Yonsei Medical Journal
Vol. 36, No. 6, 1995

Effects of Adenosine Tetraphosphate (ATPP) on Vascular Tone in the Isolated Rat Aorta

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Effects of a platelet-released, naturally occurring nucleotide, adenosine 5'-tetraphosphate (ATPP) on vascular tone were analyzed in the isolated rat aorta. Under resting tension ATPP (1~100 μM) elicited concentration-dependent contractions in endothelium-intact aortic rings in contrast to the concentration-dependent relaxation with ATP. In endothelium-denuded aortic rings, ATPP induced contraction, as ATP did, but with a greater potency. α, β-methylene ATP (APCPP 50 μM), a P_{2X}-purinoceptor antagonist, significantly inhibited ATPP- as well as ATP-induced contractions in the endothelium-denuded preparations suggesting that ATPP acts via P_{2X}-purinoceptors. ATPP (10~100 μM) relaxed precontracted aortic rings with an intact endothelium in a concentration-dependent manner. This effect of ATPP was 37 fold less potent than that of ATP. However, after P_{2X}-purinoceptor blockade, the effect became identical between the two nucleotides. Reactive blue 2, a selective antagonist of P_{2Y}-purinoceptors, significantly attenuated the ATPP-induced relaxation with no change in the ATP-induced relaxation. These results indicated that the rat aortic endothelium contains heterogeneous populations of P_2-purinoceptors (possibly P_{2Y} and nucleotide receptors). Since ATPP shows dual effects depending upon the vascular tension, it may play a significant role in the physiological regulation of vascular tone.

Key Words: Adenosine tetraphosphate, ATP, P_2-purinoceptor, nucleotide receptor, endothelium, vascular smooth muscle, α,β-methylene ATP, reactive blue 2

While intracellular nucleotides are involved in various cellular metabolisms, extracellular nucleotides and nucleosides play significant roles in the physiological regulation of the cardiovascular function (Olsson and Pearson, 1990; Ralevic and Burnstock, 1991). The extracellular actions of nucleotides are mediated mainly by purinoceptors. Subclasses of the purinoceptor have been postulated on the basis of relative potencies of nucleoside and nucleotide analogues and on the basis of selective antagonisms (Burnstock, 1978). P_1-purinoceptors are selective for adenosine and AMP and are blocked by methylxanthines, whereas P_2-purinoceptors are selective for ATP and are not blocked by methylxanthines (Burnstock, 1978). A further division of the P_2-purinoceptors into P_{2X}, P_{2Y}, and P_{2U} (nucleotide receptor) has recently been proposed (Burnstock and Kennedy, 1985; O'Connor *et al.* 1991). Generally, in a variety of vessels, the P_{2X}-purinoceptors reside exclusively in smooth muscle cells mediating vasoconstriction, whereas the P_{2Y}-purinoceptors are located both on the endothelial (De May and Vanhoutte, 1981; Martin *et al.* 1985) and smooth muscle cells (Mathieson and Burnstock, 1985; Brizzolara

Received October 6, 1995
Accepted December 9, 1995
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and Burnstock, 1991) mediating vasodilation.

Pharmacological experiments have shown that the adenine moiety as well as the number of phosphates in nucleotides are important for their interactions with P_2-purinoceptors (Fedan *et al.* 1986; Howson *et al.* 1988; Lee and Filkins, 1988). Especially, the triphosphate portion of ATP and its analogues has been identified as the key structure for binding to P_{2X}-purinoceptors (Bo and Burnstock, 1993).

Several decades ago, a novel adenine nucleotide, adenosine 5'-tetraphosphate (ATPP) which has one more phosphate than ATP, was identified in the extract of mammalian skeletal muscles (Marrian, 1954; Small and Cooper, 1966). Recently, we have established that ATPP is co-localized with other adenine nucleotides within rabbit platelets and is released by thrombin (Lee *et al.* 1995). Furthermore, ATPP was found to be relatively more resistant than ATP to the action of nucleotidases in the blood (Lee *et al.* unpublished). ATPP is released during platelet aggregation and it may play a physiologically important role in the regulation of local vascular tone. Some pharmacological studies have documented that ATPP is more potent than ATP in increasing the contractility of smooth muscle (Taylor *et al.* 1983; Lee *et al.* 1987). Interestingly, ATPP exerts dual effects on blood pressure in rats, such that in the normotensive state, aortic infusion of ATPP decreases blood pressure, whereas in the hypotensive state induced by hemorrhage, it increases blood pressure (Kong *et al.* 1991). Hence, the current study was designed to investigate i) the effects of ATPP on the relaxation and contraction of intact and endothelium-denuded rat aortas, ii) the characteristics of purinoceptors which mediate the actions of ATPP on the endothelium and vascular smooth muscle, and iii) the dual effects of ATPP at different levels of vascular tone.

