}-free solution are shown. The artery segment was incubated in the Ca^{2+}-free Tyrode solution containing 0.02 mM EGTA 30 min before the second stretch. (c) Summarized data showing the mechanical activity in response to stretch with the standard procedure at a rate of 0.44 mm s^{-1}, stimulus period of 5 min, and 65 min interval between stretches. Responses are expressed on the ordinate scale as a percentage of the first contraction produced by stretch. Columns represent means$\pm$s.e.mean for 4 experiments. $^{**}P<0.01$ vs corresponding values of vehicle.

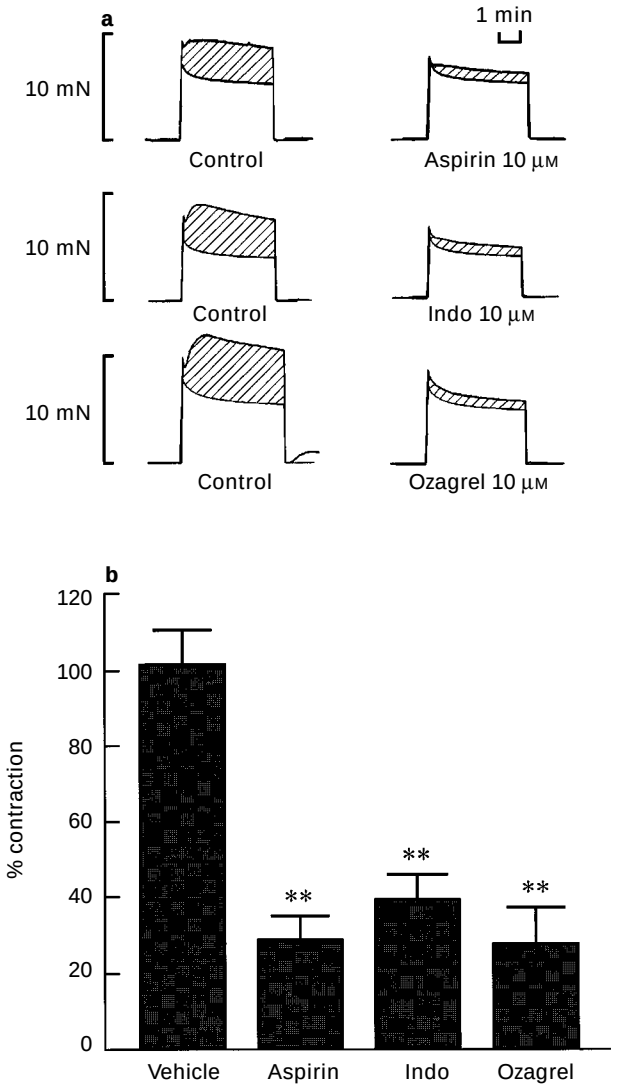


Figure 4 Effects of aspirin, indomethacin (Indo) and ozagrel, each at 10 μM, on stretch-induced contraction of rabbit pulmonary arteries. (a) Actual tracings showing the effects of aspirin, indomethacin and ozagrel on the contractile response to stretch (to 180% of the initial muscle length, a stimulus period of 5 min, with interval between stretches kept at 60 min). (b) Summarized results with all contractions normalized to the first control response to stretch. Columns show means$\pm$s.e.mean for 4 experiments. $^{**}P<0.05$ vs corresponding values of vehicle.

Aspirin and ozagrel at 10 μM significantly reduced the maximum and the slope of the concentration-response curves for ACh (Figure 5b, right and left panels, respectively). However, EC_{50} values (μM) for ACh, were not significantly altered before and after treatment with these drugs: 0.64 ± 0.13 vs 1.50 ± 0.29, each $n=4$, for aspirin; 0.57 ± 0.07 vs 1.39 ± 0.36, each $n=4$, for ozagrel.

SQ 29,548 (0.1 μM), a TXA_2/PGH_2 receptor antagonist, also inhibited the stretch-induced contraction by up to $28.4\pm4.9\%$ ($P<0.01$ vs control = 100%) (each $n=3-8$) (Figure 6a and b), and the contraction by up to $12.7\pm2.2\%$ ($P<0.01$) in response to 30 μM ACh (control = 100%, each $n=4$) (Figure 6b). However, ozagrel and SQ 29,548 (1 nM – 0.1 μM) did not show any significant effect on the contraction in response to 80 mM KCl: for instance, at the maximal concentration used in the present study, ozagrel (10 μM) and SQ 29,548 (0.1 μM) inhibited the 80 mM KCl-induced contraction by less than 10% (control = 100%) (data not shown).

