Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 5345–5348, July 1986
Physiological Sciences

Urinary kallikrein: A physiological regulator of epithelial Na^+ absorption

(Na^+ channels/transport regulation/channel hydrolysis)

SIMON A. LEWIS* AND WILLIAM P. ALLES

Department of Physiology, Yale Medical School, New Haven, CT 06510

Communicated by Gerhard Giebisch, April 3, 1986

ABSTRACT **The apical membrane of the mammalian urinary bladder contains two populations of ionic conductances—one Na^+ selective and amiloride blockable, the other cation selective and amiloride insensitive (a leak channel). Addition of kallikrein (an enzyme of unknown function normally found in urine) to the mucosal solution of the mammalian urinary bladder epithelium resulted in the loss (over a 2-hr period) of amiloride-sensitive Na^+ current and an increase in the leak current that is amiloride insensitive. The rate of hydrolysis of Na^+ channels is a first-order process that is concentration (activity) dependent and described by simple Michaelis–Menten kinetics with a maximum rate of 9.5×10^{-3} min^{-1}. At the activities measured in human urine, the corresponding rate constant will decrease Na^+ channel density by 99.5% in 24 hr. Amiloride protects the amiloride-sensitive Na^+ channels from degradation but not the leak pathway. The rate of hydrolysis of the leak pathway as well as the kinetics of hydrolysis are the same as that described for the Na^+ channel. Of interest is that the leak pathway is hydrolyzed into a form that seems to partition between the apical membrane and mucosal solution (an unstable leak pathway). These results and previous findings suggest a regulatory role for kallikrein in salt and water homeostasis.**

Unlike the plasma kallikrein-kinin system, the renal and glandular systems (although extensively studied) have an unknown physiological function. Three lines of evidence have led to speculation that the renal and glandular systems play an important (but unknown) role in water and electrolyte homeostasis (1). First, kallikrein and kininogen (the inactive precursor of kinin) are located at and released into the lumen of aldosterone-stimulated Na^+-reabsorbing epithelia, such as distal nephrons, ducts of salivary and sweat glands, and the colon of mammals. Second, the only known stimulus for the release of kallikrein into the urine is the antinatriuretic hormone aldosterone. Infusion of amiloride *in vivo* counteracts this stimulatory effect of aldosterone, inhibiting the release of kallikrein into the urine. Third, and most puzzling, urine levels of kallikrein are significantly lower in people with essential hypertension. We studied mammalian urinary bladder epithelium *in vitro* to investigate whether or not this system plays a regulatory role in transepithelial Na^+ transport (2). At physiological activities, kallikrein (but not kinin) progressively inhibited Na^+ transport. This inhibition was irreversible. Kallikrein, a serine protease, caused a progressive hydrolysis of the Na^+ channel, initially yielding a heterogenous population of channels and, ultimately, nonfunctional units. We conclude that a physiological role for the renal kallikrein system is to degrade Na^+ channels. Na^+ reabsorption is therefore regulated by balancing the rate of channel activation against degradation. A defect in the process might lead to water and electrolyte retention or loss.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Rabbit urinary bladder was dissected, the underlying muscle layer was removed, and the epithelium was mounted between temperature-controlled modified Ussing hemichambers (2 cm^2 nominal area), which are designed to eliminate edge damage (3). Transepithelial potential (V_T, serosal solution ground), transepithelial resistance (R_T), a short circuit current (I_{sc}, a measure of Na^+ transport) were monitored by a laboratory computer. Both sides of the bladder epithelium were bathed with a solution of the following composition (mM): 111.2 NaCl, 25 $NaHCO_3$, 5.8 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 1.2 KH_2PO_4, and 11.1 glucose, gassed with 95% O_2/5% CO_2 and buffered at pH 7.4 at 37°C.

