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Effects of amiloride on the neurally mediated contraction of rat mesenteric artery

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Abstract

The effects of amiloride on contraction evoked by perivascular nerve stimulation were studied in a ring preparation of rat mesenteric artery. The contraction evoked by nerve stimulation was abolished by tetrodotoxin or prazosin. Amiloride depressed the nerve-induced contraction concentration dependently. Noradrenaline induced a tonic contraction in the artery. Amiloride inhibited the noradrenaline-induced contraction concentration dependently. The excitatory junctional potential (e.j.p.) recorded intracellularly was abolished by tetrodotoxin. The amplitude of the e.j.p. was not altered by prazosin or amiloride. These results indicate that amiloride inhibits the perivascular nerve-mediated contraction of mesenteric artery mainly through postsynaptic adrenoceptor inhibition and not through mechanisms related to e.j.p.

Keywords: Mesenteric artery; Adrenergic nerve; Contraction; Excitatory junctional potential; Amiloride

1. Introduction

Amiloride, which is known to inhibit Na^+ transport in various tissues (Benos, 1982), has also been shown to inhibit contraction of smooth muscle. The inhibitory mechanism was explained by Na^+ transport inhibition (Bova et al., 1988), an α-adrenoceptor blocking action (Palaty, 1986) or inhibition of myosin light chain kinase (Ozaki et al., 1987).

Amiloride is also known to exert a hypotensive action through a decrease of vascular resistance where contraction is regulated by sympathetic nerve activity (Haddy et al., 1985; Barrett and Kau, 1986; Pamnani et al., 1988). We now studied the effect of amiloride on the contraction and membrane potential induced by transmural nerve stimulation in rat mesenteric artery, where adrenergic innervation is dense (Furness and Marshall, 1974).

2. Materials and methods

2.1. Preparations

Male Wistar Kyoto rats, 4 months old, were used. They were anesthetized with ethyl ether, stunned and bled. Second-order branches of anterior mesenteric artery were dissected free from surrounding tissue. Five-mm-long ring preparations were made. Endothelium was removed mechanically with a thin wire. Each preparation was incubated in a physiological medium aerated with 95% O_2/5% CO_2 at 37°C. The medium was a modified Tyrode's solution composed as follows (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 2.0, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, $NaH_2PO_4 \cdot 2H_2O$ 0.4, glucose 5.6, and pH 7.3. Medium with high K^+ was prepared by replacing NaCl with an equimolar amount of KCl.

2.2. Mechanical recordings

Each strip was set between two platinum electrodes with two pieces of thin tungsten wire (30 μm diameter) in

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the lumen. One of the wires was fixed to a rod and another was connected to a force-displacement transducer (U-gage, Minebea, Nagano, Japan). The preparations were set at a basal tension of 200 mg. Changes in isometric tension were recorded on a thermal pen recorder (Recti-horiz 8K, NEC-Sanei, Tokyo, Japan). The preparations were allowed to stand at least 2 h in the physiological medium before the experiments. Field stimulation with rectangular pulses (30 V pulse amplitude and 0.1 ms pulse duration) were applied with a stimulator (SEN-3201, Nihon-kohden, Tokyo, Japan) at various frequencies for 5 s.

2.3. Electrophysiological recordings

A glass microelectrode was used for intracellular membrane potential recording, as previously reported (Shimamura et al., 1993). The arteries were mounted in a chamber of 1 ml capacity with insect pins. The chamber was superfused continuously (2 ml/min) with aerated Tyrode's solution. A glass capillary electrode (Hilgenberg 1.2 mm, Germany) filled with 3 M KCl (tip resistance 40–80 MΩ) was impaled into the arterial smooth muscle through the mesenterium membrane. Membrane potential was monitored with an oscilloscope (VC-10, Nihon-kohden, Tokyo, Japan) after amplification by a preamplifier (MEZ-8201, Nihon-kohden). The data were stored in a data recorder (RMG-5304, Nihon-kohden). Impalement was considered to have been successful when a sharp drop and recovery of potential were observed at the beginning and end of recording. Perivascular nerve stimulation was performed with a suction electrode. The tissue was stimulated with 5 square pulses of 0.1 ms duration at 0.5–20 Hz. The maximum response during a stimulation period was taken as the amplitude of excitatory junctional potential (e.j.p.) evoked by the stimulation at each frequency.

