Biochemical Pharmacology, Vol. 36, No. 20, pp. 3509–3515, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

ALPHA-ADRENOCEPTOR ANTAGONISTIC ACTION OF AMILORIDE

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(*Received* 31 *December* 1986; *accepted* 23 *April* 1987)

Abstract—1. In isolated perfused rat liver, the effects of alpha-adrenergic stimulation by phenylephrine (2 μM), such as an increase of portal pressure, glucose output, Ca^{2+} release into the perfusate and the characteristic K^+ flux changes across the hepatocyte plasma membrane were almost completely abolished in the presence of amiloride (0.5 mM).

2. When the phenylephrine concentration was raised to about 100 μM, the effects of the alpha-adrenergic agonist on hepatic metabolism, Ca^{2+} and K^+ fluxes, but not on the portal venous pressure, were restored, suggesting a competitive antagonism by amiloride.

3. Amiloride antagonized in a concentration-dependent manner noradrenaline-induced isometric contractions of strips of the rabbit pulmonary artery. The concentration–response curve of noradrenaline was shifted to the right, and the maximal response obtained was also depressed, suggesting a mixed competitive and non-competitive antagonism. The estimated amiloride–adrenoceptor-dissociation constant was 8 μM.

4. The affinity of amiloride to the alpha- and β-adrenoceptor subtypes was determined by radioligand binding assays using [^{125}I]BE 2254 binding to rat liver plasma membranes (alpha$_1$-subtype), [^{3}H]yohimbine binding to human platelet membranes (alpha$_2$-subtype), (−)-[^{125}I]iodocyanopindolol (ICYP) binding to rabbit lung membranes in presence of the β_2-adrenoceptor antagonist ICI 118,551 (β_1-subtype) and ICYP binding to rat lung membranes in presence of the β_1-blocker atenolol (β_2-subtype). In all systems, amiloride inhibited specific ligand binding concentration-dependently, the K_i values for amiloride were about 25, 52, 148 and 161 μM for alpha$_1$- alpha$_2$-, beta$_1$- and beta$_2$- adrenoceptor subtypes, respectively.

5. It is concluded that amiloride in concentrations below those required for inhibition of the Na^+/H^+ exchanger is a potent antagonist of alpha- and beta-adrenoceptors in a variety of experimental systems. Whether the adrenergic antagonism of amiloride is important for antihypertensive therapy, remains to be elucidated.

Micromolar concentrations of amiloride block the Na^+ channels in frog skin and other biological systems [1]. At concentrations in the millimolar range, this compound is a potent and reversible inhibitor of the Na^+/H^+ exchanger [2, 3], which is found in the plasma membrane of most eukaryotic cells. The use of amiloride or its derivatives ethylpropylamiloride and 6-bromo-5-ethylisopropylamiloride has become an important investigative tool for pharmacologic and photoaffinity labelling studies on the Na^+/H^+ antiporter [4, 5]. Further, amiloride has been frequently employed not only in studies implicating a role of the Na^+/H^+ exchanger for regulation of cell volume, the intracellular pH and intracellular Na^+ concentrations, but also in studies on the role of intracellular pH as a signalling system (2, 6–10, for review see refs. 11–13). This signalling involves mitogen or hormone binding to plasma membrane receptors with activation of a tyrosine kinase [14] or the subsequent breakdown of inositol phospholipids and the formation of the second messengers myo-inositol-1,4,5-trisphosphate and diacylglycerol which mobilize intracellular Ca^{2+} and activate protein kinase C (for review see refs. 15–17). Receptor-mediated stimulation of tyrosine kinase or protein kinase C increases the activity of the Na^+/H^+ exchanger giving a rise in intracellular pH [18, 19].

Recent studies showed that amiloride may also inhibit protein kinase C [20] and growth factor-induced tyrosine phosphorylation [21], suggesting that this compound is not a specific inhibitor of the Na^+/H^+ exchanger. This is underlined by the data reported in this paper, demonstrating that amiloride in concentrations sufficient to inhibit the Na^+/H^+ exchanger binds to alpha- and beta-adrenergic receptors and acts as an alpha$_1$-receptor blocker in different biological systems.

