Amiloride Inhibits Mammalian Renal Kallikrein and a Kallikrein-like Enzyme from Toad Bladder and Skin

HARRY S. MARGOLIUS and JULIE CHAO, *Departments of Pharmacology and Medicine, Medical University of South Carolina, Charleston, South Carolina 29403*

ABSTRACT Renal kallikrein is localized in luminal plasma membranes of the mammalian distal nephron and gains access to urine from this site. Its activity is regulated, in part, by aldosterone. These facts led us to study the effects of amiloride, a drug known to inhibit sodium reabsorption and potassium secretion at this site, on kallikrein activity. Amiloride inhibited the esterolytic activity of purified rat or human urinary kallikrein or of rat renal cortical cells upon a synthetic substrate (ID_{50} = 0.12–0.23 mM). Kinetic analyses showed that the enzyme inhibition was noncompetitive and reversible in nature. The kinin-generating activity of kallikrein acting upon kininogen substrates was also inhibited by amiloride, as measured by bioassay in the rat uterus or guinea pig ileum or by radioimmunoassay of liberated kinins (ID_{50} = 85 μM). No other diuretic drug tested inhibited kallikrein activity, except triamterene, which did so, weakly. In addition, kallikrein-like enzyme activity was discovered in the urinary bladder or skin of *Bufo marinus* toads and this activity was also inhibited by amiloride. The localization of the enzyme and its inhibition by this drug suggest that further study of relationships amongst the glandular kallikrein-kinin system and renal ion and water transport is warranted.

INTRODUCTION

Urinary and renal kallikreins (E.C. 3.4.21.8) are serine proteinases which act upon kininogen substrates to liberate kallidin (lysyl-bradykinin), a decapeptide with a broad spectrum of biological activity including effects upon sodium and water transport across membranes (1, 2). In humans and rats, sodium-retaining steroids or maneuvers which increase aldosterone activity, such as reduced dietary sodium or increased dietary potassium increase renal and urinary kallikrein activity; whereas spironolactone reduces it (3–9). Kallikrein excretion is elevated in primary aldosteronism or Bartter's syndrome (10–13). In the rat kidney, kallikrein is localized at the apical portions of cells of the distal nephron (14, 15) and is membrane bound (16). Its active sites face the exterior of renal cortical cells in suspension (17). These findings led us to ask whether kallikrein's enzymatic activities would be affected by amiloride, an *N*-amidino pyrazine carboxamide known to block entry of sodium ions into a transport mechanism by an action at the distal nephron luminal surface (18). The results of these studies show that all of the established enzymatic properties of kallikrein are inhibited by this drug. In addition, amiloride inhibited a new kallikrein-like enzyme activity found in *Bufo marinus* urinary bladder and skin.

METHODS

Materials

The following materials were obtained from commercial sources: Sephadex G-25 (superfine) (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.); α-*N*-tosyl-L-arginine methyl ester (Tos-Arg-OMe)[1] (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.); Tos-Arg-O[^{3}H]Me (sp act ~200 Ci/mol) (Biochemical and Nuclear Corp., Burbank, Calif.); aprotinin (identical to Kunitz basic pancreatic trypsin inhibitor), phenylmethylsulfonyl fluoride, trypsin (from bovine pancreas, type IV, twice crystallized), 5,5'-dithiobis(2-nitrobenzoic acid), benzamidine (Sigma Chemical Co., St. Louis, Mo.); Nitex (Tetko Inc., Elmsford, N. Y.); sodium deoxycholate, polyethylene glycol (6,000 flake) (Fisher Scientific Co., Pittsburgh, Pa.); trypsin inhibitors from

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[1] *Abbreviations used in this paper*: EU, esterase unit; IRT-36, α,α',α''-Tris[14-amidino-2-bromophenoxy]mesitylene; IRT-63, α,α',α''-Tris[3-amidino-phenoxy]mesitylene; Tos-Arg-OMe, α-*N*-tosyl-L-arginine methyl ester.

soybean, ovomucoid, lima bean, and carboxypeptidase B (Worthington Biochemical Corp., Freehold, N. J.); synthetic bradykinin and tyrosyl-bradykinin (Peptide Institute-Protein Research Foundation, Osaka, Japan); ouabain (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.).