In experiments with a short period of drug application during membrane potential recording, a small amount of drug solution was delivered to the tissue surface through a glass capillary (inner diameter 20 μm) by positive pressure pulses (Schumann and Kreulen, 1986) using a miniature solenoid valve (General Valve, Fairfield, NJ, USA).

The drugs used were amiloride HCl, noradrenaline HCl, adenosine 5′-triphosphate 2Na (ATP), tetrodotoxin, prazosin HCl, prostaglandin $F_{2\alpha}$, all supplied by Sigma (St. Louis, MO, USA). Acetylcholine Cl was obtained from Wako (Osaka, Japan). ATP was dissolved in cold water and the pH was adjusted at 7.0 with NaOH. Prostaglandin $F_{2\alpha}$ was dissolved in dimethyl sulfoxide (DMSO). The highest final concentration of DMSO in the experiments was 0.1%.

The data were expressed as means ± S.E.M. for *n* (number of observations). Student's *t*-test was used to

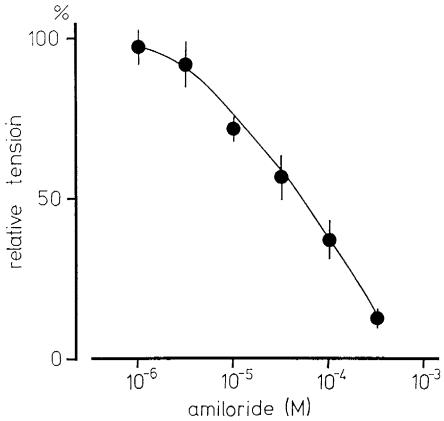

Fig. 2. A dose–response curve for the effect of amiloride on the transmural nerve stimulation-induced phasic contraction. Each phasic contraction was evoked by 5-s stimulation at 10 Hz. Each point represents the mean ± S.E.M. of 9–10 observations.

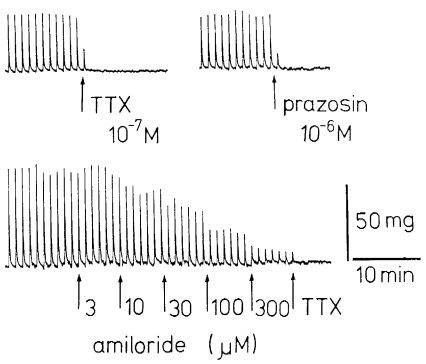

Fig. 1. A typical tracing showing the effect of amiloride, prazosin, and tetrodotoxin on transmural nerve stimulation-induced contraction in rat mesenteric artery. Electrical field stimulation was applied for 5 s at 10 Hz every minute. In the bottom tracing, amiloride was applied cumulatively at the arrows and 10^{-7} M tetrodotoxin (TTX) was applied at the last arrow.

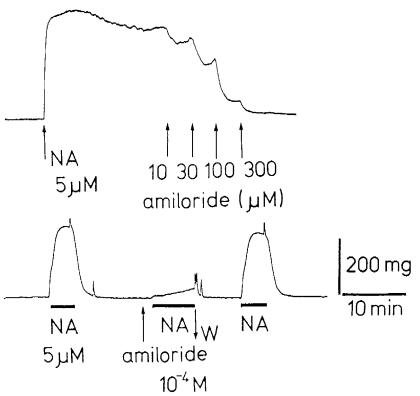

Fig. 3. Typical tracing showing the effect of amiloride on the 5 μM noradrenaline (NA)-induced contraction. In the upper tracing, amiloride was applied cumulatively during the tonic contraction induced by noradrenaline. In the lower tracing, amiloride pretreatment inhibited noradrenaline-induced contraction. The contraction recovered from the inhibition after washout of amiloride (indicated by 'W' with an arrow).

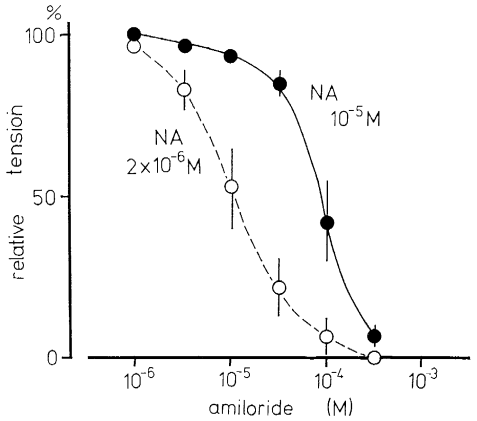

Fig. 4. Summary of data for the effect of amiloride on the noradrenaline (NA)-induced tonic contraction. Noradrenaline at two concentrations (2×10^{-6} and 10^{-5} M) was applied to develop tonic contractions.