MATERIALS AND METHODS

Hemoglobin-free liver perfusion

Livers of male Wistar rats of 100–200 g body weight, fed *ad libitum* on stock diet (Altromin), were perfused as described previously [22] without recirculation of the perfusate using the bicarbonate buffered Krebs–Henseleit saline plus L-lactate (2.1 mM) and pyruvate (0.3 mM). The influent Ca^{2+} and K^+ concentrations were 1.25 and 5.9 mM, respectively. Perfusion fluid was equilibrated with O_2/CO_2 (95/5, v/v). The temperature was 37°. Perfusate flow was 3–4 ml/min per g and was kept con-

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stant throughout the individual perfusion experiment.

The concentration of glucose in the perfusate was determined in an enzymatic optical test based on the procedures described in ref. 23. The portal pressure was recorded continuously with a pressure transducer (Hugo Sachs Elektronik, Hugstetten). Oxygen, Ca^{2+} and K^+ in the effluent perfusate were monitored continuously with a Clarke-type oxygen electrode (Bachofer, Reutlingen), a Ca^{2+} (Philips, Kassel) and a K^+ sensitive electrode (Radiometer, Munich), respectively.

Net Ca^{2+} release by the liver within the first 2–3 min after phenylephrine addition was determined by planimetry of the recorded Ca^{2+} traces. When present, a base-line drift was taken into account. Calibration of the K^+ and Ca^{2+} electrodes was performed by infusion of known amounts of KCl and $CaCl_2$ via precision micropumps, respectively.

Rabbit pulmonary artery strip superfusion

Strips of the rabbit main pulmonary artery were prepared and superfused as described previously [24, 25]. Strips of approximately 4 × 30 mm were mounted vertically under 2 g tension and were superfused with physiological salt solution at 37° and a rate of 2 ml/min. The superfusion fluid contained NaCl (118 mM), KCl (4.7 mM), $CaCl_2$ (1.6 mM), $MgSO_4$ (1.2 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (1.2 mM), glucose (11 mM), Na_2-EDTA (0.03 mM), cocaine (30 μM), corticosterone (40 μM) and propranolol (4 μM). The superfusion fluid was equilibrated with O_2/CO_2 (95/5; v/v). Isometric contractions were recorded on a Watanabe recorder by means of a Biegestab K 30 (Hugo Sachs Elektronik, Hugstetten).

After an initial superfusion for 60–80 min, two cumulative concentration–contraction response curves for (−)-noradrenaline (added to the superfusion fluid) were determined, separated by an interval of 120 min. Noradrenaline concentrations were stepwise increased by a factor of 10, when steady state contractions had been reached after 5–10 min. In experiments with amiloride, the drug was added to the superfusion fluid and was present 60 min prior to the second concentration–response curve for noradrenaline.

Radioligand binding assays

Rat liver plasma membranes, human platelet membranes, rabbit and rat lung membranes were prepared for radioligand binding assays as described previously [26–29]. The affinity of amiloride to alpha- and beta-adrenoceptor subtypes was determined by specific radioligand binding using the following models.

alpha$_1$-adrenoceptors. Binding of [^{125}I]BE 2254 (70–100 pM), a specific alpha$_1$-antagonist to rat liver plasma membranes [26, 27].

alpha$_2$-adrenoceptors. Binding of [^{3}H]yohimbine (2–3 nM), a selective alpha$_2$-antagonist to human platelet membranes [28].

beta$_1$-adrenoceptors. Binding of (−)-[^{125}I]iodocyanopindolol (ICYP) (40–60 pM) to rabbit lung membranes in the presence of the specific beta$_2$-antagonist ICI 118,551 (50 nM) [29].

beta$_2$-adrenoceptors. Binding of ICYP (40–60 nM) to rat lung membranes in the presence of the specific beta$_1$-antagonist atenolol (7.5 μM) [29].

Membranes were incubated with the respective radioligands and 20–25 concentrations of amiloride ranging from 10^{-7} to 10^{-2} M and specific binding was determined as described [26–29].

