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REGULATION OF Na^+ TRANSPORT ACROSS LEECH SKIN BY PEPTIDE HORMONES AND NEUROTRANSMITTERS

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Summary

An increase in intracellular cyclic AMP concentration stimulates transepithelial Na^+ transport across the skin of the leech *Hirudo medicinalis*, but it is unclear how cytosolic cyclic AMP levels are elevated *in vivo*. In search of this external stimulus, we performed Ussing chamber experiments to test several peptide hormones and neurotransmitters for their effect on Na^+ transport across leech dorsal integument. Although all the peptide hormones under investigation significantly affected ion transport across leech integument, none of them mimicked the effect of an experimental rise in intracellular cyclic AMP level. The invertebrate peptides conopressin and angiotensin II amide inhibited short-circuit-current- (I_{sc}) and amiloride-sensitive Na^+ transport (I_{amil}), although to slightly different degrees. The vertebrate peptide hormones 8-arginine-vasopressin and 8-lysine-vasopressin both produced an inhibition of I_{amil} comparable with that caused by angiotensin II amide. However, 8-lysine-vasopressin reduced I_{sc}, whereas 8-arginine-vasopressin induced a moderate increase in I_{sc}. The neurotransmitter dopamine, which occurs in the leech central nervous system in relatively large amounts, and its precursor L-dopamine both induced large decreases in I_{sc} and I_{amil}. However, the reactions evoked by the catecholamines showed no pronounced similarity to the effects of intracellular cyclic AMP. Two other neurotransmitters known to occur in leeches, serotonin (5-hydroxytryptamine) and γ-*n*-aminobutyric acid (GABA), had no influence on transepithelial ion transport in leech skin.

Key words: leech, *Hirudo medicinalis*, Na^+ transport, short-circuit current, amiloride, peptide, hormone, neurotransmitter, cyclic AMP, skin, angiotensin, vasopressin, dopamine.

Introduction

Living in freshwater habitats, which are strongly hypo-osmotic to its body fluids, the leech *Hirudo medicinalis* is faced continuously with the problem of water uptake and salt loss across its integument. Osmoregulation is accomplished by a collaboration between homeostatic mechanisms in the nephridia and integument. While the excretion of water and electrolytes through the nephridia has been thoroughly investigated (Zerbst-Boroffka et al., 1982; Zerbst-Boroffka and Wenning, 1986; Wenning et al., 1993), only basic information about ion transport across the leech integument is available (Weber et al., 1995; Weber et al., 1993). It has been reported that Na^+ transport across leech skin follows the classic model of Na^+ absorption through tight epithelia. Amiloride-sensitive Na^+ channels mediated apical Na^+ uptake largely by high selectivity for Na^+ over other monovalent cations; a smaller amount of Na^+ is transported by an amiloride-insensitive mechanism. A second type of Na^+ channel or unspecific cation channel could account for this amiloride-insensitive Na^+ movement, as also described for leech caecum (Milde et al., 1996). Basolaterally, Na^+ is extruded by ouabain-sensitive Na^+/K^+-ATPases.

The apical Na^+ channel has been shown to be the rate-limiting step in this process and a main target site for transport regulation. In vertebrates, the mineralocorticoid aldosterone is a major stimulus in the medium- and long-term regulation of Na^+ absorption. Peptide hormones such as the antidiuretic hormone (ADH) primarily bring about short-term regulation, which occurs within minutes. The effects of these peptides are mediated by intracellular second messengers such as adenosine 3′,5′-cyclic monophosphate (cAMP) (Orloff and Chandler, 1962). In invertebrates, aldosterone does not occur, but several peptide hormones have been identified (Osborne et al., 1982; Malecha et al., 1986; Salzet et al., 1992; Salzet et al., 1993; Salzet et al., 1994; Salzet et al., 1995; Salzet et al., 1996). The investigation of epithelial Na^+ transport and its regulation in invertebrates should be of considerable interest because details of the properties of the

putative ancestral Na^+ channel may provide information about the structure/function relationship of this transport protein. Direct comparison with the vertebrate system might clarify discrete steps in the pathways of Na^+ transport control and even disclose new regulatory principles.

Vertebrate Na^+ channels have been studied in great detail in a large number of Na^+-absorbing epithelia. The dorsal integument of the medicinal leech *Hirudo medicinalis* was recently established as a model system for the investigation of epithelial Na^+ transport in invertebrates (Weber et al., 1993). This study demonstrated that the apical Na^+ channels in this tissue seem to belong to the class of low-amiloride-affinity channels, which differ from the typical high-amiloride-affinity channels with respect to their kinetic properties and pharmacological profile (Smith and Benos, 1991). Elevation of intracellular cAMP levels increased ion transport across the leech integument; amiloride-sensitive and -insensitive Na^+ transport and the current not mediated by Na^+ movements were increased. It was shown using noise analysis that the effect of cAMP on amiloride-sensitive Na^+ transport is caused by a rise in the density of functional Na^+ channels in the apical membrane and not by an augmentation of single-channel current (Weber et al., 1993).