MATERIALS AND METHODS

Sprague-Dawley rats of either sex weighing 250 to 300 g were anesthetized with sodium pentobarbital (40 mg/kg, i.p.). The descending thoracic aorta was rapidly removed and cleaned of surrounding fat and connective tissues. Special care was taken to avoid damage to the endothelium. The aorta was cut into transverse rings of 3.5 mm in length. The aortic rings were placed onto two fine L-shaped stainless steel wires and mounted isometrically in a 10-ml organ bath containing oxygenated (95% O_2 -5% CO_2) Krebs-Ringer bicarbonate solution (KRB) at 37℃. The composition of KRB (in mM) was NaCl 117; KCl 4.7; $CaCl_2$ 1.91; KH_2PO_4 1.19; $MgSO_4$ 1.44; $NaHCO_3$ 24.8; and glucose 5.5. The ring was allowed to equilibrate for at least 1 h under a resting tension of 0.75 g which produced optimal contraction to norepinephrine (NE, 5×10^{-8} M, EC_{50}). Isometric tension was recorded by a force transducer (Grass, FT03C) connected to a polygraph (Grass, model 7E). In some cases the endothelium of the ring was denuded mechanically by gently rubbing the intimal surface with a cotton tipped rod. The denudation of the endothelium was verified by the absence of the acetylcholine (1×10^{-6} M)-induced relaxation in rings precontracted with 5×10^{-8} M NE (Furchgott and Zawadzki, 1980).

For relaxation studies, the tissue was contracted with NE (EC_{50}) that produced approximately 50% of the maximum NE-induced contraction. After the contraction had been stabilized, ATPP or ATP was added cumulatively to construct the concentration-response curve. For contraction studies, ATPP or ATP was added cumulatively under resting tension. A long interval (30 min) between treatments with different agonists was allowed to avoid possible desensitization.

The vascular responses (relaxation and contraction) to ATPP and ATP were also tested in the presence of reactive blue 2 (RB2), a P_{2Y}-antagonist, and α,β-methylene ATP (APCPP), a P_{2X}-antagonists. The tissue preparation was incubated in 100 μM RB2 for 20 min. Because RB2 slightly reduced the responsiveness of the tissue to NE, a higher concentration of NE (1×10^{-7} M) than in the control was added to obtain similar magnitude of contraction. Since the pretreatment of APCPP (50 μM) induced a transient contraction which reversed within 15 min, APCPP was added 15 min be-

fore precontraction by NE.

Adenosine 5'-tetraphosphate (ATPP), adenosine 5'-triphosphate (ATP), adenosine 5'-monophosphate (AMP), adenosine, α, β-methylene ATP (APCPP), β, γ-methylene ATP (APPCP), acetylcholine, norepinephrine, and reactive blue 2 were all obtained from Sigma Chemical (St. Louis, MO, USA) and dissolved in distilled water.

Vasodilation was expressed as a percentage of relaxation in NE-elevated tension and vasoconstriction was expressed as milligrams of tension generated. Mean responses for each concentration of a given nucleotide were calculated. Results are expressed as means ± SEM. Significance of differences between mean responses was determined using the Student t-test for unpaired observations; $p < 0.05$ was considered significant.