Calculations

Isometric contraction studies. EC_{50} values, i.e. noradrenaline concentrations that produced 50% of the maximal contraction in the respective concentration–response curves, were obtained by interpolation. From the increase in the EC_{50} caused by amiloride, the dissociation constant of the amiloride–adrenoceptor complex was calculated for each experiment as indicated by equation (3) of ref. 30.

Membrane binding studies. From the resulting competition curves IC_{50}-values, i.e. amiloride concentrations resulting in a 50% inhibition of specific radioligand binding, were determined and K_i-values for amiloride were calculated according to ref. 31.

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_D}}$$

with $[S]$ = concentration of the radioligand in the assay, and K_D = equilibrium dissociation constant of the radioligands. Scatchard-analysis [32] of radioligand saturation experiments yielded equilibrium dissociation constants (K_D) of 70 pM, 2 nM and 15 pM for [^{125}I]BE 2254, [^{3}H]yohimbine and ICYP, respectively.

Materials

Enzymes, coenzymes and dibutyryl-cyclic-AMP were from Boehringer (Mannheim). (−)-Phenylephrine HCl, (−)-noradrenaline HCl and amiloride were from Sigma (Munich). L-Lactic acid was from Roth (Karlsruhe). [^{125}I]BE 2254, ICYP and [^{3}H]yohimbine were from New England Nuclear (Dreieich) with specific radioactivities of 2175 Ci/mmol, 2175 Ci/mmol and 72 Ci/mmol, respectively. ICI 118,551 was a generous gift from ICI Company (Plankstadt). All other chemicals were from Merck (Darmstadt).

RESULTS

Effect of amiloride on alpha-adrenergic stimulation in isolated perfused rat liver

As shown in Fig. 1a, the stimulation of hepatic glucose release by phenylephrine (2 μM) was inhibited by 80–90% when amiloride (0.5 mM) was present in influent perfusate. Also the other known responses of perfused rat liver to phenylephrine [33], such as the increase of portal pressure, the stimulation of oxygen uptake and the characteristic sequence of K^+ movements across the hepatocyte plasma membrane were largely abolished in presence of amiloride (Fig. 1a, Table 1). Further, addition of phenylephrine (2 μM) in presence of amiloride was no longer accompanied by a net Ca^{2+} efflux from the liver (Fig. 1b, Table 1). This suggests that amiloride inhibited the phenylephrine-induced subcellular

