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Urinary Proteases Degrade Epithelial Sodium Channels

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Summary. The mammalian urinary bladder epithelium accommodates volume changes by the insertion and withdrawal of cytoplasmic vesicles. Both apical membrane (which is entirely composed of fused vesicles) and the cytoplasmic vesicles contain three types of ionic conductances, one amiloride sensitive, another a cation-selective conductance and the third a cation conductance which seems to partition between the apical membrane and the mucosal solution. The transport properties of the apical membrane (which has been exposed to urine in vivo) differ from the cytoplasmic vesicles by possessing a lower density of amiloride-sensitive channels and a variable level of leak conductance. It was previously shown that glandular kallikrein was able to hydrolyze epithelial sodium channels into the leak conductance and that this leak conductance was further degraded into a channel which partitioned between the apical membrane and the mucosal solution. This report investigates whether kallikrein is the only urinary constituent capable of altering the apical membrane ionic permeability or whether other proteases or ionic conditions also irreversible modify apical membrane permeability.

Alterations of mucosal pH, urea concentrations, calcium concentrations or osmolarity did not irreversible affect the apical membrane ionic conductances. However, urokinase and plasmin (both serine proteases found in mammalian urine) were found to cause an irreversible loss of amiloride-sensitive current, a variable change in the leak current as well as the appearance of a third conductance which was unstable in the apical membrane and appears to partition between the apical membrane and the mucosal solution. Amiloride protects the amiloride-sensitive conductance from hydrolysis but does not protect the leak pathway. Neither channel is protected by sodium. Fluctuation analysis demonstrated that the loss of amiloride-sensitive current was due to a decrease in the sodium-channel density and not a change in the single-channel current. Assuming a simple model of sequential degradation, estimates of single-channel currents and conductances for both the leak channel and unstable leak channel are determined.

Key Words epithelial transport · sodium channels · proteases · channel hydrolysis

Introduction

The ability of some epithelia to regulate the magnitude and direction of active transepithelial ion transport is dependent on plasma hormone levels, release of neurotransmitters, cytoplasmic concentrations of second messengers (e.g., cAMP), availability of metabolic substrates, as well as intracellular and extracellular ion activities (Scott & Goodman, 1981). However, these mediators are hard pressed to explain the large variability (in some cases by a factor greater than 100) of ion transport for a given epithelium from animals presumably in the same physiological state. This phenomenon is most notable, but seldom commented on in Na^+ transporting tight epithelia such as urinary bladders of rabbit, toad and *Necturus*, the colons of rabbit and turtle, and the ventral skin of frog and toad. Two recent observations from the mammalian urinary bladder (an epithelium whose Na^+ reabsorptive capacity is directly dependent upon apical membrane Na^+ channel density) suggest that other regulatory factors must exist in addition to those listed above. The first observation (Lewis et al., 1984) was that cytoplasmic vesicles, which are inserted into the apical membrane during bladder distension and retrieved back into the cytoplasm during bladder collapse, have an eightfold greater Na^+ transport capacity than the apical membrane, even though the apical membrane is completely composed of fused vesicles. The second observation was that the apical membrane of this epithelium contains three populations of ion channels (Lewis et al., 1984), one amiloride sensitive and Na^+ selective, another a cation channel (Na^+ and K^+ of near equal permeability) and the last a cation channel, which is unstable in the membrane and seems to partition between the apical membrane and the mucosal bath. Thus, although channel production is most probably under hormonal control, the factor(s) responsible for channel loss (i.e., degradation or internalization) has not been demonstrated. Since the cytoplasmic vesicles have a higher Na^+ channel density than the apical membrane, this difference is most easily accounted for by postulating

an extracellular factor that irreversibly inhibits this channel and in the process converts an amiloride-sensitive Na^+ channel and possibly leak channel into a form that is unstable in the membrane.

In this paper we provide evidence that two proteases (urokinase and plasmin) released by the distal nephron of the kidney into the urine (Holmbert & Astedt, 1982) hydrolyze amiloride-sensitive Na^+ channels into leak pathways that are stable in the membrane as well as pathways that are unstable.

Since similar enzymes are released into other aldosterone-stimulated Na^+ absorptive systems such as the salivary and sweat glands and lower bowel as well as the amphibian urinary bladder and ventral skin, these results might also explain, first, the wide variability in transport ability and, second, the presence of a finite cation conductance in the apical membrane of these other epithelia.

Materials and Methods

General

Male New Zealand White rabbits (2–3 kg) were sacrificed using pentobarbital. Urinary bladders were excised and mounted in a rack to remove (using blunt dissection) the underlying three layers of muscle. This "stripped" preparation was then mounted in a modified Ussing chamber specifically designed to reduce edge damage (*see* Lewis, 1977). Each chamber had a volume of 15 ml and nominal exposed area of 2 cm^2. The chambers are temperature controlled at 37°C by an external water jacket, solutions are stirred with magnetic spin bars coupled to a D.C. motor. All solutions are aerated with 95% O_2-5% CO_2 and maintained at a pH of 7.4. Stirring and aeration was briefly interrupted (3–5 min) during measurement of current fluctuations (*see below*).

Transepithelial potential (V_T) was measured using Ag-AgCl electrodes placed close to and on opposite sides of the epithelium. Current-passing electrodes (coiled Ag-AgCl wires) were placed at opposite ends of the hemi-chambers. Both voltage measuring and current passing electrodes were connected to an automatic voltage clamp interfaced to a laboratory computer. Transepithelial resistance was measured by passing a 300-msec square current pulse (ΔI_T) and measuring the voltage deflection (ΔV_T). The transepithelial resistance (R_T) was calculated using Ohm's law as $R_t = \Delta V_T/\Delta I_T$, and the short-circuit current (I_{sc}) as V_T/R_T. These measurements were performed at 10-sec intervals, allowing a continuous monitoring of the viability of the preparation during experimental perturbations.

Solution changes were performed isovolumically, using a syringe for adding fresh solution and a vacuum line for removing the solution. A 60-ml wash was adequate to exchange 97% of the old solution with fresh Ringer's in 10–20 seconds.