RESULTS

During an 1 hr equilibration period, responses to norepinephrine (NE, EC_{70}) followed by acetylcholine (Ach, 1×10^{-6} M) were occasionally elicited to confirm the functional integrity of the endothelium and smooth muscle. In endothelium-intact aortic rings with resting tension, ATPP evoked a concentration-dependent contraction while ATP elicited a concentration-dependent relaxation at 1~300 μM (Fig. 1A, and 2A). The addition of ATPP (100 μM) evoked a contraction after ATP-induced relaxation (Fig. 1A). In endothelium-denuded aortic rings, both ATPP and ATP elicited contractions (Fig. 1B and 2B). Although the EC_{50} for these responses could not be calculated in the present series of experiments, the response appeared to be significantly greater with ATPP than with ATP at all concentrations tested (Fig. 2B).

The contractile response to ATPP was also compared with those to APCPP and APPCP which are potent P_{2x}-agonists (Fig. 3). In endothelium-intact aortic rings the rank order of adenine nucleotides (50 μM) in producing contraction was APCPP > ATPP = APPCP >

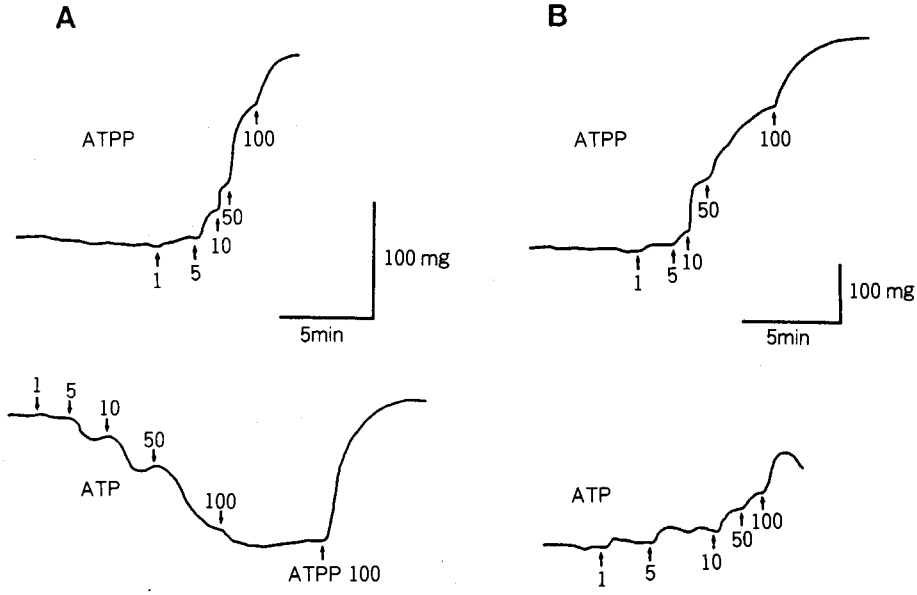

Fig. 1. *Representative traces showing responses of isolated rat aortic rings with resting tension to ATPP and ATP. Nucleotides (1~100 μM) were added cumulatively in endothelium-intact (A) and -denuded (B) preparation. The endothelium-intact aortic ring was also challenged to 100 μM ATPP after ATP-induced relaxation (A). The resting tension was 0.75 g.*

