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Amiloride selectively inhibits the urokinase-type plasminogen activator

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The diuretic drug amiloride, an inhibitor of Na^+ uptake, competitively inhibits the catalytic activity of the urokinase-type plasminogen activator (u-PA), with a K_i of 7×10^{-6} M. Generation of plasmin, cleavage of peptide substrates, and interaction of u-PA with a specific macromolecular proteinase inhibitor are all prevented in the presence of the drug. In contrast, amiloride does not affect the activity of either tissue-type plasminogen activator, plasmin, plasma kallikrein or thrombin. The inhibition of u-PA by amiloride may be related to the previously reported inhibition of u-PA-type enzymes by Na^+. Amiloride or related compounds could prove useful in selectively controlling u-PA-catalyzed extracellular proteolysis.

Plasminogen activator; Urokinase; Plasminogen; Amiloride; Protease inhibitor; Na^+

1. INTRODUCTION

The conversion of the zymogen plasminogen into plasmin, a trypsin-like extracellular protease of broad substrate specificity, is catalyzed by plasminogen activators (PAs). Two distinct PAs have been identified in mammals: urokinase-type PA (u-PA) and tissue-type PA (t-PA). The two PAs are the products of different genes [1,2], but they share a number of structural and catalytic properties. However, important functional differences between these enzymes have recently been demonstrated: t-PA binds to fibrin and to other components of extracellular matrices, and this interaction can dramatically increase its catalytic activity [3,4]; in contrast, u-PA binds to a specific plasma membrane receptor [5], a configuration which focalizes its activity to the close environment of the cell surface. Thus it is likely that the two enzymes play distinct roles in the generation of extracellular proteolysis (for a recent review on PAs, see [6]).

A wide variety of cell types synthesize and secrete one, the other, or in some cases both types of PAs. Cellular production of these enzymes is often associated with physiological and pathological processes involving cell migration and tissue remodelling, for instance in the context of ovulation [7], embryo implantation [8], mammary gland involution [9], inflammatory reactions [10], tumor invasion and metastasis [11], etc. The availability of specific inhibitors of one or the other PA would help in elucidating the role of these enzymes, and in controlling extracellular proteolysis when desirable. Besides specific antibodies that have been raised against the two enzymes, no pharmacological agent that selectively inhibits the activity of one or the other PA has been described. In this context, the inhibition of u-PA-type enzymes by physiological concentrations of NaCl [12–14] suggests an avenue of investigation for the development of specific inhibitors of u-PA.

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We report here that amiloride, an inhibitor of Na^+ transport [15], competitively inhibits the catalytic activity of u-PA, without decreasing those of t-PA, or of plasmin. Thus, amiloride may be a useful model compound for the development of u-PA-specific protease inhibitors.

2. MATERIALS AND METHODS

High-M_r (55 000) u-PA was obtained from the Green Cross Corporation (Osaka, Japan), human thrombin (T 6759) and amiloride (A 7410) from Sigma (St. Louis, MO). Low-M_r (33 000) u-PA, single chain t-PA, and human plasma kallikrein were the kind gifts of Serono (Denens, Switzerland), J. Morser (Codon, South San Francisco, CA), and M. Schapira (Division de Rhumatologie, Hôpital Cantonal, Geneva, Switzerland), respectively. Plasminogen was purified from outdated human plasma by lysine-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) affinity chromatography [16]. CNBr fragments of fibrinogen, prepared as described [17], were the gift of G. Reber (Division d'Hémostase, Hôpital Cantonal, Genève).

The thiobenzyl benzyloxycarbonyl-L-lysinate assay for trypsin-like enzymes was performed as described [18], using a substrate concentration of 7.3×10^{-5} M. *N*-Benzyloxycarbonyl-L-lysine thiobenzyl ester and 5,5′-dithio-bis-(2-nitrobenzoic acid) were obtained from Calbiochem (La Jolla, CA).