Determination of Sodium Channel and Epithelial Properties

Macroscopic amiloride binding kinetics, junctional resistance, and sodium channel selectivity were determined by measuring the effect of amiloride (over a concentration range of 0.13 to 10 μM) on the short-circuit current, the V_T and the R_T. Amiloride binding kinetics were determined by curve fitting the change in I_{sc} as a function of amiloride concentration by the Michaelis-Menten equation using a nonlinear curve fitting routine (Nfit, Island Products, Galveston, TX). The junctional resistance (R_j) and the electromotive force (emf) of the cellular pathway E_c were determined from the response of V_T and R_T to increasing amiloride concentrations (Wills, Lewis & Eaton, 1979). A plot of V_T *versus* R_T yields a linear, double intercept plot in which the intercept at the abscissa is equal to R_j and the intercept at the ordinate is equal to E_c. The sodium selectivity of the amiloride-sensitive channel was determined using the method of Lewis and Wills (1983). As previously shown E_c is the sum of the apical membrane sodium channel emf (E_a^{Na}) and the basolateral membrane emf (E_{bl}). E_a^{Na} is then calculated as the difference between E_c (as measured above) and E_{bl} (−52 mV; *see* Lewis & Wills, 1983). The sodium to potassium selectivity was calculated from E_a^{Na} using the constant field equation (*see* Lewis & Wills, 1983) and previously measured values of intracellular sodium activity (7 mM), intracellular potassium activity (90 mM), extracellular sodium activity (106 mM) and extracellular potassium activity (5.46 mM) (Wills & Lewis, 1980). The dependence of the amiloride-sensitive I_{sc} on extracellular sodium concentration was assessed by the effect of equimolar replacement of sodium with choline on the amiloride-sensitive I_{sc}.

Fluctuation Analysis

Current fluctuations under short-circuit conditions were measured using a low-noice voltage clamp similar to that used by Lindemann and Van Driessche (1977). The methods are described in detail elsewhere (*see* Lewis et al., 1984); a brief description follows. The current was high pass filtered (0.03 Hz), amplified by a low noise amplifier (Model 113, Princeton Applied Research, Princeton, NJ), and subsequently low-pass filtered at 100 Hz by a 120 dB/octave antialiasing filter (Model LP120, Unigon, Mount Vernon, NY). The signal was digitized (12 bit A-to-D converters) at 5-msec intervals using a digital oscilloscope (Model 2090, Nicolet). A total of 32,768 consecutive data points (164 sec acquisition time) were collected and stored on the oscilloscope's disk.

All subsequent computations were performed using 32-bit floating-point arithmetic. The mean power spectral density (PSD) was determined by averaging spectra computed from discrete Fourier transforms of 512-point segments of the data [for complete details, *see* Rabiner, Schafer and Dlugos (1979)]. The final mean PSD estimate consisted of 256 points in the frequency range of 0.4–100 Hz; points above ca. 80 Hz were discarded due to potential contamination resulting from aliasing.

The PSD was found to be described by the sum of a linear and Lorentzian component. At each frequency (f), the PSD is described by

$$S(f) = \frac{B}{f^{\alpha}} + \frac{S_o}{1 + (f/f_c)^2} \qquad (1)$$

where B and α are the amplitude and slope of the linear component, and S_o and f_c are the low-frequency plateau and the corner frequency of the Lorentzian component, respectively. These four parameters were determined for each PSD estimate by fitting Eq. (1) to the data using a nonlinear curve fitting algorithm (Brown & Dennis, 1972).

Interpretation of Parameters Determined from the PSD

Single-channel current (i), channel density (N), as well as the rate of amiloride association (k_{01}) and dissociation (k_{10}) were determined using the method of Lindemann and Van Driessche (1977) who assumed that amiloride blockage of channels obeys first-order kinetics. This is described by a reaction scheme where amiloride (A) binds to a channel receptor (R) to form an amiloride-blocked channel (AR) namely

$$A + R \underset{k_{10}}{\overset{k_{01}}{\rightleftharpoons}} AR.$$

The analyses which follows assumes that the amiloride concentration ([A]) greatly exceeds the total number of blocked and unblocked channels, an assumption that is appropriate in our case.

The association and dissociation rate constants are related to the corner frequency by

$$2\pi f_c = [A]k_{01} + k_{10} \tag{2}$$

a plot of the corner frequency *vs.* the amiloride concentration yields a linear relationship in which the slope equals k_{01}, and the ordinate intercept equals k_{10}. An amiloride dissociation constant (K_A: the concentration of amiloride which produces 50% inhibition of the amiloride-sensitive current) can be defined by

$$K_A = k_{10}/k_{01}. \tag{3}$$

The probability of a channel being open (unblocked) is

$$P_o = k_{10}/(k_{10} + [A]k_{01}) \tag{4}$$

and the probability of a channel being closed (blocked) is

$$P_c = 1 - P_o. \tag{5}$$

Single-channel currents are calculated from measurements of S_o and the amiloride-sensitive short-circuit current (I_{amil}) following a submaximal amiloride concentration

$$S_o = 4I_{amil}iP_c/(2\pi f_c). \tag{6}$$

Finally, the channel density (N) can be determined from the other parameters since

$$I_{amil} = iNP_o. \tag{7}$$

Solutions

Both sides of the epithelium were bathed with a solution of the following composition (in mM): 111.2 NaCl, 25 $NaHCO_3$, 5.8 KCl, 2 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4 and 11.1 glucose buffered at pH 7.4. In one series of experiments all mucosal Na^+ was replaced with the appropriate K^+ salt. Amiloride was a gift from Merck-Sharp and Dohme. Urokinase and plasmin were from Sigma. Statistical analysis was performed using either a paired or unpaired Student t test, and all values are given as means ± SEM.

Table 1. The effect of various agents on the amiloride-sensitive current and leak current

Agent	I_{amil}	I_L	Reversible
Low Ca^{2+}(~0 mM)	No change	Increase	Yes
Low pH (pH 5)	Decrease	Decrease	Yes
Hyperosmotic (1 M sucrose)	No change	No change	
1 M urea	No change	No change	
Trypsin (1 mg/ml)	Decrease	Decrease	No

I_{amil} is the amiloride-sensitive current and I_L is a cation-selective and amiloride-insensitive current. Note that only the proteolytic enzyme trypsin caused an irreversible decrease in both types of current.

Measuring Heterogeneous Channel Populations

The method of Lewis et al. (1984) was used to assess the relative contribution of amiloride-sensitive current (I_{amil}) and stable leak current (I_L) to the total short-circuit current (I_{sc}) across the mammalian urinary bladder. First the bladder was "punched" to increase the transport rate (*see* Lewis & deMoura, 1984). Next the mucosal chamber was washed to eliminate all unstable leak current from the apical membrane (I_{usL}). This results in a preparation containing only two populations of channels, one amiloride sensitive and the other not, but both are stable in the membrane. The relative magnitude of these currents was assessed using a maximal dose of amiloride. Residual current (i.e., current not eliminated by amiloride) is referred to as the stable leak current (I_L), while the quantity of current reduced by amiloride is referred to as the amiloride-sensitive current (I_{amil}).

