Activation of Epithelial Sodium Channels by Prostasin in *Xenopus* Oocytes

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**Abstract.** Prostasin, a novel serine protease, was purified from seminal fluid, and its cDNA sequence was determined. Expression of prostasin was detected in human tissues, including prostate, kidney, and lung, as well as bodily fluids, including seminal fluid and urine. However, its physiologic role in the human body is not known. Recently, a novel regulatory mechanism by which serine proteases activate epithelial sodium channel in the *Xenopus* oocyte was identified. Therefore, it was hypothesized that prostasin could activate sodium currents, and a rat prostasin cDNA clone was isolated to investigate its physiologic function. Rat prostasin mRNA is expressed predominantly in kidney, and lower levels of expression were detected in prostate, lung, colon, stomach, and skin. These all are epithelial tissues in which the epithelial sodium channel (ENaC) is expressed. Coexpression of rat prostasin and rat ENaC in *Xenopus* oocytes increased the amiloride-sensitive sodium current by twofold. Preincubation of oocytes that expressed prostasin with aprotinin did not result in an increase in sodium current, compared with the control. The removal of aprotinin from the bath solution resulted in a twofold increase of the current only in oocytes that expressed prostasin, which indicates that protease activity of prostasin is required for the ENaC activation. Expression of rat prostasin had no effect on the potassium current when expressed with rat renal outer medulla K channel, which shows specificity of prostasin action for ENaC. These results indicate that prostasin acts as an extracellular regulator of ENaC.

In many salt-reabsorbing epithelia, such as distal segments of the kidney tubule, distal colon, urinary bladder, skin, and airways, passage of sodium across the apical or outward-facing membranes through the epithelial sodium channel (ENaC) is the first and rate-limiting step in transepithelial sodium transport (1–3). This step therefore plays an important role in the regulation of sodium balance, extracellular fluid volume, and BP by the kidney and in controlled fluid reabsorption in the airways. ENaC activity must be regulated tightly to maintain homeostasis of the intracellular milieu by multiple mechanisms such as hormones, intracellular factors, and other regulatory factors that are not yet completely understood.

Recently, a novel mechanism by which serine proteases regulate ENaC activity in *Xenopus* oocyte was identified. Vallet *et al.* (4,5) cloned a new serine protease, channel-activating protease-1 (CAP-1), from the *Xenopus* kidney A6 cell line. CAP-1 is a secreted and/or glycosylphosphatidylinositol (GPI)-anchored protein and is expressed in kidney, gut, lung, skin, and ovary. They showed that coexpression of this protease and ENaC in *Xenopus* oocytes increased the activity of sodium channels by two- to threefold. Chraı́bi *et al.* (6) also reported that low concentrations of trypsin (2 μg/ml) increased the amiloride-sensitive sodium current by two- to fivefold in *Xenopus* oocytes that expressed α, β, and γ ENaC. They concluded that extracellular proteases are able to induce a large activation of ENaC through proteolysis of a protein that is either a subunit of the channel or closely associated with it.

Prostasin, a serine protease purified from human seminal fluid, has 53% homology with *Xenopus* CAP-1 and is expressed in human prostate gland, kidney, lung, colon, and liver (7,8). However, its biologic function in the human body is not known. This protease is a type Ia membrane protein (8), and its active form is secreted into seminal fluid and urine. Catalytically active prostasin was detected in urine from both men and women. Although the concentration of prostasin in urine is lower than that in seminal fluid, the production of prostasin has to be very active in the kidney, considering the relatively large amount of the urine excreted per day (7). These facts suggest the possibility that prostasin may play a key role in the regulation of renal tubular sodium transport by an autocrine/paracrine mechanism. Vuagniaux *et al.* (9) isolated a cDNA clone of mouse CAP-1 from the mouse cortical collecting duct cell line and suggested that CAP-1 is an orthologous gene for prostasin. They also demonstrated that mouse CAP-1/prostasin activates the sodium current through ENaC when it is coexpressed in *Xenopus* oocytes.