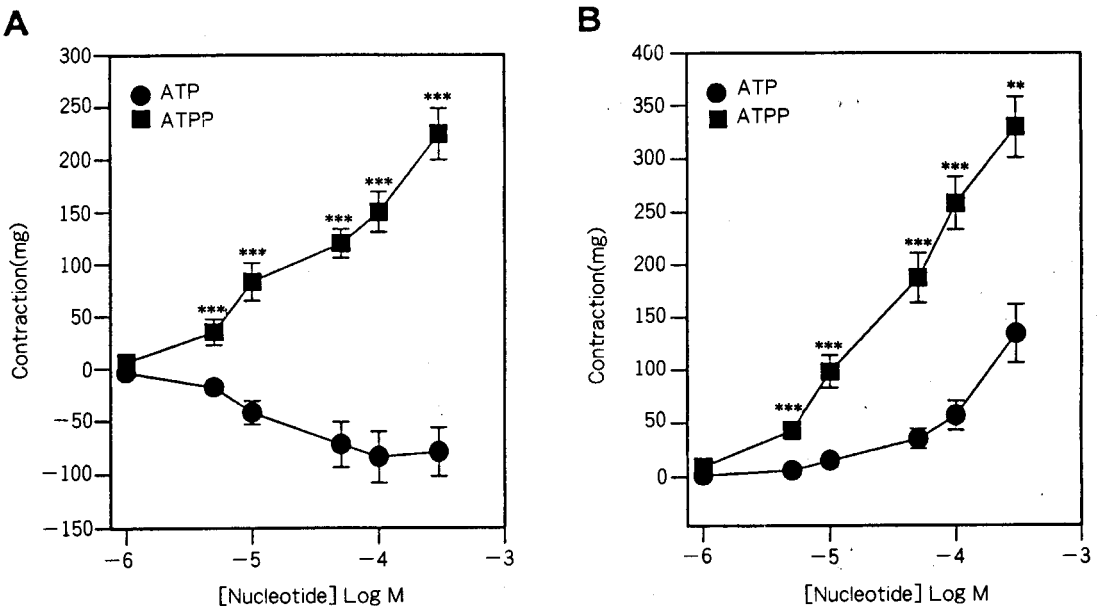

Fig. 2. *Effects of ATPP and ATP added cumulatively to endothelium-intact (A) and - denuded (B) rat aortic rings with resting tension. Results represent changes in vascular tension; (−) values indicate relaxation. Data are mean±SEM of 7 aortic rings from 7 different rats. ** and *** denote $p<0.01$ and $p<0.001$ between ATPP and ATP at each concentration. The resting tension was 0.75 g.*

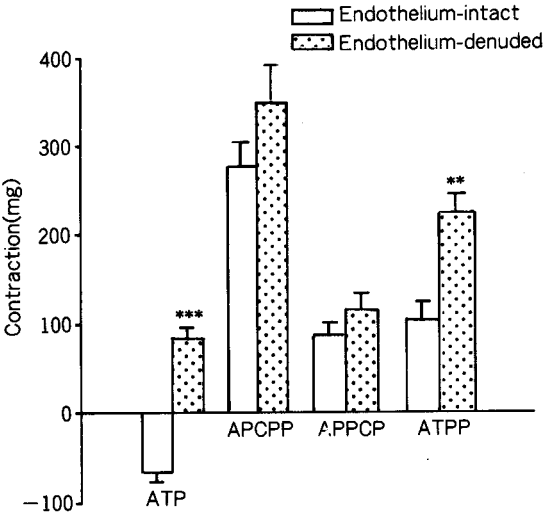

Fig. 3. *Responses of rat aortic rings to ATPP, ATP and its analogues in endothelium-intact and-denuded preparation. The concentration of nucleotides was 50 μM. To avoid desensitization, tissues were fully washed out several times after the addition of each nucleotide. Results represent changes in vascular tension; (−) value indicates relaxation. Data are mean± SEM of 8 aortic rings from 8 different rats. ** and *** denote $p<0.01$ and $p<0.001$, respectively comparisons between endothelium-intact and-denuded aortic rings. The resting tension was 0.75 g.*

> ATP. In endothelium-denuded aortic rings, the contractile responses to ATPP and ATP were significantly increased (for ATPP, 104.7± 19.8 vs. 225±21.6 mg, n=8; for ATP, −67.5± 10.6 vs. 84.4±12.7 mg, n=8) ($p<0.01$ for ATPP; $p<0.001$ for ATP) and thus, the rank order to produce contraction was APCPP > ATPP > APPCP =ATP (Fig. 3).