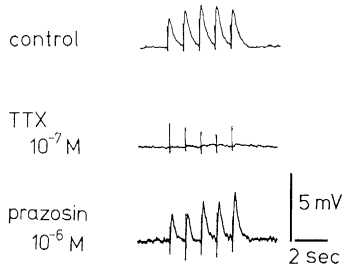

Fig. 6. Typical tracings of e.j.p. and e.j.p. recorded in the presence of tetrodotoxin (TTX) or prazosin. All tracings were obtained from the same cell. The effect of prazosin was examined after washout of tetrodotoxin, when the amplitude of e.j.p. mostly returned to the same size as control. Resting membrane potential was −68 mV. Sharp vertical deflections are stimulation artifacts.

evaluate data statistically and $P < 0.05$ was considered to be significant.

3. Results

3.1. Effect of amiloride on the contraction of mesenteric artery

A phasic contraction was evoked by each 5-s electrical stimulation. The amplitude of the contraction increased frequency dependently between 2–20 Hz. The amplitude of the phasic contraction evoked by 10 Hz stimulation was stable for 1 h when repeated at an interval of 1 or 2 min. The contraction was completely blocked by 10^{-7} M tetrodotoxin. The induced contraction was also completely inhibited by 10^{-6} M prazosin (Fig. 1). When amiloride was applied, the contraction amplitude decreased within a few minutes. Amiloride inhibited the contraction concentration dependently in the range of 10^{-6} and 3×10^{-4} M (Fig. 2).

5×10^{-6} M noradrenaline induced a tonic contraction in rat mesenteric arteries. The noradrenaline-induced contraction was inhibited immediately after amiloride was applied. When amiloride was applied cumulatively, the drug inhibited the contraction in a concentration-dependent manner between 10^{-6} M and 3×10^{-4} M (Figs. 3 and 4).

When the concentration of noradrenaline was increased cumulatively, the amplitude of the tonic contraction increased concentration dependently. The cumulative concentration-dependent contraction induced by noradrenaline was inhibited by 10^{-4} M amiloride, especially with low concentrations of noradrenaline, resulting in a rightward shift of the concentration–response curve (Fig. 5).

The above data were plotted using the equation of Schild (1949) to study the interaction of amiloride with noradrenaline at the adrenoceptor. The slope and pA_2 of the fitted line were 0.92 and 4.7, respectively.

To study if the inhibitory action of amiloride is specific to receptor-mediated contraction, the effect of amiloride on high-K^+-induced contraction was examined. The tonic

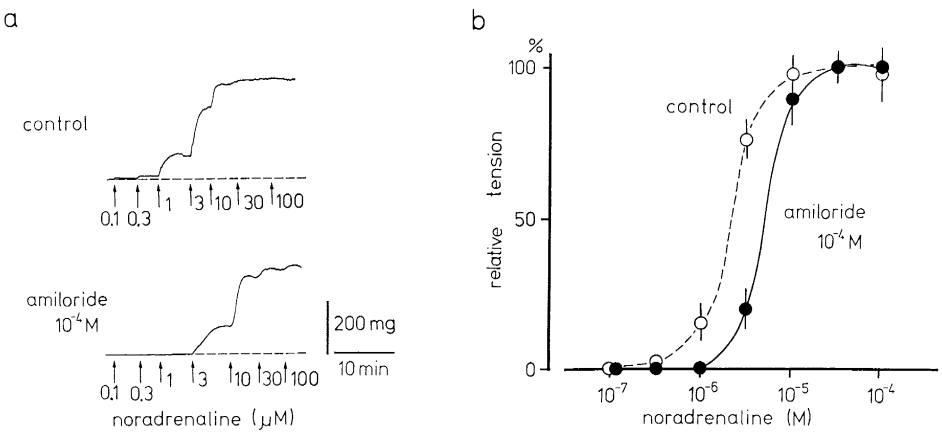

Fig. 5. (a) Typical tracings showing the effect of amiloride on the noradrenaline-induced contraction in the absence (upper trace) and presence (lower trace) of 10^{-4} M amiloride. Noradrenaline was administered cumulatively at each arrow, from 0.1 to 100 μM. (b) Dose–response curves showing the inhibitory effect of amiloride on the contraction induced by noradrenaline at different concentrations. Each point represents the mean ± S.E.M. of 8 observations.