The zymographic assay for PAs was performed as described [19]. As a source of murine u-PA and t-PA, we used the serum-free conditioned medium of MSV-3T3 [20] and PYS (a mouse parietal yolk sac-like [21] cell line that produces a PA electrophoretically, catalytically and immunologically related to t-PA, and not to u-PA) cell cultures, respectively.

Reaction of ^{125}I-u-PA with the PA-specific inhibitor of U-937 cells was analyzed as described [19].

Hydrolysis of L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide (S-2444) and D-valyl-L-glycyl-L-arginine-*p*-nitroanilide (S-2322) (KabiDiagnostica, Stockholm, Sweden) was monitored by following the increase in absorbance at 405 nm, during incubation at 37°C in 50 mM Tris-HCl, pH 8.1.

3. RESULTS

To test for a possible effect of amiloride on the catalytic activity of PAs, we used a colorimetric assay based on the cleavage of a general substrate for trypsin-like proteases. Amiloride inhibited the u-PA-catalyzed hydrolysis of this compound, but it did not affect the activity of the other enzymes tested (fig.1). In particular, neither plasmin, generated from plasminogen in the presence of t-PA, nor t-PA was affected by amiloride, even in the presence of fibrinogen peptides, which enhance the activation of plasminogen by t-PA [17].

The selective inhibition by amiloride of u-PA-catalyzed plasminogen activation was next demonstrated using a zymographic assay. In this assay, PAs diffusing out of a polyacrylamide gel activate the plasminogen present in a layer of insoluble protein substrate; the plasmin thus generated causes localized lysis of the substrate. Different human and murine PAs were subjected

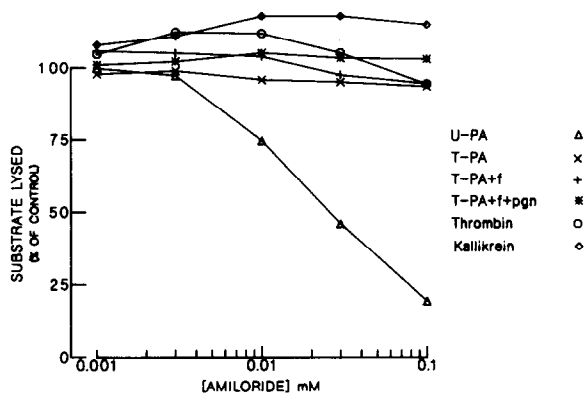

Fig.1. Thiobenzyl benzyloxycarbonyl-L-lysinate assay. The rate of substrate lysis catalyzed by u-PA (M_r 33000, 0.3 μg/ml), t-PA (0.6 μg/ml), t-PA (0.6 μg/ml) + CNBr fibrinogen fragments (200 μg/ml) (T-PA + f), t-PA (0.02 μg/ml) + CNBr fibrinogen fragments (200 μg/ml) + plasminogen (1 μg/ml) (T-PA + f + pgn), thrombin (0.1 μg/ml), and kallikrein (0.3 μg/ml) was determined in the presence of increasing concentrations of amiloride. For each enzyme, results are expressed as % of the rate of substrate lysis in the absence of amiloride. For kallikrein, plasmin, thrombin and u-PA, reactivities towards thiobenzyl benzyloxycarbonyl-L-lysinate have been compared and found to be very similar [18]; in the case of thrombin and u-PA, K_m values were reported to be 4×10^{-5} M and 3×10^{-5} M, respectively.

to SDS-PAGE, and the gels were deposited onto plasminogen-containing casein substrate layers (fig.2). When amiloride was included in the substrate, the apparition of lytic zones was markedly retarded in the samples containing both high- and low M_r forms of human u-PA, as well as in that containing murine u-PA. In contrast, the lysis areas catalyzed by either human or murine t-PA were of comparable size in the presence or absence of amiloride. This confirms that amiloride specifically inhibits the activation of plasminogen by u-PA-type enzymes, without affecting the activity of t-PAs or of plasmin.

