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Relaxant Effect of 4-Aminopyridine on the Mesenteric Artery of Rat

Se-Hoon Kim and Tae-Im Lee

Department of Physiology, College of Medicine, Konyang University, Chungnam 320−711, Korea

It has been well known that 4-aminopyridine (4-AP) has an excitatory effect on vascular smooth muscle due to causing membrane depolarization by blocking K^+-channel. However, we observed that 4-AP had an inhibitory effect on the mesenteric artery of rat. Therefore, we investigated the mechanism of 4-AP-induced vasorelaxation. The mesenteric arcuate artery and its branches were isolated and cut into ring. The ring segment was immersed in HEPES-buffered solution and its isometric tension was measured. 4-AP (0.1 ~ 10 mM) induced a concentration-dependent relaxation, which was unaffected by NO synthase inhibitor, N^G-nitro-L-arginine methylester (100 μM) or soluble guanylate cyclase inhibitor, methylene blue (10 μM). Glibenclamide (10 μM), ATP-sensitive K^+ channel blocker, did not exert any effect on the 4-AP-induced vasorelaxation. 4-AP relaxed the sustained contraction induced by 100 mM K^+ or Ca^{2+} ionophore, A23187 (10 μM) in a dose-dependent manner. In addition, 4-AP significantly decreased the phasic contractile response to norepinephrine in the absence of extracellular Ca^{2+}. However, 4-AP did not block the ^{45}Ca influx of rat aorta. From the above results, we suggest that 4-AP may not block the Ca^{2+} influx through Ca^{2+}-channel, but act as a nonspecific vasorelaxant in arterial smooth muscle.

Key Words: Mesenteric artery, 4-aminopyridine, Vasorelaxation

INTRODUCTION

It has been well known that 4-aminopyridine (4-AP) has an excitatory effect in many tissues. The excitatory effect of 4-AP is due to its potent and selective K^+-channel blocking action in neuronal cell (Hermann & Gorman, 1981; Gustafsson et al, 1982; Thompson, 1982) and in other tissues (Okabe et al, 1987; Robertson & Nelson, 1994). In neuronal cell, 4-AP causes the prolongation of action potential, resulting in a facilitation of the influx of extracellular Ca^{2+} and release of neurotransmitter (Galvan et al, 1982). Therefore, 4-AP has been shown to be effective in both myasthenia gravis (Lundh et al, 1979; Lundh et al, 1985) and Lambert-Eaton-syndrome (Agoston et al, 1978; Lundh et al, 1984). In addition, 4-AP is a potent seizure-inducing agent. Accidental poisoning of humans with this compound has been reported to induce *grand mal*-type seizures (Spyker et al, 1980).

4-AP also has an excitatory effect on vascular smooth muscle due to causing membrane depolarization by acting on K^+-channel. In vascular smooth muscle, 4-AP blocks the voltage-gated, delayed rectifier K^+-channel (Kv) (Okabe et al, 1987; Beech & Bolton, 1989; Robertson & Nelson, 1994). Kv contributes to membrane potential in vascular smooth muscle, and a blocker of this channel causes depolarization and vasoconstriction in many vessels. 4-AP pretreatment causes membrane depolarization and vasoconstriction in the coronary artery (O'Rourke, 1996; Shimizu et al, 2000), and causes membrane depolarization in the pulmonary artery (Doi et al, 2000). 4-AP causes depolarization, and increased spike duration, and firing frequency in guinea-pig portal vein (Leander et al, 1977; Hara et al, 1980), and marked depolarization and vasoconstriction in myogenic cerebral vessel (Knot & Nelson, 1995).

However, we observed that 4-AP had an inhibitory effect on the mesenteric artery of rat. This paradoxical action is unrelated to the blocking effect of 4-AP on

Correspondence to: Se-Hoon Kim, Department of Physiology, College of Medicine, Konyang University, 26 Nae-dong, Nonsan, Chungnam 320-711, Korea. (Tel) 82-41-730-5344, (Fax) 82-41-736-5318, (E-mail) sehkim@kytis.konyang.ac.kr

K^+-channel. Therefore, this study was undertaken to examine the possible mechanisms underlying the vasorelaxant action of 4-AP in the mesenteric artery of rat.