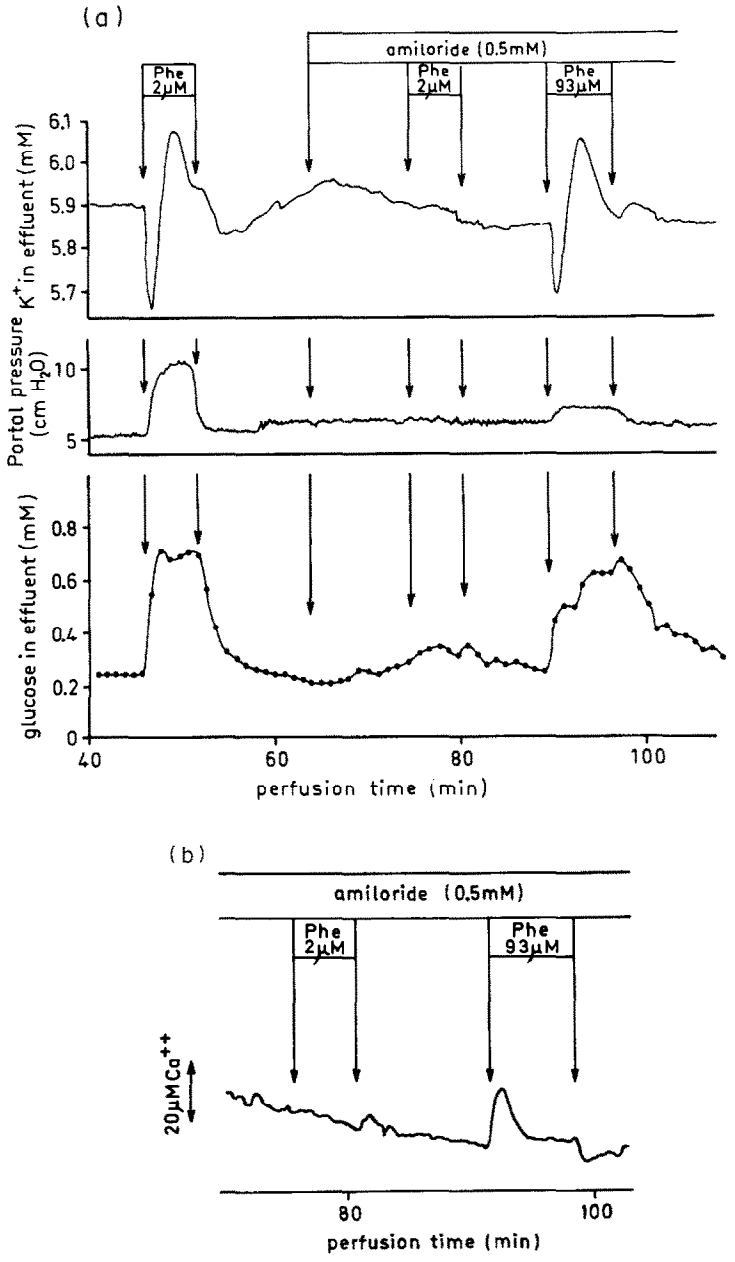

Fig. 1. (a) Effect of phenylephrine on K^+ fluxes across the plasma membrane, portal pressure and glucose output by isolated perfused rat liver in the absence and presence of amiloride (0.5 mM). One representative experiment from a series of 6 similar experiments. (b) Effect of phenylephrine (2 and 93 μM) on effluent Ca^{2+} concentration in isolated perfused rat liver in the presence of amiloride (0.5 mM). The Ca^{2+} concentration in influent perfusate was 1.25 mM. In presence of amiloride, the characteristic net Ca^{2+} efflux from the liver is observed only with the high phenylephrine concentration, and is followed by a Ca^{2+} reuptake after withdrawal of the alpha-agonist.

Ca^{2+} redistribution, which mediates the metabolic responses to alpha-adrenergic stimulation, such as increased glycogenolysis and mitochondrial oxidation [33–39]. Also when hepatic glucose release was already stimulated by phenylephrine, further addition of amiloride reversed the alpha-adrenergic stimulation of glucose release. However, glycogenolysis could be stimulated by cyclic AMP also in the presence of amiloride (Fig. 2).

When, however, the phenylephrine concentration in influent perfusate was raised from 2 μM to about 100 μM in the presence of amiloride (0.5 mM), the effects of the alpha-adrenergic agonist on glucose release, oxygen uptake and Ca^{2+} and K^+ fluxes were restored, whereas the increase in portal pressure was still largely inhibited (Fig. 1a and b, Table 1). It should be emphasized that in the absence of amiloride a phenylephrine concentration of 2 μM is already

Table 1. Effect of amiloride (0.5 mM) on phenylephrine-induced maximal extra oxygen uptake, increase in portal pressure and net Ca^{2+} efflux from perfused rat liver

	PHE (2 μM)	PHE (2 μM) + amiloride	PHE (90–110 μM) + amiloride
Extra O_2 uptake (μmol/g/min)	0.48 ± 0.08	0.01 ± 0.01	0.43 ± 0.06
Increase in portal pressure (cm H_2O)	6.4 ± 0.4	0.1 ± 0.1	1.4 ± 0.4
Extra glucose release (μmol/g/min)	1.91 ± 0.15	0.37 ± 0.08	1.66 ± 0.2
Net Ca^{2+} release (nmol/g liver wet weight)	143 ± 8	n.d.*	123 ± 9

Data are from 7 different perfusion experiments and are given as means ± SEM (N = 3–7). Phenylephrine (PHE) was added for 5–10 min and extra glucose release was measured as phenylephrine-induced increase in glucose production over a period of 3–5 min before phenylephrine withdrawal. The Ca^{2+} concentration in influent perfusate was 1.25 mM.

