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Effects of extracellular purines on ion transport across the integument of *Hirudo medicinalis*

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Summary

Little is known about the long-term regulation of epithelial ion transport in invertebrates and the specific mediators involved. For some years, we have been investigating the short-term regulation of transepithelial ion transport across the dorsal integument of the leech *Hirudo medicinalis*, and we have established a model of Na^+ uptake. In the present study, we investigated the effect of long-term acclimation on transintegumental ion transport by adapting leeches to high-salinity conditions. We dissected segments of dorsal integument and measured ion currents in Ussing chamber experiments. Electrophysiological variables, such as transepithelial potential (V_T) and short-circuit-current (I_{sc}), were profoundly affected by adaptation to high-salinity conditions. The total transepithelial Na^+ current (I_{Na}) decreased from 7.66±0.82 to 4.6±0.54 $\mu A\,cm^{-2}$ in preparations adapted to high salinity. The involvement of epithelial Na^+ channels was determined as current inhibition (I_{ami}) by apical application of amiloride; Na^+ channels were equally active in control epithelia and epithelia from leeches adapted to high salinity. Removal of Ca^{2+} from the apical solutions, which is believed to reduce intracellular Ca^{2+} concentrations, equalized transepithelial variables between high-salt-adapted integuments and control integuments.

Extracellular purines regulate transepithelial Cl^- secretion and Na^+ absorption. In a variety of tissues we tested ATP and adenosine for their effects on epithelial transport. Examination of integuments from pondwater- and high-salinity-adapted leeches revealed different sensitivities for these purines. Apical and basolateral application of ATP both stimulated transepithelial Na^+ uptake and I_{ami}. Adenosine upregulated non-Na^+ currents and acted from the basolateral side only. Apical Ca^{2+}-free conditions attenuated these effects of purines on transepithelial currents. Extracellular UTP had no effect on ion transport.

Key words: leech, *Hirudo medicinalis*, Na^+ transport, invertebrate, amiloride, cyclic AMP, ATP, adenosine, UTP.

Introduction

Fresh water represents a hypo-osmotic environment to the leech, in which it has to cope with the loss of salts and uptake of water. The nephridia and the integument collaborate in accomplishing this osmoregulation. While the homeostatic mechanisms in the nephridia have been thoroughly investigated (Nieczaj and Zerbst-Boroffka, 1993; Zerbst-Boroffka and Wenning, 1986; Zerbst-Boroffka et al., 1982), there is little information concerning integumental ion transport (Milde et al., 2001; Schnizler and Clauss, 1998; Weber et al., 1993, 1995). The absorption of extracellular Na^+ plays a prominent role in maintaining electrolyte balance. The majority of electrogenic Na^+ absorption across the leech integument is likely to be *via* mechanisms analogous to those in vertebrate tight epithelia. Recent models of ion absorption processes across frog skin from fresh water have included energization of Na^+ entry *via* apical Na^+ channels by electrogenic H^+ V-ATPases (Ehrenfeld and Klein, 1997; Nelson and Harvey, 1999). Extracellular Na^+ crosses the apical cell membrane through highly selective Na^+ channels along an electrochemical gradient that is sustained by the basolateral Na^+/K^+-ATPases. It remains to be determined whether apical Na^+ entry is driven by H^+ V-ATPases in leech integument, but epithelial Na^+ channels are, as in vertebrates, selectively blocked by amiloride and represent a rate-limiting target for the control of transcellular Na^+ uptake (Weber et al., 1993, 1995).

In the last 40 years, vertebrate epithelia have been the preferred models for investigating Na^+ transport in tight epithelia. Regulation of Na^+ absorption occurs in several ways (Garty and Palmer, 1997), the major stimulus in vertebrates being the mineralocorticoid aldosterone. This steroid hormone mediates long-term adaptation by influencing the expression of regulatory factors at both transcriptional and translational levels. Peptides, for example antidiuretic hormone (ADH), bind to G-protein-coupled receptors and exert their regulatory effects within minutes. Invertebrates such as the leech also

possess these phylogenetically old antidiuretic hormones, but they lack the mineralocorticoid system found in vertebrates (Oumi et al., 1994; Proux et al., 1987; Salzet et al., 1993, 1995; Satake et al., 1999). To investigate the processes underlying Na^+ transport across tight epithelia in invertebrates, we used the integument of the leech *Hirudo medicinalis* for electrophysiological measurements (Milde et al., 2001; Schnizler and Clauss, 1998; Weber et al., 1993, 1995).

The regulatory properties of vertebrate epithelial Na^+ channels have been subject to intensive study over the last 10 years, and regulation of these epithelial Na^+ channels occurs by modulation of channel activity within the apical cell membranes as well as by control of the total number in surface-expressed channels (Garty and Palmer, 1997). High extracellular Na^+ concentrations induce Na^+ self-inhibition, a mechanism that downregulates amiloride-sensitive apical Na^+ entry (Palmer et al., 1998; Turnheim, 1991). Furthermore, in a variety of epithelia, downregulation of apical Na^+ conductances was observed when intracellular Na^+ concentration increased. This autoregulative Na^+ feedback inhibition represents a short-term adaptive process, and current Na^+ feedback inhibition models postulate an intracellular, as yet unidentified, Na^+-sensing receptor that mediates, through G-protein-coupled mechanisms, a ubiquitin-protein ligase (Nedd4)-dependent endocytosis and degradation of the epithelial Na^+ channels (Ishibashi et al., 1999; Komwatana et al., 1998). The involvement of G proteins was not confirmed in all cases, however (Hubner et al., 1999).