Results

First the effects of reduced Ca^{2+}, pH, urea, hyperosmolarity and serine proteases on transepithelial ion transport are described. Next, we focus on the actions of serine proteases, since only these agents have an irreversible action on the amiloride-sensitive Na^+ channel. Last, the action of one of these proteases (urokinase) on both channel density as well as channel heterogeneity is quantified.

Effect of Mucosal Solution Composition on Sodium Transport

Table 1 lists the agents used and their effect on transepithelial Na^+ transport. In all cases the quantity of amiloride-sensitive Na^+ transport and stable leak current was first assessed, next the apical membrane was exposed to the agent for 0.5 to 2 hr, and last the mucosal solution bathing the apical membrane was returned to control conditions and the quantity of amiloride-sensitive current and stable

Table 2. Effect of three urinary proteases on the amiloride-sensitive current

Protease	Activity (U/ml)	% decrease of I_{amil}	Rate constant (k) (10^{-3} min^{-1})
Urokinase (n = 5)	0.013	63 ± 7	8.2 ± 1.2
Plasmin (n = 3)	0.07	52 ± 8	6.1 ± 1.4
Kallikrein[a] (n = 6)	0.07	59 ± 8	7.3 ± 1.2

[a] Data from Lewis and Alles (1986).
See Sigma Catalogue for the definition of the activity of each protease.

leak current were again measured. Of the agents listed in Table 1, only the serine protease, trypsin, caused an irreversible decrease of both the amiloride-sensitive current and the stable leak current, without the appearance of an unstable leak conductance, i.e., washing the mucosal solution does not result in a decrease in I_{sc}.

Urinary Serine Proteases

The mammalian urine contains at least three different serine proteases, which are urokinase, plasmin and kallikrein. In a previous report Lewis and Alles (1986) demonstrated that kallikrein caused (over a 2-hr period) a progressive and irreversible inhibition of the amiloride-sensitive transport. As in the case of kallikrein, both urokinase and plasmin also caused an irreversible loss of amiloride-sensitive current (Table 2). Of interest is that the decrease in amiloride-sensitive Na^+ transport is directly proportional to the initial value of the Na^+ transport, i.e., it is a first-order reaction, in which the rate constant (k_1) is described by the equation

$$I_{amil}(t) = I_{amil}(0)e^{-k_1 t} \quad (8)$$

where $I_{amil}(0)$ is the amount of amiloride-sensitive current before protease addition and $I_{amil}(t)$ is the amiloride-sensitive current remaining after t minutes of protease action. The rate constant for each protease (at the given protease activity) is listed in Table 2.

The next section will consider in more detail the effect of urokinase on Na^+ channel degradation as well as the hydrolysis by this enzyme of the nonamiloride-sensitive leak pathway.

Urokinase Hydrolyzes Both Channel Types

The proteolytic effect of urokinase on both the amiloride-sensitive leak currents was tested by comparing the amiloride-sensitive current before and 2 hr after the addition of human urokinase (at a final activity of 0.013 U/ml) to the mucosal solution. After the 2-hr exposure, the amiloride-sensitive current had decreased by 63% (Fig. 1*A*, *no amil*), while the leak current was increased by 140% (Fig. 1*C*, *no amil*). As previously reported (Lewis et al., 1984), the leak current results from two types of apical membrane channels, one that is stable in the membrane and another that can be removed by repeated washing of the mucosal bathing solution. This repeated washing reduced the leak current to a value 23% lower than the pre-urokinase value (Fig. 1*C*, *no amil*). Two conclusions can be reached from these data. First, urokinase can degrade both the amiloride-sensitive channel and the leak channel. Second, in the presence of urokinase, an apical conductance appears which can be washed out of the membrane. This latter point suggests that urokinase converts one or both of these channels into a channel that seems to partition between the apical membrane and the mucosal bathing solution.

This apparent ability of a channel to move into and out of the apical membrane has been previously reported (Lewis & Alles, 1986) and the rate constants for movement into and out of the membrane calculated. One method used to determine the sum of the rate constants for this conductance was to rapidly replace the mucosal solution with fresh solution and measure the time dependence of the decrease in the short-circuit current. Lewis and Alles (1986) found that the time-dependent decrease was well described by a single exponential with a rate constant (sum of the rate of entering and leaving the membrane) of 17.1 ± 1.6 × 10^{-3} sec^{-1}. If this unstable leak conductance is due to the proteolytic degradation of the stable leak conductance, then the time constant for washing out of the channel produced by urokinase action should be the same as for the unstable leak conductance found normally in the apical membrane of the rabbit urinary bladder. Figure 2 shows an example of the decrease of the current by a rapid and single replacement of the mucosal solution immediately following a 2-hr incubation in urokinase. The mean rate constant for the decrease in current is 16.9 ± 0.9 × 10^{-3} sec^{-1} (n = 3). This rate constant is in excellent agreement with that previously reported (*see above*) for the unstable leak current found in bladders not treated with enzyme.

Amiloride Protects the Sodium Channel

Apical membrane Na^+ channels can be blocked not only by amiloride but also by increased extracellular Na^+ activity. Can either of these agents "protect"

