Amiloride-sensitive sodium absorption is different in vertebrates and invertebrates

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Amiloride-sensitive sodium absorption is different in vertebrates and invertebrates. Am J Physiol Regul Integr Comp Physiol 292: R2318–R2327, 2007. First published March 1, 2007; doi:10.1152/ajpregu.00549.2006.—Amiloride-sensitive Na+ absorption is a well-described feature of numerous transporting epithelia in vertebrates. Yet, very little is known about this important physiological process regarding invertebrates. In the present paper, we compare vertebrate Na+ absorption mediated by the amiloride-sensitive epithelial Na+ channel (ENaC) and its invertebrate counterpart. We used the dorsal skin of the annelid Hirudo medicinalis as a model for the Na+ absorption of invertebrate epithelia. In applying electrophysiological, molecular, and biochemical techniques we found striking functional and structural differences between vertebrate and invertebrate amiloride-sensitive Na+ absorption. Using modified Ussing chambers, we analyzed the influence of different known blockers and effectors of vertebrate ENaC on leech epithelial Na+ absorption. We demonstrate that the serine protease trypsin had no effect on the Na+ transport across leech integument, while it strongly activates vertebrate ENaC. While protons, and the divalent cations Ni2+ and Zn2+ stimulate vertebrate ENaC, amiloride-sensitive Na+ currents in leech integument were substantially reduced. For molecular studies, we constructed a cDNA library of Hirudo medicinalis and screened it with specific ENaC antibodies. We performed numerous PCR approaches using a vast number of different degenerated and specific ENaC primers to identify ENaC-like structures. Yet, both strategies did not reveal any ENaC-like sequence in leech integument. From these data we conclude that amiloride-sensitive Na+ absorption in leech skin is not mediated by an ENaC-like channel but by a still unknown invertebrate member of the ENaC/DEG family that we termed INaTP (leech epithelial Na+ transporting protein).

epithelial Na+ channel/degenerin superfamily; invertebrate epithelial transport

REABSORPTION OF SODIUM (Na+) in vertebrates is essential for maintaining the volume of the extracellular compartments and is mediated by many different Na+ transport proteins. One of these is a Na+-selective channel, the amiloride-sensitive epithelial sodium channel (ENaC). Na+ enters epithelial cells through ENaC located in the apical membrane and leaves them across the basolateral membrane driven by the Na+-K+-ATPase. Those proteins enable Na+ absorption against large concentration gradients and reduce luminal Na+ to a few millimoles. This asymmetrical arrangement of transport systems allows Na+ to be actively transported through the epithelium and thus generate transepithelial potential (Vt).

The native ENaC has been shown to be a multimeric protein made up of at least three homologues subunits (α, β, γ) that together built the functional channel (11). A precise stoichiometry for the channel has not yet been determined, but a tetrameric structure of two α-, one β-, and one γ-subunit is proposed (16). Others hypothesize that ENaC is formed by nine subunits, with a stoichiometry of α3β3γ3 (47). While the α-subunit alone is able to form a functional pore, the other two subunits are necessary for proper channel gating and regulation (11). ENaC is characterized by high cation selectivity, low transepithelial conductance (Gt), and high sensitivity to the diuretic blocker amiloride and its analogs, e.g., phenamil and benzamil. The channel is regulated by a wide variety of hormones, such as aldosterone and vasopressin, by phosphorylation and proteases (2). Recently, it has been shown that serine proteases like extracellular trypsin (9) and the membrane-anchored channel-activating protease (CAP1) activate ENaC (49).

Since the first cloning of the vertebrate ENaC (10), further members of this channel family were detected in different other vertebrate epithelia like kidney, bladder, colon, and lung from amphibians and mammals. Furthermore, proteins that showed ENaC sequence homologies could be identified in several tissues from divergent species and were summarized to the ENaC/degenerin (DEG) superfamily. This group of conserved proteins encode ion channels involved in diverse cellular processes, such as Na+ reabsorption, cell homeostasis, regulatory control of blood pressure, or mechanotransduction in the perception of taste, smell, temperature, and pain (5). Known members of the ENaC/DEG family are e.g., the acid-sensing ion channels (ASICs) (51), the brain-liver-intestine ion channels (40) in vertebrates, the Phe-Met-Arg-Phe (FMRF)-amide-gated sodium channel (FaNaC) found in mollusks (32), and the DEGs of the nematode Caenorhabditis elegans (1). Yet, the number of ion channels of this superfamily is still increasing.

Up to the present time, little is known about the amiloride-sensitive Na+ absorption in invertebrates. Only some reports showed amiloride-sensitive Na+ absorption, which at the first sight, resembled the “classic” mechanisms of Na+ transport through tight epithelia in vertebrates via ENaC. An amiloride-sensitive apical Na+ transport was detected in the gill epithelium of the Chinese crab Eriocheir sinensis with saturation kinetics, suggesting apical ENaC-like Na+-channels (37, 56). In another study, amiloride-sensitive Na+ uptake was detected in the dorsal integument of the earthworm Lumbricus terrestris (41). The authors concluded from electrophysiological data that an invertebrate member of the ENaC family was responsible for the observed amiloride-sensitive Na+ absorption, yet

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there is no proof on the molecular level for that conclusion. During the last years, the dorsal skin of the medical leech Hirudo medicinalis has been established as a valuable model system for the investigation of epithelial Na\(^+\) transport in invertebrates. In thorough electrophysiological studies using modified Ussing chambers, noise, and impedance analysis (15), the amiloride-sensitive Na\(^+\) transport across the dorsal skin of Hirudo medicinalis was characterized in more detail (34, 52, 54). These studies revealed basic features of invertebrate ENaC that showed some similarities with vertebrate ENaC: 40\% of total Na\(^+\) uptake is amiloride-sensitive, but the affinity is somewhat lower [i.e., \(\sim 3 \mu M\) vs. \(\lesssim 1 \mu M\) for vertebrate ENaC (45)]. As in vertebrates, Na\(^+\) transport across leech skin is characterized by a high selectivity for Na\(^+\) over other monovalent cations with a relationship of Na\(^+\) > Li\(^+\) > NH\(_4\)^+ > K\(^+\) \(\approx\) Cs\(^+\) \(\approx\) Rb\(^+\). Ca\(^{2+}\) is involved in the regulation of leech epithelial Na\(^+\) absorption that can be activated by cAMP via recruitment of former silent channels demonstrated by noise analysis. Na\(^+\) uptake across leech skin exhibits autoregulation and is a saturable function of the extracellular Na\(^+\) concentration following a bell-shaped curve (52).