Release of thromboxane and prostacyclin

Pilot studies indicated that of the various prostanoids, the production of thromboxane and prostacyclin was enormously increased by stretch, whereas that of PGE_2 or $PGF_{2\alpha}$ was not significantly increased. Thus, the involvement of TXA_2 and/or prostacyclin in mediating the stretch-induced contraction was assessed further by measuring the amount of stable metabolites of TXA_2 and prostacyclin, i.e., TXB_2 and 6-keto $PGF_{1\alpha}$, respectively, released from the arteries. There was no significant difference between the groups in the basal release of TXA_2 and prostacyclin for the endothelium-intact arteries used in the experiments. As shown in Figure 7a, basal release of TXB_2 was 1.91 ± 0.31 pg released min^{-1} mg^{-1} wet tissue ($n=5$) for the endothelium-intact arteries. Thromboxane production in response to stretch was significantly increased up to a level of 31.52 ± 3.31 pg released min^{-1} mg^{-1} wet tissue about 17 times over the basal level ($n=5$, $P<0.01$ vs basal level). Ozagrel (10 μM) abolished the stretch-induced rise in thromboxane production in the arteries. However, the basal release of thromboxane was only slightly affected by ozagrel.

Likewise, as shown in Figure 7b, thromboxane production was significantly increased by ACh up to about 70 times over the basal level (basal level, 2.15 ± 0.25 vs after ACh, 157.0 ± 15.26 pg released min^{-1} mg^{-1} wet tissue, each $n=5$, $P<0.01$). Ozagrel (10 μM) abolished the ACh-induced rise in thromboxane production in the arteries with intact endothelium.

To determine whether endothelial cells contributed to the production of TXA_2, experiments were undertaken in which the endothelium was mechanically removed. The basal release of TXB_2 in the endothelium-denuded arteries (0.16 ± 0.03 pg released min^{-1} mg^{-1} wet tissue) was about ten times less than that of the endothelium-intact ones (1.91 ± 0.31 pg released min^{-1} mg^{-1} wet tissue, each $n=5$, $P<0.01$). Furthermore, as shown in Figure 7c the amount of TXB_2 released during stretch was not significantly changed (0.16 ± 0.03 pg released min^{-1} mg^{-1} wet tissue before stretch vs 0.26 ± 0.28 pg released min^{-1} mg^{-1} wet tissue after stretch, each $n=5$, $P>0.05$). In contrast, thromboxane production was significantly increased by ACh, as shown in Figure 7d, indicating that medial smooth muscle cells of rabbit pulmonary artery can also produce/release TXB_2 (0.16 ± 0.03 pg released min^{-1} mg^{-1} wet tissue before ACh vs 28.20 ± 2.16 pg released min^{-1} mg^{-1} wet tissue after ACh, each $n=5$, $P<0.01$), although the amount of TXB_2 released was about 10 to 15 times less than that observed in endothelium-intact arteries (2.15 ± 0.25 vs 0.16 ± 0.02-pg min^{-1} mg^{-1} wet tissue, each $n=5$).

Since prostacyclin is generally considered to possess biological properties including vasodilatation, which opposes the effects of TXA_2, we investigated whether the release of 6-keto $PGF_{1\alpha}$, a stable metabolite of prostacyclin, was affected during the response to stretch or ACh. Figure 8 shows that stretch

