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EFFECTS OF AMILORIDE ON CONTRACTIONS AND THE RELEASE OF TRITIUM FROM RAT VAS DEFERENS PRELOADED WITH [^{3}H]-NORADRENALINE

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1 The effect of amiloride was studied on contractions and tritium release from rat vas deferens preloaded with [^{3}H]-noradrenaline.

2 Amiloride had no effect on the resting tension and maximal contractile force of the vas deferens and did not alter the ED_{50} of noradrenaline.

3 Amiloride (10^{-4}–10^{-3} M) decreased the response of vas deferens to electrical stimulation dose-dependently without inhibiting the response to KCl (60 mM).

4 The effect of amiloride was not prevented by preincubation of the tissue with phentolamine, propranolol, atropine or indomethacin.

5 Amiloride did not alter the spontaneous outflow of radioactivity from [^{3}H]-noradrenaline labelled vasa deferentia.

6 Amiloride decreased the release of tritium induced by electrical stimulation or nicotine but did not inhibit the release of radioactivity induced by KCl or tyramine.

7 It is concluded that amiloride may inhibit the contractions of rat vas deferens by inhibiting the release of noradrenaline.

Introduction

Amiloride is a potassium sparing diuretic. In toad bladder (Bentley, 1968; Gatzy, 1971), frog skin (Nagel & Dorge, 1970) and human red cells (Aceves & Cereijido, 1973), amiloride is known to inhibit the entry of sodium ions into cells. Like tetrodotoxin and guanethidine, amiloride contains a guanidine group and may possibly affect neural function. Tetrodotoxin blocks the fast sodium channel (Kao, 1966) and guanethidine blocks the release of noradrenaline, most probably by stabilizing the membrane of sympathetic nerve endings (Hausler & Haefely, 1979). The effect of amiloride on adrenergic nerve function has not been studied. In the present experiments, the effect of amiloride on the contractions of the rat vas deferens and the release of [^{3}H]-noradrenaline ([^{3}H]-NA) have been investigated. The results show that amiloride inhibits the release of tritium from sympathetic nerve endings.

Methods

Male albino rats, weighing 200–300 g, were killed by a blow on the head and exsanguinated. Vasa deferentia were removed and cleaned of vascular and connective tissue while being kept in oxygenated Tyrode solution.

Transmural stimulation

The isolated vas deferens was threaded through a ring stimulating electrode fixed on a glass holder, and secured to the bottom hook of the holder. The upper end of the tissue was attached to an F-50 microdisplacement transducer connected to a Type DMP-4B Physiograph recorder (Narco Biosystems Inc.). The electrode along with the tissue was placed in a 10 ml bath containing oxygenated Tyrode solution (37°C) of the following composition (mM): NaCl 136.8, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9 and glucose 5.5. The indifferent electrode was left in the bath fluid. The vas deferens was subjected to a resting tension of 0.5 g. Stimulation was carried out with 1 ms pulses, 2–50 Hz, 150 V for 1 s every 50 s using an S-88 Grass stimulator. Stimulation with KCl was achieved by exposure of the tissue to a Tyrode solution in which KCl concentration was increased to 60 mM and NaCl concentration was reduced to 79.5 mM in order to maintain isotonicity.

[^{3}H]-noradrenaline incubation and release

The vas deferens was mounted on a glass holder with upper and lower hooks (and a ring electrode, when necessary) and incubated in 10 ml oxygenated

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Tyrode solution for 30 min. The solution contained disodium ethylenediamine tetraacetic acid (6×10^{-5} M) and ascorbic acid (1.1×10^{-4} M) to prevent oxidation of [^{3}H]-NA. The tissues were then incubated with (±)-[^{3}H]-NA 1 μCi/ml (sp. act. 10 Ci/mmol, The Radiochemical Centre, Amersham) for 30 min. They were then washed every 10 min for 90 min before being used to study the release of tritium. The medium was then changed at 5 min intervals and samples retained for the determination of radioactivity. The following stimuli were used to release [^{3}H]-NA: electrical stimulation (900 pulses, 3 Hz, 1 ms, 150 V), KCl (60 mM), nicotine (6×10^{-5} M) and tyramine (6×10^{-5} M).

Measurement of radioactivity

A small volume (0.5 ml) of each tissue-bathing Tyrode sample was added to 15 ml scintillant with the following composition: 2,5-diphenyloxazole (PPO) 19.5 g, *p*-bis-2-(5-phenyloxazoyl))benzene (POPOP) 390 mg, toluene 1500 ml, naphtalene 312 g, 1,4-dioxane 1500 ml and methanol 900 ml. The tissues were dissolved in 2 ml Soluene-350 (Packard) and the Soluene was then neutralized with 6 M acetic acid. Radioactivity of the dissolved material was determined after appropriate dilution. Radioactivity was measured in a Beckman liquid scintillation spectrometer LS233. The magnitude of stimulation-induced tritium release is expressed as a percentage of the radioactivity present in the tissue at the start of stimulation.