The amiloride-sensitive Na\(^+\) absorption of Hirudo medicinalis dorsal integument shows similarities to ENaC-mediated Na\(^+\) absorption in vertebrates, and it is tempting to assume that there is also an ENaC-like channel in invertebrates. Yet, there are some differences that lead us to investigate leech Na\(^+\) absorption more closely. Therefore, we performed comparative investigations between the Na\(^+\) transport in vertebrates and invertebrates by focusing on already described ENaC properties. First we created a cDNA library and performed PCR approaches with a lot of specific and degenerate ENaC primers. Then we used ENaC-specific antibodies to detect an ENaC-like protein in the leech. Furthermore, we applied known effectors and blockers of vertebrate ENaC to the dorsal skin of Hirudo medicinalis, e.g., the protease trypsin, cAMP, Ni\(^{2+}\), Zn\(^{2+}\), extracellular protons and the nitric oxide (NO) donor sodium nitroprusside (SNP) to compare leech Na\(^+\) uptake with Na\(^+\) absorption in vertebrate epithelia.

For ENaC in vertebrate kidney, it was shown that exposure to endogenous serine proteases can activate the channel (49) and even enhance its open probability (9). It was also shown that vertebrate ENaC is activated by cAMP via two different pathways: increasing the number of active channels already present in the plasma membrane and PKA-dependent exocytotic delivery of preformed ENaC to the plasma membrane (42). Recent studies revealed that a newly described ENaC-\(\delta\)-subunit expressed in human brain, heart, and pancreas can be activated by extracellular protons (28). ENaC derived from A6 cells is activated by Ni\(^{2+}\) (14), whereas rat ENaC expressed in Xenopus laevis oocytes is inhibited by Ni\(^{2+}\) (42). Very recent studies using oocytes of X. leavis expressing rat ENaC revealed that extracellular Zn\(^{2+}\) activates epithelial Na\(^+\) channels by eliminating the Na\(^+\) self-inhibition (43). Furthermore, it was shown for vertebrates that NO inhibits sodium absorption, which appears to occur via amiloride-sensitive ENaC channels (48). Although this NO inhibition was shown for the “classic” ENaC, e.g., in A6 cells and in murine M1 cortical collecting duct cells (21), NO has no inhibitory effect on ENaC of human nasal epithelium (38).

From the literature cited above and from our own contributions, it becomes more and more clear that the invertebrate amiloride-sensitive Na\(^+\) transporter cannot be compared with the structure of ENaC known from vertebrates. Our results show striking functional and molecular differences between the Na\(^+\) absorption in vertebrates and in the invertebrate Hirudo medicinalis. Therefore, we conclude that amiloride-sensitive Na\(^+\) absorption is mediated by different molecules in vertebrates and invertebrates, at least in the medical leech. Consequently, we termed the putative amiloride-sensitive Na\(^+\) transporter in leech skin ENaTP (leech epithelial Na\(^+\) transporting protein) to clearly distinguish it from the ENaC of vertebrates. ENaTP might be a new member of the fast-growing ENaC/DEG superfamily. Parts of these data were published in abstract form (46).

**MATERIALS AND METHODS**

**Animals and tissue preparation.** The experimental procedures were similar to those described in previous studies (52, 54). Briefly, leeches (H. medicinalis) obtained from ZAUG (Biebertal, Germany) were kept at room temperature (22–25°C) and without feeding in artificial tap water (0.5\% sea salt; 0.016 osmol/l). After a time period between 2 and 7 wk, animals were killed, the integument was dissected by two lateral incisions, and the dorsal skin was subsequently detached from the intestines. The internal muscular layers were stripped off by gentle scraping with a scalpel until the diagonal muscle layer could be distinguished. Further dissection was impossible without damaging the tissue. Afterward, a piece of tissue was fixed with tissue adhesive (Histoacryl blue; Braun Melsungen, Germany) with its internal side to a Lucite ring. The tissue was then mounted into a Ussing chamber, with a 0.5 cm\(^2\) aperture, specially designed to minimize edge damage. Silicone grease was used to seal the edges on both sides of the tissue. During the whole experiment, the two compartments were continuously perfused with Ringer solutions. All electrical parameters were normalized to an area of 1 cm\(^2\). All procedures involving animals were carried out under the guidelines of and approved by the Institutional Animal Care and Use Committee of the Westphalian Wilhelms-University Muenster.

**Electrical measurements.** The electrophysiological measurements were performed in modified Ussing chambers designed by Prof. Dr. Willy Van Driessche (KU Leuven, Belgium). For voltage- and current-measurements we used KCl electrodes, which were connected to the Ringer solution through agar bridges. For transepithelial measurements, the \(V_t\) was clamped to 0 mV with a low-noise voltage clamp. The transepithelial short-circuit current (\(I_{sc}\)), \(G_t\), and capacitance (\(C_t\)) were continuously and simultaneously recorded (ImpDsp 1.4; KU Leuven). The principles of \(G_t\) and \(C_t\) measurements are identical to those described in detail by Van Driessche et al. (50).