Since the purpose of this study was to investigate the effects of kallikrein and kinin on transepithelial Na^+ transport, we optimized the level of Na^+ transport by using the technique introduced by Lewis and deMoura (4) and confirmed by Lewis *et al.* (5). In brief, a series of hydrostatic pressure pulses (termed punching) are applied to the apical membrane. This induces the fusion into the apical membrane of cytoplasmic vesicles, which contain in their lipid bilayer a higher density of Na^+ channels (by a factor of 8) and a variable level of non-amiloride-sensitive current, a fraction of which can be eliminated by simply washing the mucosal chamber with fresh solution. This latter phenomenon is interesting and has been investigated in more detail in this paper. Under these conditions, the preparation contains two populations of channels in the apical membrane—one amiloride sensitive and Na^+ selective, the other amiloride insensitive with nearly equal Na^+ and K^+ permeability.

RESULTS

Effect of Kallikrein on Transport. To assess the effect of kallikrein and kinin (Lys-bradykinin) (Sigma) on the apical membrane Na^+ transport properties, we first treated the tissue with 0.01 mM amiloride and measured the amiloride-sensitive and the amiloride-insensitive Na^+ currents. Amiloride was then washed off and either 50 μM Lys-bradykinin or 0.07 esterase unit (EU) of kallikrein per ml (Sigma) was added to the mucosal solution. After a 2-hr incubation the magnitudes of these currents were again measured using 0.01 mM amiloride. Lys-bradykinin, whether added to only the mucosal solution or both mucosal and serosal solutions, caused no change in either amiloride-sensitive or leak current. In the remainder of this paper we will address the effect of the mucosal addition of kallikrein on Na^+ transport across the urinary bladder.

Abbreviation: EU, esterase unit(s).

*To whom all correspondence should be addressed.

Kallikrein at a concentration of 0.07 EU/ml reduced the amiloride-sensitive Na^+ transport by 59% ± 7.5% and increased the non-amiloride-sensitive transport—i.e., leak current—by 131% ± 25%. Extensive washing reduced this leak to a value of 22% ± 12% lower than the pre-kallikrein control. These data suggest that (*i*) kallikrein degrades amiloride-sensitive Na^+ channels, (*ii*) it hydrolyzes "stable" leak channels into unstable channels, and (*iii*) amiloride-sensitive and stable leak channels (either or both) are converted into a leak conductance that can partition between the apical membrane and the mucosal solution. The decrease in amiloride-sensitive Na^+ transport is directly proportional to the initial value of Na^+ transport (i.e., a first-order reaction), where the rate constant of degradation (k) is described by the equation:

$$I_{amil}(t) = I_{amil}(o)\exp(-kt), \quad [1]$$

where $I_{amil}(o)$ is total amiloride-sensitive current before kallikrein addition and $I_{amil}(t)$ is the current remaining after "t" minutes of incubation in kallikrein and equals (7.3 ± 1.2) × 10^{-3} min^{-1} at a kallikrein activity of 0.07 EU/ml.

Addition of amiloride (10 μM) to the mucosal solution, before and during kallikrein treatment (0.07 EU/ml), inhibits degradation of the amiloride-sensitive channel (Table 1). However, it does not inhibit the degradation of the stable leak pathway into one that partitions between the mucosal solution and apical membrane. The rate of degradation is the same as for the amiloride-sensitive channel [(7.3 ± 1.2) × 10^{-3} min^{-1} at a kallikrein activity of 0.07 EU/ml].

Kinetics of Channel Hydrolysis. The rate of channel degradation was determined at two additional kallikrein activities—namely, 1.2 and 0.007 EU/ml. This latter activity of kallikrein is close to the average measured in urine from normal humans (6). After 2 hr of exposure to mucosal kallikrein (0.007 EU/ml), amiloride-sensitive current had decreased by 27% (k' = (2.98 ± 1.2) × 10^{-3} min^{-1}) and total leak current had increased by 35%. This increase in leak current was reduced to a value 7% lower than control after extensive washing of the mucosal solution. Thus, as with the higher dose, kallikrein (at physiological activities) reduces amiloride-sensitive current, producing a leak pathway, part of which is unstable in the apical membrane. At the higher activity (1.2 EU/ml) the rate constant was (9.3 ± 0.7) × 10^{-3} min^{-1}.