Results were compared by Student's *t* test. A probability of 0.05 was taken as the minimum level of significance.

Drugs

The following drugs were used: amiloride (Merck, Sharp & Dohme Co.), atropine sulphate (E. Merck), cocaine hydrochloride (May & Baker), indomethacin (Merck, Sharp & Dohme Co., dissolved in 0.004 N NaOH) nicotine dihydrogen tartrate (BDH), (−)-noradrenaline bitartrate (Winthrop), phentolamine mesylate (Ciba Geigy) propranolol hydrochloride (ICI) and tyramine hydrochloride (Sigma).

Results

Effect of amiloride on contraction of vas deferens

Amiloride had no effect on the resting tone of the rat vas deferens. The vas deferens was contracted by a high concentration of noradrenaline (4.9×10^{-4} M) before and after 15 min incubation with amiloride 3×10^{-4} M. Amiloride did not inhibit the maximum contractile response of the vas deferens to noradrenaline. In another group of experiments, maximum contractile force of the vasa was potentiated by preincubation of the tissue with cocaine (3×10^{-6} M). Amiloride did not inhibit the maximum contractile response of the vasa pretreated with cocaine. In the absence of cocaine the $-\log ED_{50}$ of noradrenaline was 5.02 ± 0.16 and 5.10 ± 0.09 ($n = 8$) before and after addition of amiloride (3×10^{-4} M) respectively.

Vasa deferentia were electrically stimulated for 1 s every 50 s with frequencies of 2, 5, 10, 20 and 50 Hz. Second and third series of stimulation were carried out 15 min after the last stimulation of the previous cycle. Control experiments showed that the force of

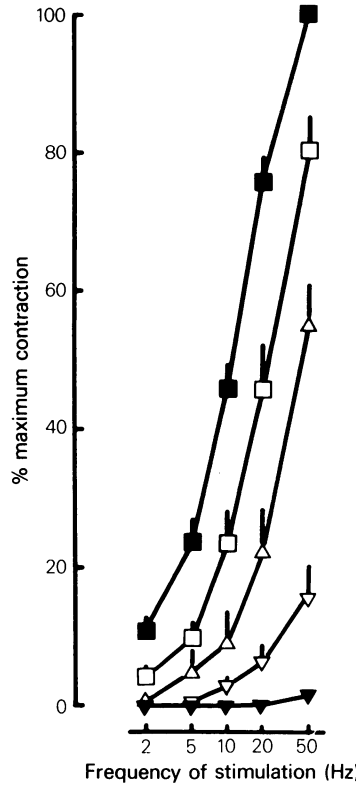

Figure 1 Effect of amiloride on the electrically induced contractions of rat vas deferens. The tissue was stimulated with frequencies of 2, 5, 10, 20 and 50 Hz for 1 s every 50 s. After 2 series of stimulations (S_1 and S_2), the tissue was exposed to only one concentration of amiloride and the third series of stimulations was carried out 15 min later. (■) Control (S_2 stimulation); (□) 10^{-4} M, (△) 3×10^{-4} M, (▽) 5.4×10^{-4} M and (▼) 1.1×10^{-3} M amiloride. Ordinate scale is percentage of maximal contraction evoked during S_2. Abscissa scale denotes frequency of stimulation. Each point represents the mean of at least 3 observations; vertical lines show s.e.mean.

contraction decreased after the first cycle, but there was no significant difference in the force of contractions when the second and third cycles were compared. Amiloride was added immediately after the second cycle in the experimental groups. Amiloride ($10^{-4}-10^{-3}$ M) decreased the response of the tissue to electrical stimulation dose-dependently (Figure 1). With a concentration of 3×10^{-4} M, the effect appeared in about 1 min and was maximal in approx. 15 min. It was reversed partially or completely on washing the preparation. When the preparation was stimulated with a low frequency (0.02–0.1 Hz or 2 Hz for 1 s every 50 s) lower concentrations of amiloride (10^{-5} and 3×10^{-5} M sometimes inhibited the contractions, but this was not always reproducible. Pretreatment of the tissue with propranolol (5×10^{-6} M), phentolamine (3×10^{-6} M), atropine (3×10^{-8} M) or for 1 h with indomethacin (10 μg/ml) did not prevent the inhibitory effect of amiloride on electrically induced contractions.

The contractile response of the tissue to KCl (60 mM) was inhibited by phentolamine (3×10^{-6} M) indicating that the contraction was at least partially due to the release of noradrenaline. The contraction induced by KCl was not inhibited by amiloride suggesting that, under these conditions, the release of noradrenaline is not inhibited by amiloride.