The following drugs and chemicals were gifts: amiloride (a gift of Dr. Edward J. Cragoe, Jr.), hydrochlorothiazide, ethacrynic acid (Merck Institute for Therapeutic Research, West Point, Pa.); furosemide (Hoechst-Raussel Pharmaceuticals, Inc., Somerville, N. J.); triamterene (SK. & F Co., Carolina, P. R.); spironolactone (Searle & Co., San Juan, P. R). Pentamidine and the tris-amidine derivatives: α,α',α''-Tris-[14-amidino-2-bromophenoxy]mesitylene (IRT-36) and α,α',α''-tris[3-amidino-phenoxy]mesitylene (IRT-63); were gifts from Dr. J. D. Geratz and Dr. R. R. Tidwell, University of North Carolina. SQ20881 was a gift from Dr. Z. Horowitz of the Squibb Institute for Medical Research, New Brunswick, N. J. Thiobenzyl benzyloxycarbonyl-L-lysinate was a gift from Dr. C. Kettner and Dr. E. Shaw, Brookhaven National Laboratory, Upton, N. Y. Partially purified human urinary kallikrein was a gift from Dr. J. V. Pierce, National Institutes of Health, and purified bovine low molecular weight kininogen (17.4 U/A_{280})[2] was supplied by Dr. H. Kato of the Protein Research Institute, University of Osaka, Osaka, Japan.

Rat urinary kallikrein purification and assays

Rat urinary kallikrein B was purified to homogeneity as described previously (19) and its purity was determined by polyacrylamide slab gel electrophoresis and isoelectric focusing. The molecular weight of this enzyme is 35,500 daltons and its isoelectric point is 4.26. Its activity was measured with four separate assay systems as described below.

Radiochemical Tos-Arg-OMe esterase assay

A modification (3) of the method of Beaven et al. (20) was used routinely to determine Tos-Arg-OMe esterase activity, a measure of kallikrein-like enzyme activity. The assay was carried out in 1.5-ml polypropylene tubes to which 0.03 ml of 0.2 M Tris-HCl (pH 8) (assay buffer) and 0.02 ml of the enzyme solution with or without drug were added, mixed, and allowed to stand for 30 min at 25°C. Then 0.01 ml Tos-Arg-O[^{3}H]Me (0.047 μCi, ~200 Ci/mol) was added, mixed, and incubated for 30 min. The [^{3}H]methanol released was counted in a Beckman LS-355 liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif. [counting efficiency for ^{3}H, 40%]). 1 Tos-Arg-OMe esterase unit (EU) is defined as that amount of enzyme that hydrolyzes 1 μmol of Tos-Arg-OMe/min at pH 8 and 30°C using a human urinary kallikrein as a standard in a titrametric assay (21).

Kininogenase assay

The method of Shimamoto et al. (22) was used to determine kallikrein's kinin-releasing activity. Generated kinins were measured by a kinin radioimmunoassay (23). The reaction was carried out at 37°C. 0.05 ml of purified rat urinary kallikrein (0.02 EU/ml), 0.05 ml of water or water plus amiloride, and 0.2 ml of 0.1 M phosphate buffer (pH 8.5) were mixed and incubated for 30 min at 37°C, and then 0.2 ml of bovine low molecular weight kininogen (7.5 μg/ml) was added. The reaction tubes were incubated for 20 min at 37°C before stopping the reaction by immersion in boiling water for 10 min. The nonincubated control tubes were immediately placed in boiling water for 10 min. From the nonincubated and incubated tubes, 0.01-ml aliquots were removed and added to 0.19 ml of 0.01 M Na phosphate, 0.14 M NaCl (pH 7) containing 1% egg albumin, 0.01 M Na_2 EDTA, and 0.003 M phenanthroline (assay buffer) in plastic tubes. Then, 0.1 ml of ^{125}I-tyrosyl-bradykinin (~8,000 cpm), prepared monthly as previously described (23), in assay buffer was added and the contents were then mixed. Next, 0.1 ml of rabbit antibradykinin antiserum (1:20,000 dilution) in assay buffer was added to each tube, mixed, and incubated at 4°C for 12 h. After incubation, 0.4 ml of ice cold 1% bovine gamma globulin in phosphate-buffered saline was added to each tube followed by 0.8 ml of ice cold 25% polyethylene glycol in phosphate-buffered saline. After mixing, the tubes were centrifuged at 3,000 *g* for 20 min at 4°C, the supernate was carefully aspirated, and then the radioactivity in the unwashed precipitates was counted in a Beckman automatic well-type gamma spectrometer. Values of nonincubated control tubes were subtracted from respective incubated sample values. A seven-point standard curve of synthetic bradykinin assayed in duplicate in concentrations ranging from 2 to 125 pg kinin per tube was used for each assay. In 20 assays, the antibody gave 38.7 ± 1.7% (mean ± 1 SE) binding of ^{125}I-tyrosyl-bradykinin.