Michaelis–Menten kinetics adequately describes the activity dependence of the rate of degradation (Fig. 1). The maximum rate of degradation (at infinite enzyme activity) is 68%/2 hr (k_{max} = 9.5 × 10^{-3} min^{-1}) and the activity required for a half-maximal rate of degradation is 0.017 EU/ml (a kallikrein concentration of about 5 nM).

Properties of the Apical Leak Pathways. As determined by using the method of Lewis *et al.* (5), stable and unstable pathways (produced by the proteolytic effect of kallikrein) have the same selectivity for K^+ to Na^+ of 1.4 (i.e., near free solution mobilities). The gain in unstable leak current compared to the loss of stable leak current (in the presence of amiloride) indicates that the unstable leak pathway is 3.9 ± 1.2 times more conductive than the stable pathway. This increase in conductance is a minimal estimate since it is calculated under the assumptions that (*i*) all stable leak pathways are hydrolyzed into unstable pathways, (*ii*) unstable pathways are resistant to further proteolytic attack, and (*iii*) all unstable pathways are in the apical membrane.

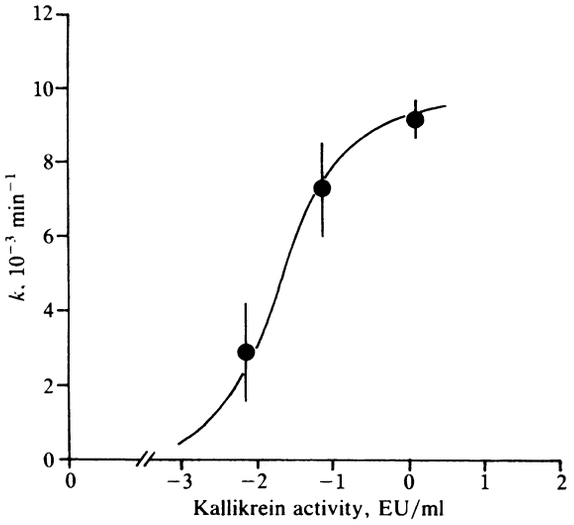

FIG. 1. The rate constant (k) for hydrolysis of amiloride-sensitive current (see Eq. 1) as a function of kallikrein activity (EU/ml). The best-fit k_{max} is 9.5 × 10^{-3} min^{-1} and K_m (activity for half-maximal rate of hydrolysis) is 0.017 EU/ml. Note this is a semilogarithmic plot.

To test the possibility that the unstable "channel" (or part of the channel—i.e., an activating fragment) partitions between the apical lipid bilayer and the mucosal solution, two experimental approaches were used. First, a rapid replacement of the mucosal bath with fresh Ringer's solution will initially deplete the mucosal bath of these pathways and then cause a time-dependent redistribution of these pathways between the apical membrane and the mucosal solution. Thus, the magnitude of the unstable leak current remaining after each solution change [$I_{usL}(n)$] compared to the total amount of unstable leak current [$I_{usL}(T)$] will be described by the equation:

$$I_{usL}(n) = I_{usL}(T)\cdot P^n, \quad [2]$$

where P is the ratio of unstable leak current after a wash to before a wash (the probability that the channel is in the apical membrane), and n is the wash number. Fig. 2 shows the effect of successive rapid washing of the mucosal chamber on the unstable leak current. The best fit value for P is 0.61. Since P is the ratio of the number of pathways in the apical

Table 1. Amiloride-sensitive Na^+ current and leak current are decreased by kallikrein