METHODS

Male Sprague-Dawley rats were anesthetized by urethane and killed by bleeding. The mesenteric arcuate artery and its branches were isolated and cleaned of surrounding connective tissue. Arterial rings about 2 mm in length were prepared and mounted between two pieces of stainless steel in 5 ml organ bath containing HEPES-buffered solution of the following composition (mM): NaCl, 143; KCl, 5.4; $CaCl_2$, 1.8; $MgCl_2$, 1.0; glucose, 5; HEPES, 10. The HEPES-buffered solution was titrated with NaOH to pH 7.4. In experiments using high-K^+ solution, Na^+ in the bathing medium was replaced by equimolar concentration of K^+ to maintain a constant ion strength. The bath solution was constantly bubbled with 100% O_2. The isometric tension was measured with myograph force-displacement transducer (Myo-interface, Model 410A) and recorded with strip chart recorder (Linear).

The arterial ring was allowed to equilibrate for 60 minutes under 4 mN resting tension. The arterial ring was contracted with single concentration of norepinephrine (NE, 3 μM) three times before experiment. After we confirmed that the amplitude of NE-induced contraction was constant, experiments were performed. Arterial ring was incubated with each inhibitor for 15 minutes before they were contracted with agonists. then 4-AP was added cumulatively to induce concentration-dependent relaxation. The effects of 4-AP on the sustained tone were expressed as percentages of the control value. Cumulative concentration-relaxation relationships were analyzed with non-linear curve fitting by means of a logistic equation and ED_{50} values were calculated as the drug concentration causing the half maximum relaxation.

Rat aorta was isolated and cut into longitudinally. Endothelium was removed by rubbing internal surface. Aortic strip was cut into 5 pieces, and incubated in HEPES-buffered Tyroids solution for 1 hour at room temperature and incubated in Ca^{2+}-free solution at 37°C for 1 hour. The aortic strips were transferred to the control solution, 100 mM K^+ containing HEPES-buffered solution (K100-solution), K100-solution with 10 μM verapamil, K100 solution with 10 mM 4-AP, and K100-solution with 100 mM La^{3+}, respectively. After incubating the strips in each solution for 10 minutes, 20 μCi/ml of ^{45}Ca was added to the incubating solution. Incubation was continued for 40 minutes in ^{45}Ca contained solution and stopped by addition of ice-cold 30 mM La^{3+}-solution. Arterial strips were dried at 70°C for 30 minutes and weighed. Arterial strips were lysed with 0.5 ml of 0.5 N NaOH and 10 ml of scintillation cocktail (Luma-gel) was added. Radioactivity was measured with a liquid scintillation counter and divided by weight of arterial strip.

The following compounds were used: norepnephrine hydrochloride, A23187, 4-AP, N^G-nitro-L-