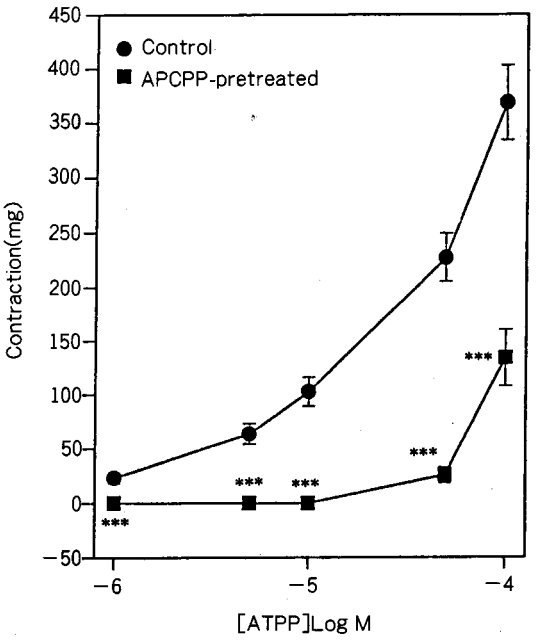

*Fig. 4. Effects of α, β-methylene ATP (APCPP) on ATPP-induced contraction of endothelium-intact aortic rings. Under resting tension of 0.75 g, tissues were preincubated with 50 μM APCPP to desensitize the P_{2X}-purinoceptor. APCPP itself induced a contraction which returned to the resting level within 15 min. Thus, after 15 min preincubation ATPP was added cumulatively. Results represent changes in vascular tension. Data are mean±SEM of 8 aortic rings from different rats. Note that at low concentrations (1~10 μM) ATPP-induced contractions were completely blocked. *** denotes p<0.001: comparisons between control and preincubation with APCPP at each concentration.*

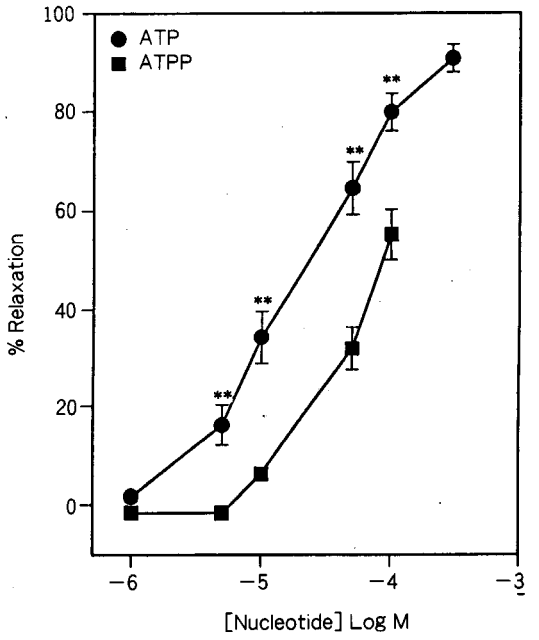

*Fig. 5. Cumulative concentration-response curves for ATPP and ATP in NE- precontracted rat aortic rings with intact endothelium. Results represent percentage changes of relaxation in NE (EC_{50})-elevated tension. Data are mean±SEM of 13 aortic rings from different rats. ** denotes p<0.01: comparisons between ATP-and ATPP-induced responses at each concentration. Note that ATPP induced weak contraction (below 0%) at low concentrations (1~5 μM).*

To define the subtype of P_2-purinoceptors which mediate the contractile response to ATPP, endothelium-denuded aortic rings were preincubated with 50 μM APCPP, a selective desensitizer of P_{2X}-purinoceptors. APCPP itself elicited contraction (Fig. 3) but the tension completely reversed within 15 minutes. After returning to the resting tension, the ability of ATPP to induce contraction of the smooth muscle was tested (Fig. 4). The APCPP pretreatment completely abolished the contractile response to 1 -10 μM ATPP and reduced the response to 50 and 100 μM ATPP ($p<0.001$). Similarly, the APCPP pretreatment also resulted in a complete inhibition of ATP-induced contractions (data not shown).

Tonic contractions of aortic rings were evoked by NE (EC_{50}) then at a plateau tension various concentrations of ATPP and ATP were added cumulatively (Fig. 5). In endothelium-intact aortic rings, ATPP did not changd the tension at 1~5 μM but it induced concentration-dependent relaxation at above 10 μM. At all concentrations tested, ATP caused concentration-dependent relaxation which was significantly greater than that by ATPP. Be-