**Protein biochemistry.** For detection of ENaTP on the protein level we carried out Western blot analysis with specific polyclonal ENaC antibodies directed against the whole bovine \(\alpha\)-ENaC subunit (a kind gift from Dr. M. S. Awayda, Department of Physiology and Biophysics, Buffalo University School of Medicine, Buffalo, NY). We followed the standard protocol as described elsewhere (30). Briefly, membrane proteins were isolated by homogenizing leeches in PBS containing 2\% Triton X-100. Nonsoluble proteins were removed by centrifugation of the homogenate at 4,000 \(\times\) g for 10 min at 4°C. SDS-PAGE was performed using 10–20 \(\mu\)g protein per lane on a 7.5\% gel according to Laemml (31). For Western blot analysis, proteins were transferred onto a nitrocellulose membrane at 0.8 mA/cm\(^2\). Nonspecific binding sites were blocked for 2 h at room temperature using 5\% nonfat dry milk in Tris-buffered saline/Tween (TBST). The membrane was incubated overnight at 4°C with a rabbit anti-\(\alpha\)-ENaC antibody, diluted 1:5,000 in 5\% nonfat dry milk/TBST. The membrane was then washed three times for 15 min in TBST, and subsequently incubated for 1 h at room temperature with the secondary antibody (goat anti-rabbit IgGs conjugated with alkaline phospha-
tase (Dianova, Hamburg, Germany) and diluted in 5% nonfat dry milk (TBST). The membrane was washed as described above and stained with nitro-blue-tetrazolium and bromo-chloro-indolyl-phosphosphate. NH2-terminal amino acid sequencing was performed by a commercial service (B. Scheding, Institute for Physiological Chemistry and Pathobiotechnology, Muenster, Germany) using proteins transferred to a polyvinylidene difluoride membrane.

Molecular cloning. Total RNA was extracted from three adult H. medicinalis. Poly(A)+ RNA was then isolated using the PolyATract kit (Promega, Mannheim, Germany). Poly(A)+ RNA (5 µg) was used to construct a directionally cloned cDNA expression library applying the Lambda ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA). For amplification of the putative ENaTTP, we designed many different degenerate and specific primers according to a broad spectrum of published invertebrate and vertebrate ENaC, FaNaC, and MEC sequences (http://www.ncbi.nlm.nih.gov). We carried out PCR with the cDNA library as a template and RT-PCR in parallel. PCR products were run on a 0.8% agarose gel, stained with ethidium bromide, and appropriate bands were cut out, extracted, cloned into the pCR II-TOPO TA vector (Invitrogen, La Jolla, CA), and sequenced by a commercial sequencing service (Genterprise, Mainz, Germany). Additionally, we screened the cDNA library with two anti-α-ENaC antibodies (kind gifts of Dr. Edith Hummeler, Institute of Physiology, University of Lausanne, Switzerland, and Dr. M. S. Awayda). Putative positive phage clones were converted to plasmid vectors by using material provided by Stratagene and sequenced on both strands by Genterprise.

Antibodies. The used polyclonal antibody was generated against a near full-length bovine α-ENaC fusion protein. The predominant immunogen was a 50-kDa α-ENaC protein containing the NH2 terminus and the extracellular loop. The exact procedure is described in detail by Ismailov et al. (26). The rationale behind this approach was that this antibody would recognize multiple sites on α-ENaC, which would allow its use as a probe of the α-ENaC subunit across many species. Thus, the design of this antibody is ideal for probing for α-ENaC homologues in various species, including the leech. The efficacy of this antibody in detecting α-ENaC from various species was demonstrated by Ismailov et al. (26) (Western blot analysis with bovine and rat α-ENaC). Awayda et al. (45) (immunofluorescence with bovine and rat α-ENaC), Wilson et al. (55) (Western blot analysis and immunofluorescence in teleost fish), and by Western blot analysis in frog, and by competition with the original 50-kDa immunogen in ELISA (M. S. Awayda, personal communication).

Results are presented as means ± SE; N is the number of animals and n the amount of preparations. Statistical analyses were carried out by the Student’s t-test (Origin 6.1; Origin-Lab), and a significance level of P ≤ 0.05 was assigned to be significant and a P Value of ≤ 0.01 to be highly significant.

RESULTS

Basic parameters of Na+ absorption across leech skin. After mounting the tissue into the Ussing chamber and equilibration with mucosal and serosal NaCl Ringer on the respective sides of the epithelium, the transepithelial Isc, Gt, Ct, and reached stable values within 20–40 min and remained stable for several hours under control conditions. The initial electrophysiological parameters showed considerable variations between epithelia from different animals but exhibited only minor differences between tissue preparations from different regions of the dorsal integument of a single animal. Table 1 summarizes these initial parameters of leech dorsal integument measured under control conditions at the beginning of each experiment. Despite the variety of the initial values of different animals, the relative response of each parameter to altered experimental conditions was comparable for all tissue preparations under examination. All preparations were routinely tested for amiloride inhibition. When amiloride (100 µM) was added to the apical side of the epithelium, Isc began to drop instantaneously. This amount of the inhibitor is saturating and blocks >99% of the amiloride-sensitive Na+ absorption (54). The Isc makes up about 50.04 ± 3.98% (n = 45). Additionally, in some experiments, the amount of Na+-dependent portion of the total Isc (IscNa+) was measured by replacing Na+ with TMA in the mucosal Ringer. We found that 63.43 ± 4.18% of total Isc was Na+ dependent (n = 15). Thus, at least two different systems mediate Na+ absorption across leech integument: one that is sensitive to amiloride and its analogs and another one that cannot be blocked by the typical ENaC inhibitors as also reported earlier (52, 54).