Effect of amiloride on the release of tritium

Field stimulation of [^{3}H]-NA labelled vasa deferentia was carried out with trains of 900 pulses at 15 min intervals. The sample collected during the first train of stimulation was discarded. The results on tritium outflow during the stimulation periods S_2 and S_3 are shown in Figure 2. In the control group, the outflow of radioactivity during S_2 and S_3 were not significantly different. In the experimental group, S_3 was carried out after 15 min exposure to amiloride. Spontaneous outflow of radioactivity was not altered by amiloride (3×10^{-4} M, $P>0.10$). Amiloride 10^{-4} M decreased the mean outflow of radioactivity induced by electrical stimulation by about 25%. The difference was significant only when paired comparisons were made ($P<0.025$, Figure 2b). A higher concentration of amiloride (3×10^{-4} M) completely prevented the increase of outflow induced by electrical stimulation ($P<0.02$, Figure 2c).

The increase in the outflow of radioactivity induced by nicotine (6×10^{-5} M) in the control group (231 ± 50%) was completely prevented by 3×10^{-4} M amiloride (6.9 ± 7.3%, $P<0.001$, $n=4$, Figure 3a).

Tyramine (6×10^{-5} M) increased the outflow of radioactivity by 156 ± 49% ($n=4$) and this was unaffected by the presence of amiloride (3×10^{-4} M; 131 ± 41%, $n=4$, $P>0.05$, Figure 3b).

Exposure of the tissue to 60 mM KCl (with 1.8 mM $CaCl_2$) increased the outflow of radioactivity by 171 ± 21% ($n=8$). In the presence of 3×10^{-4} M amiloride the increase in radioactivity was

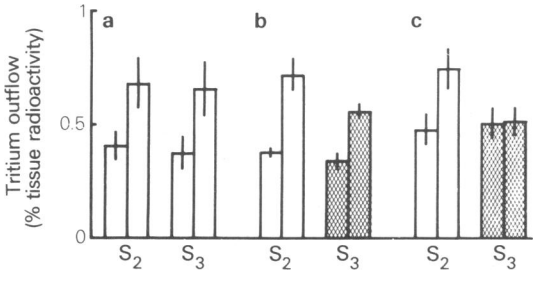

Figure 2 Effect of amiloride on tritium outflow from [^{3}H]-noradrenaline labelled vasa deferentia. The tissues were stimulated with 900 pulses, 3 Hz, 1 ms duration every 15 min. In each pair, the first column is spontaneous outflow before stimulation and the second column is outflow during electrical stimulation. Open columns: release in Tyrode solution, cross-hatched columns: release during exposure to amiloride. Tritium outflow is expressed as a percentage of tissue radioactivity at the onset of sampling period. (a) S_2 and S_3 in the absence of amiloride, 5 experiments; (b) S_2 before and S_3 after 15 min exposure to 10^{-4} M amiloride, 4 experiments; and (c) S_2 before and S_3 after 15 min exposure to 3×10^{-4} M amiloride, 6 experiments. Vertical lines are s.e.mean.

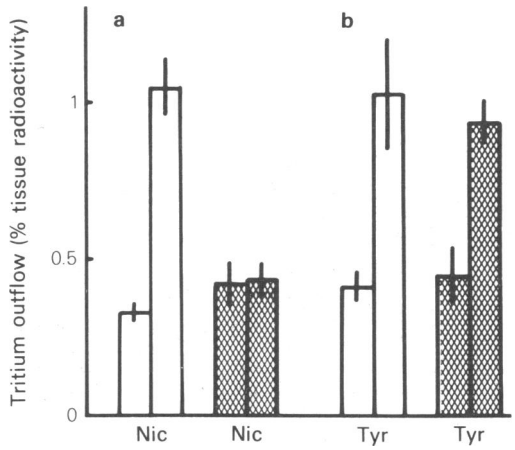

Figure 3 Effect of amiloride on the outflow of radioactivity from [^{3}H]-noradrenaline labelled vasa deferentia. Tritium outflow is expressed as a percentage of tissue radioactivity at the onset of sampling period. In each pair, the first column is the spontaneous outflow before and the second column is the outflow during exposure to the stimulant agent. Open columns: release in Tyrode solution, cross-hatched columns: release during exposure to 3×10^{-4} M amiloride. Release was induced by (a) nicotine (Nic, 6×10^{-5} M) and (b) tyramine (Tyr, 6×10^{-5} M). Vertical lines are s.e.mean of 4 experiments.

$182 \pm 19\%$ ($n = 6$, Figure 4a). In two preparations, 2×10^{-3} M amiloride was also unable to prevent the effect of KCl. Depolarization by KCl can cause a massive influx of calcium. Amiloride may not be able to prevent this massive influx to an extent that would hinder the release of [^{3}H]-NA. To find out whether amiloride has any inhibitory effect on calcium influx, the concentration of calcium in Tyrode solution was decreased to 0.25 mM. Under these conditions, KCl increased the outflow of radioactivity only by $44 \pm 4.2\%$ in the control group. The outflow in the presence of amiloride was $71 \pm 8.2\%$ (Figure 4b).