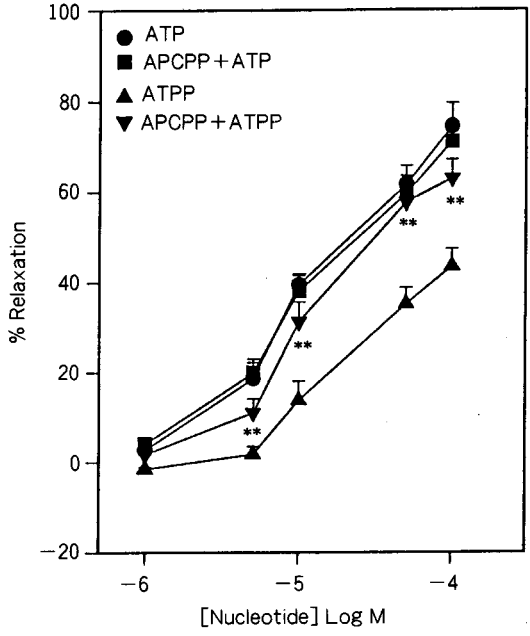

Fig. 6. *Effects of APCPP on ATPP- and ATP-induced relaxations in rat aortic rings with intact endothelium. Tissues were preincubated with APCPP (50 μM) to block P_{2X}-purinoceptors on smooth muscles for 15 min (see Fig. 4), and then precontracted with NE (EC_{50}). At a plateau in the developed tension, nucleotides were added cumulatively. Results represent as percentage changes of relaxation in NE-elevated tension. (−) values indicate contraction. Data are mean±SEM of 7 aortic rings from different rats. ** denotes $p<0.01$: comparisons between control and preincubation of APCPP for ATPP at each concentration. For ATP no significant difference was observed between control and preincubation of APCPP.*

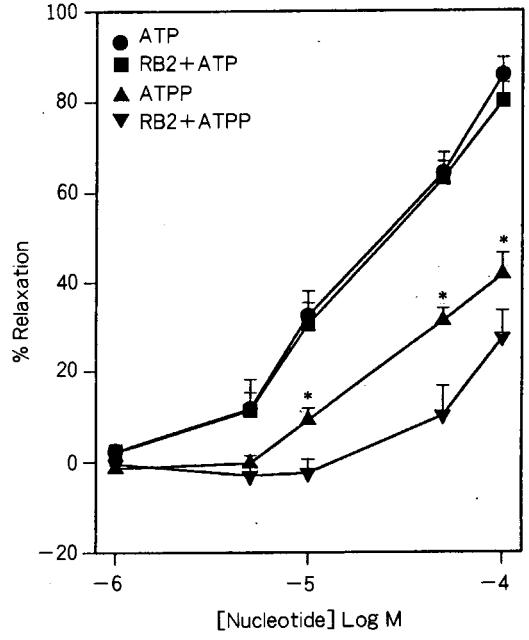

Fig. 7. *Effects of reactive blue 2 on ATPP- and ATP-induced relaxations in rat aortic rings with intact endothelium. Twenty minutes after preincubation with 100 μM reactive blue 2 (RB2), aortic rings were precontracted with NE (1×10^{-7} M). At a plateau in the developed tension, nucleotides were added cumulatively. Results represent as percentage changes of relaxation of NE-elevated tension. (−) values indicate contraction. Data are mean±SEM of 7 aortic rings from different rats. * denotes $p<0.05$: comparisons between control and preincubation of RB2 for ATPP. No significant difference was observed between control and preincubation of RB2 for ATP.*

cause the highest concentration (300 μM) of ATPP used evoked contraction rather than relaxation, the magnitude of relaxation at 100 μM was assumed to be the maximum in the calculation of EC_{50} value. The value of EC_{50} for ATPP (93 μM) was 3.7 times higher than that for ATP (25 μM) indicating that ATP is 3.7 times more potent than ATPP in producing relaxation.

Since ATPP may act both on the endothelium and smooth muscle, the relaxation by ATPP was compared with that of ATP after blocking the P_{2X}-purinoceptors with 50 μM APCPP (Fig. 6). In endothelium-intact aortic rings, the response to ATPP (over 10 μM) was significantly increased by the APCPP treatment. However, the response to ATP was not altered by APCPP (Fig. 6). Adenosine and AMP did not mimic the effect of ATPP in producing relaxation and contraction (data not shown). When the endothelium was intact, APCPP and APPCP, potent P_{2X} agonists, in-

duced further contraction of precontracted aortic rings (at 50 μM, 10.1±2.6% and 4.8±3.2% of the NE-induced contractions respectively. n =8). In endothelium-denuded preparations, neither ATPP nor ATP produced detectable relaxation of precontracted smooth muscle at 1 to 100 μM.