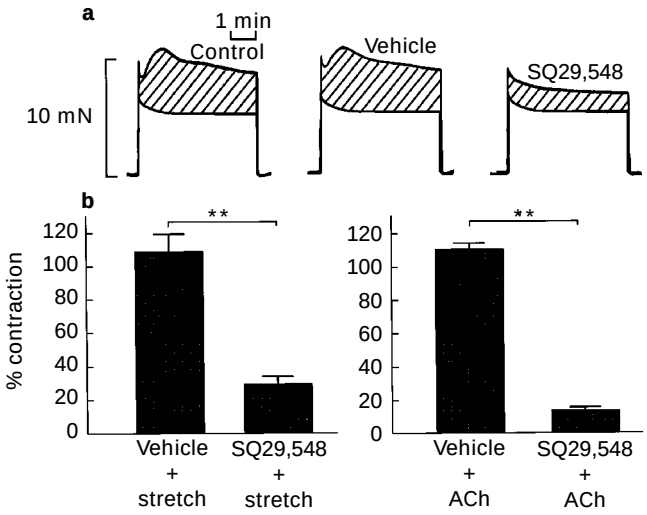
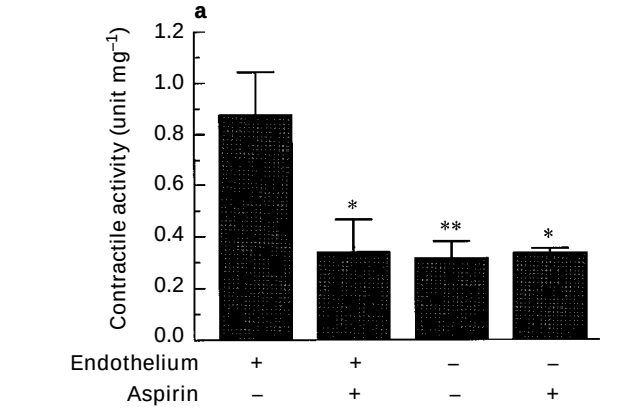


Figure 6 Attenuation of mechanical responses of rabbit pulmonary artery by SQ29,548. (a) Tracings indicate a typical effect of SQ29,548 (0.1 μM) on stretch-induced contraction of rabbit pulmonary artery. Active tension is shown as the hatched area of the mechanogram as in Figure 1. (b) Summarized data showing the mechanical activity in response to stretch (left panel) with the standard procedure at a rate of 0.44 mm s^{-1}, stimulus period of 5 min and 65 min interval and in response to ACh (30 μM) (right panel). Responses are expressed on the ordinate scale as a percentage of the first contraction produced by stretch of ACh. Columns represent means $\pm$ s.e.mean for 3 to 8 experiments. $^{**}P<0.01$ vs corresponding values for vehicle.



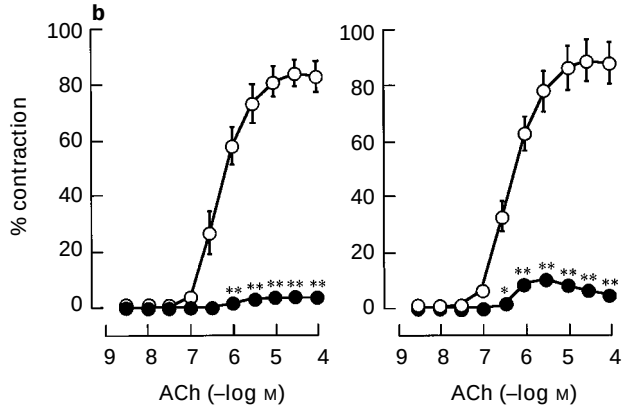


Figure 5 Differential mechanical responses of rabbit pulmonary artery with and without endothelium. (a) Effects of aspirin (10 μM) on stretch-induced contraction in rabbit pulmonary arteries with (+) and without (−) endothelium. Contractile activity was normalized to the first response to stretch. Separate artery segments were used for endothelium-intact and -denuded preparations and the segments were contracted in the presence (+) or absence (−) of 10 μM aspirin. Each column represents the mean $\pm$ s.e.mean for 3 to 5 experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs corresponding value for endothelium intact artery. (b) Effects of aspirin (left panel) and ozagrel (right panel) at 10 μM on ACh-induced contraction in rabbit pulmonary arteries. All contractions are expressed as a % of contraction induced by 80 mM KCl before (○) and after (●) treatment with inhibitory drugs. Points and vertical lines indicate the mean $\pm$ s.e.mean for 4 experiments. $^{*}P<0.05$, $^{**}P<0.01$ vs corresponding control value.