In the present study, we cloned rat prostasin cDNA and investigated its effect on rat ENaC activity when both are expressed in *Xenopus* oocytes. Isolation of the rat prostasin
cDNA revealed that it encodes a protein of 342 amino acids and that its mRNA is expressed in the kidney, lung, colon, and skin, where ENaC is known to be expressed. We also demonstrated that coexpression of rat prostasin cRNA, together with rat α, β, and γ ENaC cRNA in Xenopus oocytes, increased the ENaC activity by twofold. These results strongly support the hypothesis proposed by Vuagniaux et al. (9) that prostasin functions as an extracellular regulator of the ENaC activity in renal tubules in an autocrine/paracrine manner. This study suggests the importance of this serine protease in the control of sodium balance and BP.

Materials and Methods

**cDNA Cloning of Rat Prostasin**

A partial cDNA fragment of rat prostasin was obtained by PCR with the use of degenerate oligonucleotide primers. Two degenerate primers were designed on the basis of the conserved amino acid sequence of human prostasin (8). The sense primer was 5'-CTI-ACIGCICICTGTYTTCGACAATG-3', which corresponds to amino acid sequence LSAAHCPFS of human prostasin, and the antisense primer was 5'-GGIGCICCRCAYCTCRTCTCCCAATAG-3', which corresponds to WGDACGAR (I: Inosine; Y: C or T; R: A or G). Polya (A)^+ rat kidney RNA was reverse-transcribed and amplified with these primers by the use of the following PCR protocol: 94°C for 3 min; 27 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 45 s; and 72°C for 10 min. PCR products were subcloned into pBluescript vector (Stratagene, La Jolla, CA) and sequenced by the dideoxy chain termination method with the use of fluorescent dye–labeled terminators on an automated sequencer. The sequence was determined at the Genomics and Proteomics Core Facility of the National Institute of Diabetes and Digestive and Kidney Diseases. Rat prostasin cDNA fragments were amplified with specific oligonucleotide primers (sense 5'-CACAGCAAGGAAGTATG-1-3') and antisense 5'-TGTGGGGTTCTTCAGGCAC-3') by use of the following PCR protocol: 94°C for 3 min; 27 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min; and 72°C for 7 min. The predicted size of the cDNA amplification product was 376 bp. Specificity of the amplified products was confirmed by direct sequencing, and the sample without RT was used as a negative control.

**Electrophysiologic Studies in Xenopus Oocytes that Express ENaC and Prostasin**

In vitro–transcribed cRNA for the α, β, and γ subunits of rat ENaC (generously provided by Dr. B. C. Rossier, University of Lausanne, Lausanne, Switzerland) with or without rat prostasin cRNA were injected into stage V–VI Xenopus oocytes (0.5 ng of cRNA for each subunit of ENaC and 1 ng of cRNA of prostasin in a total volume of 50 nl) (10, 13). Electrophysiologic studies were performed 16 to 24 h after cRNA injection. The amiloride-sensitive sodium current was measured by use of the two-electrode voltage clamp technique. The current was recorded with CEZ-1250 voltage clamp apparatus (Nihon Kohden, Tokyo, Japan) at room temperature and at a holding potential of −100 mV in a solution containing 96 mM sodium gluconate, 2 mM potassium gluconate, 1.8 mM CaCl_2, and 10 mM HEPES (pH 7.2), 5 mM BaCl_2, and 10 mM tetraethylammonium chloride. Amiloride-sensitive sodium currents were determined by the difference in current before and after the addition of 5 μM amiloride to the bath solution. The current signal was filtered at 1 kHz by use of the internal filter of the CEZ-1250. Current-voltage (I-V) curves were determined by setting the command potential for 500 ms (at 2-s intervals) from a −100 mV holding potential to values ranging from −100 to +40 mV in increments of 20 mV. Steady-state currents were measured 1 s after the voltage change and continuously recorded on a paper chart. For single-channel current measurements, Xenopus oocytes that expressed ENaC α, β, and γ subunits with or without rat prostasin were prepared for patch-clamp experiments by manual removal of the vitelline membrane after exposure to a hypertonic solution (6). Single-channel recordings were made in the cell-attached configuration at room temperature. Currents were recorded with EPC-8 amplifiers (HEKA, Lambrecht, Germany) and stored on a VR-10B digital tape recorder (Instrutech, Great Neck, NY). Cell-attached patches were obtained with pipettes (resistance of 10 to 20 MΩ) filled with a solution containing 110 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl_2, and 10 mM HEPES (pH 7.4). The bath solution was identical to the pipette solution, except that KCl replaced NaCl. Data were filtered at 100 Hz and sampled at 2 kHz by use of an ITC-16 analog-digital interface (Instrutech). Current records were analyzed with Patch Analyst Pro software (MT Corp., Nishinomiya, Japan). Channel open probability and once with 0.1× standard saline citrate, 0.5% sodium dodecyl sulfate at 65°C for 20 min (10).