To identify the subtype of P_2-purinoceptors which mediates relaxation responses to ATPP and ATP, 100 μM RB2, a selective P_{2Y}-purinoceptor antagonist, was added before precontraction of aortic rings with intact endothelium. The results indicated that the blocking of P_{2Y}-purinoceptors significantly attenuated the ATPP (10~100 μM) -induced relaxation but had no effect on the ATP-induced relaxation (Fig. 7).

DISCUSSION

The present study demonstrated that ATPP is a strong agonist to P_2-purinoceptors in the rat aortic vascular smooth muscle and endothelium. The contractile response to ATPP was probably mediated by P_{2X}-purinoceptors as suggested by the blocking of the response by a selective desensitizer of the P_2-purinoceptor, APCPP.

Although ATP induced contraction similar to that by APPCP, in endothelium-denuded aortic rings, the ATP effect was much smaller than that of ATPP. Furthermore, APCPP prevented activation by a weaker agonist ATP. Such response has also been demonstrated in the saphenous vein (Houston *et al.* 1987). On the other hand, APCPP did not abolish the ATPP-induced contraction at high concentrations. Two possibilities may explain the greater effect of ATPP on the P_{2X}-purinoceptors as compared with ATP. First, ATPP may have a higher affinity than ATP to the P_{2X}-purinoceptor due to higher number of phosphates. Several studies have shown that the number of phosphates in a side chain of an adenine nucleotide determines the relative affinity to the P_2-purinoceptors (Lee *et al.* 1987; Howson *et al.* 1988; Lee and Filkins, 1988; Bo and Burnstock, 1993). Secondly, ATPP seems to be more resistant to the enzymatic degradation in the circulation than ATP resulting in a higher concentration around the purinoceptors (Lee *et al.* umpublished data).

In isolated rat aortic rings, ATP was 3.7 fold more potent than ATPP in evoking the endothelium-dependent relaxation. Since this may be due to a potent action of ATPP on P_{2X}-purinoceptors, we also assessed the effects of ATPP and ATP after blocking the P_{2X}-purinoceptors with APCPP. Blocking of P_{2Y}-purinoceptor has been reported to have no effect on the ATP-induced relaxation via P_{2Y}-purinoceptors (Mathieson and Burnstock, 1985). Our results showed that the APCPP treatment significantly increased ATPP- but not ATP-induced relaxation and that ATPP was almost equipotent to ATP in inducing relaxation. The desensitization of P_{2X}-purinoceptor by APCPP seemed to eliminate the contractile effect of ATPP allowing its relaxing effect to be pronounced. We therefore conclude that ATPP may strongly activate purinoceptors both on the endothelium and smooth muscle while ATP activates purinoceptors on the endothelium.

Adenosine has been known to induce remarkable relaxation of endothelium-denuded vascular smooth muscle (Furchgott and Zawadzki, 1980; Chinellato *et al.* 1991). In our experiments, however, ATPP and ATP did not elicit relaxation at all concentrations tested in endothelium-denuded aortic rings. These results may eliminate the possibility that ATPP- and ATP-induced relaxations are mediated by P_1-purinoceptors. In addition, ATPP-induced relaxation is not likely to be resulted from a breakdown of ATPP to ATP because ATPP is metabolized directly to AMP and phosphates (Lee *et al.* 1995).

The exclusive role of P_{2Y}-purinoceptors in mediating endothelium-dependent responses to ATP has been challenged by the findings that the pyrimidine nucleotide UTP is also active and potent as ATP (Needam *et al.* 1987; Carter *et al.* 1988; O'Conner *et al.* 1991). Dainty et al. (1991) have suggested that the rat aorta may contain a heterogeneous population of receptors (possibly both P_{2Y} and nucleotide receptors) which mediate endothelium-dependent relaxation. To identify the subtypes of P_2-