Living in fresh water and only occasionally entering brackish water (Herter, 1939), *Hirudo medicinalis* cannot be expected to be a euryhaline organism. Nevertheless, our previous studies showed that the leech integument reduces electrogenic Na^+ uptake in the presence of high apical Na^+ concentrations. This short-term adaptive downregulation was maintained for several hours (as long as the experiments lasted) and quickly became inoperative after a return to low-[Na^+] conditions (Weber et al., 1995). Adult leeches survive in water of up to 16 ‰ salinity for several months (Boroffka, 1968). There, they behave as hyperosmotic osmoconformers, with hyporegulated [Cl^-] in the blood but an accumulation of short-chain carboxylic acids (Nieczaj and Zerbst-Boroffka, 1993). In the initial phase (within 4 h) of acclimation, osmotic loss of water and uptake of salt were the prominent passive events. Concentrations of Na^+, K^+, Cl^- and of organic anions in the blood were greatly increased (Nieczaj and Zerbst-Boroffka, 1993). After a few days, extracellular volume was restored. Our interest in investigating whether there is long-term acclimation of transintegumental ion transport during extended exposures to high-saline conditions arose from these findings. We exposed leeches for several days to a high-salinity environment (200 mmol l^{-1} NaCl) to elicit an osmoregulatory long-term adaptation of Na^+ uptake; the annelids appeared to cope with this excessive physiological stress. The plasma Na^+ concentration of *Hirudo medicinalis* is approximately 115 mmol l^{-1} (Zerbst-Boroffka and Wenning, 1986), and an unhindered influx of Na^+ from the 200 mmol l^{-1} Na^+ environment would therefore change the physiological situation from absorption to excretion of excess Na^+.

For comparative purposes, dorsal segments of these high-salt-adapted and of pondwater-adapted integuments were dissected for Ussing chamber experiments. The preparations were voltage-clamped, and the initial electrophysiological properties determined to expose any differences attributable to high-salt-adaptation at the level of integumental Na^+ conductances. We measured total transepithelial Na^+ uptake and the amiloride-sensitive Na^+ current.

Extracellular nucleotides serve as agonists for a variety of membrane receptors. Besides ionotropic P2X-type receptors, which comprise intrinsic ion channels, two other large families of G-protein-coupled purine receptors, the P2Y- and the P1-receptors with a multitude of subtypes, have been characterized and cloned (Ralevic and Burnstock, 1998; Surprenant et al., 1995). Expression of these receptors has been detected in a variety of epithelia in which they function in the para- and/or autocrine control of transepithelial ion-transport processes (Ralevic and Burnstock, 1998). ATP, for example, may be released from epithelial cells under physiological conditions (Schwiebert, 1999). A reciprocal regulation, stimulation of Cl^- secretion accompanied by a reduction in Na^+ absorption, by extracellular trinucleotides may be a common and cytosolic Ca^{2+}-dependent principle of ion-transport control, e.g. in renal or pulmonary epithelia (Cuffe et al., 2000; Hayslett et al., 1995; McCoy et al., 1993). Nucleotides can regulate NaCl transport *via* P2Y- and P2X-type receptors (McCoy et al., 1999). The distribution of some epithelial purinoceptors is restricted to either the apical or the basolateral membrane, and this accounts for the ‘side-specific’ regulatory effects of extracellular nucleotides (Casavola et al., 1996, 1997). In the present study, we evaluate the regulatory impact of intercellular nucleotide messengers on the control of ion transport across the leech integument. For this purpose, we measured the effects of adenosine, ATP and the pyrimidine UTP on Na^+ transport and also investigated whether the removal of apical Ca^{2+}, which is known greatly to stimulate transintegumental current (Prusch and Otter, 1977; Weber et al., 1995), interferes with purinergic control. Examination of pondwater- and high-salt-adapted integuments indicated different sensitivities to extracellular nucleotides.

Materials and methods

Animals and tissue preparation

Hirudo medicinalis L. were obtained from Zaug (Biebertal, Germany) and were kept without food for 4–10 days either in artificial pondwater (APW; 1 mmol l^{-1} NaCl) or in high-salinity water (HSW; 200 mmol l^{-1} NaCl) at room temperature (18–24 °C). Before preparation, animals were made torpid by exposure to low temperature (0 °C).

A ventral incision was made in the leech body wall, and the intestine was detached and muscular layers were carefully scraped off. The dorsal integument of the subclitellar region was fixed on a needle-spiked ring. The Ussing chamber had an

aperture of 0.5 cm^2. The edges of the tissue were sealed with silicone grease. During experiments, both compartments of the Ussing chamber were continuously perfused (apical, at approximately 7 ml min^{-1}; basolateral, at approximately 3 ml min^{-1}). All experiments were performed at room temperature (18–24 °C). A preparation of the dorsal integument (subclitellar region) was mounted in an Ussing type chamber. After measurement of the initial potential, V_{init}, the transepithelial potential, V_T, was allowed to equilibrate and then clamped to 0 mV. The short-circuit current (I_{sc}) was recorded continuously, and the transepithelial resistance (R_T) was calculated from the effects of 20 mV pulses on I_{sc}. The amiloride-sensitive current (I_{ami}) was measured as the decrease in I_{sc} in the apical presence of apical 100 μmol l^{-1} amiloride. I_{Na}, the transepithelial Na^+ current, was when apical NaCl was substituted with equimolar tetramethylammonium chloride (TMA-Cl). Readdition of apical Na^+ re-established the I_{sc}.