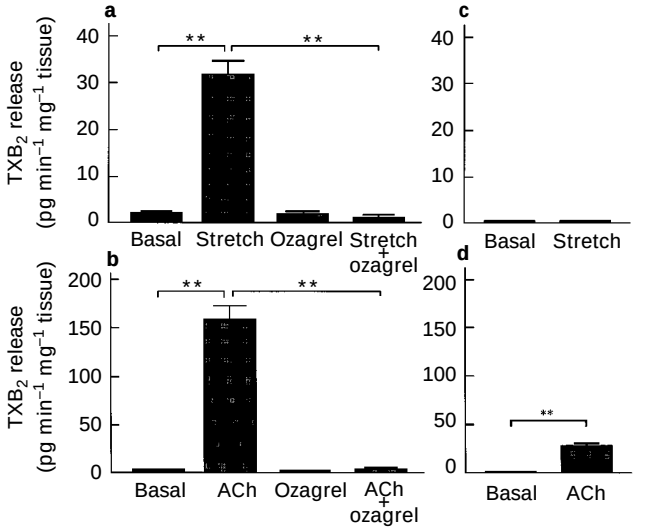


Figure 7 Release of thromboxane B_2 (TXB_2) from rabbit pulmonary arteries with and without endothelium. TXB_2 levels in the presence and absence of 10 μM ozagrel were measured in the medium surrounding the vessels with intact endothelium before and 5 min after application of stretch (a) or 30 μM ACh (b). TXB_2 levels were also measured in the medium surrounding the vessels without endothelium before and 5 min after application of stretch (c) or 30 μM ACh (d). Amount of TXB_2 released is expressed as pg min^{-1} mg^{-1} tissue wet weight. Each column represents the mean $\pm$ s.e.mean for 5 experiments. $^{**}P<0.01$ vs corresponding value indicated in the figure.

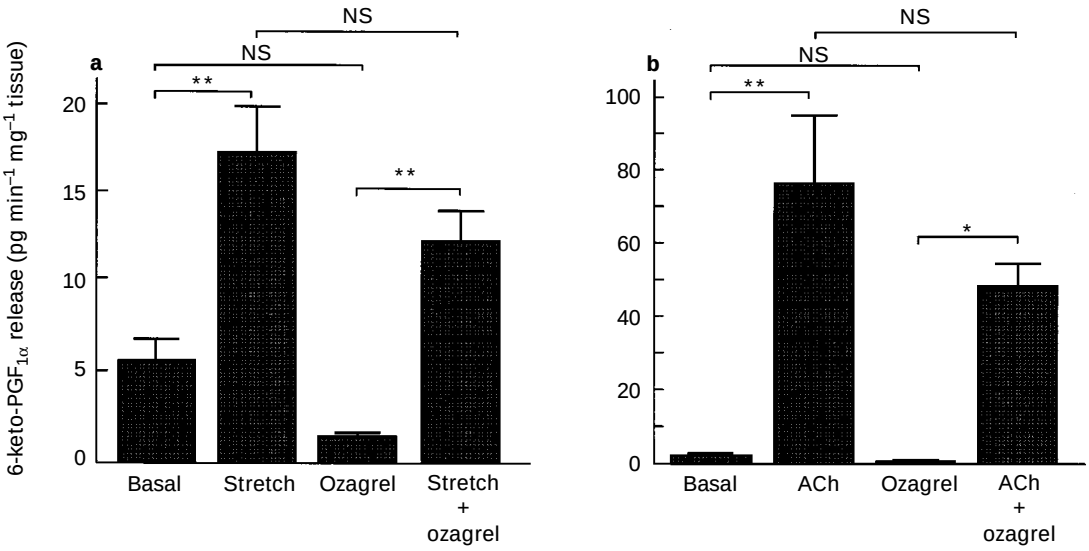


Figure 8 Effects of ozagrel on release of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) from rabbit pulmonary arteries with intact endothelium. 6-Keto-$PGF_{1\alpha}$ levels were measured as those of TXB_2 in Figure 7, and are expressed as pg min^{-1} mg^{-1} tissue wet weight. 6-Keto-$PGF_{1\alpha}$ levels in the presence and absence of 10 μM ozagrel were measured in the medium surrounding the vessels with intact endothelium before and 5 min after application of stretch (a) or 30 μM ACh (b). Each column represents the mean $\pm$ s.e.mean for 4 to 5 experiments. $^{**}P<0.01$ vs corresponding basal value. NS, not statistically significant.