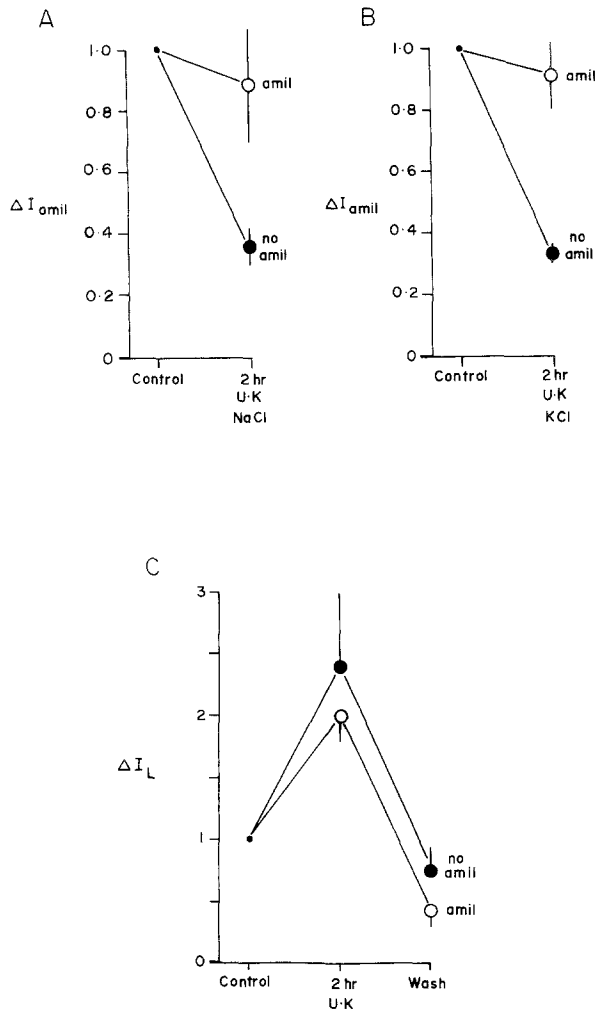

Fig. 1. (*A*) The effect of a 2-hr mucosal urokinase (U.K.) incubation on the amiloride-sensitive current across the rabbit urinary bladder. Both sides of the epithelium were bathed with a NaCl Ringer's. In the absence of mucosal amiloride (*no amil*, filled circle; $n = 5$) urokinase decreased amiloride-sensitive Na^+ transport by 63%. This irreversible decrease by urokinase was blocked by 10^{-5} M mucosal amiloride (*amil*, open circle; $n = 3$), suggesting that amiloride protects the channel from degradation. (*B*) Same as *A* except all mucosal Na^+ was replaced by K^+ after measuring the level of amiloride-sensitive current. In the absence of amiloride (*no amil*, filled circle; $n = 4$) and 2 hr after urokinase addition, the amiloride-sensitive current was decreased by 66%. This latter measurement of amiloride-sensitive current was performed by replacing KCl with NaCl after a 2-hr urokinase incubation. As in *A*, amiloride protects the channel from urokinase degradation (*amil*, open circle; $n = 3$). (*C*) In parallel with the amiloride-sensitive current, there is an amiloride-insensitive current (here called a leak current). A 2-hr incubation in urokinase, in the absence of amiloride, resulted in a 140% increase in the leak current; extensive washing of the mucosal solution reduced this current to 40% of control. The smaller increase in leak current in the absence of amiloride indicates that even though urokinase is reducing the magnitude of the leak pathway in the membrane, at the same time it is converting amiloride-sensitive channels to leak pathways. Such a phenomenon does not occur in the presence of amiloride. Vertical bars are SEM

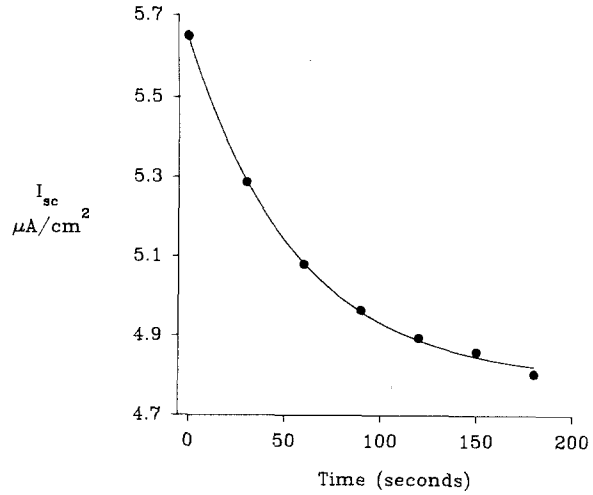

Fig. 2. A linear plot of the decrease in total short-circuit current as a function of a single rapid wash of the mucosal solution. These data were fit by an equation which is the sum of a baseline (constant) current plus an inverse exponential in which the rate constant represents the sum of the rate at which the channel or part thereof can associate with or dissociate from the apical membrane. The best fit value for the rate constant is 17.7×10^{-3} sec^{-1}

the channel from urokinase degradation? This question was investigated by the application of a saturating dose of mucosal amiloride and/or the replacement of all mucosal Na^+ by K^+ prior to urokinase application. If amiloride or Na^+ can protect the channels, then one would expect that the urokinase-induced loss of current would be less in the presence of amiloride and greater in the absence of Na^+ when compared to the control levels. Replacement of Na^+ by K^+ did not alter the loss of amiloride-sensitive current (66% decrease in the presence of K^+ compared to 63% for Na^+; *compare* Fig. 1*A* and *B*, *no amil*). However, in the presence of 0.01 mM amiloride, the amiloride-sensitive current was decreased by only 14 and 11% (Na^+ and K^+ mucosal solutions, respectively; Fig. 1*A*,*B amil*). This indicates that amiloride protects the amiloride-sensitive channel, while Na^+ does not. In the amiloride-protected preparations, the leak current increased by 100%, and after washing decreased by 60% compared to pre-urokinase level (Fig. 1*C*, *amil*). The decrease in the leak current resulting from washing the mucosal bathing solution is greater in the presence of amiloride than the absence (Fig. 1*C*, *no amil*), suggesting that amiloride inhibits the degradation of an amiloride-sensitve channel to a leak pathway, but does not inhibit the degradation of a stable leak pathway to one that is not stable and can be washed from the apical membrane. Using Eq. (8) we calculate a rate constant for hydrolysis of the

Table 3. Effect of urokinase (U.K.) on epithelial and sodium channel properties

	E_c (mV)	R_j $\Omega \cdot cm^2$	k_m^{Na} (mM)	k_i^{amil} (μM)
Before U.K.	-102 ± 8	22.2 ± 9	162 ± 25	0.28 ± 0.04
After U.K.	-53 ± 8	34.9 ± 14	140 ± 18	0.34 ± 0.08
p	0.04	NS	NS	NS

Mean ± SEM (n = 5), paired analysis.
See Materials and Methods and the text for the calculation of the above parameters.

leak channel (into a channel which is unstable in the apical membrane) of $7.6 \pm 1.6 \times 10^{-3}$ min^{-1} (n = 5).