Solutions and chemicals

The basolateral Ringer's solution contained (in mmol l^{-1}): 115 NaCl, 4 KCl and 1.8 $CaCl_2$. In the apical solution, KCl was replaced with TMA-Cl. In Na^+-free solutions, NaCl was substituted by equimolar concentrations of TMA-Cl. Ca^{2+}-free solutions contained 0.5 mmol l^{-1} EDTA. All solutions were buffered with 5 mmol l^{-1} Hepes and adjusted to pH 7.4 with Tris (Trizma-base).

APW contained (in mmol l^{-1}): 1 NaCl, 0.05 KCl, 0.4 $CaCl_2$ and 0.2 $NaHCO_3$, pH 7.4. HSW contained (in mmol l^{-1}): 200 NaCl, 1 KCl, 0.4 $CaCl_2$ and 0.2 $NaHCO_3$, pH 7.4.

Adenosine, ATP, UTP, cyclic AMP [8-(4-chlorophenylthio)-cAMP, cpt-cAMP] and 3-isobutyl-1-methyl-xanthine (IBMX) were obtained from Sigma.

Electrical measurements

Ag/AgCl wires in 1 mol l^{-1} KCl served as current- or voltage-measuring electrodes. For conductive connection to bathing compartments, 1 mol l^{-1} KCl/agar bridges were used. During measurements, the transepithelial potential was voltage-clamped to 0 mV (voltage-clamp amplifier; Nagel, Munich, Germany). The short-circuit current (I_{sc}) was recorded continuously on a stripchart recorder and on computer (Apple IIsi, MacLab interface, chart recorder program: Analog Digital Instruments). R_T was calculated according to Ohm's law from changes in I_{sc} during superimposed 20 mV pulses.

Statistical analyses

Data are presented as means ± S.E.M. *N* is the number of experiments and animals. Statistical analyses were performed using paired or independent Student's *t*-tests (Microcal Origin).

Results

Electrophysiological variables

After an equilibration period, the electrophysiological parameters V_T, I_{sc} and R_T were measured from both groups of adapted integuments (Table 1). The values of most of the initial variables were significantly reduced in HSW-adapted compared with APW-adapted integuments. Interestingly, although I_{sc} and total I_{Na} were greatly decreased by adaptation to 200 mmol l^{-1} NaCl, I_{ami} was not reduced significantly. Removal of apical Ca^{2+} increased I_{sc} by a factor of approximately 1.4 in APW-adapted and approximately 2.4 in HSW-adapted integuments (Table 1). The contribution of I_{Na} to I_{sc} increased from 40 % in APW and 48 % in HSW preparations to 75 % and 70 %, respectively, under Ca^{2+}-free conditions (Table 1); however, the amiloride-sensitive portion of total I_{Na} remained at approximately 60 % for both groups. The non-Na^+ currents, calculated as the I_{sc}–I_{Na}, even increased slightly in HSW-adapted integuments but decreased in the APW-adapted integuments upon removal of apical Ca^{2+}.

Table 1. *Electrophysiological variables of pondwater- and high-salinity-adapted leech dorsal integuments*

	APW	HSW
Control conditions		
V_{init} (mV)	17.5±2.4	8.4±1.5*
V_T (mV)	32.7±3.9	16.2±2.5*
R_T (Ω cm^2)	2038±346	1701±176
I_{sc} (μA cm^{-2})	18.96±1.76	9.56±1.1*
I_{ami} (μA cm^{-2})	4.34±0.68	3.12±0.48
I_{Na} (μA cm^{-2})	7.66±0.82	4.6±0.54*
Apical Ca^{2+}-free conditions		
R_T (Ω cm^2)	1735±179	1706±109
I_{sc} (μA cm^{-2})	25.88±1.82†	22.66±1.92†
I_{ami} (μA cm^{-2})	13.54±1.36†	10.16±1.72†
I_{Na} (μA cm^{-2})	19.44±1.1†	15.96±1.62†

APW, artificial pondwater; HSW, high-salinity water.

Values are means ± S.E.M., *N*=16.

*Values of HSW-adapted integuments significantly different from values of APW-adapted integuments, $P<0.05$.

†Ca^{2+}-free values significantly different from control values, $P<0.05$.

V_{init}, initial transepithelial potential; V_T, transepithelial potential; R_T, transepithelial resistance; I_{sc}, short-circuit current; I_{ami}, amiloride-sensitive current; I_{Na}, transepithelial Na^+ current.

Regulation of epithelial Na^+ uptake via *purinergic receptors*

We tested different concentrations of ATP for their effect on transepithelial ion conductances of APW- and HSW-adapted integuments. ATP was applied to the basolateral side at concentrations of 10 μmol l^{-1} to 10 mmol l^{-1} (Fig. 1). Maximal stimulation was observed in all preparations with 1 mmol l^{-1} ATP, giving a concentration required for 50 % stimulation (EC_{50}) of approximately 80 μmol l^{-1}. Exposure to 1 mmol l^{-1} ATP did not affect I_{sc} in APW-adapted integuments (98.7±6.9 %; *N*=5) (Fig. 2). In HSW-adapted integuments ATP induced a twofold (214.7±42.1 %) increase in I_{sc}, which resulted from the activation of Na^+ conductances (Fig. 2). This, in turn, could be attributed to a more than threefold (364±103.9 %) increase in the amiloride-sensitive current. In comparison, in APW-adapted integuments, I_{ami} increased only