In the present study, we focused on amiloride-sensitive Na^+ transport since the transport systems underlying the residual Na^+ current and the Na^+-independent current are unknown. We first tested two peptide hormones that we have isolated from brain of the rhynchobdellid leech *Theromyzon tessulatum* (Salzet et al., 1992; Salzet et al., 1993; Salzet et al., 1994; Salzet et al., 1995; Salzet et al., 1996; Salzet, 2001). The peptides lysine-conopressin and angiotensin II amide can be considered as homologues of the vertebrate hormones vasopressin and angiotensin II, respectively, and may be involved in osmoregulation in leeches (Salzet et al., 1992; Salzet et al., 1993; Salzet et al., 1994; Salzet et al., 1995; Salzet et al., 1996; Salzet et al., 2000). In our study, we found well-defined effects of these peptides on ion transport across leech skin.

In addition to the peptides isolated from *Theromyzon tessulatum*, we tested two forms of vasopressin, which is known for its ability to influence Na^+ transport in vertebrate epithelia (Chalfant et al., 1993). In leech skin, both 8-arginine-vasopressin and 8-lysine-vasopressin evoked complex changes in transepithelial ion transport that showed striking parallelism to the effects caused by the invertebrate peptides. This conformity strongly underlines the suitability of the leech integument as a model system for investigations concerning the regulation of epithelial Na^+ transport.

Finally, we examined whether some well-known neurotransmitters in leeches, dopamine, its precursor L-Dopa, serotonin and γ-*n*-aminobutyric acid (GABA), affected ion transport across the dorsal integument. Two of these (dopamine and L-Dopa) had a significant inhibitory effect on transepithelial currents, while serotonin and GABA were ineffective. Examination of the mechanisms by which neurotransmitters change the transport characteristics of epithelia may greatly extend our knowledge about the transport molecules involved.

Materials and methods

Animals and tissue preparation

The methods and materials were similar to those described in detail by Weber et al. (Weber et al., 1993). Briefly, they were as follows. Leeches (*Hirudo medicinalis*) were obtained from Zaug (Giessen, Germany) and kept, without feeding, in tapwater at room temperature (20–24 °C). After hypothermal anaesthesia (10–15 min at −20 °C), the integument was dissected by two lateral incisions and the dorsal skin subsequently detached from the intestines. The internal muscular layers were removed by careful scraping with a scalpel until the diagonal muscle layers became visible. Further dissection was impossible without damaging the integument. A piece of tissue was then glued, by its serosal side, to a Lucite ring using tissue adhesive (Histoacryl blau; Braun Melsungen, Germany). This ring was then mounted in an Ussing chamber specially designed to minimize edge damage, with an aperture of 0.5 cm^2. Silicone grease was used to seal the edges on both sides of the tissue, so that the only path for ion exchange between the two half-chambers was through the tissue mounted between them. Throughout the experiment, the two compartments were continuously perfused with Ringer's solution at a flow rate of approximately 7 ml min^{-1} in the apical half-chamber and 3 ml min^{-1} in the basolateral half-chamber. All electrical variables are normalized to an area of 1 cm^2. All experiments were performed at room temperature (20–24 °C).

Solutions and chemicals

The serosal NaCl Ringer's solution contained (in mmol l^{-1}): 115 NaCl, 4 KCl, 1.8 $CaCl_2$; in the mucosal Ringer, KCl was replaced by tetramethylammonium chloride (TMA-Cl). For Na^+-free solutions, Na^+ was substituted by equimolar concentrations of the nonpermeant TMA^+. All solutions were buffered with 5 mmol l^{-1} Hepes and adjusted to pH 7.4 with 1 mmol l^{-1} Trizma-base. All these compounds were obtained from Sigma (Deisenhofen, Germany) and were, unless stated otherwise, of analytical grade. Peptide hormones (conopressin and angiotensin II amide) isolated from *Theromyzon tessulatum* (Rhynchobdellidae) were synthesized by Neosystem (France).

Electrical measurements

Current- and voltage-measuring electrodes were prepared by placing Ag/AgCl wires in 1 mol l^{-1} KCl and connecting them to the bathing compartments through 1 mol l^{-1} KCl/agar bridges. During transepithelial measurements, the tissue was voltage-clamped to 0 mV using a low-noise voltage-clamp (Van Driessche and Lindemann, 1978). The short-circuit current (I_{sc}) was recorded continuously on a stripchart recorder and a computer (Apple IIsi) with a MacLab interface and a chart recorder program (Analog Digital Instruments). The transepithelial resistance (R_t) was calculated according to

Ohm's law from the changes in I_{sc} induced by short 10 mV pulses.

Statistical analyses

All data are given as means ± standard error of the mean (S.E.M.); N is the number of experiments. Only one tissue preparation from each animal was used to analyse the experiments. Statistical significance was tested using Student's t-test (SigmaPlot, Jandel Scientific); a significance level of $P<0.05$ was accepted.