purinoceptors which mediate ATPP- and ATP-induced relaxation, RB2, an anthraquinone-sulphonic acid derivative, was used as a selective P_{2Y}-purinoceptor antagonist. RB2 is known to antagonize the ATP-induced relaxation of strips of guinea-pig distal colon (Kerr and Krantis, 1979) and rat duodenum (Manzini *et al.* 1985). In the rat mesenteric artery and perfused coronary circulation, RB2 attenuates the vasodilation via P_{2Y} but has no effect on the vasoconstriction via P_{2X} (Burnstock and Warland, 1987). In our experiments, RB2 had little effect on the response to ATP. This observation confirmed that in rat aorta ATP induces relaxation mainly via nucleotide receptors for UTP rather than P_{2Y}-purinoceptors (O' Conner *et al.* 1991). Interestingly, ATPP-induced relaxation was significantly attenuated by reactive blue 2 indicating that the response was mediated by a P_{2Y}-purinoceptor. It is, however, unlikely that ATPP acted solely through P_{2Y}-purinoceptors because prior exposure to UTP resulted in a marked inhibition of the ATPP-induced relaxation (Park, unpublished data). Therefore, ATPP seems to induce relaxation via heterogeneous purinoceptors (P_{2Y} and nucleotide receptors) coexisting in the rat aortic endothelium. However, it is not understood why ATP acts mainly on nucleotide receptors while ATPP acts on both P_{2Y} and nucleotide receptors.

The action of ATPP seemed to change depending upon the vascular tone. At a low tone (resting tension), ATPP exerted a contraction via P_{2X}-purinoceptors on the smooth muscle; while at a NE-elevated tone, it induced relaxation via heterogeneous purinoceptors on the endothelium. Ralevic and Burnstock (1991) have shown that in the isolated mesenteric artery, the vascular response to ATP at a low tone is contraction, whereas the response at a high tone is relaxation. However, in the rat aorta with intact endothelium, ATP exerts only relaxation at all levels of vascular tone (Lee *at al.* unpublished data). This observation suggests that the vascular response to agonists of P_2-purinoceptors may differ from one blood vessel to another. The dual effects of ATPP in the rat aorta may be relevant to the physiological regulation of blood pressure. Kong *et al.* (1991) have reported that in *in vivo* experiments an aortic infusion of ATPP decreased the mean arterial pressure during normotensive state, whereas it increased mean arterial pressure in hypotensive states induced by hemorrhage. Furthermore, ATP lowered the blood pressure under both normo- and hypotensive states (Kong *et al.* 1991) which is consistent with present *in vitro* findings. Possible explanation may include an increased sensitivity of P_{2X}-purinoceptors in the smooth muscle to ATPP at low levels of vascular tone (Kong *et al.* 1991). However, the precise mechanisms responsible for the dual effects of ATPP remain to be elucidated.

Regulation of vascular tone by an agonist has a physiological relevance only if there are sources of the agonist around the receptors. Evidence that for several different sources of ATPP have been accumulated. First, ATPP has been detected in extracts of mammalian skeletal muscles (Marrian, 1954; Small and Cooper, 1966). However, little attention has been paid to possible physiological and pharmacological roles of this novel nucleotide until it was identified in rabbit platelets (Lee *et al.* 1995) Although the amount of ATPP released by thrombin is less than that of ATP, ATPP may play a significant role in the regulation of local vascular tone during platelet aggregation and thrombus formation due to its strong resistance against degradation by blood nucleotidases (Lee *et al.* unpublished data). Furthermore, the detection of ATPP in the adrenal medulla (Jeong, unpublished observation) suggests the possibility that ATPP may be released as a co-transmitter with noradrenalin from perivascular sympathetic nerves and may be as a transmitter released from purinergic and sensory nerves similar to the release of ATP (Kugelgen and Starke, 1991).

In summary, our results indicate that ATPP is a novel adenine nucleotide which acts on both the endothelium and smooth muscle of the isolated rat aorta. ATPP elicited a contraction via P_{2X}-purinoceptor with a potency greater than that of ATP. In addition, ATPP was as effective as ATP in eliciting relaxation effects after the blocking of P_{2X}-purino-

ceptors in smooth muscle. The ATPP-induced relaxation might be mediated by P_{2Y} and nucleotide receptors on the rat aortic endothelium. Finally, ATPP showed dual effects depending upon the vascular tone implying that ATPP may participate in the physiological regulation of blood pressure.

ACKNOWLEDGEMENTS

We thank Drs. R.D. Wurster and J.P. Filkins and for carefully correcting the manuscript. This work was supported by a grant from the Korea Science and Engineering Foundation (*901-0408-061-1).

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