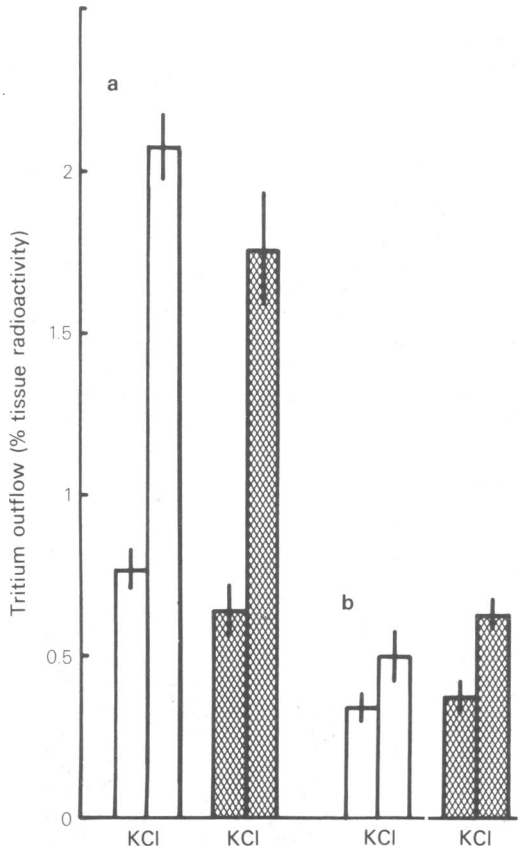

Figure 4 Effect of amiloride on the outflow of radioactivity induced by 60 mM KCl from [^{3}H]-noradrenaline labelled vasa deferentia. Tritium outflow is expressed as a percentage of tissue radioactivity at the onset of sampling period. In each pair, the first column is the spontaneous outflow before and the second column is outflow during exposure to KCl. Open columns: outflow in Tyrode solution; cross-hatched columns: outflow during exposure to 3×10^{-4} M amiloride. The concentration of Ca in Tyrode solution was 1.8 mM in (a) and 0.25 mM in (b). Vertical lines are s.e.mean of 6 experiments in (a) and 4 experiments in (b).

Discussion

The absence of effect of amiloride on the ED_{50} and maximum contraction for noradrenaline in the vas deferens excludes any inhibitory effect of the drug on the amine pump, postjunctional α-adrenoceptors or the availability of calcium to the muscle. The present results show that the inhibitory effect of amiloride is independent of α- and β-adrenoceptors, muscarinic receptors and prostaglandins.

The response of the tissue to KCl was inhibited by phentolamine suggesting that the contraction induced by KCl is at least partially due to the release of noradrenaline. Depolarization by KCl opens membrane channels that are permeable to calcium ions (Blaustein, 1975) and this results in noradrenaline release by exocytosis (Thoa, Wooten, Axelrod & Kopin, 1975). Amiloride did not inhibit the response of the tissue to KCl nor the release of tritium induced by KCl. This suggests that amiloride does not inhibit the entry of calcium ions into adrenergic nerve endings.

Amiloride inhibited the response of the tissue to electrical stimulation. It also decreased or abolished the release of tritium induced by nicotine or electrical stimulation. These stimuli increase the permeability of neuronal membranes to Na ions and depolarize the nerve endings by an inward sodium current. This results in increased membrane calcium conductance, net Ca entry and release of noradrenaline by exocytosis (Jayasundar & Vohra, 1977; Loffelholz, 1979). As amiloride seems not to interfere with the entry of Ca ions and does not stimulate the prejunctional receptors, the results of the present experiments are compatible with a blockade of sodium channels by amiloride in adrenergic nerve endings. This suggestion is in accordance with the observation that amiloride inhibits the entry of sodium ions into other cells (Bentley, 1968; Nagel & Dorge, 1970; Gatzy, 1971; Aceves & Cereijido, 1973). It is interesting that guanidine increases the release of noradrenaline induced by electric nerve stimulation without affecting the release induced by KCl (Hirsch, Kirpekar & Prat, 1979). It is possible that guanidine increases the permeability of sodium channels, while its derivatives (amiloride and tetrodotoxin) block the sodium channel. This is reminiscent of the similarity of structure of many agonists with their antagonists.

Unlike guanethidine, amiloride does not increase the spontaneous outflow of noradrenaline in the rat vas deferens. As amiloride releases acetylcholine from the myenteric plexus of the guinea-pig ileum (personal observation), the results of the present experiments cannot be extrapolated to parasympathetic nerves.

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