produced a significant increase in the amount of 6-keto $PGF_{1\alpha}$ released from the artery segments. ACh (30 μM) also increased the release much more so, giving a value about 30 fold over the basal one after an incubation period of 5 min. Furthermore, in order to clarify the specific action of ozagrel, we examined the effect of the drug on the release of prostacyclin in response to either stretch or ACh. The release of 6-keto-$PGF_{1\alpha}$ in response to stretch (Figure 8a) and ACh (Figure 8b) was significantly increased, even after treatment with ozagrel for 40 min. However, ozagrel (10 μM) itself inhibited only slightly the basal release of 6-keto-$PGF_{1\alpha}$.

Discussion

The present experiments showed that stretch-induced contraction of rabbit intrapulmonary arteries was dependent on the presence of endothelium, and was susceptible to inhibitors of cyclo-oxygenase (aspirin and indomethacin) and TXA_2 synthase (ozagrel), as well as to SQ 29,548, a TXA_2/PGH_2 receptor antagonist (Hunt *et al*., 1992; Heygate *et al*., 1995). In support of this finding, the biochemical assay indicated that the release of TXB_2, a stable metabolite of TXA_2, was increased in response to stretch only when the endothelium was intact. Although it has been found that vascular resistance in the blood-perfused rabbit pulmonary bed is increased by prostacyclin, given exogenously, via stimulation of TXA_2 synthesis (Kaapa *et al*., 1991), prostacyclin is generally considered to act as an antagonist of TXA_2 (Moncada & Vane, 1979). Our study showed that stretch also increased the production of 6-keto $PGF_{1\alpha}$, an inactive metabolite of prostacyclin. Therefore, it is likely that the stretch can act on endothelial cells of rabbit pulmonary artery and produce contraction via augmentation of the release of TXA_2 and/or an increase in the ratio of TXA_2/prostacyclin. The balance of production between these two prostanoids acting mutually antagonistically may play an important role in the regulation of contraction of the rabbit pulmonary artery.

Ozagrel has been shown to inhibit TXA_2 synthesis but augment prostacyclin synthesis in several preparations such as bronchoalveola of the guinea-pig (Nambu *et al*., 1990) and rat peritoneal cells (Hiraku *et al*., 1986). However, we found that ozagrel (10 μM) inhibited the production of TXA_2 without having any significant effect on the production of prostacyclin in the rabbit pulmonary artery. Alternatively, SQ 29,548, a TXA_2/PGH_2 receptor antagonist, could also attenuate the contractile response to both stretch and ACh. The combined use of inhibitors of cyclo-oxygenase (aspirin or indomethacin) and those of TXA_2 synthesis, including ozagrel, is, nevertheless, appropriate at present to assess the involvement of TXA_2 in the biological process, including vascular contraction.

As to the endothelium-dependence of stretch-induced contraction, our findings are in strong contrast to those obtained by Belik (1994), who studied large pulmonary arteries of the guinea-pig. He found that the contraction in response to quick stretch was myogenic and completely suppressed by gallopamil but unaltered by addition of the nitric oxide synthase inhibitor N^G-methyl-L-arginine or indomethacin. Kulik *et al*. (1988) have shown that stretch can act, probably directly, on smooth muscle in small pulmonary arteries of the cat to elicit contraction. The discrepancy of our results with others seems to be attributable to not only species difference but also different experimental conditions: a mechanical stimulus was applied to pulmonary arteries in the study of Belik (1994) (the artery was rapidly stretched in less than 3 s to 200% of the optimal length) and in our previous studies (Nakayama, 1982; Nakayama *et al*., 1989; Tanaka & Nakayama, 1991) a 'quick/rapid stretch' i.e., a dynamic stretch was used instead of a static/slow stretch (Johnson & Mellander, 1975) as in the present experiments. We have shown that the contraction of various vascular segments, including cerebral ones, obtained from different animal species, such as dogs (Nakayama *et al*., 1989) and cats (Tanaka & Nakayama, 1991) in response to a quick stretch at a rate of 10 cm s^{-1} is myogenic in nature and not affected by the removal of the endothelium. Interestingly, the contraction of rabbit pulmonary artery was also not susceptible to the removal of endothelium, i.e., it is apparently myogenic, when the artery was quickly stretched at a rate of over 10 cm s^{-1} (our unpublished observations). Therefore, it is likely that the rate of stretch or its acceleration greatly dictates whether a myogenic response or an endothelium-dependent one ensues.