The leech integument preparations had a transepithelial resistance of 1.78 ± 0.21 kΩ (n = 43), similar to values as previously reported (52) and therefore belonging to the class of tight epithelia. Interestingly, the electrophysiological properties like IscNa+ of the dorsal skin of H. medicinalis resembled those of measured human respiratory epithelium (39).

Table 1. Initial electrophysiological parameters of Na+ absorption across leech skin measured after an equilibration period of 30 min

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Initial Value</th>
<th>No. Animals</th>
<th>Amount of Preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isc</td>
<td>26.35 ± 1.97 µA/cm2</td>
<td>43</td>
<td>60</td>
</tr>
<tr>
<td>R0</td>
<td>1.78 ± 0.21 kΩ·cm-2</td>
<td>43</td>
<td>60</td>
</tr>
<tr>
<td>IscNa+</td>
<td>13.28 ± 0.07 µA/cm2</td>
<td>43</td>
<td>60</td>
</tr>
<tr>
<td>IscNa−</td>
<td>16.71 ± 0.08 µA/cm2</td>
<td>15</td>
<td>21</td>
</tr>
</tbody>
</table>

Results are presented as means ± SE. Isc, short-circuit current; R0, trans-epithelial resistance; IscNa+, amiloride-sensitive portion of Isc; IscNa−, Na+ dependent portion of the total Isc.
serine protease trypsin was added to the apical side of the epithelium after the initial equilibration period. Addition of 10 μM trypsin had no significant effect on $I_{sc}$ (37.64 ± 5.7 μA/cm²) compared with the control values without trypsin in these particular experiments (36.63 ± 5.44 μA/cm²). This indicates that there is no trypsin activation of any electrogenic transport system in leech skin including the putative lENaTP as it was shown for vertebrate ENaC (46). However, from our data we cannot definitely rule out regulation of lENaTP by proteases, because the cuticula on the external side of the integument might inhibit a possible effect of trypsin on lENaTP in our experiments.

**Inhibition of lENaTP by extracellular H⁺.** We analyzed the effect of extracellular protons in the external medium on $I_{sc}$ and $I_{ami}$ across leech skin by using mucosal Ringer of different pH values. Figure 1 illustrates the time courses of $I_{sc}$, $G_t$, and $C_t$ during a typical pH experiment. Statistical evaluation of 21 experiments revealed that total $I_{sc}$ at pH 4 is significantly lower than at pH 5.4 and at pH 7.4, but not significantly different from $I_{sc}$ at pH 8.4. Figure 2 summarizes the data of the pH dependence of the epithelial Na⁺ absorption in leech integument. To detect the dependence of $I_{ami}$ on the pH of the external medium, amiloride (100 μM) was added to the mucosal Ringer in every step after equilibration to the respective pH. A highly significant decrease in $I_{ami}$ at pH 4 could be observed in relation to all other pH values (Fig. 3). Obviously, high concentration of extracellular H⁺ ([H⁺]o) inhibits the amiloride-sensitive lENaTP, while its vertebrate counterpart ENaC is activated by high [H⁺]o (28). This different behavior of lENaTP and ENaC in response to [H⁺]o is another hint that these two transport systems are completely different.

**cAMP stimulation increases $I_{sc}$ and $C_t$.** Stimulation experiments with a cAMP “cocktail” (8-CTP-cAMP and IBMX) were performed to test whether cAMP exhibits effects on $C_t$ of the tissue. Membrane $C_t$ is a direct measure of the membrane surface area and increasing or decreasing $C_t$ reflects changes in the membrane surface area induced by exocytotic or endocytotic events, respectively (53). First, the integument was equilibrated to physiological pH 7.4 on both sides and then the cAMP “cocktail” was added to the serosal Ringer. cAMP increased $I_{sc}$ by 56.75 ± 15.10% and $G_t$ 28.90 ± 15.45% (Fig. 4; $n = 7$). Moreover, $C_t$ showed a significant increase after cAMP stimulation in every experiment (on average, 9.63 ± 3.64%). From this, we conclude that at least a certain part of the increase in $I_{sc}$ and $G_t$ is evoked by exocytic translocation of preformed Na⁺ transporters from intracellular stores and functional insertion into the plasma membrane.

**Ni²⁺ and Zn²⁺ have an inhibitory effect on $I_{ami}$.** ENaC from different vertebrate tissue react on Ni²⁺ quite distinctly. Therefore, we investigated the influence of Ni²⁺ on leech Na⁺ absorption. Figure 5 illustrates the time course of transepithelial $I_{sc}$ and $G_t$ during a typical Ni²⁺ experiment. Increasing Ni²⁺ concentrations on the apical side of the epithelium caused marked inhibition of $I_{sc}$ as well as $G_t$. The inhibition of $I_{sc}$ followed Michaelis-Menten kinetics with a $K_i$ value of 1.17 ±
0.13 mM (Fig. 6). Obviously, complete inhibition was not reached in the experiments using Ni$^{2+}$/H$^{+}$ concentrations up to 5 mM; therefore, some further experiments were performed with Ni$^{2+}$/H$^{+}$ concentrations up to 10 mM, which caused complete inhibition but left $K_{i}$ unchanged. From our data, we conclude that Ni$^{2+}$ at the apical side of the epithelium has an inhibitory effect on lENaTP, which is dependent on the Ni$^{2+}$/H$^{+}$ concentration and shows saturation at $\approx 10$ mM Ni$^{2+}$.