* n.d. = not detectable.

sufficient to obtain the maximal stimulation of Ca^{2+} release, glucose output or oxygen consumption in isolated perfused rat liver [38]. The amount of Ca^{2+} mobilized (Table 1) by phenylephrine (2 μM) compares quite well with the values of 120–160 nmol/g liver wet weight reported in other studies [33, 38, 39]. In the presence of amiloride, a similar amount of Ca^{2+} could be mobilized by phenylephrine. However, much higher concentrations of the alpha-agonist were required, with virtually no Ca^{2+} efflux being detectable with 2 μM phenylephrine (Table 1).

These data suggest that the inhibition by amiloride of the phenylephrine (2 μM)-induced effects on metabolism and ion fluxes is not due to an inhibition of the Na^+/H^+ exchanger in the liver plasma membrane or an effect on the glycogenolytic enzymes, but is due to competition with phenylephrine for the $alpha_1$ receptor of the hepatocyte plasma membrane. Studies with specific antagonists have shown that the effects of phenylephrine on hepatic metabolism and Ca^{2+} fluxes are mediated by $alpha_1$, but not by $alpha_2$ receptors [39, 40]. Amiloride (0.5 mM) itself exhibited no $alpha_1$-adrenergic activity in isolated perfused rat liver, since amiloride was without effect on portal pressure (compare Fig. 1a), oxygen uptake or hepatic glucose release: hepatic glucose output was 1.03 ± 0.06 and 1.01 ± 0.05 μmol/g/min (five different perfusion experiments) in the absence and presence of amiloride (0.5 mM), respectively. Thus amiloride acts as an alpha-receptor blocker in isolated perfused rat liver.

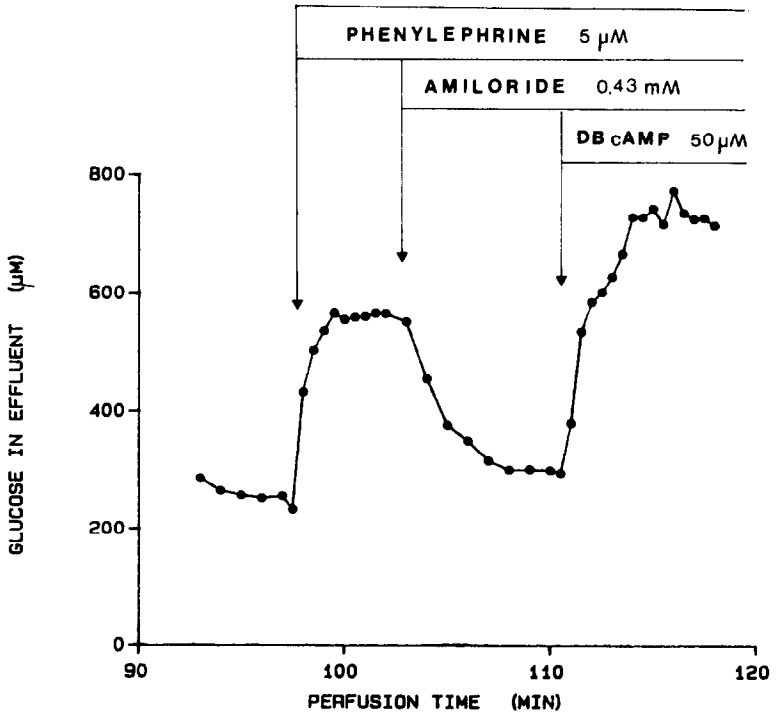

Fig. 2. Inhibition of phenylephrine-stimulation of glycogenolysis by amiloride in perfused rat liver and the effect of cyclic AMP. One representative experiment from a series of 4 similar experiments.