There are several ideas as to the role of endothelium in the contractile activation of vascular tissue in response to stretch: (i) the endothelium liberates constrictor substances, including cyclo-oxygenase-mediated products, since the response to stretch/pressure was inhibited by prior treatment of the artery with indomethacin (Katsusic *et al*., 1987; Rubanyi, 1988); (ii) the endothelium accelerates cell-to-cell signal transduction (Harder, 1987); and (iii) the primary sensor initiating myogenic

tone lies in the endothelium or in the myoendothelial junction (Halpern *et al*., 1987). The first suggestion is supported by some of our present findings: our functional study showed endothelium-dependent contraction in response to stretch and its specific attenuation by either indomethacin, aspirin or ozagrel. In addition, the production/release of TXB_2, a major metabolite of TXA_2, increased in response to stretch only when the endothelium was present.

It has been hypothesized that cellular responses to direct mechanical stress involve an interplay between structural elements and biochemical second messengers (Osol, 1995). As to the augmentation of TXA_2 production in the endothelial cells in response to stretch, there is a possibility that stretch can activate the enzyme cascade concerned with TXA_2 production (this cascade includes phospholipase A_2, cyclo-oxygenase and TXA_2 synthase), through an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in endothelial cells. It has been shown that the stretching cellular membranes increase $[Ca^{2+}]_i$ in cultured endothelial cells obtained from human umbilical vein (Naruse & Sokabe, 1993). The intracellular Ca^{2+} mobilization was not sensitive to nifedipine but totally blocked by Gd^{3+}, a putative blocker of stretch-activated channels, and was partially blocked by ryanodine, an inhibitor of Ca^{2+}-induced Ca^{2+} release. Therefore, it was concluded that stretching the membrane primarily induces extracellular Ca^{2+} entry through stretch-activated channels followed by Ca^{2+} release from intracellular Ca^{2+} stores. Our observations also indicated that the removal of Ca^{2+} from the incubation medium, but not nifedipine, inhibited the production of prostanoids such as TXA_2 in the rabbit pulmonary artery (Figure 3).

In our preliminary experiments, Gd^{3+} (5 mM) abolished the stretch-induced contraction of rabbit pulmonary artery, whereas the ion at 2 mM did not. However, Gd^{3+} in such high concentrations inhibited 80 mM KCl-induced contraction in the same preparation; i.e., complete inhibition occurred at 5 mM, and 20 to 50% inhibition at 1 to 2 mM (unpublished observations), indicating that Gd^{3+} shows a nonspecific inhibition of the contraction of the pulmonary artery. Therefore, whether the Gd^{3+}-sensitive stretch-activated channels are involved in the stretch-induced contraction of the rabbit pulmonary artery remains to be elucidated. Nevertheless, the cascade enzymes are considered to be Ca^{2+}-dependent (Smith, 1992). Therefore, it is possible that stretch-activated channels in endothelial cells play an important role as a mechano-sensor component in the production/release of TXA_2 through Ca^{2+} mobilization.

With regard to the question as to which cell type(s) in the rabbit pulmonary artery produces TXA_2 in response to a muscarinic agonist such as methacholine or arachidonic acid, Buzzard and Pfister (1993) showed that TXA_2 is not produced by endothelial cells but may arise from cells that adhere to the luminal surfaces or infiltrate into vascular tissues, since no TXA_2 synthase activity was found in cultured endothelial cells obtained from rabbit pulmonary artery. In the present study, we frequently surveyed the intimal surface of the rabbit pulmonary artery segments with and without endothelium by using a scanning microscope, according to the procedure described previously in detail (Nakayama, 1988). However, we could not find any residual platelets or macrophages when the artery segments were properly treated as described in the Methods section. The present study implicates the endothelial cell as the primary source of not only mechanical stretch- but also ACh-induced production/release of vasoactive prostanoids. However, we cannot rule out the possibility that adherent platelets or macrophages or such cells that have infiltrated the vascular tissue are also involved in the stretch-induced contraction. Therefore, further studies are needed to elucidate the mechanisms for the interaction of blood components with endothelium and medial smooth muscles.