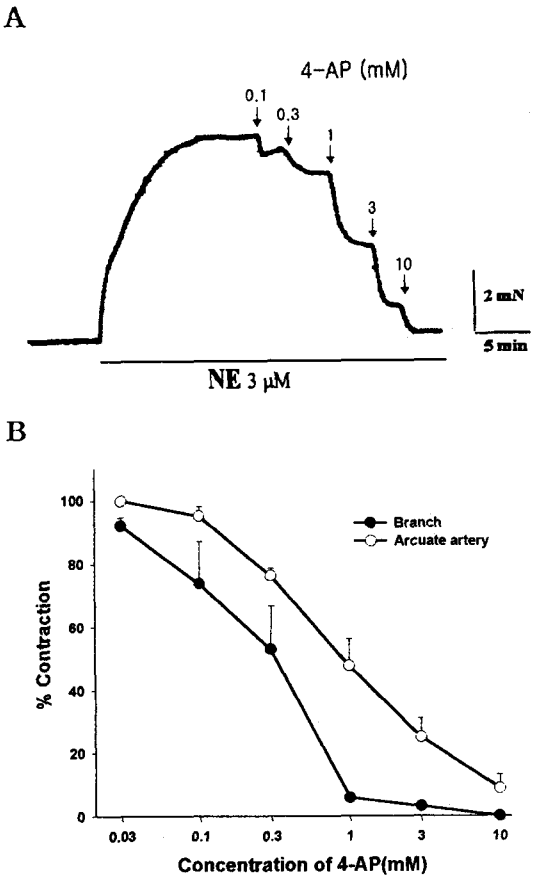

Fig. 1. Effect of 4-aminopyridine (4-AP) on the norepinephrine (NE)-induced contraction in the mesenteric artery of rat. A: Typical tracing of 4-AP-induced vasorelaxation of mesenteric arcuate artery. Mesenteric arcuate artery was contracted by NE and cumulative addition of 4-AP caused the vasorelaxation dose-dependently. B: Mean concentration-response curves for 4-AP in NE-precontracted artery. Points are the mean and vertical lines show S.E. from 5 experiments.

arginine methylester (L-NAME), glibenclamide and pinacidil. All compounds except A23187 and glibenclamide were dissolved in distilled water. A23187 was dissolved in ethanol and glibenclamide was dissolved in DMSO. A23187 and glibenclamide were diluted in HEPES-buffered solution before use.

RESULTS

NE induced a sustained contraction in isolated mesenteric arcuate artery and its branch. Fig. 1 shows concentration-dependent relaxation of mesenteric arcuate artery and its branch by cumulative addition of 4-AP. The branch of arcuate artery was more sensitive to 4-AP than arcuate artery. The concentration (ED_{50}) at which 4-AP produced the half-maximal relaxation was 0.95 ± 0.19 mM and 0.26 ± 0.03 mM in arcuate artery and its branch, respectively. 2-aminopyridine (2-AP) and 3,4-diaminopyridine (3,4-DAP) also caused the relaxation of mesenteric arcuate artery. ED_{50} values of 2-AP and 3,4-DAP were 2.40 ± 0.41 mM and 0.88 ± 0.20 mM, respectively (Fig. 2).

Fig. 3A shows the effect of L-NAME, nitric oxide synthase inhibitor, on the 4-AP-induced vasorelaxation. 100 μM L-NAME was pretreated for 15 minutes before the mesenteric arcuate artery was contracted by NE. As shown in Fig. 3A, pretreatment of L-NAME did not exert any effect on the relaxation induced by 4-AP. Pretreatment of methylene blue (10 μM), guanylate adenylase inhibitor, also did not influence the inhibitory action of 4-AP on NE-precontracted artery (Fig. 3B). Fig. 4 shows the effect of ATP-sensitive K^+ channel blocker on the vasorelaxation evoked by 4-AP. NE-induced contraction was relaxed by K^+ channel opener, pinacidil and the relaxation was reversed by glibenclamide, ATP-sensitive K^+-channel blocker. However, pretreatment of glibenclamide did not alter the relaxant effect of 4-AP.