Bioassays

Guinea pig ileum preparations (24). 0.1-ml aliquots of heated (60°C, 60 min) dog plasma were added to the ileum in a 10-ml isolated tissue bath at 27°C and incubated for 1–2 min. 0.1-ml aliquots of rat urinary kallikrein (0.1 EU/ml) preincubated with or without amiloride for 30 min at 37°C were added to the bath. Kinin-releasing activity of kallikrein was measured with a Grass force-displacement transducer FT03C and Grass polygraph (Grass Instrument Co., Quincy, Mass.).

Rat uterus preparations (25). Virgin female Sprague-Dawley rats (150–200 g) were injected with 200 μg/kg 17β-estradiol (2 mg/ml in 95% ethanol) 20–24 h before sacrifice by cervical dislocation. Uterine horns were immediately removed and placed in de Jalon's solution aerated with 95% O_2/5% CO_2. Aliquots of rat urinary kallikrein (0.1 EU/ml) preincubated with or without amiloride for 30 min at 37°C were added to the isolated, estrogen-primed rat uterus in a 10-ml tissue bath at 27°C. Contractile responses of the tissue induced by kallikrein were recorded with the force-displacement transducer and polygraph.

Spectrophotometric assay

The initial velocity of 2-*N*-thiobenzyl benzyloxycarbonyl-L-lysinate hydrolysis by rat urinary kallikrein was measured with a Cary 15 spectrophotometer (Varian Associates, Instrument Div., Palo Alto, Calif.) using the 0.1-absorbance slide wire. The reactions were carried out at 25°C by circulating water from a temperature-controlled bath (Haake Inc., Saddle Brook, N. J.) through thermospacers. Tubes containing either 2.7 ml of 0.2 M Tris-HCl buffer, pH 7.5, and 0.05 ml of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M sodium phosphate buffer, pH 7, or containing rat urinary kallikrein (0.1 EU/ml) with and without amiloride in varying concentrations (0.4 ml total volume), were preincubated for 30 min at 25°C. The substrate, thiobenzyl benzyloxycarbonyl-L-lysinate, was added to the buffer-5,5′-dithiobis(2-nitroben-

[2] 1 U of kininogen is defined as that amount of low molecular weight kininogen which, after treatment with trypsin, produces contractile activity equivalent to 1 μg synthetic bradykinin on the isolated rat uterus.

zoic acid) mixture to make final substrate concentrations of from 12 to 80 μM and a total volume of 2.95 ml. After 5 min, the reactions were initiated by addition of either 0.05 ml of enzyme alone or 0.05 ml of enzyme-inhibitor solution, mixed, and then immediately transferred to the cuvette. Kallikrein activity was determined by measuring the linear production of 3-carboxy-4-nitrothiophenoxide ion (yellow anion) at 412 nm for 5 min and corrected for spontaneous hydrolysis in the reference cuvette containing the substrate, 5,5'-dithiobis(2-nitrobenzoic acid) and amiloride, when indicated. Velocity is expressed as the molarity of product formed per minute using a molar extinction coefficient of 1.36×10^4 for 3-carboxy-4-nitrothiophenoxide ion (26). The reversibility of kallikrein inhibition by amiloride was determined by measuring V_{max} (V_{max} = the maximal velocity of thiobenzyl benzyloxycarbonyl-L-lysinate hydrolysis obtained from intercepts of Lineweaver-Burk plots as described above) at various kallikrein concentrations (2–4 mEU/3-ml reaction mixture) in the presence or absence of (20 μM) amiloride. Irreversible inhibition can be distinguished from reversible noncompetitive inhibition by plotting V_{max} vs. the total amount of kallikrein in the assay, in the presence or absence of inhibitor. With reversible noncompetitive inhibition, the "plus inhibitor" curves will have smaller slopes than control, but pass through the origin. With irreversible inhibition, the plus inhibitor curves will have slopes similar to control, but will intersect the horizontal axis at a point equivalent to the amount of enzyme that is irreversibly inactivated.

Renal cortical cell suspension preparations

Renal cortical cell suspensions were prepared from Sprague-Dawley rats according to the procedures described by Chao and Margolius (17). Cell counts were performed with a hemocytometer and viability was determined using the trypan blue exclusion method (27). Intact viable renal cortical cells in suspension are capable of hydrolyzing Tos-Arg-OMe and this activity was assayed as described previously (17).