Condition	$I_{amil}(o)$, μA/μF	$I_{amil}(t)$, μA/μF	$I_{amil}(t)/I_{amil}(o)$	$I_L(o)$, μA/μF	$I_L(t)$, μA/μF	$I_L(t)/I_L(o)$	$I_L^{wash}(t)$, μA/μF	$I_L^{wash}(t)/I_L(o)$
Kallikrein	2.98 ± 0.6	1.4 ± 0.4	0.41 ± 0.07	2.7 ± 0.36	5.5 ± 0.6	2.3 ± 0.25	2.3 ± 0.2	0.78 ± 0.12
Kallikrein + amiloride	2.3 ± 0.7	2.2 ± 0.7	0.96 ± 0.1	2.7 ± 0.43	5.3 ± 0.8	2.0 ± 0.5	1.4 ± 0.3	0.45 ± 0.05

The effect of kallikrein on the amiloride-sensitive Na^+ current and leak current 2 hr after the addition of 0.07 EU of this enzyme per ml to the mucosal solution in the absence or presence of 10 μM mucosal amiloride. $I_{amil}(o)$ and $I_L(o)$ are the values of the amiloride-sensitive current and leak current before the addition of kallikrein, respectively, and $I_{amil}(t)$ and $I_L(t)$ are the current values after 2 hr of incubation in kallikrein. $I_L^{wash}(t)$ is the amount of stable leak current after an extensive washing of the mucosal chamber; the difference between $I_L(t)$ and $I_L^{wash}(t)$ is the amount of unstable leak current generated by kallikrein. Note that amiloride inhibited the degradation of the amiloride-sensitive Na^+ current but not the leak current.

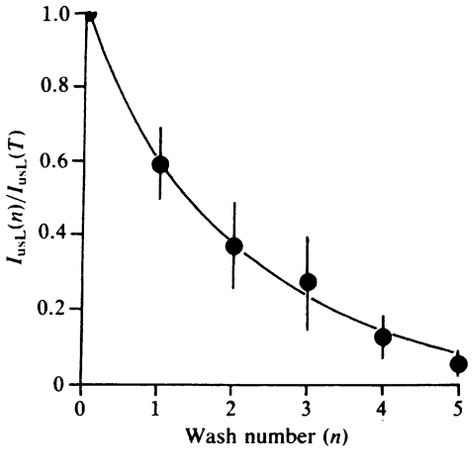

FIG. 2. The decrease in unstable leak current after rapidly (10–20 sec) replacing (isovolumetrically) the mucosal solution (15 ml) with fresh Ringer's solution (60 ml). Each isovolumetric wash replaces 97% of the prewash mucosal solution. Total unstable leak current [$I_{usL}(T)$] was the difference in transepithelial current before and after at least eight separate washes. $I_{usL}(n)$ is the unstable current remaining after each successive wash (n). Interwash time was 5–10 min (until the current had stabilized to a new and lower level). In each experiment $I_{usL}(n)$ was normalized to $I_{usL}(T)$. The smooth curve was fit by Eq. 2, where P is the probability that the channel is in the membrane; for six experiments, P had a mean value of 0.61.

membrane (N apical) to the sum of the number of pathways in the apical membrane and mucosal solution (N bath), we calculated a partition coefficient (N apical/N bath) of 1.6:1. Although this seems like an extremely low value considering that the pathway is hydrophobic, one must take into account the relative volume of the mucosal solution (15 ml) to the apical membrane volume ($2\ cm^2 \times 4 \times 10^{-7}$ cm). This increases the value of the partition coefficient to $\approx 30 \times 10^6$:1, supporting the notion of the hydrophobic nature of this pathway.

From the time course of the change (decrease) in leak current after a single mucosal wash, one can calculate the sum of the rate constants of channel association (K_2) and dissociation (K_1) at the apical membrane, using the equation

$$\Delta I_{usL}(t) = \Delta I_{usL} \exp[-(K_1 + K_2)t], \quad [3]$$

where ΔI_{usL} is the total leak current lost with a single wash and $\Delta I_{usL}(t)$ is the amount of ΔI_{usL} remaining as a function of time (t) after the wash. The value of the sum of rate constants ($K_1 + K_2$) is $(17.13 \pm 1.6) \times 10^{-3}\ sec^{-1}$ ($n = 6$). This sum of rate constants can be separated into the individual values by using the relationship

$$P = \frac{K_2}{K_1 + K_2},$$

where P is the probability that the channel is in the apical membrane (see Eq. 2). The rate of association (rate at which the channel enters the apical membrane) K_2 is $9.4 \pm 1.1 \times 10^{-3}\ sec^{-1}$ ($n = 6$) and the rate of dissociation (i.e., the rate at which the channel exits from the apical membrane) K_1 is $7.75 \pm 0.7 \times 10^{-3}\ sec^{-1}$.