To study the possible inhibitory action of 4-AP on

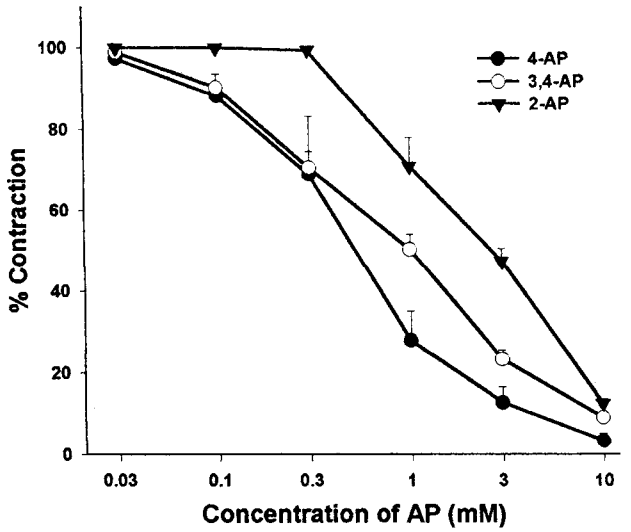

Fig. 2. B: Mean concentration-response curves for aminopyridine-analogues in mesenteric arcuate artery precontracted by norepinephrine (3uM). 2-aminopyridine (2-AP) and 3,4 diaminopyridine (3,4-AP) also relax the mesenteric artery. Points are the mean and vertical lines show S.E. from 5 experiments.

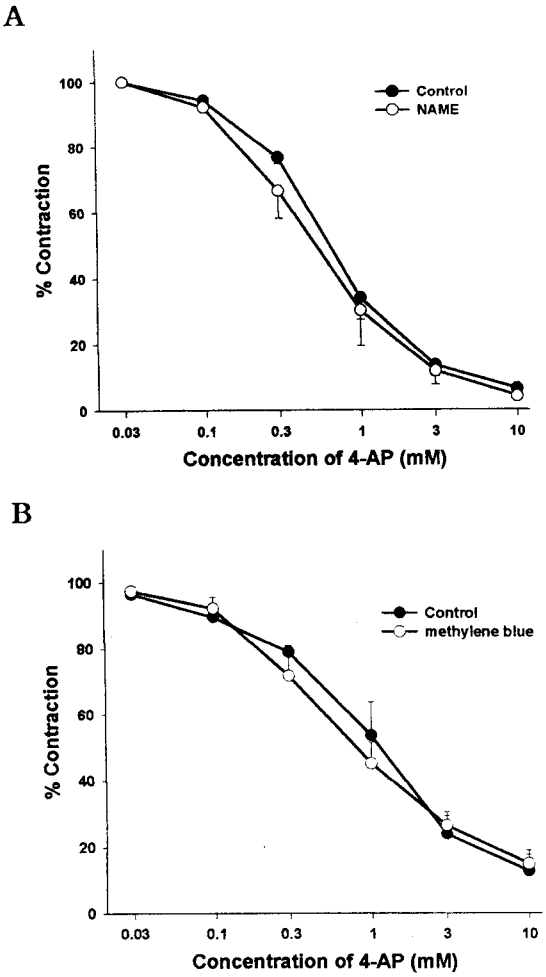

Fig. 3. Mean concentration-response curves for 4-aminopyridine (4-AP) in mesenteric arcuate artery precontracted by norepinephrine (3 μM) in the presence of 100 μM NAME (A) or 10 μM methylene blue (B). Points are the mean and vertical lines show S.E. from 5 experiments.

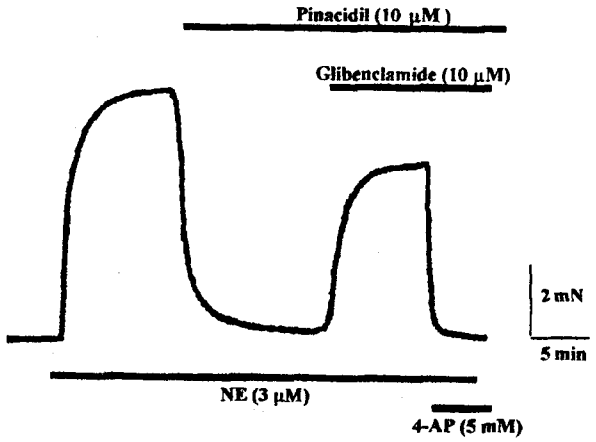

Fig. 4. Effect of glibenclamide on the 4-aminopyridine (4-AP)-induced vasorelaxation. Mesenteric arcuate artery precontracted by norepinephrine (NE) was relaxed by pinacidil. Pinacidil-induced relaxation was reversed by glibenclamide, but 4-AP-induced relaxation was not affected by glibenclamide.