As to the Ca^{2+} handling mechanisms underlying TXA_2-induced contraction of the rabbit pulmonary artery, Ca^{2+} antagonists including verapamil or a calcium-free perfusate prevented the thromboxane-induced pulmonary vasoconstriction assessed in the rabbit perfused lung (Farrukth *et al*., 1985). These results suggest that TXA_2 causes pulmonary vasoconstriction by increasing the cytosolic Ca^{2+} concentration. TXA_2 and its stable breakdown metabolite, TXB_2, are known to be pulmonary vasoconstrictors (Friedman *et al*., 1979). We showed previously that 9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$ (U46619), a stable TXA_2 analogue, increased not only the release of Ca^{2+} from the intracellular storage sites but also the sensitivity of the myofilament to Ca^{2+} in the canine cerebral artery (Tanaka *et al*., 1995), similar to data obtained in other vascular smooth muscle cells, such as the rabbit pulmonary artery (Himpens *et al*., 1990). Furthermore, we showed that U46619 increased the Ca^{2+} sensitivity of the contractile elements in the canine basilar artery permeabilized by *Staphylococcus aureus* toxin (α-toxin) in the presence of guanosine 5′-triphosphate (GTP). This effect was reversed by a putative TXA_2 antagonist, 7-[2α,4α-(dimethylmethano)-6β-(2-cyclopentyl-2β-hydroxy acetamido)-1α-cyclohexyl]-5(Z)-heptenoic acid (ONO-3708) or guanosine 5′-(β-th-io)diphosphate (GDPβs), indicating the possible involvement of pharmaco-mechanical coupling via a GTP binding protein (Tanaka *et al*., 1995). As to the vasoconstrictor action of TXA_2, U46619 has been shown to inhibit directly Ca^{2+}-activated-K^+ channels (K_{Ca}) prepared from pig coronary artery smooth muscle and incorporated into lipid bilayers (Scornik & Toro, 1992). Inhibition of K^+ channels would lead to depolarization of the plasma membrane and contraction of the coronary artery. Furthermore, these authors suggested that the inhibition of K_{Ca} was mediated not by a mechanism involving GTP-binding protein or protein kinase but by the binding site for TXA_2 itself.

The strong dependence on extracellular Ca^{2+} of the stretch-induced contraction of the rabbit pulmonary artery suggests that the regulation of $[Ca^{2+}]_i$ involves a complex interaction between Ca^{2+} entry and extrusion across the plasma membrane and Ca^{2+} release and re-uptake from the sarcoplasmic reticulum in both medial smooth muscle contraction or relaxation and the initiation of other cellular responses, including production/release of TXA_2 in endothelial cells.

It is known that GTP-binding protein acts as a signal transducer in intracellular signalling when the pharmacological receptor is the seven-times membrane-perforated type. As to the muscarinic receptor stimulation in the guinea-pig atrium, it is well recognized that the negative inotropic effect of ACh on isoprenaline-induced augmentation of contraction occurs primarily through M_2 muscarinic receptors and is pertussis toxin sensitive, indicating the participation of a coupling protein, Gi, which is a GTP-binding protein (Hulme *et al*., 1990). Our unpublished observations suggest that the endothelium-dependent contraction in response to both stretch and ACh is at least pertusis toxin insensitive. Moreover, our previous findings indicated that the contractile reaction in response to quick stretch of cerebral arteries of various animal species including rabbits and dogs, is pertussis toxin and cholera toxin insensitive (Nakayama & Tanaka, 1993). In the present study, if we presume a process by which stretch or ACh augments the production/release of TXA_2 from the endothelium and the released TXA_2 contracts medial smooth muscles, then it seems very likely that the process is pertussis toxin-insensitive. Further studies are necessary to assess the role of GTP-binding protein(s) in the signal transduction mechanisms for the stretch-induced contraction of vascular tissues.

The present study clearly gives a uniform insight into both mechanical stimulus- and muscarinic agonist-mediated pulmonary vascular tone in which the intra- and intercellular signalling mechanisms, linking endothelium, arachidonic acid metabolism and medial smooth muscle, are likely to be well integrated.

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