**Microdissection of Collecting Tubules and Reverse Transcription-PCR**

The microlocalization of rat prostasin mRNA expression in the nephron segments was determined by reverse transcription-PCR (RT-PCR) combined with the microdissection techniques, as described elsewhere (11). Cortical collecting ducts, outer medullary collecting ducts, and inner medullary collecting ducts were microdissected and dissolved in 3.5 μl of 2% Triton X-100 in the presence of 1 U/μl RNase inhibitor and 5 mM/L dithiothreitol. Each sample was reverse-transcribed by use of a cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany), according to the manufacturer’s protocol. Rat prostasin cDNA fragments were amplified with specific oligonucleotide primers (sense 5'-CACAGCAAGGAAGTATG-1-3') and antisense 5'-TGTGGGGTTCTTCAGGCAC-3') by use of the following PCR protocol: 94°C for 3 min; 27 cycles of 94°C for 1 min, 59°C for 1 min, and 72°C for 1.5 min; and 72°C for 7 min. The predicted size of the cDNA amplification product was 376 bp. Specificity of the amplified products was confirmed by direct sequencing, and the sample without RT was used as a negative control.
was determined by use of 50% threshold analysis. All results are the mean of at least three separate experiments, in each of which the amiloride-sensitive sodium currents were measured in 10 to 20 individual oocytes.

**Electrophysiologic Studies in Xenopus Oocytes that Express ROMK2 and Prostasin**

Oocytes received an injection of 2 ng of renal outer medulla K channel 2 (ROMK2) cRNA in the presence or absence of 1 ng of prostasin cRNA. The ROMK2-induced potassium currents were measured as the inward current in a solution containing 98 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES (pH 7.6) at a holding potential of −100 mV at room temperature (14). I-V curves were determined by setting the command potential for 500 ms (at 2-s intervals) from a −100 mV holding potential to values ranging from −100 to +40 mV in increments of 20 mV. All results are the mean of at least three separate experiments, in each of which the potassium currents were measured in 10 to 20 individual oocytes.

**Results**

**Cloning of the Rat Prostasin cDNA**

We isolated a cDNA clone of rat prostasin from rat kidney to study its physiologic function. RT-PCR, 3′-RACE, and 5′-RACE methods were used to obtain a full-length cDNA. The sequence contains a 1029-bp coding region corresponding to a 342-amino acid polypeptide, a 223-bp 5′ noncoding region, and a 956-bp 3′ noncoding region. The coding region starts with an ATG codon, which is present in a sequence of GTC-CGGGGCCATGG, similar to the consensus sequence for the eukaryotic translation initiation site. Protein secondary structure analysis revealed that rat prostasin is presumed to be GPI-anchored type Ia membrane protein. A potential N-glycosylation site is present at Asn159 in a sequence of Asn-Ala-Ser. A common polyadenylation signal, AATAAA, and variant polyadenylation signal, AACAAA, were found in the 3′ noncoding region, suggesting that alternate splice variants may exist. The catalytic triad that is highly conserved among the serine proteases is also found in rat prostasin (His⁸⁵, Asp¹³⁴, and Ser²³⁸). The deduced amino acid sequence of rat prostasin and sequence comparison with several members of the serine proteases is shown in Figure 1. Rat prostasin shares 77 and 91% identity with human and mouse CAP-1/prostasin, respectively. Vuagniaux et al. (9) proposed that mCAP-1, xCAP-1, and human prostasin are orthologous genes, and our data also support their initial observation.