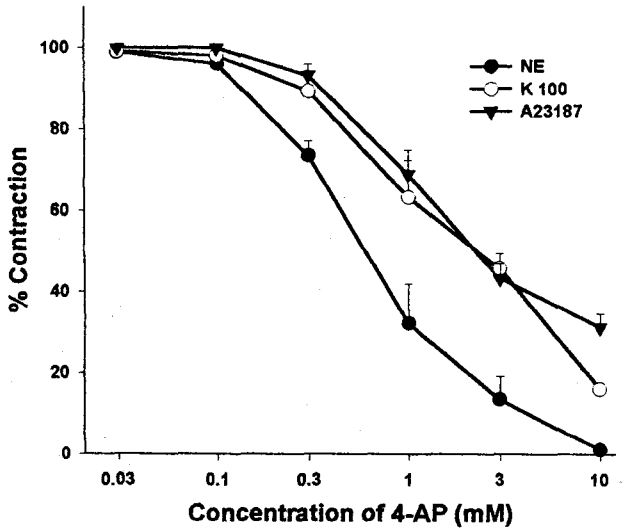

Fig. 5. Mean concentration-response curves for 4-aminopyridine (4-AP) in mesenteric arcuate artery precontracted by norepinephrine (3 μM), 100 mM K^+ or A23187 (10 μM). A23187-induced contraction was performed in the presence of 100 μM NAME. Points are the mean and vertical lines show S.E. from 5 experiments.

Ca^{2+} influx, its effect on contractions induced by 100 mM K^+ or A23187 was examined (Fig. 5). In the 100 mM K^+-contracted artery, cumulative addition of 4-AP induced a concentration-dependent relaxation. A23187, Ca^{2+} ionophore, induced the sustained contraction in the presence of L-NAME. Since A23187

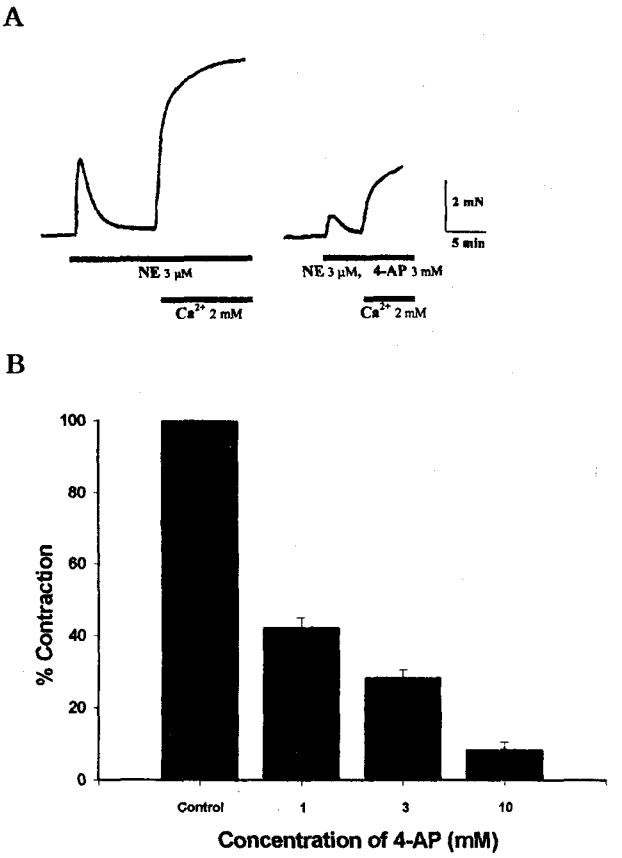

Fig. 6. Effect of 4-aminopyridine (4-AP) on the norepinephrine (NE)-induced contraction in Ca^{2+}-free bathing solution. Typical tracing of inhibitory effect of 4-AP on the NE-induced contraction in Ca^{2+}-free bathing solution (A). Columns are the mean and vertical lines show S.E. from 5 experiments (B).

is known to release the nitric oxide from endothelium, L-NAME (100 uM) was pretreated to abolish the effect of nitric oxide. In the A23187-induced contraction, 4-AP relaxed the sustained contraction in a dose-dependent manner. In Ca^{2+}-free bath solution containing 0.1 mM EGTA, NE caused the phasic contraction transiently and addition of Ca^{2+} induced the sustained tonic contraction. Pretreatment of 4-AP decreased the amplitude of phasic contraction. The phasic contractions were 42.4±2.7 (1 mM), 28.5± 2.1 (3 mM) and 8.6±2.0% (10 mM) in the presence of 4-AP compared with control, respectively (Fig. 6).