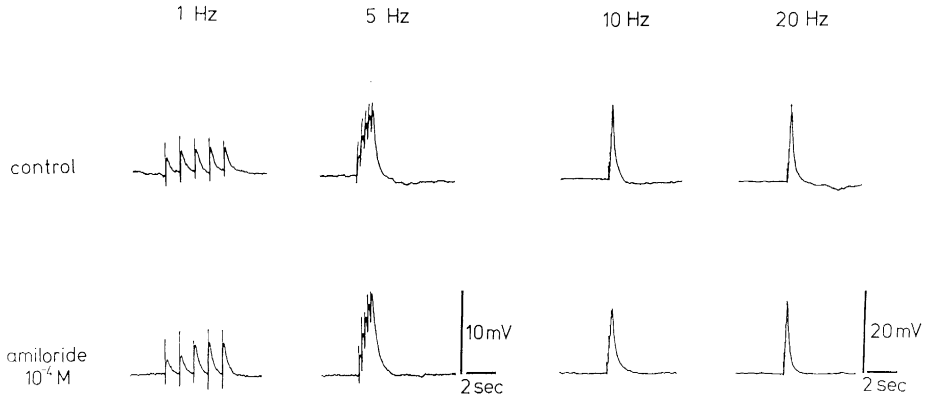

Fig. 7. Typical tracings showing the effect of 10^{-4} M amiloride on e.j.p. evoked at different frequencies (1, 5, 10, 20 Hz). All tracings were obtained from the same cell whose resting membrane potential was −72 mV. Calibration scale for membrane potential is 10 mV for 1 and 5 Hz and 20 mV for 10 and 20 Hz. Sharp vertical deflections preceding each e.j.p. are electrical stimulation artifacts.

phase of 80 mM K^+-induced contraction was inhibited partially (10–20%, $n = 5$) by amiloride 10^{-4} M. 10^{-6} M prazosin also inhibited the contraction partially (10–20%, $n = 5$). In the presence of 10^{-6} M prazosin, amiloride did not inhibit the contraction further ($n = 5$).

The effect of acetylcholine was tested on noradrenaline (5×10^{-6} M)-induced tonic contraction in 5 preparations. 10^{-6} M acetylcholine did not induce any relaxation.

To examine whether the amiloride-induced inhibition of the contraction was α-adrenoceptor specific, we applied amiloride during the prostaglandin $F_{2\alpha}$-induced contraction, where the TP prostanoid receptor was involved. Prostaglandin $F_{2\alpha}$, 3×10^{-6} M and 10^{-5} M, induced tonic contraction with an amplitude of 7% and 18% of that of the 80 mM K^+-induced contraction, respectively. The contraction in the presence of 10^{-4} M had an amplitude $99.3 \pm 3.5\%$ ($n = 5$) and $97.4 \pm 1.5\%$ ($n = 5$) of the control contraction which was induced by prostaglandin $F_{2\alpha}$ 3×10^{-6} M and 10^{-5} M, respectively. Therefore, no shift in prostaglandin $F_{2\alpha}$-induced contraction was observed in the presence of 10^{-4} M amiloride.

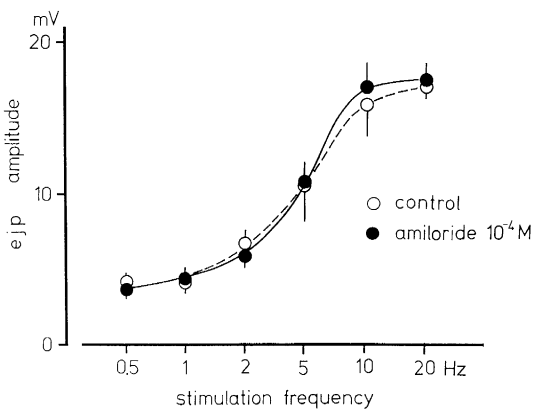

Fig. 8. Summary of data showing that 10^{-4} M amiloride did not affect e.j.p. amplitude evoked by 5 pulses at 0.5 to 20-Hz stimulation. Each point represents the mean ± S.E.M. of 5–8 observations.