**Tissue Distribution of Rat Prostasin mRNA**

Distribution of rat prostasin mRNA expression was determined by Northern blot analysis of total RNA from various rat tissues. As shown in Figure 2A, rat prostasin transcripts were expressed predominantly in kidney cortex and medulla and at lower levels in prostate, lung, colon, stomach, and skin. No expression was observed in heart, liver, testis, brain, or pancreas. Other transcripts with a lower molecular mass were detected in the same tissues. This might be an alternate splice variant, as is suggested by the dual polyadenylation signal in the 3′ noncoding region. RT-PCR also was performed, to confirm the Northern blot analysis data (Figure 2B). Data obtained by RT-PCR were similar to the Northern blot analysis. A smaller transcript in testis was detected by Northern blot but not by RT-PCR. This band might be another kind of serine protease closely related to prostasin.

Because prostasin mRNA is expressed predominantly in the rat kidney and mouse CAP-1/prostasin has been shown to activate ENaC in *Xenopus* oocytes, we next studied the expression of rat prostasin in the collecting tubules of the rat kidney, where ENaC is known to be expressed (15,16).
Collecting Tubules

Expression of Rat Prostasin mRNA in Rat Kidney Collecting Tubules

Expression of rat prostasin mRNA in the rat collecting tubules was studied by RT-PCR with the use of microdissected collecting tubule segments. The amplified products of rat prostasin mRNA were detected as a single band of 376 bp. As shown in Figure 3, prostasin is expressed in cortical collecting ducts, outer medullary collecting ducts, and inner medullary collecting ducts. Because rat prostasin is presumed to be a GPI-anchored protein, theoretically it should localize to the apical membrane of the collecting tubules (17). ENaC is also expressed on the apical membrane (15,16). These findings strongly suggest the possibility that ENaC and prostasin may co-localize on the same membrane and that prostasin may have an effect on the ENaC activity in an autocrine/paracrine manner. Therefore, we used the Xenopus oocyte expression system to coexpress these two proteins on the same membrane and studied the effect of prostasin on the ENaC activity.

Activation of ENaC by Prostasin

To determine the functional properties of the prostasin, we used the two-electrode voltage-clamp method on Xenopus oocytes. As shown in Figure 4A, expression of ENaC α, β, and γ subunits in Xenopus oocytes produced a large inward current (2.0 ± 0.5 μA at -100 mV [n = 12]) that was blocked by 5 μM amiloride. Current produced by oocytes that expressed rat ENaC showed no time-dependent voltage effects and had a linear I-V relationship, as was reported elsewhere by Canessa et al. (12,13) (Figure 4, A and C). Coexpression of prostasin together with rat ENaC led to an approximately twofold increase in the amiloride-sensitive sodium current (control, 2.0 ± 0.5 μA at -100 mV [n = 12]; prostasin, 3.9 ± 0.7 μA at -100 mV [n = 14]; P < 0.05; Figure 4, B and C). Coexpression of prostasin and ENaC could increase the amiloride-sensitive sodium current by increasing the conductance of sodium current through each channel, by increasing the open probability (Po) of each channel, or by increasing the number of active channels in the membrane. To investigate these possibilities, we recorded single-channel current in cell-attached patches of oocyte that expressed ENaC in the presence or absence of prostasin. Representative tracings are shown in Figure 5A. Oocytes that expressed both ENaC and ENaC with prostasin displayed the characteristic slow kinetics, as reported by Canessa et al. (12,13). Single-channel conductance of ENaC was not changed by the expression of prostasin (control, 4.60 ± 0.07 pS [n = 12]; prostasin, 4.57 ± 0.08 pS [n = 14]; NS). The Po of rat ENaC was variable, as is shown in Figure 5B, and this is consistent with the findings by Canessa et al. (12,13). Coexpression of prostasin had no effect on the Po and the single-channel conductance. These results indicate that an increase in whole-cell sodium current caused by prostasin cannot be attributed to an increase in single-channel conductance or an increase in Po. Thus, by exclusion, we speculate that prostasin increases the number of functional channels in the plasma membrane.