In order to get the direct evidence about inhibitory action of 4-AP on Ca^{2+} influx, its effect on ^{45}Ca influx in rat aorta was examined (Fig. 7). Verapamil has been known to inhibit the Ca^{2+} influx via blocking Ca^{2+}-channel. Therefore we compared the effect of

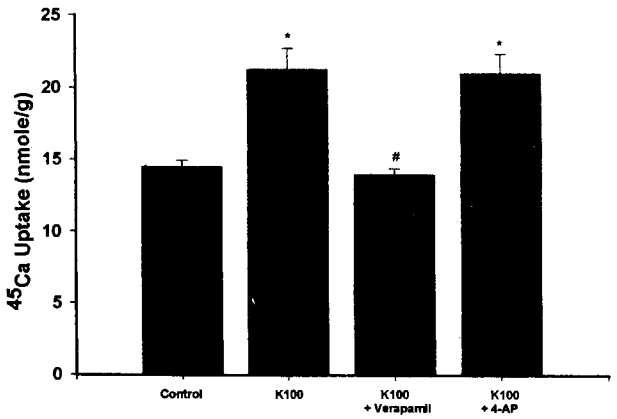

Fig. 7. Effect of 4-aminopyridine (4-AP) on the ^{45}Ca uptake in rat aorta. Control solution is HEPES-buffered physiological salt solution and K100 means 100 mM K^+ solution. Columns are the mean and vertical lines show S.E. from 5 experiments. *value significantly different from control ($p<0.05$). #value significantly different from K100 ($p<0.05$).

4-AP on ^{45}Ca influx with verapamil. ^{45}Ca influx was increased from 14.5 ± 0.48 to 21.3 ± 1.46 nmole/g by raising the extracellular K^+ concentration to 100 mM. Pretreatment of verapamil inhibited the 100 mM K^+-induced ^{45}Ca influx as expected. However, 4-AP did not inhibit the 100 mM K^+-induced ^{45}Ca influx.

DISCUSSION

It has been well known that 4-AP inhibits voltage-dependent K^+ channel of the vascular smooth muscle (Okabe et al, 1987; Beech & Bolton, 1989; Robertson & Nelson, 1994). These channels conduct outward, hyperpolarizing K^+ current that influences resting membrane potential. Therefore, blocking of voltage-dependent K^+ channel with 4-AP leads to membrane depolarization and increment of Ca^{2+} influx, thereby causes smooth muscle to contract (O'Rourke, 1996; Doi et al, 2000; Shimizu et al, 2000). However, in our study, 4-AP caused the vasorelaxation in the mesenteric artery of rat. The inhibitory action of 4-AP was not previously shown in blood vessel. The observed effects of 4-AP on mesenteric artery of rat therefore seem both paradoxical and intriguing.

The contraction-relaxation cycle of vascular smooth muscle is dependent on the concentration of intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and the sensitivity of the contractile apparatus to Ca^{2+}. $[Ca^{2+}]_i$ is regulated by many factors such as influx of Ca^{2+} across the membrane, release of Ca^{2+} from the sarcoplasmic reticulum (SR), extrusion of Ca^{2+} from the sarcoplasm and re-uptake into the SR. Many factors, such as endothelial cell derived factor, exitatory and inhibitory neurotransmitters and also other substances, determine the $[Ca^{2+}]_i$ by regulation of the voltage-dependent Ca^{2+} channel directly or indirectly through depolarization or hyperpolarization of the membrane (Bolton et al, 1984; Garland, 1987). Regulation of the sensitivity of the contractile apparatus to Ca^{2+} appears to be an another important mechanism in regulating the contraction-relaxation cycle of vascular smooth muscle (Nishimura et al, 1988; Karaki, 1989; Kitazawa et al, 1989). Excitatory and inhibitory substances such as norepinephrine, prostaglandin and endothelin regulate the sensitivity of contractile protein to Ca^{2+} (Ozaki et al, 1989, 1990; Lee et al, 1997).