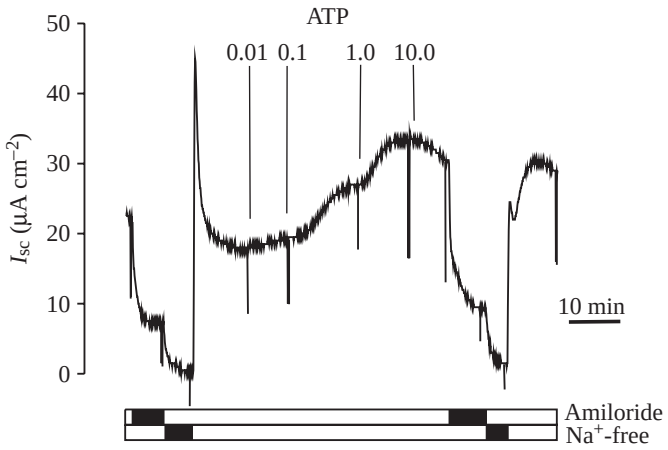

Fig. 1. Original recording of short-circuit current (I_{sc}) from leech integument adapted to high-salinity water. Increasing concentrations of ATP (in mmol l^{-1}) were added to the basolateral compartment at the indicated times. Note the weak decrease in I_{sc} in response to 10 mmol l^{-1} ATP. The current sensitive to 100 μmol l^{-1} amiloride was determined before superfusion with Na$^+$-free Ringer's solution; periods of superfusion are shown below the recording.

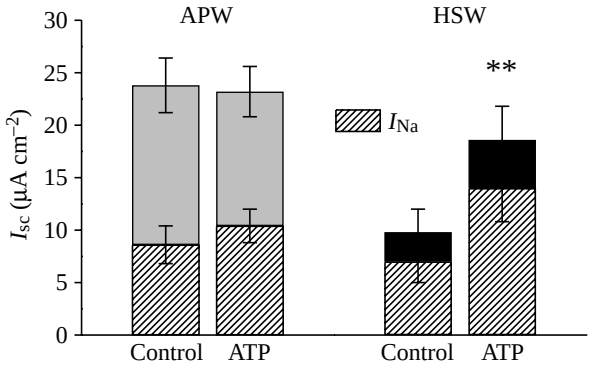

Fig. 2. Effect of basolateral ATP on short-circuit current (I_{sc}) of artificial pondwater (APW)- and high-salinity (HSW)-adapted integuments. Transepithelial Na$^+$ current was determined after I_{sc} had reached steady state (control). Subsequently 1 mmol l^{-1} ATP was added to the basolateral compartment and transepithelial Na$^+$ current (I_{Na}) was determined again (ATP) when I_{sc} had stabilized. Values are means ± S.E.M. (N=5). ATP significantly increased I_{Na} in HSW-adapted preparations, **P<0.005. Hatched columns, transepithelial Na$^+$ current; filled columns, residual non-Na$^+$ currents.

slightly (by 187.5±33 %) at the cost of other Na$^+$ conductances (not shown). The ATP-induced I_{ami} was larger than the subsequently determined net Na$^+$ transport (I_{Na}; Table 2). This paradoxical finding may be explained by additional effects of apical Na$^+$-free conditions on, for example, Cl$^-$ conductances. Higher concentrations, such as 10 mmol l^{-1} ATP, partially deactivated I_{sc} again. This downregulation phenomenon in the presence of 10 mmol l^{-1} ATP was observed in all experiments (data not shown). Apical application of ATP (1 mmol l^{-1}) also greatly stimulated I_{sc}, a result that was not further investigated.

Under apical Ca^{2+}-free conditions, ATP had no effect on I_{sc} across APW-adapted integuments (112.8±6.8 %). In contrast, upregulated I_{sc} across HSW-adapted integuments under Ca^{2+}-free conditions responded to 1 mmol l^{-1} ATP with a significant increase to 136.3±6.4 %. ATP preferentially stimulated Na$^+$ uptake and had very little effect on non-Na$^+$ conductances. This effect of ATP on transepithelial Na$^+$ transport in HSW-adapted integuments was largely due to an upregulation of I_{ami} by a factor of 235.9±49.5 %, whereas I_{ami} increased to 185.4±27.9 % in APW-adapted integuments (not shown).

Certain types of P2Y receptor selectively bind uridine nucleotides but not adenosine nucleotides (Lazarowski and Boucher, 2001; Ralevic and Burnstock, 1998). UTP (1 mmol l^{-1}) was added either to the apical or basolateral compartment and tested for its effect on macroscopic

Table 2. *Effect of basolateral application of extracellular nucleotides on different currents in leech dorsal integuments under control and apical Ca^{2+}-free conditions*

	ATP		UTP		Adenosine	
	APW	HSW	APW	HSW	APW	HSW
Control conditions						
I_{sc} (μA cm^{-2})	23.10±2.48	18.60±3.22	11.30±1.12	9.50±1.38	14.50±2.30	13.0±1.40
I_{ami} (μA cm^{-2})	13.50±1.02	14.30±2.68	2.76±1.16	5.76±1.46	5.15±1.58	3.80±0.70
I_{Na} (μA cm^{-2})	10.30±1.60	14.0±3.24	3.76±0.78	6.88±1.68	7.70±1.58	5.20±0.82
R_T (Ω cm^2)	3739±366	4221±330	8400±1615	6537±1816	5354±1259	3214±424
Apical Ca^{2+}-free conditions						
I_{sc} (μA cm^{-2})	27.10±4.58	31.62±3.24*	19.20±6.44	17.95±2.70	19.40±1.86	16.62±2.04
I_{ami} (μA cm^{-2})	22.10±1.52*	24.50±3.54*	11.40±1.94	10.20±1.30	8.20±1.30	6.76±1.56
I_{Na} (μA cm^{-2})	24.50±1.44*	28.88±3.40*	15.10±3.06	15.30±0.98	14.70±1.20*	11.38±2.10
R_T (Ω cm^2)	2701±343	3028±394	5709±1067	4659±740	3980±708	3897±230