Amphibian enzyme extraction and assay

Female *Bufo marinus* toads said to be of Mexican or Puerto Rican origin were obtained from Lemberger Co., Oshkosh, Wis., and kept upon moist pads at room temperature. The urinary bladders or skin were removed from doubly-pithed toads. Usually, four hemibladders were removed from two toads and rinsed several times with phosphate-buffered saline (pH 7.4). Each hemibladder was placed in a petri dish, covered with phosphate-buffered saline and the surface was then scraped with a glass slide. The harvested cells were pooled and suspended in 5 ml of the same buffer and filtered through three layers of gauze upon Nitex (100-μm pore size). These cell suspensions were homogenized twice in an Omni-mixer (Iowa Mfg. Co., Cedar Rapids, Iowa) at 4°C for 30 s each at top speed. The homogenates were treated with deoxycholate (0.5%, wt/vol) at 4°C for 30 min and then centrifuged at 20,000 *g* for 30 min at 4°C. The supernate (~5 ml) was desalted by gel filtration through Sephadex G-25 (20). The filtrate was acid-treated by the procedure of Furtado (2) and kininase II activity inhibited with 200 μg/ml SQ20881. Pieces of weighed ventral skin were minced, homogenized, and treated as the bladders. The protein concentration of these extracts was determined by the method of Lowry et al. (28). Tos-Arg-OMe esterase activity of these toad bladder or skin extracts was measured as described previously (17). The contractile responses of the isolated guinea pig ileum or estrogen-primed rat uterus were measured in response to toad skin or bladder extracts before or after the latter were incubated with heated dog plasma for 30–60 min at 37°C. The Tos-Arg-OMe esterase activity of intact pieces of total bladder were measured in the following fashion. After toads were doubly-pithed, the thoracic cavity was opened, the vena cava was cut, and the ventricle was punctured and perfused with a standard frog Ringer's solution until the urinary hemibladder vessels appeared free of blood (usually 100–150 ml of Ringer's solution). The hemibladders were then removed and rinsed in Ringer's solution and transferred to 50 ml of Tris-HCl buffer 0.2 M, pH 8. Each hemibladder was then loosely mounted upon a wooden block with the mucosal surface upward and kept moist with the Tris-HCl buffer. 25 0.25-cm^2 sections were punched out of each hemibladder with an Osborne arch punch (C. S. Osborne & Co., Harrison, N. J.) and individually placed in 1.5-ml polypropylene tubes containing 0.03–0.04 ml of the Tris-HCl buffer, 0.01 ml of vehicle or vehicle plus amiloride (final concentrations as indicated) and allowed to stand at room temperature for 20 min. Tos-Arg-O[^{3}H]Me (0.047 μCi, 0.01 ml) was added, mixed, and incubated for 30 min, the reaction was stopped and then [^{3}H]methanol was measured as described above. Activity was measured in quadruplicate in vehicle-incubated pieces and in triplicate in amiloride-treated pieces.

Results are expressed as means±1 SE where appropriate. Student's *t* test for unpaired data was used to assess statistical significance.

RESULTS

Amiloride inhibition of mammalian kallikrein. Fig. 1 shows that the Tos-Arg-O[^{3}H]Me esterase activity of either rat or human urinary kallikrein was inhibited 50% by amiloride, 0.23 mM. In this assay system, all other diuretic drugs tested including: furosemide, hydrochlorothiazide, ethacrynic acid, spironolactone, and ouabain in concentrations ranging from 1 μM to 1.7 mM did not inhibit either rat or human urinary kallikrein activity to any degree. Triamterene, 1.7 mM, produced slight inhibition (22.5±4.2%, mean±1 SE, n = 6) of rat urinary kallikrein activity. Fig. 1 also shows that the kallikrein-like esterase activity of isolated, viable renal cortical cells in suspension was also inhibited by amiloride with 50% inhibition seen at a drug concentration of 0.12 mM.

Inhibition of the activity of purified rat urinary kallikrein was also observed when its kinin-releasing activity was measured with a kinin radioimmunoassay. Fig. 2 shows that amiloride inhibits this activity of kallikrein with an ID_{50} of ~85 μM. The slow contraction of the isolated guinea pig ileum seen when kallikrein was added to the tissue bath containing a source of kininogen was inhibited by preincubation of the enzyme with amiloride (Fig. 3). The contractile responses of the isolated rat uterus induced by rat urinary kallikrein alone were abolished by preincubating the enzyme with amiloride (Fig. 3). Amiloride never inhibited bradykinin-induced contractile responses of either the guinea pig ileum or rat uterus.