Effect of amiloride on rabbit pulmonary artery contraction

The rabbit pulmonary artery possesses an almost pure population of alpha$_1$-adrenoceptors [41], although alpha$_1$- and alpha$_2$-adrenoceptor agonists may interact with this common alpha$_1$-recognition site in a different manner [42], and the effect of amiloride on the noradrenaline-induced contraction of pulmonary artery strips was studied. As shown in Fig. 3, in which contractions are expressed as the percentage of the maximal contraction obtained in the first noradrenaline concentration–response curve, half-maximal contractions were observed at noradrenaline concentrations of 56 ± 8.5 nM (N = 18), in agreement with earlier studies [24]. The maximal response amounted to 2.9 ± 0.3 g (N = 18) tension. In control experiments, greater contractions were elicited in the second noradrenaline concentration–response curve (Fig. 3); a phenomenon typical for rabbit pulmonary artery [24]. Amiloride at concentrations of 50 μM (N = 6) and 500 μM (N = 6) reduced the maximal response and shifted the concentration–response curve to the right, suggesting a mixed competitive and non-competitive antagonism. From the shifts to the right, the dissociation constant of the amiloride–alpha$_1$-receptor complex was estimated to be 8.4 ± 1.0 μM (N = 12) (for details see Methods and ref. 24).

These data indicate an alpha$_1$-antagonistic action of amiloride also in rabbit pulmonary artery.

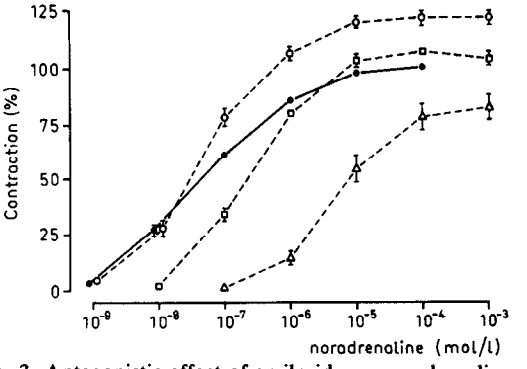

Fig. 3. Antagonistic effect of amiloride on noradrenaline-induced contractions of the rabbit pulmonary artery. Two concentration–response curves were determined on each artery strip and contractions are expressed as the percentage of the maximal contraction obtained in the first concentration–response curve: ●, first concentration–response curve (control); ○, second concentration–response curve in the absence of amiloride; □, second concentration–response curve in presence of amiloride (50 μM); △, second concentration–response curve in the presence of amiloride (500 μM). Data are given as means ± SEM (from 18 and 6 different experiments for the control curve and each other condition, respectively).

Studies on the affinity of amiloride to alpha and beta-adrenoceptor subtypes

To determine the affinity of amiloride to alpha- (for review see ref. 43) and beta-adrenergic receptor