Extracellular Zn$^{2+}$ was reported to activate ENaC by eliminating Na$^{+}$/H$^{+}$ self-inhibition (44). We were interested how Zn$^{2+}$ would interact with lENaTP; therefore, we added different concentrations of Zn$^{2+}$ from 0.1 mM to 5 mM to the mucosal Ringer solution. Contrary to vertebrate ENaC, leech Na$^{+}$ absorption was inhibited by Zn$^{2+}$. The strongest effect could be observed at 2.5 mM Zn$^{2+}$. At this external Zn$^{2+}$ concentration, 42.55 ± 13.56% of $I_{sc}$ was inhibited, quite similar to the described Ni$^{2+}$ block. These data indicate that divalent cations in general have an inhibitory effect on lENaTP. Again, invertebrate lENaTP and vertebrate ENaC showed different characteristics in response to the divalent cations.

NO inhibits Na$^{+}$ absorption across leech skin. Several previous studies reported that NO shows inhibitory effects on Na$^{+}$ transport through ENaC in different tissues (21). In most of the studies, SNP was used as a NO donor. It was shown that 1 mM of the NO donor SNP was able to release sufficient amounts of NO to block Na$^{+}$ absorption in A6 cells (38). Therefore, we investigated the potential influence of NO on the Na$^{+}$ absorption of leech integument by using the NO donor SNP. Figure 7 shows the time course of transepithelial $I_{sc}$ and $G_{t}$ during an experiment with the NO donor SNP. First, the epithelium was tested for amiloride inhibition, and then 2 mM SNP was applied to the apical side of the epithelium. The released NO inhibited $I_{sc}$ in a biphasic way: a quick, transient inhibition was followed by a lower, plateau value. Interest-
ingly, $G_i$ was first transiently stimulated and then markedly inhibited. Dose response curves with increasing concentrations of SNP and NO, respectively, showed increasing inhibition of Na$^+$ absorption with increasing NO concentration. Steady-state NO inhibition follows Michaelis-Menten kinetics with a $K_i$ value of 0.31 ± 0.12 mM ($n = 10, N = 6$) shown in Fig. 8.

**Protein biochemistry and NH$_2$-terminal sequencing.** To determine whether an ENaC-like protein is present in the integument of *H. medicinalis*, we carried out Western blot analysis with an α-ENaC-specific polyclonal primary antibody directed against the whole bovine α-ENaC subunit and an alkaline phosphatase-conjugated secondary antibody. For separating the membrane proteins, we loaded either 10 μg or 20 μg protein onto the SDS gel. The anti-α-bENaC antibody recognized a distinct band of ~66 kDa (Fig. 9), which is in broad agreement with the size published for the ENaC α-subunit (2).

Determination of the amino-terminal ends of the band by NH$_2$-terminal amino acid sequencing revealed the first 8 amino acids of the detected protein: DTHK(Se)(I)A. With this amino acid sequence we performed a protein search for short, nearly exact matches (www.ncbi.nlm.nih.gov/BLAST), which did not result in a specific hit for an ENaC-like protein. From this we conclude that there is no ENaC-like sequence that could be detected by the specific antibody.

**Molecular identification of lENaTP.** Since ENaC-like proteins are described in vertebrates and some invertebrates (29), we carried out many different approaches to identify ENaC in *H. medicinalis* and *L. terrestris* with a variety of molecular biological methods. Therefore, a cDNA expression library from whole tissue of *H. medicinalis* was screened with a polyclonal-specific anti-α-ENaC antibody directed against the whole protein, which recognized a distinct band in Western blot analyses before (see Fig. 9).

However, in different screening repetitions we obtained ~30 positive phage clones, which did not carry ENaC-related cDNA. According to published sequences from vertebrate and invertebrate ENaC-like sodium channels, we designed a huge set of degenerate and specific primers and carried out PCR (see supplementary material for oligonucleotide sequences of the primers). All primers cover highly conserved regions of ENaC or ENaC-related proteins and were also used in combination with primers binding to the promoter region of the vector used for cDNA library synthesis (PBK cytomegalovirus (CMV); Stratagene, La Jolla, CA). As a template we used the *H. medicinalis* cDNA expression library and RNA isolated from *L. terrestris*. Putative positive fragments were cloned into the TOPO TA vector (Invitrogen), transformed into competent *E. coli*, and sequenced by a commercial sequencing service (Genentype). In all approaches, we used low-stringency annealing conditions for DNA amplification and increased the number of cycles to avoid missing specific PCR fragments. Although such approaches have been successful for the molecular cloning of other low-abundance annelid cDNAs like the Na$^+$-$K^+$-ATPase α-subunit (30), in this case we were not able to identify an ENaC-like sequence in annelids. In addition to all our attempts to clone an ENaC-like gene for *H. medicinalis*, we periodically searched the *L. rubellus* Expressed Sequence Tags database (www.ncbi.nlm.nih.gov) for appropriate sequences (α-, β-, γ-, δ-ENaC, FaNaCh, MEC-4, MEC10, ASIC).

**DISCUSSION**

Amiloride-sensitive Na$^+$ absorption has been demonstrated for various epithelial tissues of different species, including vertebrates and invertebrates. Although there is a vast number of groups that investigate mammalian epithelial Na$^+$ absorption, only a few groups are working on Na$^+$ uptake across invertebrate epithelia. Yet, epithelial Na$^+$ absorption across the integument of the invertebrate *H. medicinalis* has been closely investigated (52, 54). On first sight, the functional properties of leech epithelial amiloride-sensitive Na$^+$ transport resembled the “classic” mechanisms of Na$^+$ absorption through tight epithelia of vertebrates mediated by the epithelial Na$^+$ channel ENaC. Therefore, somewhat rashly, it was presumed that the invertebrate leech epithelial Na$^+$ transporter (lENaTP) and vertebrate ENaC might be functionally and structurally identical. Here we summarize the data from some invertebrate studies and combine these data with new findings from our
group. These studies and our own work during the last years revealed some similarities, although there are substantial differences between the Na\(^+\) absorption mediated by lENaTP and ENaC, respectively.