Effect of Urokinase on Macroscopic Sodium Channel Properties

The above data demonstrates that urokinase can hydrolyze the amiloride-sensitive sodium channel into a stable leak channel and the stable leak channel into an unstable leak channel. In addition to this conversion of channels, we also investigated the effect of urokinase on the amiloride-binding kinetics, channel selectivity and the sodium concentration dependence of the amiloride-sensitive short-circuit current. Table 3 shows the effect of urokinase on the above parameters as well as its effect on the junctional resistance. Of the above parameters measured, urokinase did not alter the junctional resistance (R_j), amiloride inhibition constant (k_i^{amil}) nor the dependence of the amiloride-sensitive I_{sc} on mucosal sodium concentration (k_m^{Na}). However, it caused a significant decrease in E_c (cellular emf) from -102 to -53 mV. Using the previously published value for E_{bl} of -52 mV (Lewis & Wills, 1983), the change in E_c reflects a change in the amiloride-sensitive emf (E_a^{Na}), which decreased from -50.5 ± 3.6 mV (n = 5) to -1.3 ± 7.0 mV (n = 5). The sodium-to-potassium selectivity of the amiloride-sensitive conductance was then calculated using the constant field equation and known values for extracellular and intracellular sodium and potassium activities (*see* Materials and Methods). The permeability ratio (P_{Na}/P_K) was significantly decreased from 10.5 ± 3.4 (n = 5) before urokinase to 0.88 ± 0.35 2 hr after urokinase. These data then suggest that not only does urokinase convert an amiloride-sensitive sodium-selective channel into one that is not blocked by amiloride and is cation selective (stable leak channel), but it can cause a loss of sodium selectivity without altering k_i^{amil}.

Urokinase Decreases Channel Density

To determine whether the urokinase-induced decrease in I_{amil} was caused by the alteration of either the single-channel current, the channel density or both, we used fluctuation analysis to calculate both the single-channel currents and channel density before and after urokinase treatment. Figure 3*A* shows the power spectral density before (control) and after a 2-hr incubation with urokinase (Fig. 3*B*). The best fit curves for the Lorentzian component before and after urokinase (induced by 1.4 μM amiloride) is shown in Fig. 3*C*. The magnitude of the depression of the plateau value and constancy of the corner frequency suggests that both the amiloride-binding kinetics and the single-channel current are unaltered by urokinase. Thus the decrease in Na^+ transport can be accounted for by a decrease in the channel density. The increase in the power at 1 Hz of the linear component (Fig. 3*D*) is consistent with the increase in leak current that occurred in this particular preparation and supports the findings of Lewis et al. (1984) that the leak current and the power at 1 Hz are linearly related. Table 4 summarizes the effect of urokinase on amiloride-sensitive short-circuit current (I_{amil}), single-channel current (i), channel density (N), and the amiloride dissociation (k_{10}) and association rate constant (k_{01}) and the microscopic rate constant (k_A). The important aspect of this table is that urokinase decreases the channel density by the same fraction that it decreases the amiloride-sensitive short-circuit current, i.e., single-channel current remains near constant. In addition, urokinase does not alter amiloride-binding kinetics.

Effect of Aldosterone on Apical Membrane Currents

Lewis and Diamond (1976) demonstrated that aldosterone increased the I_{sc} of the rabbit urinary epithelium by approximately 88%; however, they did not determine whether the increase was due only to an increase in amiloride-sensitive current or whether there was also an increase in the nonamiloride-sensitive leak current. In this last section we investigate whether aldosterone (at a final concentration of 20 μM) increases the amiloride-sensitive current, the leak current or both types of current after a 4-hr incubation. First, aldosterone produced a $150 \pm 23\%$ (n = 7) increase in the amiloride-sensitive current. Since the percent increase was independent of the initial level of I_{amil} (a linear regression of I_{amil} before and after aldosterone yielded a slope of 2.45, an intercept not significantly different from zero and a correlation coefficient of 0.8986, *data not shown*),