Given that this pathway partitions between a hydrophobic and a hydrophilic phase, then an increase in the available lipid (by the addition of lipid vesicles to the mucosal solution) should cause a time-dependent redistribution of this pathway. The density of these channels is expected to decrease in the apical membrane and mucosal solution and increase in the added vesicles. Such a decrease in density will be reflected by a decrease in amiloride-insensitive current. Thirty minutes after the addition of 1 mg of asolectin (soybean phospholipid) vesicles to the mucosal solution, the unstable leak pathway had decreased to 63% ± 12% ($n = 3$) of the control value, whereas the amiloride-sensitive current was unaffected. Thus, the mucosal addition of lipid vesicles to the mucosal solution causes a redistribution of channels such that the probability that the channel is in the apical membrane is 0.37 ± 0.08 ($n = 3$), in the mucosal solution is 0.25, and in the added vesicles is 0.38. These values were calculated by assuming that channels from the vesicles had to first enter the mucosal solution before gaining access to the apical membrane, and vice versa.

DISCUSSION

There are four important aspects to this communication. First, the degradation of the Na^+ channel by kallikrein offers an explanation for why the mammalian urinary 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. Na^+ channel degradation is a first-order process, where only a given fraction of channels is degraded per unit time; thus, Na^+ channel density decreases as a single inverse exponential. The rate of decrease of Na^+ transport is dependent on the concentration of kallikrein. At physiological levels, kallikrein can reduce urinary bladder transport by 74% in an 8-hr period. The alteration of the stable leak pathway is complex and its time course is a function of the ratio of initial Na^+ channel density to leak pathway density. Hydrolysis of the two types of channels occurs at similar rates, suggesting a progressive, serial degradation of "healthy" channels into nonselective "sick" channels to unstable channels to "lost" channels.

Second, it is likely that Na^+ channels in the apical membrane of distal renal tubules such as the cortical collecting duct are also hydrolyzed (but at a slower rate). Two lines of evidence suggest that this is the case. First, the apical membrane of the cortical collecting duct (CCD) (of rabbit) possesses, in addition to amiloride-sensitive Na^+ channels and a Ba^{2+}-blockable K^+ channel, a leak conductance whose magnitude is almost equal to that of the Na^+ conductance (7). Five to 7 days after adrenalectomy, Na^+ and K^+ conductances are reduced by half, whereas the leak conductance is very low and within the measuring error of the experimental equipment.† Since kallikrein activity is reduced [by at least 50% (8)] after adrenalectomy, a possible explanation for this decrease in leak conductance (following adrenalectomy) is a reduced rate of hydrolysis of Na^+ channels into a leak conductance. The second line of evidence is that the time response and magnitude of the apical membrane Na^+ and leak conductance following DOCA (deoxycorticosterone acetate) administration to control or adrenalectomized rabbits differ. Short-term DOCA administration (18 hr) to adrenalectomized rabbits resulted in larger final apical conductances to Na^+ and K^+ when compared to DOCA-treated, nonadrenalectomized rabbits (ref. 7; †). Although DOCA administration increases the apical leak conductance in CCD from nonadrenalectomized rabbits, there is no evidence for this leak conductance in the apical membrane of CCD from DOCA-challenged adrenalectomized rabbits. Again, the lack of a leak current in the CCD apical membrane of adrenalectomized rabbits following DOCA administration as well as the larger Na^+ conductance (compared to DOCA-treated control rabbits) suggest that kallikrein might be hydrolyzing not only active but also quiescent Na^+ channels in the apical