To determine whether the prostasin-induced increase in the sodium current was caused by the protease activity of prostasin, we incubated oocytes with 50 μg/ml aprotinin, a serine protease inhibitor, after injection of cRNA until current measurement. Both control and oocytes that received an injection of prostasin exhibited similar sodium currents in the presence of aprotinin. In oocytes that expressed prostasin, a significant increase in the sodium current was observed after the bath solution was washed out for 30 min (control, 1.1 ± 0.08-fold [n = 10]; prostasin, 1.9 ± 0.2-fold [n = 12]; P < 0.05), as shown in Figure 6. These results indicate that the proteolytic activity of prostasin is involved in the activation of ENaC.
Effect of Prostasin on ROMK2 Activity

To evaluate the specificity of the action of prostasin on the ENaC, we also studied the effect of prostasin on ROMK2 activity. ROMK2 was chosen because it is probably expressed in the apical membrane of the collecting ducts, the same membrane as ENaC (14). The inward potassium current was measured in oocytes that expressed ROMK2 in the presence or absence of prostasin. As shown in Figure 7, A and C, expression of ROMK2 produced a large inward current (3.9 ± 0.3 μA at −100 mV [n = 10]), and the I-V relationship of the current was linear. These findings are consistent with the findings reported by Zhou et al. (14). Coexpression of prostasin had no effect on the whole-cell current of ROMK2 (Figure 7, B and C). These data demonstrate the selectivity of the action of prostasin on ENaC.

Discussion

In the present study, we isolated rat prostasin cDNA and demonstrated that expression of prostasin increases the amiloride-sensitive sodium current through the activation of ENaC in Xenopus oocytes. Our studies confirm the initial observation by Vuagniaux et al. (9) that prostasin is an extracellular regulator of ENaC.

Human prostasin, a novel serine protease, first was purified from seminal fluid, and its cDNA was cloned from a human prostate cDNA library (7,8). Human prostasin has a 32–amino acid signal sequence at the amino terminal and is presumed to be a GPI-anchored type Ia membrane protein. It is cleaved by some mechanism and secreted into seminal fluid and urine (7). Although human prostasin has been shown to have trypsin-like serine protease activity in vitro, its physiologic function in the human body has not been elucidated. Existence of prostasin in urine from both men and women strongly suggests the possibility that prostasin plays an important role in the kidney, rather than only in the prostate gland (7). Therefore, we cloned rat prostasin cDNA from the kidney to study its function in renal tubules. Rat prostasin encodes a 342–amino acid polypeptide that is 77 and 91% identical to human prostasin and mouse CAP-1/prostasin at the amino acid level, respectively. The mRNA distribution pattern of rat prostasin was similar to mouse CAP-1/prostasin but slightly different from human prostasin (8,9). The difference in the species or in the body fluid status used for the experiments might be the reason for this discrepancy.

In contrast to several earlier reports that indicated an inactivating effect of proteases on the epithelial sodium channel (18–20), Vallet et al. (4) and Chraı ¨bi et al. (6) reported that serine proteases such as trypsin, chymotrypsin, and CAP-1 induce significant activation of ENaC in Xenopus oocytes. In addition, Vuagniaux et al. (9) demonstrated that mouse CAP-1/prostasin could increase the amiloride-sensitive sodium current through activation of ENaC. In the present study, we demonstrated that coexpression of ENaC and rat prostasin in Xenopus oocytes resulted in a twofold increase in the amiloride-sensitive sodium current and that the activation of the channel activity is selective for ENaC, because prostasin had no effect on ROMK2 activity. Single-channel conductance and single-channel Po of ENaC expressed in oocytes were not modified significantly by the expression of prostasin. This stimulatory effect of prostasin on ENaC is blocked completely by preincubation of oocytes with aprotinin, a serine protease inhibitor, which indicates that the proteolytic activity of prostasin is indeed involved in the activation of ENaC. How does prostasin activate ENaC? One reasonable explanation is that the active channel number of ENaC at the cell surface is increased by prostasin, because both single-channel conduc-
Distance and single-channel $P_o$ of ENaC are not significantly changed by prostasin. Because we are unable to measure the expression level of ENaC at the cell surface of oocytes, we cannot assess this possibility at this point. A quantitative approach to study the expression of ENaC at the cell surface by use of a FLAG insertion mutant revealed that either trypsin or CAP-1 does not increase the channel density of ENaC at the cell surface (6,7,9). If prostasin works in the same way as trypsin or CAP-1, then these findings do not support our hypothesis that prostasin increases the number of channels at the membrane. However, Firsov et al. (21) suggested that the majority of the channels expressed at the plasma membrane of oocytes are either nonconducting channels or channels with an extremely low $P_o$ because the number of channels estimated by the FLAG mutant is at least 10 times more than that calculated by the electrophysiologic studies. Therefore, we speculated that these channels might be activated by the protease activity of prostasin. A similar conclusion was reached by Chraibi et al. (6). They suggested that there might be a significant fraction of “silent channels” that are not seen in patch-clamp experiments but are present at the oocyte surface and can be activated by trypsin treatment. We think that this silent channel hypothesis proposed by Chraibi et al. is the most favorable mechanism to explain the effect of prostasin on ENaC at this point. Although additional studies are needed to elucidate that this kind of silent channels does exist, this hypothesis reasonably explains all of our findings on the activation of ENaC by prostasin.