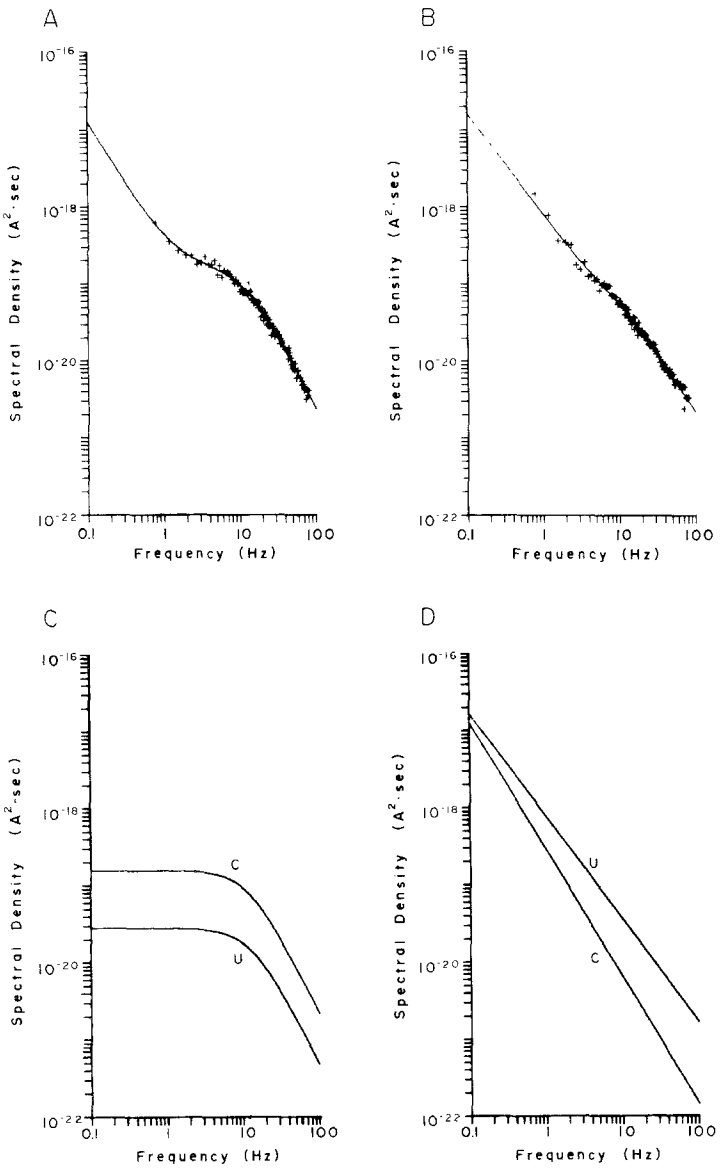

Fig. 3. (*A*) Power spectral density of rabbit urinary bladder with 1.4 μM amiloride before the addition of mucosal urokinase. The amiloride-sensitive current was 26 μA, with a leak current of 2.9 μA. The best fit parameters to the Lorentzian was a plateau value of 15.8 $\times$ 10^{-20} A^2 sec and a corner frequency of 11.97 Hz, and the best fit values to the linear component was 29 $\times$ 10^{-20} A^2 sec and a slope of 1.65. Data are for a 2 cm^2 area of bladder epithelium. (*B*) Same as *A* except for a 2-hr incubation in urokinase with a NaCl Ringer's solution with no amiloride. The amiloride-sensitive current was reduced to 6.6 μA, and the leak current increased to 4.7 μA. The best fit parameters to the Lorentzian was a plateau value of 2.8 $\times$ 10^{-20} A^2 sec and a corner frequency of 13.2 Hz, and the best fit values to the linear component was 77.5 $\times$ 10^{-20} A^2 sec and a slope of 1.34. (*C*) Best fit curves for the Lorentzian component before (*C*) and after urokinase (*U*). The large decrease in the plateau value, but insignificant change in the corner frequency indicates that urokinase is decreasing channel density without modification to the single channel currents. (*D*) Best fit curves for the linear component of the power spectral density before (*C*) and after urokinase (*U*). The increase in the value for the power at 1 Hz after urokinase (*compare* curve *U* to *C*) is consistent with the observation of Loo et al. (1983) that there is a linear and direct correlation between the leak current and the power at 1 Hz

this suggests that quiescent amiloride-sensitive channels are being activated by aldosterone. This observation is in agreement with that previously reported by Garty and Edelman (1983) for the toad urinary bladder. In addition to the increase in I_{amil} there was also a proportional increase in the nonamiloride-sensitive current (I_L) by 95 $\pm$ 10% (n = 7). A linear regression of I_L before and after aldosterone yielded a slope of 2.03, an intercept not significantly different from zero and a regression coefficient of 0.999. Using a paired t test, the percent increase in I_{amil} and I_L were found not to be significantly different at the 0.05 level. These data suggest that urinary proteases can degrade a quiescent sodium channel into a stable leak channel which is also quiescent but can be activated by aldosterone. We found no evidence for the presence of an aldosterone-activated unstable leak current. There are two possible explanations for this. Either urinary proteases cannot degrade a stable leak current into one that partitions between the membrane and mucosal solution, or a nonconductive channel is lost into the urine. Further studies are needed to separate out these two possibilities.

Discussion

The results of this study are relevant to two aspects of epithelial Na^+ transport: (i) epithelial Na^+ channels are not static entities but can be hydrolyzed by endogenous enzymes, and (ii) these endogenous

Table 4. Effect of urokinase on single sodium channel properties

Condition	I_{amil}	i (pA)	N	k_{10} (sec^{-1})	k_{01} ($\mu M^{-} sec^{-1}$)
Control	1	0.72 ± 0.04	1	13.1 ± 1.2	47.5 ± 4.9
NaCl + U.K.	0.37 ± 0.06	0.79 ± 0.09	0.33 ± 0.09	12.1 ± 1.1	45.9 ± 6.3
(n = 5) p	0.01	NS	0.001	NS	NS
Control	1	0.72 ± 0.07	1	8.4 ± 1.7	38.1 ± 3.4
KCl + U.K.	0.33 ± 0.05	0.77 ± 0.05	0.30 ± 0.03	9.4 ± 0.1	27.6 ± 1.7
(n = 4) p	0.01	NS	0.001	NS	NS
Control	1	0.66 ± 0.06	1	10.6 ± 1.8	37.8 ± 1.6
NaCl + U.K. + amil	0.86 ± 0.2	0.68 ± 0.07	0.79 ± 0.32	19.1 ± 5.3	41.8 ± 5.3
(n = 3) p	NS	NS	NS	NS	NS
Control	1	0.78 ± 0.07	1	10.0 ± 0.1	39.4 ± 3.1
KCl + U.K. + amil	0.89 ± 0.13	0.74 ± 0.03	0.95 ± 0.22	10.2 ± 0.7	37.5 ± 1.9
(n = 3) p	NS	NS	NS	NS	NS

See Materials and Methods for the calculation of the single channel current (i) and the channel density (N). I_{amil} and N have been normalized to their control values.

enzymes come into contact with these channels in vivo and as a consequence might play a physiological role in transport regulation by channel turnover.

We will consider two aspects of this study in detail. The first will be to model the stages or steps involved in hydrolysis of the Na^+ channel. Then, we will speculate about the level of channel hydrolysis that might occur in vivo and determine whether such enzyme levels can explain the difference between the density of Na^+ channels in the apical membrane of the rabbit urinary bladder compared to the channel density in the cytoplasmic vesicles.

Steps in Channel Degradation

In addition to the present study which has quantified the actions of urokinase on the urinary bladder Na^+ channels, a previous report (Lewis & Alles, 1986) also demonstrated that the urinary protease, kallikrein, was also able to degrade the Na^+ channel. We propose that the Na^+ channels in the apical membrane of the rabbit urinary bladder undergoes a stepwise degradation process involving the loss of channel selectivity, the loss of amiloride binding ability, and ending with a channel that is unstable in the apical membrane. A possible scheme is shown in Fig. 4, and was derived from the present studies as well as previous observations.

One observation made by Lewis and deMoura (1982) and confirmed by Loo et al. (1983) and Lewis et al. (1984) was that the density of Na^+ channels is less in the apical membrane than in the cytoplasmic vesicles, even though the apical membrane is formed by the fusion of cytoplasmic vesicles during the normal expansion cycle of the bladder. This indicates that once these channels are incorporated into the apical membrane, they undergo a degradation process. Our data suggest that proteases are responsible (at least in part) for this degradation and the resulting differences observed in channel densities.

Lewis and Wills (1983) and Lewis and deMoura (1984) showed that the amiloride-sensitive channel has a Na^+ to K^+ permeability ratio of 2 : 1 for bladders from animals on control (normal Na^+) diets, 9 : 1 for animals on low Na^+ diets, and greater than 30 : 1 for the cytoplasmic vesicles. However, the amiloride-binding kinetics are the same for all conditions. It appears, then, that proteases can modify the selectivity of the channel for cations without modifying the amiloride binding kinetics.

This study shows that urokinase causes a decrease in the sodium-to-potassium selectivity ratio (from 10.5 : 1 to 1 : 1.6) of the amiloride-sensitive channels, a loss of amiloride-sensitive current and a concomitant increase in the magnitude of the amiloride-insensitive leak pathway with a Na^+ to K^+ permeability rate of 1 : 1.5. This suggests that, apart from altering the selectivity of the channel, urokinase can abolish amiloride binding capability without destroying the channel's conductive properties. The magnitude of the unstable leak current increases, suggesting that urokinase degrades the leak channels to the extent that they can be removed by washing the mucosal bathing solution.