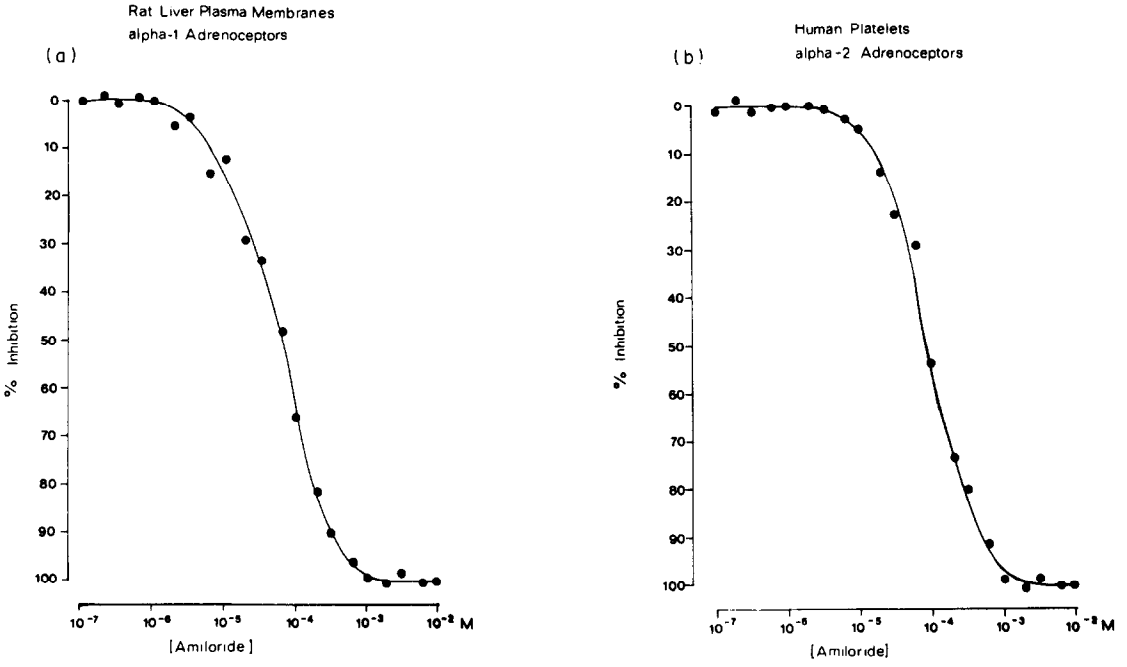

Fig. 4. (a) Inhibition of specific [^{125}I]-BE 2254 binding to alpha$_1$-adrenoceptors in rat liver plasma membranes by amiloride. Membranes were incubated with [^{125}I]-BE 2254 (70–100 pM) in the presence or absence of 21 different concentrations of amiloride and specific binding was determined as described in Methods. "100% inhibition" refers to inhibition of binding by 10 μM phentolamine. Data are given as means of 5 experiments with a SEM less than 10%. (b) Inhibition of specific ^{3}H-yohimbine binding to alpha$_2$-adrenoceptors in human platelet membranes by amiloride. Membranes were incubated with ^{3}[H]yohimbine (2–3 nM) in the presence or absence of 21 different concentrations of amiloride and specific binding was determined as described in Methods. "100% inhibition" refers to inhibition of binding by 10 μM phentolamine. Data are given as means of 5 different experiments with a SEM less than 10%.

subtypes, radioligand binding assays were performed using rat liver plasma membranes ($alpha_1$), human platelet membranes ($alpha_2$) and rabbit ($beta_1$) and rat ($beta_2$) lung membranes (for details see Materials and Methods). As shown in Fig. 4 (a, b) amiloride at concentrations above 1 mM completely inhibited specific binding of the selective $alpha_1$-antagonist [^{125}I]BE 2254 to rat liver plasma membranes and also the binding of the selective $alpha_2$ antagonist [^{3}H]yohimbine to human platelets, when these ligands were added at concentrations near their dissociation constant. From these competition curves K_i values for amiloride can be calculated; they amounted to about 25 μM and 52 μM for $alpha_1$ and $alpha_2$-receptor subtypes, respectively (Table 2). These values obtained in specific membrane binding studies are somewhat higher than those observed in rabbit pulmonary artery strips; a phenomenon which was also observed in other systems when antagonist affinities determined by radioligand binding to membrane fragments were compared to functionally determined affinities in intact tissues (e.g. ref. 43).

In addition to the amiloride-inhibition of specific ligand binding to alpha-receptor subtypes, specific binding of amiloride to $beta_1$- and $beta_2$-receptors could also be demonstrated. This was shown in studies on the effect of amiloride on ICYP binding to rabbit and rat lung membranes in presence of the specific $beta_2$-antagonist ICI 118,551 (50 nM) or the specific $beta_1$-antagonist atenolol (7.5 μM), respectively. However, the calculated K_i values of amiloride for $beta_1$- and $beta_2$-adrenoceptors were 3–6-fold higher than those for alpha-adrenoceptors (Table 2). In all membrane preparations amiloride inhibited ligand binding with steep monophasic competition curves and pseudo-Hill coefficients (n_H) were not significantly different from 1.0 (Table 2).