Values are means ± S.E.M., N=5.
*Ca^{2+}-free values significantly different from control values, P<0.05.
Note that there are no significant differences between the artificial pondwater-adapted (APW) and high-salinity-adapted (HSW) integuments.
I_{sc}, short-circuit current; I_{ami}, amiloride-sensitive current; I_{Na}, transepithelial Na$^+$ current; R_T, transepithelial resistance.

transepithelial ion conductances across APW- and HSW-adapted integuments. Apical treatment with 1 mmol l^{-1} UTP left I_{sc}, I_{Na} and its amiloride-sensitive component I_{ami} unaffected in both integuments (data not shown). While I_{ami} of APW-adapted integuments responded somewhat inconsistently to basolateral UTP, the I_{sc} was reduced to 87 % compared with the control value and total I_{Na} was not significantly affected. Under apical Ca^{2+}-free conditions, UTP had no effect on I_{sc}. In addition, the basolateral side of HSW-adapted integuments was insensitive to 1 mmol l^{-1} UTP under both normal and Ca^{2+}-free conditions.

Regulation of Na^+ transport by adenosine /P1-receptors

We examined the effects of extracellular adenosine in the apical and the basolateral compartments. I_{sc} and Na^+ currents across APW- and HSW-adapted integuments were insensitive to apical adenosine (data not shown). While I_{sc}, I_{Na} and I_{ami} in APW-adapted integuments were largely unaffected by basolateral application of adenosine, the I_{sc} of HSW-adapted integuments increased in a concentration-dependent manner (Fig. 3). A maximal, stable and non-transient activation of I_{sc} was observed in the presence of 10 mmol l^{-1} adenosine (204±35 % of control amplitude; N=5; EC_{50}=0.17 mmol l^{-1}). In contrast to the effect of ATP described above, the net increase in I_{sc} was due to a stimulation of non-Na^+ conductances (Fig. 4). Interestingly, in HSW-adapted integuments, adenosine increased I_{ami} current to 188.1±18.7 % without changing total I_{Na} (Fig. 4). Basolateral treatment with 1 mmol l^{-1} adenosine, after upregulation of Na^+ currents by Ca^{2+}-free conditions, had no significant effect on I_{sc} or on the component of I_{sc} carried by Na^+. I_{ami}, however was slightly reduced to 79.5±5.6 % in APW-adapted integuments, whereas it increased in HSW-adapted integuments to 175.6±65.2 % in response to adenosine. The size of this effect varied substantially, but it was observed in all preparations tested.

Table 2 summarizes the currents and the effects of apical Ca^{2+}-free conditions in the basolateral presence of 1 mmol l^{-1}

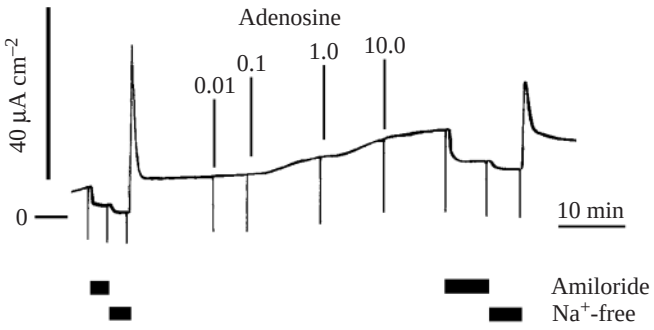

Fig. 3. Original recording of short-circuit current (I_{sc}) from high-salinity-adapted integument. Adenosine (1 mmol l^{-1}) was added in increasing concentrations to the basolateral compartment to give the final concentrations (mmol l^{-1}) indicated. The current sensitive to 100 μmol l^{-1} amiloride was determined before superfusion with Na^+-free Ringer's solution; periods of superfusion are shown below the recording.

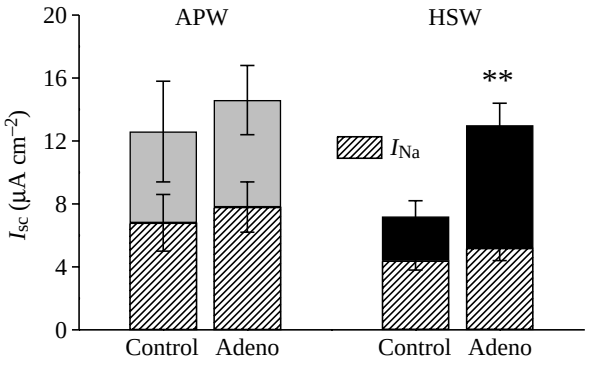

Fig. 4. Effect of basolateral adenosine upon transepithelial currents of leech dorsal integuments. Integuments adapted to artificial pondwater (APW) or high-salinity (HSW) were clamped to 0 mV. Transepithelial Na^+ current (I_{Na}) was determined after equilibration (Control). Subsequently, 1 mmol l^{-1} adenosine was added to the basolateral compartment and, when the short-circuit current (I_{sc}) had stabilized, I_{Na} was determined again (Adeno). Values are means ± S.E.M. (N=5). Addition of 1 mmol l^{-1} adenosine to the basolateral compartment significantly increased I_{sc} but not I_{Na} in HSW-adapted integuments, $^{**}P$<0.005. Hatched columns, transepithelial Na^+ current; filled columns, residual non-Na^+ currents.