The degradation process also reveals information regarding the structure of the Na^+ channels.

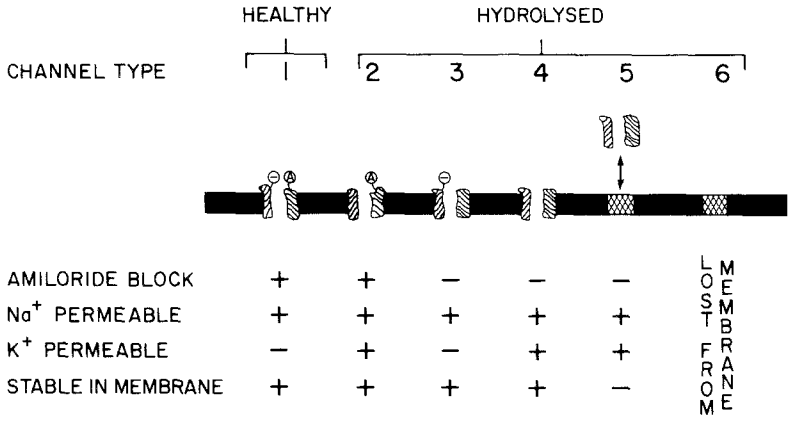

Fig. 4. Possible channel states resulting from the hydrolysis of a "healthy" amiloride-sensitive and sodium-selective channel. There is evidence for three intermediate states, with the fourth representing loss of the channel from the membrane. Although not observed to date, a possible intermediate state is one that is highly sodium selective but not blocked by amiloride

First, amiloride effectively protects the channel against hydrolysis. Second, the amiloride binding site and the Na^+ selectivity "filter" are on different sites of the channel, since selectivity is altered in the absence of a change in microscopic amiloride binding rate constants. Third, the proteases do not seem to act (at short times) as reversible blockers. Last, all three proteases which have been tested in this report, i.e., trypsin, urokinase and plasmin, as well as kallikrein (Lewis & Alles, 1986), are all serine proteases that cleave at the carbonyl site of an arginyl residue.

The degradation of the Na^+ channel by proteases offers an explanation for why the mammalian bladder has three channel populations: a Na^+ channel, a stable leak channel and an unstable leak channel. The latter two channels are simple degradation products of the (amiloride-sensitive) Na^+ channel and are not channels that the cell synthesizes and inserts into the apical membrane. In addition, it offers an explanation of why aldosterone increases not only the amount of amiloride-sensitive current but also the amount of the nonamiloride-sensitive current in the rabbit urinary bladder.

Kinetic Properties of Channel Degradation

The rate of degradation of the Na^+ and the leak pathways are equal and is a first order reaction where the loss of channels as a function of time is described by a single inverse exponential [see Eq. (8)]. In the remainder of this section we shall describe the kinetics of the loss of Na^+ channels, the loss of the stable leak pathway and, last, the appearance of the unstable leak pathway. We will consider the simplest model as shown below.

$$N_{Na} \xrightarrow{k_1} N_L \xrightarrow{k_2} N_{usL}$$

where N_{Na} is a number of Na^+ channels, N_L is the number of leak channels and N_{usL} is the number of unstable leak channels. This model assumes that the total number of channels (i.e., $N_{Na} + N_L + N_{usL}$) is constant and, for simplicity, that channel hydrolysis is sequential.

The Na^+ Channel

As first described by Fuch, Hviid Larsen and Lindemann (1977) for frog skin and later for toad urinary bladder (Li et al., 1982), hen coprodaeum (Christensen & Bindslev, 1982) and rabbit urinary bladder (Lewis et al., 1984), external Na^+ blocks amiloride-sensitive Na^+ channels in a reversible manner. From the data of Lewis et al. (1984), some 77% of the Na^+ channels are blocked by 136.2 mM Na^+ in a reversible manner while the remaining 23% are conductive. Since the percent loss of amiloride-sensitive current is independent of the bathing solution cation (*see* Fig. 1*A* and *B*) this suggests that Na^+ does not protect the channel nor change the rate of channel hydrolysis; i.e. both conducting and blocked channels can be degraded.

The last point is that amiloride completely inhibits channel degradation, suggesting that both conductive and blocked channels are protected by a saturating level of amiloride. The equation which describes the loss of Na^+ channels is

$$N_{Na}(t) = N_{Na}(o)e^{-k_1 t}. \tag{9}$$

The Leak Pathways

Unlike the amiloride-sensitive pathway which exists in two states (Na^+ blocked and conductive), the stable leak pathway has lost the Na^+ self-inhibition site. This conclusion is based on the observation

that the K^+ permeability of this pathway in the presence of Na^+ is not increased when all mucosal Na^+ is replaced with K^+.

The percent decrease of stable leak current caused by urokinase in the absence of mucosal amiloride is much less than the decrease in the presence of mucosal amiloride. The most straightforward interpretation of this observation is that the Na^+ channel is first hydrolyzed into a stable leak pathway followed by a hydrolysis into the unstable form. Thus the percentage change in stable leak current caused by a protease will depend upon the initial ratio of Na^+ current to leak current, the rate of hydrolysis of the channels and the ratio of the current flow through a single Na^+ channel to that through a leak pathway.

From a simple irreversible kinetic model (*see* p. 85) the number of leak channels N_L at any time (t) after protease addition is given by the differential rate equation

$$dN_L/dt = N_{Na}(o)k_1e^{-k_1t} - k_2N_L. \quad (10)$$

Integration of Eq. (10) gives

$$N_L(t) = \frac{N_{Na}(o)k_1}{k_2 - k_1}(e^{-k_1t} - e^{-k_2t}) + N_L(o)e^{-k_2t}. \quad (11)$$

Since in these studies $k_2 = k_1$, i.e., the rate of degradation of amiloride-sensitive current equals the rate of degradation of the leak current, then substitution of k_1 for k_2 and integration of Eq. (10) yields the following:

$$N_L(t) = N_{Na}(o)k_1te^{-k_1t} + N_L(o)e^{-k_1t}. \quad (12)$$

Similarly for the production of unstable leak channels as a function of time (using the above kinetic scheme and assuming that $N_{usL}(o) = 0$), the differential equation is (when $k_2 = k_1$)

$$dN_{usL}/dt = k_1(N_{Na}(o)k_1te^{-k_1t}) + k_1N_L(o)e^{-k_1t}. \quad (13)$$

Integration of Eq. (13) yields

$$N_{usL}(t) = N_{Na}(o)(1 - k_1te^{-k_1t} - e^{-k_1t}) + N_L(o)(1 - e^{-k_1t}). \quad (14)$$

The above set of equations describe the number of amiloride-sensitive channels [Eq. (9)], stable leak channels [Eq. (12)] and unstable leak channels [Eq. (14)] as a function of time after protease addition.