3.2. Effect of amiloride on the membrane potential of smooth muscle cells of mesenteric artery

The effect of amiloride on the electrical activity of smooth muscle was studied from recordings of membrane potentials before and 5 min after application of the drug. The resting membrane potential of the artery was -70.3 ± 2.1 mV ($n = 12$) and -70.2 ± 1.2 mV ($n = 12$) in the absence and presence of amiloride ($P > 0.05$), respectively. Thus, amiloride did not affect the resting membrane potential. When five pulses were applied through the suction electrode, each electrical stimulation evoked an e.j.p. The e.j.p. was not followed by slow depolarization in this preparation. The e.j.p. was abolished by 10^{-7} M tetrodotoxin in 3 min but was not affected by 10^{-6} M prazosin (Fig. 6). When the stimulation frequency was increased, the amplitude of e.j.p. increased frequency dependently. 10^{-4} M amiloride did not affect the peak amplitude of e.j.p. at frequencies between 0.5–20 Hz (Figs. 7 and 8).

To examine the effect of amiloride on the postsynaptic response to ATP, 1 M ATP was applied for 10 ms at 200 μm upstream of the recording site. A transient depolarization of 1–2 s duration was observed immediately after each application. The amplitude of the depolarization in-

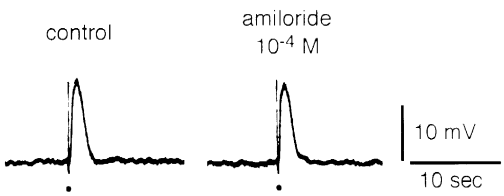

Fig. 9. Typical tracings showing ATP-induced transient depolarization in the absence (control) and presence of 10^{-4} M amiloride. Tracings were obtained from the same cell whose resting membrane potential was −67 mV. In each tracing, 10-ms positive pressure was applied to a capillary containing 1 M ATP at the time indicated by a dot at the bottom of the tracing. Sharp vertical deflections preceding the depolarization are artifacts related to opening of the solenoid valve.

creased when ATP was applied with a longer pulse duration. Repeated application of ATP with 10-ms pulse duration, at an interval of 1 min, evoked depolarizations with constant peak amplitude. The effect of amiloride was evaluated by measurement of the peak amplitude of the depolarization 5 min after the start of amiloride perfusion. The amplitude of the ATP-evoked depolarization was 16.8 $\pm$ 3.3 mV ($n = 5$) and 17.2 $\pm$ 0.9 mV ($n = 5$) in the absence and presence of 10^{-4} M amiloride, respectively (Fig. 9), with no statistical significance of the difference between the values.

4. Discussion

4.1. Transmural nerve stimulation-induced contraction in rat mesenteric artery

The rat mesenteric vessels are richly innervated with perivascular nerves (Furness and Marshall, 1974). The transmural nerve stimulation-induced contraction in arteries has been shown to be mediated mainly by the release of ATP and noradrenaline (Cheung, 1984; Von Kügelgen and Starke, 1985; Sneddon and Burnstock, 1985). In the present study, the transmural nerve stimulation-induced contraction in rat mesenteric artery was abolished by prazosin, an α_1-adrenoceptor antagonist. This shows clearly that the transmural nerve stimulation-mediated contraction in rat mesenteric artery is mainly mediated by endogenous noradrenaline which activates α_1-adrenoceptors (Angus et al., 1988).

Although amiloride may relax smooth muscle through the Na^+ transport inhibition (Pinon and Fabre, 1985; Bova et al., 1988; Krampetz and Bose, 1988), previous reports revealed several other mechanisms in the relaxation by amiloride (Ozaki et al., 1987; Reynolds et al., 1988; Sharma et al., 1988). In the present experiments, amiloride inhibited the transmural nerve stimulation-induced contraction in rat mesenteric artery. As the contraction is preferentially adrenergic, we examined adrenergic mechanisms first.

4.2. Effects of amiloride on adrenergic mechanisms

Exogenous noradrenaline contracted the rat mesenteric artery concentration dependently. The contraction was inhibited by prazosin. Amiloride shifted the cumulative concentration-response curve for the noradrenaline-induced contraction to the right. The clear rightward displacement of the curve by amiloride, the calculated value for slope, which was close to 1, and the dissociation constant from the Schild plot indicated its competitive α-adrenoceptor blocking action as has been reported previously (Palaty, 1986; Haussinger et al., 1987; Periyasamy, 1987; Reynolds et al., 1988; Sharma et al., 1988).