DISCUSSION

Adrenoceptor antagonistic action of amiloride

The data in this paper show that amiloride in concentrations below those required to inhibit the Na^+/H^+ exchanger in biological systems, binds specifically to alpha- and beta-adrenoceptor subtypes (Fig. 4, Table 2) and exerts an $alpha_1$-antagonistic action. This is suggested by the finding that amiloride itself did not mimick the biological effects of $alpha_1$-adrenoceptor agonists, like stimulation of glycogenolysis and oxygen uptake, K^+ movements and portal pressure increase in perfused rat liver or contractions of pulmonary artery strips. This antagonism was further demonstrated by the inhibition of the noradrenaline-induced contractions of rabbit pulmonary artery strips by amiloride and its inhibitory effect on the phenylephrine-induced metabolic response in perfused rat liver, when phenylephrine was added at a maximally stimulating concentration of 2 μM [39]. This inhibition could largely be overcome in both experimental systems (Table 1, Figs 1–3) by increasing the concentration of the agonists noradrenaline and phenylephrine, respectively, indicating a competitive component of inhibition, in addition to a non-competitive one (Fig. 3). Interestingly, the amiloride-inhibition of the alpha-agonist-induced increase of portal pressure was not abolished upon raising the phenylephrine concentration to values which restored the metabolic response towards $alpha_1$-receptor activation, like Ca^{2+} and K^+ fluxes, glucose output and oxygen consumption (Table 1, Fig. 1). This might reflect the non-competitive component of amiloride-inhibition. However, it should be noted that in contrast to the metabolic effects [39] the hemodynamic response is not maximal at a phenylephrine concentration of 2 μM (D. Häussinger, unpublished).

Recent studies [45] showed that the $alpha_2$-receptor agonist guanabenz stimulated Na^+ uptake in rabbit renal proximal tubular cells, an effect which was inhibited by amiloride (1 mM), suggesting that the effect of $alpha_2$-stimulation was mediated by the Na^+/H^+ exchanger. However, amiloride was also found to competitively inhibit ^{3}H-rauwolscine binding to $alpha_2$-adrenoceptors in renal cortical membranes with a K_i value similar to that reported in our study and it was suggested that $alpha_2$-receptor blockade by amiloride may contribute to the amiloride-mediated inhibition of response to guanabenz [45].

Thus, amiloride must be considered as an alpha- and beta-adrenoceptor antagonist in a variety of experimental systems, such as rat liver, human platelets, rabbit pulmonary artery, rat and rabbit lung membranes and rat renal cortex membranes.

Possible significance

The adrenoceptor-antagonism of amiloride reported in this paper shows that this compound is not a specific inhibitor of Na^+ channels or of the Na^+/H^+ exchanger. Our findings may be important for the interpretation of experimental data using amiloride in order to study the role of the Na^+/H^+

Table 2. K_i-values for inhibition by amiloride of radioligand binding to $alpha_1$-, $alpha_2$-, $beta_1$- and $beta_2$-adrenoceptors

Experimental model	Adrenoceptor subtype	K_i-value (μM)	n_H
Rat liver membranes	$alpha_1$	25.0 ± 3.8	1.00 ± 0.11
Human platelet membranes	$alpha_2$	51.8 ± 3.1	1.18 ± 0.16
Rabbit lung membranes	$beta_1$	148 ± 15.7	0.94 ± 0.17
Rat lung membranes	$beta_2$	161 ± 21.1	1.07 ± 0.09

Data are given as means ± SEM of 5 different experiments for each condition and were performed on different membrane preparations. n_H, pseudo-Hill coefficient.

exchanger in intact cell systems. In this respect, it would be of interest whether the newer inhibitors of the Na^+/H^+ exchanger that do not belong to the amiloride series, like guanidinium or its derivatives [46], are also adrenoceptor blockers.

Amiloride is clinically used as K^+-sparing diuretic. Whether its antihypertensive effect *in vivo* is also due to its alpha-blocking potency remains to be established. This, however, seems unlikely in view of the reported therapeutic plasma concentrations of amiloride below 1 μM [47].

Acknowledgements—Expert technical assistance was provided by Mr Th. Stehle and Mr E. Schöffel. This study was supported by Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 154 "Experimentelle und Klinische Hepatologie" and by the SANDOZ Stiftung für Therpeutische Forschung (O.-E. B.).

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