ATP, UTP and adenosine. Transepithelial ion conductances have been reported to be regulated by extracellular adenosine and through cyclic AMP/protein kinase A (PKA) signalling mechanisms. We treated APW- and HSW-adapted integuments in the absence of apical Ca^{2+} with membrane-permeant 100 μmol l^{-1} cpt-cAMP in combination with 1 mmol l^{-1} IBMX (an inhibitor of phosphodiesterases). However, this effect of cyclic AMP was abolished under Ca^{2+}-free conditions. Both I_{Na} and the non-Na^+ current were

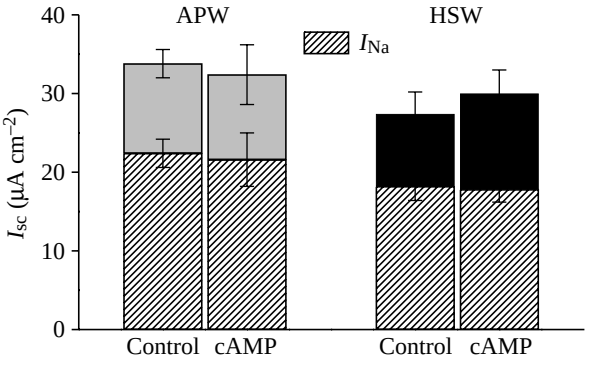

Fig. 5. Effect of apical 8-(4-chlorophenylthio)-cyclic AMP (cAMP) on short-circuit current (I_{sc}) of artificial pondwater (APW)-adapted or high-salinity (HSW)-adapted leech integuments under apical Ca^{2+}-free conditions. Ca^{2+} was removed from the apical compartment and, when I_{sc} had stabilized, transepithelial Na^+ current (I_{Na}) and non-Na^+ currents were determined (Control). cAMP (100 μmol l^{-1}) and 3-isobutyl-1-methyl-xanthine (1 mmol l^{-1}) were added. I_{Na} was determined by apical perfusion with Na^+-free Ringer (cAMP). Values are means ± S.E.M. (N=6). Hatched columns, I_{Na}; filled columns, residual non-Na^+ currents.

unaltered by cyclic AMP (Fig. 5). The amiloride-sensitive part of I_{Na} did not change in APW-adapted (94±6 %) or HSW-adapted integuments (102±8 %). The apical epithelial Na^+ channels were therefore not affected.

Discussion

Hirudo medicinalis proved to be reasonably tolerant of transfer to a high-salinity environment (Boroffka, 1968; Nieczaj and Zerbst-Boroffka, 1993). The initial phase, consisting of loss of water and accumulation of Na^+, Cl^- and organic anions, is followed by long-term acclimation, as for a hyperosmotic osmoconformer (Nieczaj and Zerbst-Boroffka, 1993). After several days, plasma volume is restored, while intracellular water content is not (Nieczaj and Zerbst-Boroffka, 1993). How this physiological acclimation affects transintegumental ion transport was the subject of the present study.

Pondwater presents the leech integument with the problem of absorbing ions against considerable electrochemical gradients. The apical transport of Na^+ requires energy and may be driven by proton-motive forces from, for example, H^+ V-ATPases (Ehrenfeld and Klein, 1997; Nelson and Harvey, 1999). In high-salinity conditions the situation for overall ion regulation is reversed. Extracellular 200 mmol l^{-1} Na^+ exceeds plasma concentrations (115–140 mmol l^{-1}) (Prusch and Otter, 1977; Zerbst-Boroffka and Wenning, 1986) and should therefore result in increased levels of body salts. Shutdown of apical Na^+ conductances and extrusion of excess salt by, for example, nephridia may then balance total Na^+ uptake (Zerbst-Boroffka and Wenning, 1986). To determine when long-term (transcriptional and/or translational) regulation of ion transport sets in, one would have to perform a series of time-course experiments that specify time required for changes in electrical properties of the leech integument. However, such an approach is difficult because this epithelium shuts down ion transport in the presence of high apical Na^+ concentrations. Na^+ uptake recovers soon after a return to low-[Na^+] conditions (Weber et al., 1995; M. Schnizler, personal observations). The recovery of Na^+ transport after a shift from apical high-Na^+ conditions to low-Na^+ conditions is repeatably reproducible and the phenomenon remains unchanged even after exposure to high-Na^+ conditions for more than 5 h. We therefore decided to extend the high-salt challenge over several days, by which time long-term adaptive processes could be expected to have occurred (Nieczaj and Zerbst-Boroffka, 1993). Recent models attribute triggering of the autoregulative feedback inhibition of Na^+ transport in tight epithelia to an elevation in intracellular [Na^+] (Palmer et al., 1998; Turnheim, 1991). The phenomenon is a short-term adaptive process that starts immediately and is reproducible for several cycles of transfer from low to high salinity. There is evidence for Na^+-sensing receptors that mediate the endocytosis of epithelial Na^+ channels and their subsequent degradation (Hubner et al., 1999; Ishibashi et al., 1999; Komwatana et al., 1998). The feedback inhibition is therefore due to a reduction in the total number of surface-expressed channels. We expected the high-salt exposure of several days not only drastically to reduce transcellular Na^+ transport but also to affect the number of new vesicle-stored channels available for apical membrane insertion. Long-term adaptation to high-salt conditions reduced V_T and I_{sc} (Table 1). However, although transepithelial Na^+ uptake was downregulated, the amiloride-sensitive transcellular Na^+ current (I_{ami}) was not affected (Table 1).