†Sansom, S. C., Muto, S. & Giebisch, G., 18th Meeting, American Society of Nephrology, Dec. 15–18, 1985, New Orleans, abstr. 257.

membrane and that aldosterone (or DOCA) activates these channels. Evidence for this latter mechanism has been reported for the toad urinary bladder (9), in which trypsin (also a serine protease) hydrolyzed active and quiescent Na^+ channels. It is interesting to speculate that cytoplasmic vesicles containing active and quiescent channels are inserted into the apical membrane at a constant rate and serve a finite residency time in this membrane before they are endocytosed (at a rate equal to that of insertion) and degraded in lysosomes. Such a futile cycle offers a number of advantages. First, aldosterone stimulation of transport is greatly simplified: the target cell does not have to increase channel synthesis, insertion, and activation; it only has to synthesize an activating agent [perhaps a methylating agent as suggested by Sariban-Sohraby *et al.* (10)]. Second, after plasma aldosterone levels return to control, the target cell does not have to decrease synthesis, insertion, and activation of channels while increasing the rate of channel removal; the cell only has to decrease the rate of synthesis of activating agent. Thus, down-regulation is greatly facilitated by increased channel degradation, insertion of vesicles containing a higher density of quiescent channels, and withdrawal of vesicles containing a higher density of active channels. Although data from the CCD (ref. 7; †), toad urinary bladder (9), and A6 cells (10) support such a scheme, one cannot rule out the possibility that altered rates of channel synthesis, insertion, and withdrawal are also occurring.

Third, since aldosterone stimulates and amiloride (11) or adrenalectomy (12) inhibits Na^+ reabsorption and kallikrein release, the regulation of urine kallikrein activity might involve not only aldosterone but also cell Na^+ content. In this manner, one role of kallikrein might be to balance Na^+ entry rate against basolateral Na^+ exit.

Last, we suggest that a physiological role of renal kallikrein is to regulate the reabsorptive capacity of distal nephron tubules. Alterations in the stoichiometry between plasma aldosterone and kallikrein secretion will result in either salt and water retention or loss. In this regard, it is interesting to note that the rate of kallikrein secretion in people with essential (primary) hypertension is lower than that of normotensive people (6). This increased reabsorptive capacity will result in salt and water retention, increased blood volume, and, ultimately, increased blood pressure.

We thank Dr. N. K. Wills for critical review of this manuscript. This work was supported by National Institutes of Health Grant AM33243.

1. Margolius, H. S. (1984) *Annu. Rev. Physiol.* **46**, 309–326.
2. Lewis, S. A. & Wills, N. K. (1983) *J. Physiol. (London)* **341**, 169–184.
3. Lewis, S. A. (1977) *Am. J. Physiol.* **232**, F187–F195.
4. Lewis, S. A. & deMoura, J. L. C. (1982) *Nature (London)* **297**, 685–688.
5. Lewis, S. A., Ifshin, M. S., Loo, D. D. F. & Diamond, J. M. (1984) *J. Membr. Biol.* **80**, 135–151.
6. Margolius, H. S., Howitz, D., Pisano, J. J. & Keiser, H. R. (1974) *Circ. Res.* **35**, 820–825.
7. Sansom, S. C. & O'Neil, R. G. (1981) *Am. J. Physiol.* **248**, F858–F868.
8. Marchetti, J., Imbert-Teboul, M., Ilhenc-Gelas, F., Allegrini, J., Monard, J. & Morel, F. (1984) *Pflügers Arch.* **401**, 27–33.
9. Garty, H. & Edelman, I. S. (1983) *J. Gen. Physiol.* **81**, 785–803.
10. Sariban-Sohraby, S., Brug, M., Wiesmann, W. G., Chiang, P. K. & Johnson, J. P. (1984) *Science* **225**, 745–746.
11. Scicli, A. G., Diaz, M. A. & Carretero, O. A. (1983) *Am. J. Physiol.* **245**, F198–F203.
12. Noda, Y., Yamada, K., Igic, R. & Erdos, E. G. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 3059–3063.