In addition to catalytically activating plasminogen, u-PA may also react stoichiometrically with protease inhibitors and form covalent, inactive, enzyme-inhibitor complexes. To determine whether amiloride also prevented reaction of u-PA with such an inhibitor, ^{125}I-u-PA was added to conditioned medium from a culture of monocyte-like U-937 cells, which produce a high affinity, PA-specific, protease inhibitor [19]. Amiloride prevented the formation of a 72 kDa u-PA-inhibitor complex (fig.3). This demonstrated that amiloride inhibits not only the catalytic activity of u-PA on plasminogen, but also its reaction with macromolecular antiproteases.

Finally, the mode of inhibition of u-PA by amiloride was investigated using L-pyroglutamyl-

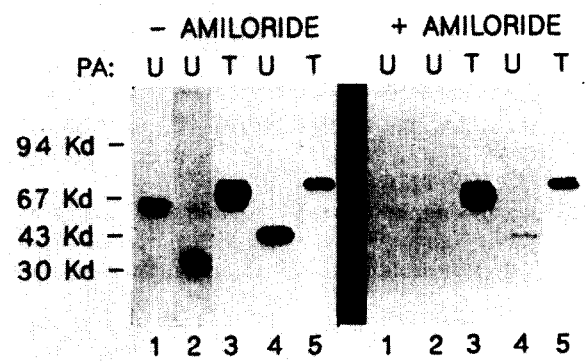

Fig.2. Zymographic assay of PAs. Equivalent catalytic amounts (0.1 Ploug units) of 55 kDa human u-PA (lanes 1), 33 kDa human u-PA (lanes 2), human t-PA (lanes 3), murine u-PA (lanes 4) and murine t-PA (lanes 5) were subjected to SDS-PAGE. The electrophoretic gels were layered onto casein-agar-plasminogen substrate gels; amiloride (1 mM) was included in one of the substrate gels (right panel). Photographs were taken under dark-ground illumination, after 3 h of incubation at 37°C. The molecular masses of marker proteins, electrophoresed in adjacent lanes and stained with Coomassie blue, are indicated.

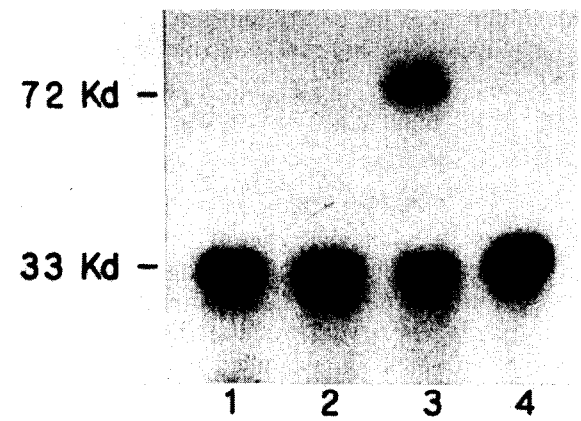

Fig.3. Autoradiographic analysis of u-PA-inhibitor complex formation. Amiloride (0.2 mM final concentration) was added to fresh serum-free culture medium and to inhibitor-rich conditioned medium from U-937 cells. 33 kDa ^{125}I-u-PA (2 ng) was then added to 10 μl samples of fresh medium (lane 1), amiloride-containing fresh medium (lane 2), conditioned medium (lane 3), and amiloride-containing conditioned medium (lane 4). After 1 h at 4°C, the samples were subjected to SDS-PAGE, and the gel was analyzed by autoradiography.