Effect of Ca^{2+} and purinergic signalling

High extracellular Na^+ concentrations are generally believed to elevate cytosolic Ca^{2+} concentrations which, in turn, inhibit the amiloride-sensitive Na^+ channels, either directly or by protein-kinase-C-mediated mechanisms (Garty and Palmer, 1997). Despite persistent evidence for the importance of Ca^{2+} in short-term Na^+ feedback inhibition, their direct involvement could not be verified (Abriel and Horisberger, 1999). It is not clear whether the downregulation of transcellular Na^+ uptake by apical Ca^{2+} results from interactions with elements in the apical membranes or by Ca^{2+} entering the cells and increasing cytosolic Ca^{2+} concentrations (Abriel and Horisberger, 1999; Palmer et al., 1998; Turnheim, 1991). In addition to a direct and possibly non-specific action upon Na^+ channels or regulatory factors, G-protein-coupled Ca^{2+}-sensing mechanisms may come into play (Komwatana et al., 1998). Surface expression of such Ca^{2+} receptors has been reported for several tissues (Brown et al., 1993; Riccardi, 2000), but their presence in the leech integument must first be verified.

One objective of our study was to investigate whether long-term acclimation to high-salt conditions affects this short-term regulation of transcellular Na^+ uptake and its sensitivity to apical Ca^{2+}. Both high-salt- and pondwater-adapted integument responded to the removal of apical Ca^{2+} with an increased I_{sc} to values of similar magnitude, and this was mainly due to an upregulation of I_{ami} (Table 1). Thus, the amiloride-sensitive Na^+ conductances are partly inhibited by the extracellular presence of Ca^{2+}. This stimulating effect of Ca^{2+} removal in the leech skin may be correlated with downregulated Na^+ transport since only under apical high Na^+-conditions could I_{sc} be stimulated by the removal of apical Ca^{2+} (Weber et al., 1995).

Transcellular transport of Ca^{2+} occurs in a variety of epithelia, and apical entry of Ca^{2+} greatly affects intracellular Ca^{2+} concentrations (Hoenderop et al., 2000). In vertebrates, for example, there are epithelial Ca^{2+} channels that lose their selectivity and transport monovalent cations in the absence of extracellular Ca^{2+} (Vassilev et al., 2001). Total removal of apical Ca^{2+} can be expected to reduce cytosolic [Ca^{2+}]. Basolateral electrogenic $3Na^+/1Ca^{2+}$ exchange processes are considerably downregulated in this situation, and this may explain upregulation of transcellular Na^+ transport in Ca^{2+}-free conditions (Blaustein and Lederer, 1999; Hoenderop et al., 2000). Interestingly, the stimulating effect of a removal of Ca^{2+} was greater in HSW-adapted than in APW-adapted integuments, but the final upregulated currents from HSW-adapted integuments were equal to those from APW-adapted

integuments (Table 1). We do not know the extent to which vesicular translocation of new channels contributed to the effect of Ca^{2+}, but as a result of long-term adaptation, one would expect a marked reduction in *de novo* synthesis of channels rather than the formation of channel-containing membrane vesicles. The rapid upregulation observed indicated *in situ* activation of silent, but surface-expressed, Na^+ channels.

P2 receptors

Locally released ATP has a direct paracrine and/or autocrine effect modulating epithelial transporters and channels. Reciprocal regulation of Cl^- secretion and Na^+ absorption by extracellular nucleotides appears to be a common feature of mammalian respiratory and renal epithelia (Cuffe et al., 2000; Mall et al., 2000; McCoy et al., 1999). ATP and UTP stimulate Cl^- secretion, whereas apical Na^+ entry is downregulated (Cuffe et al., 2000; Inglis et al., 1999). In frog skin epithelium, the classic model for NaCl absorption from fresh water, ATP and UTP affect the short-term regulation of transepithelial ion transport (Brodin and Nielsen, 2000b). However, in this case, transcellular amiloride-sensitive Na^+ transport increased in response to extracellular nucleotides, but Cl^- conductances seemed not to be affected (Brodin and Nielsen, 2000a). Testing whether trinucleotides mediate transintegumental ion regulation in our limnic invertebrate, we found a different situation. We were unable to detect any significant ATP sensitivity of I_{sc} in APW-adapted integuments. In contrast, adaptation to high-salt conditions increased the sensitivity of I_{sc} to 1 mmol l^{-1} ATP applied from the basolateral side (Fig. 2). Adaptation to high-salt conditions resulted in the recruitment of ATP-triggered transduction mechanisms that are not present or remain silent in a pondwater environment. I_{ami} was upregulated in integuments adapted to both conditions although the HSW-adapted preparations responded more strongly (Fig. 2, Table 2). The inhibition of this basolateral effect of ATP by amiloride clearly identified epithelial Na^+ channels localized in the apical membrane as the upregulated target, suggesting the involvement of transduction mechanisms that trigger the release of diffusible second messengers. The time course of upregulation of I_{sc} was a typical response to ATP (Fig. 1), supporting the idea of a slower messenger-mediated process.