From the present available data we do not know anything about the molecular nature of this protein. It could be a novel Na\(^+\) channel, a channel made of one or several subunits that belongs to the ENaC family or the ENaC/Deg superfamily, or an electrogenic transport system that is not a channel at all. Therefore, the somewhat vague designation lENaTP is advisable until we will have more molecular data on the amiloride-sensitive Na\(^+\) transport protein.

The first hint for a putative relationship between lENaTP and ENaC certainly comes from the sensitivity of both transporters to the diuretic blocker amiloride. However, it was demonstrated that lENaTP has a lower affinity to amiloride than ENaC with a \(K_i\) of about 2.9 \(\mu\)M amiloride for lENaTP (54) and <1 \(\mu\)M for ENaC (45). The low affinity of lENaTP for amiloride indicates that this Na\(^+\) transporter could belong to the class of low-affinity channels according the classification of Smith and Benos (45).

Ion selectivity, one of the basic features of ion channels, is directly dependent on the molecular structure of the particular channel. Therefore, different selectivity profiles could argue for different molecular channel structures. Whereas ENaC prefers Li\(^+\) over Na\(^+\) (Li\(^+\)/Na\(^+\) = 1.3–1.5; Ref. 19), lENaTP is less selective for Li\(^+\) than for Na\(^+\) (Na\(^+\)/Li\(^+\) = 5;1; Ref. 52). Furthermore, it could be demonstrated that lENaTP shows a lower selectivity for Na\(^+\) compared with K\(^+\) (Na\(^+\)/K\(^+\) = 30;1; Ref. 52) than does ENaC (Na\(^+\)/K\(^+\) = 100;1; Ref. 52). From these different selectivity profiles of ENaC and lENaTP, it could be speculated that there might be two different molecular structures. Since ENaC is highly tissue specific, there are also differences in the selectivity profiles of ENaC from different tissues, and there is no overall selectivity profile. Thus, selectivity is a difficult criterion for discrimination between ENaC and lENaTP.

Both lENaTP and ENaC transport systems are inhibited by calcium. In vertebrates, Ca\(^{2+}\) acts via various poorly understood intracellular signaling pathways or directly on the channel (24). Both Na\(^+\) transport systems are sensitive to changes in intracellular Ca\(^{2+}\) concentration (7, 52). Yet, lENaTP is inhibited from Ca\(^{2+}\) on the external side, as also reported from toad urinary bladder (17), while ENaC is blocked by an increase of intracellular Ca\(^{2+}\) (7). This fact is another piece of evidence that lENaTP and ENaC are different transport systems, although probably both being members of the ENaC/Deg superfamily.

However, there is one feature that both Na\(^+\) transporters share: autoregulation by external Na\(^+\). This is one of the mechanisms that prevent epithelial cells from being overcharged by Na\(^+\). While ENaC is regulated by Na\(^+\) self-inhibition and by Na\(^+\) feedback inhibition (36), lENaTP seems to be regulated only by Na\(^+\) self-inhibition (54).

Effects of the serine protease trypsin on Na\(^+\) transport in lENaTP and ENaC. Recently, it was shown that ENaC is activated by serine proteases, such as trypsin and the extracellular–located serine protease CAP1 (9). In the ENaC-expressing epithelial cell line A6, derived from X. laevis kidney, the exposure of the apical membrane to the protease inhibitor aprotinin reduces transepithelial Na\(^+\) transport measured as the \(I_{\text{ami}}\) (49). Subsequent application of trypsin resulted in a significant increase in \(I_{\text{ami}}\), demonstrating that trypsin is a potent ENaC activator, a fact that has been also demonstrated for Xenopus ENaC (12, 49). Moreover, in a recent abstract, it was shown that trypsin stimulates exocytotic delivery of preformed rat ENaC molecules to and functional insertion into the plasma membrane in the X. laevis oocyte expression system (6).

Furthermore, endogenous CAP1 increased the amiloride-sensitive current twofold, when coexpressed with ENaC in oocytes. The authors concluded that CAP1 and trypsin act through a common mechanism to enhance ENaC activity and that CAP1 has to be involved in the regulation of blood pressure and sodium balance, suggesting a regulation of epithelial Na\(^+\) transport in vivo by proteases (49). The exact mechanism of this activation process has not been fully understood, but a recent abstract showed that trypsin stimulates the exocytotic insertion of preformed ENaC molecules into the plasma membrane (6). However, ENaC regulation via proteases is highly tissue specific (9, 49).

In the present study we demonstrate that the application of the serine protease trypsin to the dorsal integument of H. medicinalis had no effect on the Na\(^+\) absorption mediated by the leech epithelial Na\(^+\) transporter lENaTP. The transepithelial measurements showed no significant changes in the electrophysiological parameters, revealing that lENaTP in the dorsal integument of the leech, contrary to ENaC, is not regulated by trypsin.

On the other hand, different members of the ENaC family show different reactions on protease treatment. For instance, proteases have been shown to inhibit amphibian urinary bladder ENaC (18), while X. laevis ENaC is activated (49). In addition, in many cases, the effects of proteases in a native tissue could be observed only after previous exposure to a protease inhibitor. Thus, our findings of the absence of a direct effect of trypsin on leech skin \(I_{\text{sc}}\) might give only weak support of our hypothesis that the putative protein mediating electrogenic Na\(^+\) transport across leech skin does not belong to the ENaC family. However, the lack of a trypsin effect is another small piece in this rather complicated jigsaw puzzle. Furthermore, it will be interesting to see what effects trypsin will have on leech skin after exposure of the tissue to a protease inhibitor. But even if trypsin fails to affect \(I_{\text{sc}}\) after protease inhibitor treatment, this fact would also not exclude definitely that the lENaTP would belong to the ENaC family.