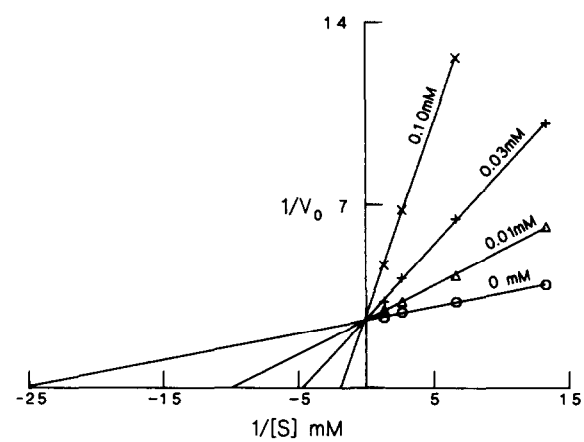

Fig.4. Lineweaver-Burk plot of initial reaction velocity, V_0, versus substrate concentration, S, of u-PA-catalyzed hydrolysis of L-pyroglutamyl-glycyl-L-arginine-*p*-nitroanilide, in the presence and absence of amiloride. The concentrations of amiloride used are indicated on the graph. K_m in this experiment: 4×10^{-5} M; $K_i = 7 \times 10^{-6}$ M.

glycyl-L-arginine-*p*-nitroanilide, a chromogenic peptide substrate for the enzyme. A Lineweaver-Burk plot (fig.4) of the data demonstrated that the inhibition is competitive, with a K_i of 7×10^{-6} M. Concentrations of amiloride up to 1×10^{-3} M did not inhibit cleavage of this substrate, nor that of D-valyl-L-glycyl-L-arginine-*p*-nitroanilide, by t-PA.

4. DISCUSSION

We have shown here that amiloride competitively inhibits the catalytic activity of u-PA on a variety of substrates, including plasminogen, its natural macromolecular target. This appears to be related to a general property of the catalytic domain of u-PA-type enzymes, since human, murine and porcine (not shown) u-PAs were similarly affected. The non-catalytic A-chain of the enzyme is not implicated in this phenomenon since both high-M_r u-PA and low-M_r u-PA (a cleavage product that lacks most of the A-chain but retains full catalytic activity [22]) were inhibited by amiloride. The selectivity of this inhibition is illustrated by the lack of effect of amiloride on t-PA-catalyzed cleavage of both plasminogen and synthetic substrates. In fact, of the serine proteases tested in the present work, which belong to the coagulation, kinin formation, and fibrinolytic pathways, only u-PA was inhibited by amiloride.

Macromolecular antiproteases that are essentially PA specific, i.e. PA-I 1 and PA-I 2, have recently been identified and characterized [23,24]; unlike amiloride, these proteins, which belong to the serpin family of antiproteases and are produced by endothelial cells and mononuclear phagocytes respectively, inhibit both u-PA and t-PA.

The guanidino group of amiloride is probably involved in its inhibitory activity; indeed, another compound that contains this functional group, i.e. *p*-nitro-guanidino-benzoate, is a very potent inhibitor of tryptic proteases, including u-PA [12]. The selective inhibition of u-PA by the Na^+ transport blocker amiloride may in some way be related to the inhibition of this enzyme, but not of most other tryptic proteases, by relatively low concentrations of Na^+ [12–14]. This contention is supported by the fact that urinary kallikrein, a trypsin-like serine protease of similar catalytic specificity but with a different preferred macromolecular substrate (i.e. kininogens), is also inhibited by both Na^+ and amiloride [25,26]; however, it must be noted that inhibition of urinary kallikrein by amiloride is a controversial issue [27], and that the mode of kallikrein inhibition by amiloride was reported to be non-competitive [25].

In conclusion, our results suggest that a study of amiloride analogs, as well as of other agents known to affect transport and other aspects of Na^+ metabolism, may reveal interesting compounds that could be used to control u-PA-catalyzed proteolysis. Indeed, although amiloride itself may not be specific enough to be useful as an anti-u-PA in vivo, a drug that would block the activation of plasminogen by u-PA, without affecting the activity of t-PA or of plasmin, could be a valuable therapeutic tool.

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