The principal agonists for P2X receptors appear to be ATP and its derivatives, but not UTP. Neither basolateral nor apical application of 1 mmol l^{-1} UTP had a dramatic effect on I_{sc} or I_{Na} in integuments adapted to both conditions (Table 2), which suggests the existence of P2X-mediated processes. Following activation by ATP, ligand-gated P2X receptors open their intrinsic non-selective cation channel, allowing Na^+ and Ca^{2+} to pass from the extracellular fluid into the cell. Cellular release of ATP therefore provides one mechanism for controlling intracellular Ca^{2+} concentration and downstream transduction processes (Bean, 1992; Dubyak and el-Moatassim, 1993). In general, P2X- and P2Y-receptor-induced regulation of transepithelial Na^+ absorption and/or Cl^- secretion is mediated by an increase in cytosolic [Ca^{2+}] (Cuffe et al., 2000; Mall et al., 2000; McCoy et al., 1999) as a result of extracellular Ca^{2+} entering the cell *via* P2X receptors or a P2Y-induced release of Ca^{2+} from intracellular stores. However, the absence of Ca^{2+} from apical solution did not prevent the stimulating effect of basolateral ATP on Na^+ conductances in HSW-adapted integuments (Table 2). I_{ami} was again upregulated, although the response was less pronounced. A significant contribution of Ca^{2+} influx to the transduction of purinergic stimulation of I_{ami} cannot be ruled out since, for example, the previously observed upregulation of I_{sc} or the total I_{Na} in HSW-adapted integuments by ATP are reduced or in some cases even prevented after removal of apical Ca^{2+}. A further explanation may be that Ca^{2+} greatly prestimulates amiloride-sensitive Na^+ conductances, leaving fewer quiescent apical Na^+ channels to be activated in situ by further, i.e. purine-induced, mechanisms. Hydrolysis or the conversion of exogenous nucleotides by ecto-nucleotidases (Harden et al., 1997) may be one explanation for the requirement of concentrations as high as 1 mmol l^{-1} ATP to evoke the maximal effect.

At present, no agonists or antagonists are available that discriminate effectively between the families and subtypes of P2X and P2Y receptors (Norenberg and Illes, 2000; Ralevic and Burnstock, 1998; von Kugelgen and Wetter, 2000) and little is known about invertebrate orthologues and their pharmacology. Thus, it is difficult to characterize the basolateral leech ATP receptors and to prove a correlation between their relative expression levels and environmental salinity using northern blots or quantitative reverse transcriptase/ polymerase chain reactions.

P1 receptors

Adenosine has been reported to have pleiotropic actions in epithelia (McCoy et al., 1993; Olah and Stiles, 1992). Na^+ uptake by tight epithelia, e.g. in the medullary collecting duct cells of the rat kidney, is controlled by extracellular adenosine (Yagil, 1994). In A6 cell monolayers (cells from *Xenopus laevis* nephron), A1 receptors are located on the apical surface and regulate Cl^- secretion or inhibition of transepithelial Na^+ transport *via* stimulation of phospholipase C activity. On the basolateral surface, there are A_{2A} receptors, which stimulate Na^+ uptake (Casavola et al., 1996, 1997). This positive effect is believed to be due to upregulation of cyclic AMP production followed by stimulation of Na^+/H^+ exchange activity. The restricted distribution of receptors to the apical or basolateral membrane and the distinct post-receptor mechanisms are both responsible for the dual-control regulation by adenosine of transepithelial ion conductances.

In the leech integument, addition of adenosine to the apical compartment failed to have any effect on macroscopic I_{sc}. Instead, we found that activation of basolateral adenosine receptors only stimulated I_{sc} in HSW-adapted integuments and not in pondwater-adapted controls. Net Na^+ transport was not upregulated, and we assume that Cl^- conductances are targets for this effect of adenosine (Fig. 4). The slope of the current traces indicated that this activation of HSW-adapted

integuments was a monophasic but rather slow process (Fig. 3). Interestingly, the amiloride-sensitive portion of I_{Na} was enlarged at the expense of other Na^+ conductances. This effect was abolished under Ca^{2+}-free conditions.

After ligand-binding by certain purinoceptors, the signal is propagated in some cases by cyclic AMP/PKA-mediated pathways (Ralevic and Burnstock, 1998). Under normal conditions, membrane-permeant cyclic AMP activates amiloride-sensitive currents across leech integuments (Weber et al., 1995). We applied cpt-cAMP after the effect of the removal of Ca^{2+} had concluded, but could detect no convincing effect of this compound on transintegumental ion conductances, however (Fig. 5). I_{ami} across APW-adapted integuments was slightly reduced rather than stimulated, and I_{ami} across HSW-adapted integuments was unchanged. Interestingly, the overall Na^+ current, I_{Na} was unaltered by the addition of cpt-cAMP (Fig. 5), possibly indicating that apical Ca^{2+}-free conditions had stimulated the Na^+ conductances to their maximum. High concentrations of adenosine were required to induce the maximal response, so mechanisms other than binding to metabotropic receptors must be taken into consideration. The nucleosides may have entered the cells and initiated further processes.

In conclusion, the leech integument provides a useful model for investigating the regulation of ion transport in an invertebrate tight epithelium. Adaptation of leeches to high-salinity conditions altered most of the initial electrophyiological properties of the integuments but left the amiloride-sensitive Na^+ current unaffected. Absence of apical Ca^{2+} stimulated the bioelectrical activities in integuments adapted to high-salinity conditions and pondwater to a similar extent. Long-term exposure to high ($200\,mmol\,l^{-1}$) NaCl concentration conferred ATP-sensitivity to the I_{sc} and the I_{Na}, although there was little effect on control integuments. Our findings provided no evidence that UTP has any relevance in the control of ion-transport processes across the leech integument. Adenosine upregulated transepithelial non-Na^+ currents in HSW-integuments, while APW-adapted preparations were largely unaffected by this nucleoside. The surface expression of adenosine/P1 receptors was restricted to the basolateral membrane. These disparities in sensitivity to extracellular purines may be caused by differentially expressed receptors or regulatory factors during adaptation to high-salt conditions. Finally, one can argue that high-salt stress is a situation that a leech will never face under natural conditions. Nevertheless, exposure to such physiological challenges provides an opportunity for detecting variances of macroscopic variables, for instance transepithelial ion conductances, which may indicate assimilative processes in a whole-animal model and initiate more fine-tuned approaches in future projects.

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