Fig. 4*a* shows measurement of kallikrein activity upon the synthetic substrate, thiobenzyl benzyloxy-

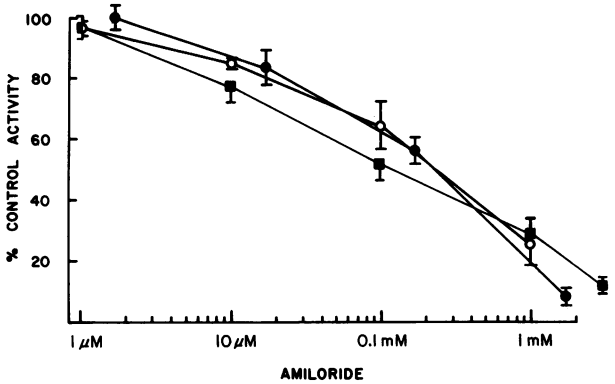

FIGURE 1 Inhibition of rat or human urinary kallikrein or of renal cortical cell suspension Tos-Arg-O[^{3}H]Me esterase activity by amiloride. 10 μl (11 nM) of either rat (●) or human (○) urinary kallikrein (20 mEU/ml, 1 EU defined as in Methods), 10 μl of water or water plus amiloride (final concentrations as indicated) and 30 μl of 0.2 M Tris-HCl buffer, pH 8 were mixed and allowed to stand for 30 min at 25°C. 20 μl of freshly prepared renal cortical cells (■) (5.0×10^6 cells/ml) in phosphate-buffered saline containing 2 mM Ca^{2+}, 20 μl of 0.2 M Tris-HCl buffer, pH 8 and 10 μl of water or water containing amiloride (final concentrations as indicated) were mixed and also allowed to stand for 30 min at 25°C. Tos-Arg-O[^{3}H]Me (3.0×10^4 cpm, 10 μl) was added, mixed and allowed to incubate for 30 min. The reaction was stopped and the [^{3}H]methanol released was measured as described in Methods. Activity is expressed as the percent of control esterase activity in the absence of amiloride. Each value represents the mean±1 SE of 12 experiments in duplicate for rat urinary kallikrein, six in duplicate with human urinary kallikrein and six in duplicate with separate rat renal cortical cell preparations.

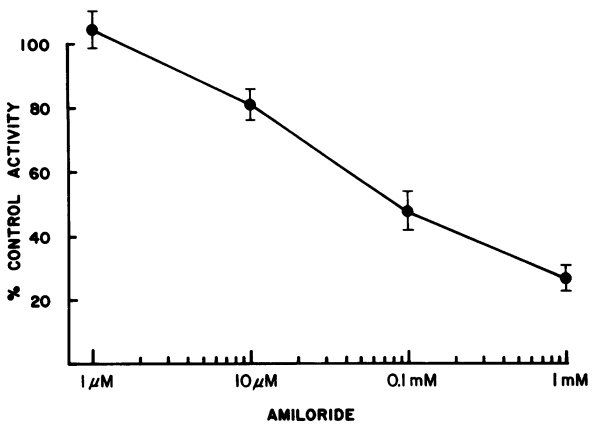

FIGURE 2 Inhibition of the kininogenase activity of rat urinary kallikrein by amiloride. Kinin-releasing activity was measured with a kinin radioimmunoassay. 50 μl of rat urinary kallikrein (40 mEU/ml) and 50 μl of water or water containing amiloride (final concentrations as indicated) were mixed and incubated at 37°C for 30 min. The rest of the procedure is as described in Methods. Activity is expressed as the percent of control kinin generation in the absence of amiloride. Each value represents the mean±1 SE of seven experiments in duplicate.

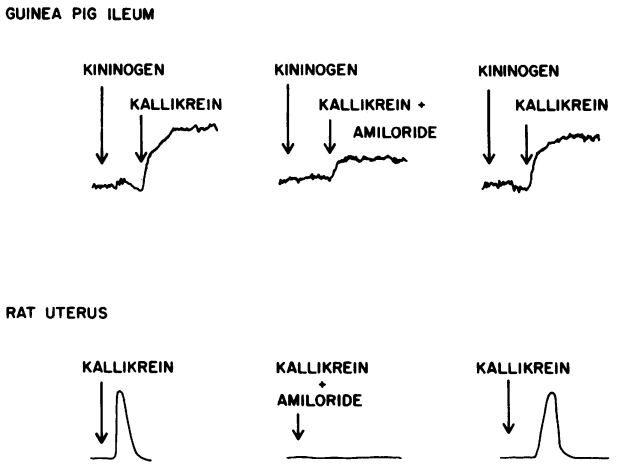

FIGURE 3 Bioassays of kallikrein in the absence and presence of amiloride. (Upper panel): The slow contractile response of the guinea pig ileum produced by kinin liberation from 0.1 ml of heated dog plasma kininogen substrate incubated within the isolated tissue bath with 0.1 ml of purified rat urinary kallikrein (0.1 EU/ml) is seen. The same quantity of enzyme was preincubated with 3 mM amiloride for 30 min at 37°C and a 0.1-ml aliquot was added to the bath. The response was inhibited by amiloride (bath concentration, 30 μM). After washing, the response returns. (Lower panel) The contractile response of the rat uterus was induced by 0.1 ml of rat urinary kallikrein (0.1 EU/ml). The same quantity of enzyme was preincubated with 3 mM amiloride for 30 min at 37°C and a 0.1-ml aliquot was added to the bath. The response was inhibited by amiloride (bath concentration, 30 μM). After washing, the response returns.