To clarify whether amiloride acted on the contraction at the receptor or at other sites, we examined the effect of amiloride on the contraction induced by a TP prostanoid receptor agonist which is independent of α_1-adrenoceptors. Our data showed that amiloride failed to inhibit the contraction induced by a TP prostanoid agonist. It has been shown that both α_1-adrenoceptor- and TP prostanoid receptor-mediated contractions are mediated by G_{q11} (Watson and Girdlestone, 1996). Because amiloride inhibited only the α_1-adrenoceptor-mediated contraction, the inhibition may not be related to intracellular signal transduction following α_1-adrenoceptor activation, which is the same as TP prostanoid receptor activation. Therefore it can be concluded that amiloride inhibited the noradrenaline-induced contraction by acting on the receptor.

The depression of the high-K^+-induced contraction by amiloride can be explained by its α-adrenoceptor blocking action, since it has been reported that the release of noradrenaline is induced in the artery by high-K^+ (Shimamura et al., 1987). The extent of the depression by amiloride was comparable to that produced by prazosin in the high-K^+-induced contraction. Moreover, amiloride failed to depress the high-K^+-induced contraction in the presence of prazosin, indicating the involvement of an α_1-adrenoceptor blocking action in the depression by amiloride.

Thus, the results now obtained indicate that amiloride inhibits the transmural nerve stimulation-induced contraction by inhibiting the postsynaptic response to noradrenaline released from perivascular nerves and acting on the receptor.

4.3. Effect of amiloride on mechanisms related to e.j.p.

It has been reported that amiloride decreases noradrenaline release from nerve terminals in vas deferens (Akhtar-Khavari et al., 1981). We were interested to find if a similar decrease in transmitter release is involved in the inhibition of the transmural nerve stimulation-induced contraction by amiloride. Perivascular nerve stimulation in the rat tail artery evoked two kinds of responses, mediated by ATP and by noradrenaline, respectively (Cheung, 1984). Suzuki and Kou (1983) reported that sympathetic nerve stimulation evoked in rabbit ear artery an e.j.p. which is resistant to α-adrenoceptor antagonists. In the present study with rat mesenteric artery, we confirmed that the e.j.p. is resistant to prazosin. Therefore, the e.j.p. response in this preparation is not mediated by α-adrenoceptors, but may be mediated by purinoceptors (Sneddon and Burnstock, 1985; Hirst and Edwards, 1989).

To clarify the effect of amiloride on the postsynaptic mechanism related to e.j.p., we examined the effect of amiloride on ATP-evoked depolarization. Repeated application of ATP to the organ bath during mechanical recording from the artery induced transient unstable contractions, possibly due to desensitization. To obtain stable responses,

we applied ATP for a very short period, on a very small area of the preparation close to the recording electrode. ATP evoked a transient depolarization with an amplitude comparable to that of the e.j.p. Amiloride did not affect the depolarization amplitude. This may indicate that amiloride does not have any effect on the electrical response of postsynaptic purinergic receptors. In addition, amiloride did not change either the resting membrane potential of smooth muscle cells or the amplitude of the e.j.p. Therefore, it can be concluded that amiloride does not alter the release of or the response to the transmitter which induces e.j.p.

It has been shown that noradrenaline and ATP seem to be released from the same vesicle of a neuron as both responses were inhibited by guanethidine or 6-OH dopamine (Von Kügelgen and Starke, 1985). The finding that e.j.p. was not altered by amiloride may then indicate that the release of noradrenaline in rat mesenteric artery is not altered by amiloride. However, we do not have any conclusive evidence concerning the effect of amiloride on noradrenaline release.

Recently, the separate control of noradrenaline and ATP release by presynaptic receptors has been reported (Driessen et al., 1994). Therefore, although a postsynaptic inhibitory effect may play the dominant role, a presynaptic inhibitory effect of amiloride on noradrenaline release in the present study cannot be excluded.

Amiloride has been reported to inhibit contraction also when an endothelium-dependent mechanism is involved (Cocks et al., 1988). In the present experiments, contributions of endothelium-mediated responses were minimized by endothelium removal as confirmed by the absence of acetylcholine-induced relaxation in the tonic component of noradrenaline-induced contraction.

In conclusion, the present study showed that amiloride inhibited the sympathetic nerve-mediated contraction of rat mesenteric artery by acting as an antagonist at the α_1-adrenoceptor. Decrease of transmitter release by amiloride was not supported by observation of the e.j.p.

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