The present results showed that the 4-AP relaxed the mesenteric artery and its vasorelaxant action was independent on L-NAME, nitric oxide synthase inhibitor. Endothelial removal did not also exert any effect on the 4-AP-induced vasorelaxation (data not shown). Hence, vascular smooth muscle appears to be the principal target for the actions of 4-AP. Membrane hyperpolarization of vascular smooth muscle is one of the main causes of vasorelaxation. K^+ channel openers such as cromakalim and pinacidil are known to activate ATP-sensitive K^+ channel, thereby hyperpolarize the membrane potential (Standen et al, 1989). In our study, pinacidil relaxed the rat mesenteric artery and glibenclamide, ATP-sensitive K^+ channel blocker, reversed the pinacidil-induced vasorelaxation but glibenclamide did not reverse the 4-AP-induced vasorelaxation. These results mean that 4-AP-induced vasorelaxation is not related to membrane hyperpolarization. Furthermore, the ability of 4-AP to relax mesenteric artery bathed in 100 mM K^+ solution minimizes the possibility that the vasorelaxation by 4-AP is due to membrane hyperpolarization. If the main mechanism of 4-AP-induced vasorelaxation is membrane hyperpolarization, the contraction by 100 mM K^+ must not be relaxed by 4-AP. Since extracelluar 100 mM K^+ is high enough as compared to intracellular K^+ concentration, increased K^+ conductance do not exert any effect on membrane potential.

100 mM K^+ depolarizes the membrane and membrane depolarization increases the open probability of the voltage-gated Ca^{2+} channel. On the other hand,

A23187 makes the membrane permeable to Ca^{2+} and increase the $[Ca^{2+}]_i$. Therefore, extracellular Ca^{2+} and Ca^{2+} influx are essential for the contraction induced by 100 mM K^+ or A23187. 4-AP suppressed the 100 mM K^+-induced contraction and also suppressed the A23187-induced contraction. These data suggest that 4-AP may reduce the Ca^{2+} entry across sarcolemma or may lower the sensitivity of contractile apparatus to influxed Ca^{2+}. However, the possibility that 4-AP may reduce the Ca^{2+} entry across sarcolemma is very low, since 4-AP did not inhibit 100 mmM K^+-induced ^{45}Ca uptake, which is accomplished via voltage-gated Ca^{2+} channel. The present result that 4-AP suppressed the phasic contractile response to NE in the absence of extracellular Ca^{2+} also minimize the possibility that 4-AP reduce the Ca^{2+} entry across sarcolemma. The phasic contraction by NE in the Ca^{2+}-free solution is not related to extracellular Ca^{2+} and mediated by Ca^{2+} released from SR. If the 4-AP reduced the Ca^{2+} entry across sarcolemma and relaxed the mesenteric artery, phasic contractile response should not be affected by 4-AP.

Although there is no direct evidence about the effect of 4-AP on Ca^{2+}-sensitivity of contractile apparatus, above results support the idea that 4-AP-induced vasorelaxation is not related to blocking of Ca^{2+} influx, and may be related to the sensitivity of contractile apparatus to Ca^{2+}. If 4-AP reduced the sensitivity of contractile apparatus to Ca^{2+}, both of phasic contraction and tonic contraction might be attenuated by 4-AP without influence on the Ca^{2+} influx and release. However, many problems remain to be solved before we fully understand the mechanism of 4-AP-induced vasorelaxation.

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