carbonyl-L-lysinate at pH 7.5. In this typical Lineweaver-Burk plot, the rate of substrate hydrolysis varied as a function of substrate concentration according to the Michaelis-Menten model. Amiloride, 6.67–33 μM, did not affect K_m (calculated to be 0.125 mM when the line was fitted to the experimental points by linear regression analysis, $r = 0.99$) but increased $1/V_{max}$ indicating a noncompetitive inhibition of the enzyme. Secondary plots of the intercepts (not shown) or slopes (Fig. 4*b*) vs. amiloride concentration were also linear, and support the interpretation of noncompetitive inhibition of kallikrein by amiloride. The calculated K_i for kallikrein inhibition by amiloride (using linear regression analysis, $r = 0.98$) was 26 μM. Data obtained at pH 8 also indicated noncompetitive inhibition with a K_i of 25 μM and the lower K_m (67 μM) expected as the enzyme approaches its pH optimum (8.5–9.0) (20). The experiments in Fig. 4*c* show that the slope of V_{max} vs. various concentrations of enzyme is smaller in the presence of amiloride but the intercept of this plot is at the origin, indicating reversible inhibition.

Amphibian kallikrein-like activity. Toad bladder or skin extracts (12 preparations at various times of the year) contained from 0.03 to 0.09 EU/mg protein

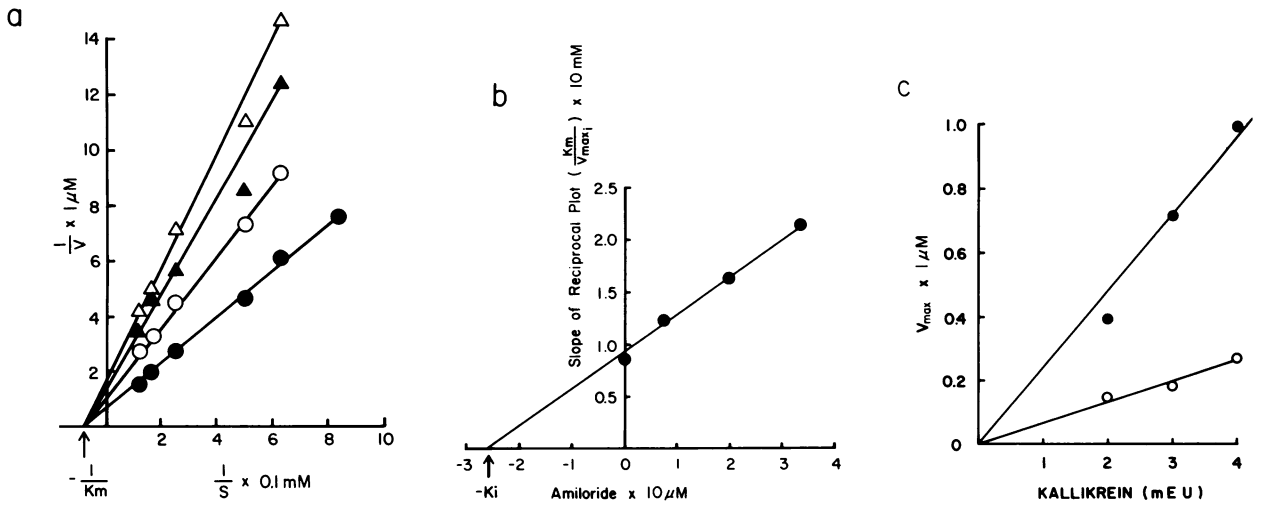

FIGURE 4(*a*) Lineweaver-Burk plot of initial reaction velocity, V, vs. substrate concentrations, S, in the absence (●) and in the presence of amiloride [6.7 μM, (○); 20 μM, (▲); 33 μM, (△)]. (*b*) Secondary plot of the slopes from the primary Lineweaver-Burk plot vs. amiloride concentration. (*c*) Plot of V_{max} vs. kallikrein concentration. (●) control; (○) 20 μM amiloride. Velocities are expressed as the molar concentration of 3-carboxy-4-nitrothiophenoxide ion formed in the reaction mixture over a 1-min period after addition of kallikrein.

against Tos-Arg-O[^{3}H]Me using human urinary kallikrein as the standard. The pH profile of this esterase activity from either urinary bladder or skin followed a Gaussian distribution with an optimum of 9.