**Stimulation of lENaTP and ENaC by cAMP.** Stimulation by cAMP could be demonstrated for both invertebrate and vertebrate Na\(^+\) transport systems. In the case of vertebrate ENaC, this stimulation is mediated by two different mechanisms revealed by cAMP measurements: cAMP initiates not only the PKA-dependent phosphorylation of channels already located in the membrane (25) but also exocytotic insertion of ENaC molecules into the apical membrane by vesicle fusion from intracellular pools (8, 42). For lENaTP, it was demonstrated that the effect of cAMP on amiloride-sensitive Na\(^+\) transport is caused by activation of former silent Na\(^+\) transporters in the apical membrane by a still unknown mechanism (54).

In this study, we showed that the application of cAMP increases the transepithelial Na\(^+\) current within 30 min up to 57%. Moreover, we showed that transepithelial \(C_{\text{sc}}\) increases about 9.6% following cAMP stimulation. This indicates that
fusion of vesicles containing preformed lENaTP molecules with the apical membrane is involved in the process of cAMP stimulation of transepithelial Na⁺ transport across leech skin, as well as in vertebrates. Nevertheless, for vertebrate ENaC, as well as for lENaTP, both mechanisms are still far from being completely understood and therefore need further investigation.

Regulation by extracellular protons. Epithelial Na⁺ channels in the apical membrane of many tight Na⁺-absorbing epithelia are exposed to a highly dynamic pH environment and have to react with a proper regulation mechanism for self-protection. Different members of the ENaC/DEG superfamily, e.g., ASIC, are activated by decreasing extracellular pH values (29). A6 cells respond to a small decrease of extracellular pH from 7.4 to 6 with a stimulated Iₘₐₙ (3). Recent studies revealed that the newly described ENaC δ-subunit expressed in human brain, heart, and pancreas is activated by extracellular protons (28). Coexpression of this δ-subunit with human β- and γ-ENaC in Xenopus oocytes revealed a maximal activation of Na⁺ current of ~50% at pH 4 and a half-maximal activation by protons at pH 6.

Therefore, we investigated whether leech lENaTP could be activated by extracellular protons like the above-mentioned members of the ENaC/DEG superfamily. This approach should give further information about putative structural similarities between these transporters. Interestingly, our investigations showed differences in the regulation of lENaTP by extracellular protons, which are in contrast to the reported properties of the ENaC/DEG members. Decreasing pH values of the mucosal Ringer resulted in an inhibition of the amiloride-sensitive part of the transepithelial Na⁺ current. Statistical evaluation of the experiments revealed that the whole Iₘₑ at pH 4 is significantly lower than at pH 5.4 and at pH 7.4, but not significantly different from Iₘₑ at pH 8.4 (see Fig. 2). At pH 4, a highly significant decrease in the amiloride-sensitive Na⁺ current (Iₘₐₙ) could be observed in relation to all other investigated pH values (see Fig. 3). The effect of low pH values (pH 4) on Iₘₐₙ is quite pronounced and is apparently responsible for the decrease of total Iₘₑ under this pH condition. These series of experiments again revealed putative differences between ENaC and lENaTP. lENaTP is inhibited by high [H⁺]o in contrast to a marked activation of ASIC and vertebrate ENaC. However, since large variations in the reactions of different ENaC specimens from diverse tissues and species in response to varying proton concentrations are described, our findings on the pH sensitivity cannot finally rule out that the lENaTP might be a member of the ENaC superfamily.

Influence of the divalent cations Ni²⁺ and Zn²⁺ on epithelial Na⁺ transport. The divalent cation Ni²⁺ is an essential trace element without pharmacological importance that has manifold effects on several cation channels (35). Studies with external Ni²⁺ revealed contrary effects on epithelial Na⁺ absorption in vertebrates. Na⁺ absorption via ENaC is stimulated by Ni²⁺ in A6 cells with a Kᵢ of 0.1 mM (14), whereas heterologously expressed rat ENaC in Xenopus oocytes is inhibited by Ni²⁺ (42). In A6 cells, Ni²⁺ evokes an increase in the density of active channels and accelerates the open probability, independent of endo- or exocytotic processes and therefore constitutes a direct effect of Ni²⁺ on the channel activity (14). Na⁺ transport through the amphibian frog skin is also stimulated by Ni²⁺ (20), whereas mouse ENaC expressing oocytes show an inhibition in Na⁺ absorption by Ni²⁺ (43). These opposite Ni²⁺ effects could be due to species and organ dependent effects, but also might be associated with the fact of whether ENaC is native or heterologously expressed (14).

In our hands, leech epithelial sodium transporter lENaTP was inhibited by Ni²⁺ with an apparent Kᵢ of 1.17 mM and thus needs obviously higher Ni²⁺ concentrations for a half-maximal inhibition compared with the Kᵢ of 0.1 mM Ni²⁺ in A6 cells. Investigations on mouse ENaC expressed in X. laevis oocytes revealed two Kᵢ values (representing two binding sites for Ni²⁺) of 0.05 mM and 11 mM (43). All reported Kᵢ values are quite different from our findings in leech integument. Obviously, vertebrate ENaC is only inhibited by Ni²⁺ when expressed in oocytes. This could argue for an effect of Ni²⁺ on the oocyte plasma membrane that secondarily leads to ENaC inhibition. ENaC in its native environment is probably stimulated by extracellular Ni²⁺. From our data, we have to conclude that native lENaTP is inhibited by Ni²⁺ in contrast to stimulation of the vertebrate ENaC. These controversial effects of Ni²⁺ in different species, organs, or expression systems in

Table 2. Summary of the differences and similarities between the apical Na⁺ absorption mediated by the invertebrate lENaTP and the vertebrate ENaC