The distribution of rat prostasin mRNA expression supports the hypothesis that prostasin affects the ENaC activity. cRNA of rat ENaC $\alpha$, $\beta$, and $\gamma$ subunits were injected into oocytes in the presence or absence of rat prostasin cRNA. Oocytes that received an injection were incubated for 16 h in MBS solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 15 mM HEPES [pH 7.6], 0.3 mM Ca(NO)$_3$, 0.41 mM CaCl$_2$, and 0.82 mM MgSO$_4$) in the presence of aprotinin (50 $\mu$g/ml). Amiloride-sensitive sodium currents were recorded in the presence of aprotinin, then oocytes were washed with MBS solution to remove the aprotinin. Thirty min later, sodium currents were remeasured in the same oocytes. Error bars represent the SEM.

**Figure 5.** Single-channel patch-clamp analysis of oocytes that expressed rat ENaC with and without rat prostasin. (A) Representative traces of oocyte that expressed rat ENaC (upper panel) and rat ENaC with prostasin (lower panel). These patches of membrane contained at least one active channel. The solid bar on the left indicates the closed state, and downward deflections represent openings. The holding potential was $-100$ mV. (B) Open probability ($P_o$): circles (○, ●) show values from individual experiments, and squares (□, ■) show mean ± SEM. $P_o$ was measured at a holding potential of $-100$ mV. $P_o$ for rat ENaC and rat ENaC with prostasin were not significantly different ($P = 0.25$).

**Figure 6.** Effect of aprotinin on prostasin-induced ENaC activity. cRNA of rat ENaC $\alpha$, $\beta$, and $\gamma$ subunits were injected into oocytes in the presence or absence of rat prostasin cRNA. Oocytes that received an injection were incubated for 16 h in MBS solution (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO$_3$, 15 mM HEPES [pH 7.6], 0.3 mM Ca(NO)$_3$, 0.41 mM CaCl$_2$, and 0.82 mM MgSO$_4$) in the presence of aprotinin (50 $\mu$g/ml). Amiloride-sensitive sodium currents were recorded in the presence of aprotinin, then oocytes were washed with MBS solution to remove the aprotinin. Thirty min later, sodium currents were remeasured in the same oocytes. Error bars represent the SEM.
of mRNA expression revealed that it is expressed predominantly in rat kidney and, to some extent, in other epithelial tissues, most of which express ENaC. In addition, we demonstrated that rat prostasin increases the sodium current in Xenopus oocytes that express ENaC and that it functions as an extracellular regulator of ENaC activity in an autocrine/paracrine manner. However, the precise mechanisms by which prostasin increases ENaC activity still need to be elucidated in future studies. Our findings presented in this article suggest that prostasin may be involved in the regulation of BP and sodium reabsorption in the kidney. An understanding of the regulation of prostasin expression and the mechanism of its action on ENaC would provide a new insight into the treatment of hypertension.

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References