A typical illustration of the biological activity of toad skin extract is shown in Fig. 5. The guinea pig ileum responded to the extract plus kininogen from heated dog plasma with characteristic slow contraction. This response was prevented by preincubation with carboxypeptidase B. When carboxypeptidase B was placed in the tissue bath (2–3 U/ml), the response was also abolished.

Table I shows that this kallikrein-like alkaline esterase activity was inhibited by several inhibitors of mammalian glandular kallikrein. Aprotinin, a potent inhibitor of mammalian glandular kallikrein (19, 29), inhibited this esterase activity (ID_{50} = 28 μM). Phenylmethylsulfonyl fluoride, a specific serine proteinase

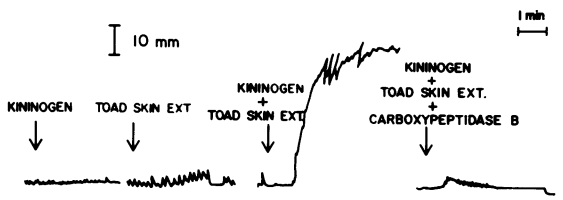

FIGURE 5 Bioassay of kinin-releasing activity of toad skin extract. The isolated guinea pig ileum was bathed in 10 ml of Tyrode's solution. Neither heated dog plasma (kininogen) nor the toad skin acid-treated extract alone produced contractile responses. 0.1 ml of substrate was incubated with 0.1 ml of toad skin extract (0.1 EU/ml, 0.05 EU/mg protein) in Tris buffer, 0.2 M pH 8 (0.6 ml total volume) for 30 min at 37°C. After the reaction was stopped by boiling for 5 min and the tubes cooled, a 0.05-ml aliquot produced a contraction equivalent to that seen with 10 ng of synthetic bradykinin. Addition of 21.5 U of carboxypeptidase B after cooling abolished the contractile response.

TABLE I
Inhibition of Toad Bladder Tos-Arg-O[^{3}H]Me Esterase Activity

Inhibitor	Inhibition, %			
	39 μM	3.9 μM		0.39 μM
Aprotinin	56	17		12
	1.7 mM	0.17 mM	17.0 μM	1.7 μM
Phenylmethylsulfonyl fluoride	90	32	22	2
Benzamidine	98	76	24	7
Pentamidine	100	99	96	63
IRT-36	100	89	46	30
IRT-63	100	100	93	55
Amiloride	100	78	20	2

10 μl of the toad bladder extract (20 mEU/ml), 10 μl of vehicle or vehicle plus inhibitor (final concentrations as shown) and 30 μl of 0.2 M Tris-HCl buffer, pH 8, were mixed and allowed to stand for 30 min. Tos-Arg-O[^{3}H]Me esterase activity was measured as described in Fig. 1 and Methods. Activity is expressed as percent inhibition compared to controls. Each value represents the average of three experiments in duplicate. Identical inhibitory patterns exist for toad skin extract alkaline esterase activity.

inhibitor (29), inhibits bladder esterase activity at an ID_{50} of 0.35 mM. Benzamidine and pentamidine are strong inhibitors of trypsin and kallikrein, and IRT-36 and IRT-63 are more potent inhibitors of kallikrein than other serine proteinases (30). The ID_{50} of benzamidine, pentamidine, IRT-36 and IRT-63 were 56, 1, 20, and 1 μM, respectively. Trypsin inhibitors from lima bean or ovomucoid do not inhibit purified mammalian glandular kallikrein (19) and soybean trypsin inhibitor does so, weakly; they did not inhibit toad bladder extract esterase activity in concentrations up to 1.7 mg/ml. Finally, the diuretic drug amiloride also inhibited this extract alkaline esterase activity (ID_{50} = 50 μM). All other diuretics tested including: furosemide, hydrochlorothiazide, ethacrynic acid, spironolactone, and ouabain, in concentrations ranging from 1 μM to 1.7 mM, did not inhibit this activity to any degree.

Tos-Arg-O[^{3}H]Me esterase activity of toad bladder pieces was measured in the absence and presence of amiloride, 1 nM–1 mM. Fig. 6 shows that significant inhibition was detected at an amiloride concentration of 10 nM although the ID_{50} was ~0.1 mM.

DISCUSSION

The results show that amiloride can inhibit kallikrein and kallikrein-like enzyme activity. The inhibition appears to be of a noncompetitive and reversible type. The inhibitory effects of amiloride can be detected with a variety of assay systems and can be observed using a purified homogenous rat urinary kallikrein, a partially purified human urinary kallikrein, or kallikrein-like activities upon the surface of rat renal cortical cells in suspension, in toad bladder pieces, or in extracts of toad bladder or skin. The concentration of amiloride required to inhibit enzyme activity 50% ranges from 85 to 230 μM depending upon substrate, enzyme preparation, or assay system. A minimal, but significant effect was detected at 10 nM amiloride using toad bladder pieces. Of the other diuretic agents tested, using the most convenient assay system (Tos-Arg-OMe esterase activity) and the most pure enzyme preparation (rat urinary kallikrein B purified to homogeneity) (19), only triamterene produced any detectable inhibition, and this was minimal. Furosemide, hydrochlorothiazide, ethacrynic acid, spironolactone, and ouabain were without inhibitory effects over a broad range of concentrations.