<table>
<thead>
<tr>
<th>ENaC (vertebrates)</th>
<th>lENaTP (invertebrates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amiloride-sensitivity</td>
<td>High affinity (Kᵢ &lt; 1 μM) (45)</td>
</tr>
<tr>
<td>Selectivity</td>
<td>Li⁺ &lt; Na⁺ &lt; K⁺ (19)</td>
</tr>
<tr>
<td>Ca²⁺-sensitivity</td>
<td>Intracellular Ca²⁺ (7)</td>
</tr>
<tr>
<td>Stimulation by cAMP</td>
<td>Vesicle fusion and activation of silent channels (8, 25)</td>
</tr>
<tr>
<td>Molecular data</td>
<td>+</td>
</tr>
<tr>
<td>Activation by trypsin</td>
<td></td>
</tr>
<tr>
<td>Effect of extracellular protons</td>
<td></td>
</tr>
<tr>
<td>Effect of extracellular Ni²⁺</td>
<td>Activation [A6 cells and frog skin (28)]</td>
</tr>
<tr>
<td>Effect of extracellular Zn²⁺</td>
<td>Activation (44)</td>
</tr>
<tr>
<td>Effect of NO</td>
<td>Inhibition of “classic” ENaC, e.g., M1 and A6 cells (21), no influence on human nasal ENaC (38)</td>
</tr>
</tbody>
</table>

lENaTP, leech epithelial Na⁺ transporting protein; ENaC, amiloride-sensitive epithelial Na⁺ channel; NO, nitric oxide. Numbers in parentheses are published studies found in REFERENCES.
general and especially concerning lENaTP are poorly understood, and it is clear that much additional work is required before a complete understanding.

It has been reported that external Zn$^{2+}$ activates ENaC by eliminating Na$^+$ self-inhibition (44). The authors demonstrated that extracellular Zn$^{2+}$ increases Na$^+$ transport through ENaC, measured as amiloride-sensitive current in ENaC expressing Xenopus oocytes. Similar to the results obtained with Ni$^{2+}$, Zn$^{2+}$ inhibits Na$^+$ transport in IENaTP in a concentration-dependent manner. This fact gives a further hint for the divergent properties of the invertebrate IENaTP in contrast to the vertebrate ENaC.

As a single argument, the dependence on pH and divalent cations would not prove our overall finding that IENaTP does not belong to the ENaC family. Taking together all data, we believe that we can be rather sure that IENaTP does not belong to the ENaC family.

NO inhibits sodium absorption across leech integument. Independent studies reported that the free radical NO, which is synthesized from the amino acid L-arginine by nearly every cell type, inhibits Na$^+$ absorption in vertebrates (27, 48). Furthermore, it was shown that NO decreases Lam by 60% in cultured rat distal lung epithelial monolayers (13). Additionally, NO is very effective in decreasing the amiloride-sensitive ENaC Na$^+$ current in murine M1 cells and in Xenopus A6 cells through a cGMP-dependent pathway (21). However, a recent study demonstrated that NO has no inhibitory potency on amiloride-sensitive ENaC derived from nasal epithelia and seems to differ from all previously reported NO investigations concerning the epithelial Na$^+$ channel ENaC (38). In the present study, we demonstrate that the application of the NO donor SNP to the apical side of the integument of H. medicinalis inhibits the Na$^+$ absorption via IENaTP, since it was already shown for the “classic” vertebrate ENaC like in M1 and A6 cells and differs from the reported NO effects on human nasal ENaC. One explanation for these apparently controversial findings in human nasal ENaC and other ENaC might be that the ENaC properties could be strictly tissue specific and dependent on the respective ENaC subunit stoichiometry in a given tissue (22).

Molecular and biochemical detection of ENaC-like proteins in the leech. Molecular cloning revealed that vertebrate ENaC is composed of three subunits and belongs to the ENaC/DEG superfamily. However, this indicates that the antibody showed nonspecific cross-reaction in the Western blot with another protein, which is not related to ENaC.

In previous studies, our group demonstrated that in leech the Na$^+$/K$^+$/ATPase is a low-abundance protein compared with vertebrates (33). Therefore, the molecular cloning and protein biochemical detection of the Na$^+$/K$^+$/ATPase α-subunit from H. medicinalis proves that the used methods are appropriate (30). For our investigations of the Na$^+$/K$^+$/ATPase, we used exactly the same H. medicinalis cDNA expression library and carried out PCR and RT-PCR with the same preparation of RNA. Furthermore, for the identification of the Na$^+$/K$^+$/ATPase at the protein level, we performed Western blot analysis with antibodies directed against rat Na$^+$/K$^+$/ATPase α-subunit and received a distinct protein band. In contrast to the trials for the identification of an ENaC-like protein, we were successful with the pump in all disciplines of molecular biology.

**Perspectives**

In conclusion, our results show profound functional differences between the Na$^+$ absorption in vertebrates and in the invertebrate H. medicinalis. Taken together with the already published data (all summarized in Table 2) we conclude that amiloride-sensitive Na$^+$ absorption might be mediated by different transport systems in the invertebrate H. medicinalis in contrast to vertebrates. Our data indicate that IENaTP might be a member of the ENaC/DEG superfamily but is certainly structurally different to the epithelial Na$^+$ channel ENaC described from vertebrates. Since we do not have any single channel data, we do not know whether IENaTP is an ion channel or another type of transport protein. Yet, the amiloride sensitivity, the marked ion selectivity, stimulation by cAMP, and regulation by Ca$^{2+}$ argue for a transport protein rather than unspecific Na$^+$ absorption.

To point out that the amiloride-sensitive Na$^+$ absorption is obviously different in invertebrates and vertebrates, we named the putative amiloride-sensitive Na$^+$ transporter in leech skin IENaTP to clearly distinguish it from the ENaC of vertebrates.

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