For the sodium channel, the relationship between channel number and (the measured parameter) current is

$$N_{Na} = \frac{I_{Na}}{i_{Na}P_o^{Na}} \quad (15)$$

where I_{Na} is the measured amiloride-sensitive Na^+ current, i_{Na} is the single-channel current and P_o^{Na} is the probability that the channel is open.

For the leak channel

$$N_L = \frac{I_L}{i_LP_o^L} \quad (16)$$

where I_L is the measured stable leak current, i_L is the single-channel current and P_o^L is the probability that the channel is open.

For the unstable leak channel

$$N_{usL} = \frac{I_{usL}}{i_{usL}P_o^{usL}} \quad (17)$$

where I_{usL} is the measured unstable leak current, i_{usL} is the single channel current and P_o^{usL} is the probability that the channel is open.

An interesting feature of Eqs. (12) and (14)–(17) is that by substitution and rearrangement one can estimate the single-channel current of the leak pathway and the unstable leak pathway. Thus substituting Eqs. (15) and (16) into Eq. (12) yields

$$i_L = \frac{i_{Na}}{P_o^L}\frac{P_o^{Na}(I_L(t) - I_L(o)e^{-k_1t})}{I_{Na}(o)k_1te^{-k_1t}}. \quad (18)$$

Similarly, substituting Eqs. (15)–(18) into Eq. (14) and rearranging yields

$$i_{usL} = \frac{i_{Na}P_o^{Na}}{I_{Na}(o)} \frac{I_{usL}(t)/P_o^{usL}}{1 - k_1te^{-k_1t} - e^{-k_1t} + \left(\frac{I_L(o)(1 - e^{-k_1t})k_1te^{-k_1t}}{I_L(t) - I_L(o)e^{-k_1t}}\right)}. \quad (19)$$

In order to calculate i_L and i_{usL} using Eqs. (18) and (19) and the experimental data, we must have estimates of P_o^{Na}, i_{Na}, P_o^L and P_o^{usL}. Lewis et al. (1984) estimate a P_o^{Na} (at 136.2 mM Na^+ concentration) of 0.23 and a i_{Na} of 0.7 pA (also the present study). P_o^L is estimated from the present study as 1 and P_o^{usL} from a previous study as 0.18 (*see* Lewis & Alles, 1986). Using the above equations and the measured values for $I_{Na}(o)$, $I_L(o)$, $I_L(t)$ and $I_{usL}(t)$ from this paper, the value for i_L is 0.11 ± 0.05 pA (n = 5) and for i_{usL} is 0.45 ± 0.18 pA, which can be compared to the value for i_{Na} of 0.7 pA. Single-channel

conductances were subsequently calculated using a net electrochemical driving force of 47 mV (*see* Lewis et al., 1984). The calculated single leak channel conductance of 2.4 ± 1 pS (n = 5) is lower than the single-channel conductance of the Na^+ channel of 5.8 pS, while the single-channel unstable leak conductance is 9.7 ± 3.8 pS (n = 5). The above values must be considered as first estimates and, as such, an explanation for the apparent changes in channel conductance is not justified. In a recent paper (Zweifach & Lewis, 1988), the usL conductance was concentrated and incorporated into a lipid bilayer. At room temperature (21°C) the single-channel conductance was calculated to be 9 pS, a value in reasonable agreement with that calculated using the most simple of kinetic models. Further verification of the model must await measurement of the single-channel current of the stable leak channel using either the patch-clamp technique, reconstitution, or once a reversible blocker is available, fluctuation analysis.

Channel Degradation at Physiological Protease Activity

At the enzyme activity used in this study, urokinase will, in an 8-hr period, reduce Na^+ channel density by about 98.4% in the rabbit urinary bladder. What is essential to determine, however, is the activity of this enzyme in urine and from this activity calculate the time-dependent decrease in channel density (in this case amiloride-sensitive Na^+ current) for the urinary bladder.

Using the data for urine from normal humans (Holmberg & Astedt, 1982), the urine activity of urokinase is 136×10^{-6} U/ml, giving a rate constant (k_1) of 0.48×10^{-3} min^{-1} (assuming a maximum rate of hydrolysis of the sodium channel, by urokinase, of 10.5×10^{-3} min^{-1}. This value translates into a decrease in Na^+ channel density of about 50% in a 24-hr period. This loss of channel activity by protease action is a minimal value since we have not included the effect of urine plasmin levels nor the effect of urine kallikrein levels. In a previous report (Lewis & Alles, 1986) we demonstrated that kallikrein was also capable of degrading Na^+ channels in a manner similar to that reported for urokinase. Of interest is that the calculated rate constant based on measured values of kallikrein activities in urine is about 10 times higher than for urokinase, i.e., 3.72×10^{-3} min^{-1} for kallikrein. This urine activity will reduce the Na^+ channel density by 99.5% in a 24-hr period. Thus for the rabbit urinary bladder, the presence of urine proteases can have profound effects on Na^+ channel density.

An important aspect of this study is that the existence of the cation channel and the unstable leak channel in the apical membrane of the rabbit urinary bladder is not due to the synthesis and insertion of these channels into the apical membrane, but rather that these two channel populations are produced by the degradation of amiloride-sensitive and sodium-selective channels.

Do Urinary Proteases Degrade Renal Sodium Channels?

As noted above, one of the hallmarks of channel degradation by urinary proteases is that in addition to the amiloride-sensitive sodium channel there is a parallel leak channel. Such a conductance has been reported in the rabbit cortical collecting duct and represents approximately 10% of the native apical membrane conductance (Sansom & O'Neil, 1985). After 7 days of in vivo administration of the mineralocorticoid DOCA, these authors noted that the fraction of leak conductance to total membrane conductance remained constant; however, the absolute value of the sodium conductance and the leak conductance had increased twofold. A possible interpretation of this data is that aldosterone stimulates the synthesis not only of the amiloride-sensitive conductance but also of this leak conductance. Our data, however, would suggest an alternate explanation, namely that the increase in the parallel leak conductance might be due to the activity of urinary proteases (e.g. urokinase, plasmin and kallikrein) on the renal sodium channel. Whether this is indeed the case must await the direct measurement of protease activity on the renal sodium channel.

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