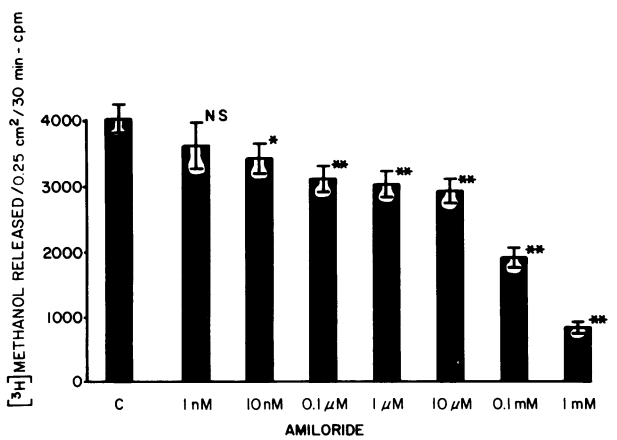

FIGURE 6 Tos-Arg-O[^{3}H]Me esterase activity of toad bladder pieces in the absence (*C*) or presence of amiloride. Each bar represents the mean ± 1 SE of the Tos-Arg-O[^{3}H]Me esterase activity (expressed as counts per minute of [^{3}H]methanol released/0.25 cm^2/per 30 min) of no less than 45 separate pieces from 15 to 20 hemibladders. NS-not significantly different from control. *, **, differ from control with $P < 0.05$ or 0.001, respectively.

Amiloride is a substituted pyrazine carboxamide developed as a result of a search for a nonsteroidal natriuretic, antikaluretic agent (31). It has become useful as a probe of membrane ion transport since its effects upon short-circuit current, a reflection of active sodium transport, were first demonstrated in amphibian tissues (32, 33). It has been shown subsequently that amiloride acts upon susceptible amphibian (bladder, colon, skin) and mammalian (distal nephron, colon, salivary duct) sites to rapidly and reversibly inhibit permeability to sodium at the mucosal surface or apical cell borders (34). The local concentrations required to reduce permeability to sodium ions 50% vary widely, and range from 20 nM in frog skin bathed in low sodium (35) to 0.1 mM or higher in micropunctured, perfused rat distal tubules (36). Thus, the concentrations of this drug which reduce membrane sodium permeability are similar to those which inhibit kallikrein or kallikrein-like enzyme activity to an equivalent degree.

Although it must be emphasized that the present data do not indicate that mammalian glandular kallikrein plays any role in the mechanism of action of amiloride, there are reasons to support further investigation of this notion. First, the tissues in which amiloride is known to act are also those containing kallikrein or a kallikrein-like activity, e.g., kidney, salivary glands, sweat glands, colon and now, amphibian bladder and skin. Second, recent biochemical (16), histochemical (14, 15), and physiologic (37) evidence has suggested that the enzyme is bound within apical plasma membranes of the distal nephron or salivary ducts and gains access to tubular fluid from these sites where amiloride is known to act. Third, the studies of Crocker and Willavoys (1) have shown that low concentrations of bradykinin (10 pM) can increase Na^+ and water transfer from mucosal to serosal surface of the rat jejunum when basal transfer rates are low. Finally, our own preliminary studies (38) have shown that inhibitors of mammalian glandular kallikrein reduce short-circuit current in the isolated toad

bladder. On the other hand, no published studies have examined comprehensively the effects of kallikrein-kinin system components upon electrolyte and water transport in tissues where these components reside. Thus, studies of the direct effects of kinin peptides, glandular kallikrein substrates, and inhibitors or antibodies to the enzyme and peptides, upon ion and water transport in the distal nephron or amphibian bladder must be carried out to determine if the system has a role in ion transport or the mechanism of action of amiloride.

In summary, the results of the present study prove that a drug known to inhibit Na^+ reabsorption reversibly at specific sites of glandular kallikrein localization can, in similar concentrations, noncompetitively and reversibly inhibit kallikrein or an amphibian kallikrein-like activity. The facts suggest that further investigation of the actions of components of the glandular kallikrein-kinin system upon ion and water transport at pertinent mammalian and amphibian sites would be of interest, as would be studies of relations between the effects of amiloride and its analogues upon kallikrein, and renal electrolyte and water transport.

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