Results

Electrophysiological variables

After mounting the tissue in Ussing chambers with mucosal and serosal NaCl Ringer on the respective sides of the epithelium, transepithelial potential (V_t), short-circuit current (I_{sc}) and resistance (R_t) stabilized within approximately 60 min and remained stable for several hours. Electrophysiological variables under control conditions showed considerable variations for epithelia from different animals but exhibited only minor differences between tissue preparations from different regions of dorsal integument of a single animal. Despite the varying control values, the responses to altered experimental conditions were comparable for all tissue preparations under examination. Table 1 summarizes the electrophysiological variables of leech dorsal integument measured under control conditions.

Following the equilibration period, the amiloride-sensitive current (I_{amil}) was measured in all epithelial preparations by mucosal application of 100 μmol l^{-1} amiloride. Directly after the addition of amiloride, I_{sc} decreased by 57±4 %; the difference in I_{sc} before and during amiloride application is termed I_{amil}. A further decrease in I_{sc} of 10 % was observed when Na^+ was removed from the mucosal Ringer's solution after the application of amiloride. Transepithelial Na^+ transport (I_{Na}) was calculated as the difference between the values for I_{sc} with 115 mmol l^{-1} Na^+ and 0 mmol l^{-1} Na^+ in the mucosal Ringer's solution and made up approximately 67 % of total I_{sc}. It is not known which ions mediate the remaining 33 % of I_{sc}. I_{amil} made up 69±2 % of I_{Na}; some unknown type of amiloride-insensitive transporter must mediate the 31 % of the Na^+-dependent current remaining. Re-addition of mucosal Na^+ led to a large transient overshoot in I_{sc}. For most of the present study, we concentrated on the effects of the test substances on the amiloride-inhibitable Na^+ transport, which is the most prominent apical ion conductance in the dorsal integument of the leech.

Table 1. *Control values for the electrophysiological values of leech dorsal integument*

Short-circuit current, I_{sc} (μA cm^2)	20.6±1.1
Transepithelial potential difference, V_t (mV)	–34.6±2.1
Transepithelial resistance, R_t (kΩ cm^2)	1.7±0.1

The data are given as means ± S.E.M. out of a pool of 43 experiments.

Table 2. *Peptide sequences tested in this study*

Peptide	Sequence
Angiotensin II amide	DRVYIHPFamide
LORF	IPEPYVWDamide
GDPFLRFamide	GDPFLRFamide
Lysine-conopressin	CFIRNCPKGamide
Lysine-vasopressin	CYFQNCPKGamide
Arginine-vasopressin	CYFQNCPRGamide
Oxytocin	CYIQNCPLGamide

Leech peptide hormones

The peptide hormones lysine-conopressin, GDPFLRFamide, IPEPYVWDamide and angiotensin II amide were purified from the central nervous system of the rhynchobdellid leech *T. tessulatum* (Table 2, Salzet et al., 1992; Salzet et al., 1993; Salzet et al., 1994; Salzet et al., 1995; Salzet et al., 1996; Salzet, 2000). All peptides were added to the serosal Ringer's solution at a concentration of 5 μmol l^{-1}. We have demonstrated a diuretic effect for angiotensin II amide and GDPFLRFamide (Salzet et al., 1992), and these peptide hormones can be assumed to affect epithelial ion transport. The effects of the peptide hormones isolated from *T. tessulatum* are summarized in Table 3, together with the data for 8-arginine-vasopressin and 8-lysine-vasopressin (see below).

We have previously demonstrated that, shortly after serosal application of the leech osmoregulator factor (LORF; IPEPYVWDamide; Salzet et al., 1996, 5 μmol l^{-1}), I_{sc} decreased by 18.15±0.94 % (N=3). Of the amiloride-sensitive portion of the short-circuit current, 28.19±2.74 % was inhibited by LORF; 34.8±9.5 % of the whole Na^+-mediated current was blocked (Salzet et al., 1996).

Similarly, in Ussing chamber experiments, we investigated

Table 3. *Percentage changes in I_{sc} and I_{amil} caused by serosal application of the leech peptides lysine-conopressin, LORF and angiotensin II amide (5 μmol l^{-1}) and two forms of vertebrate vasopressin (20 μmol ml^{-1})*

	Percentage change		
Peptide	I_{sc}	I_{amil}	N
Lysine-conopressin	–27.8±7.6*	–26.8±9.6*	3
LORF[a]	–18.2±0.9*	–28.2±2.7*	3
Angiotensin II amide	–28.6±5.8*	–44.8±11.5*	3
8-Arginine-vasopressin	+24.0±9.6*	–44.7±10.4*	4
8-Lysine-vasopressin	–31.1±7.6*	–49.5±10.1*	5

Values are means ± S.E.M.

[a]Salzet et al. (1996).

I_{sc}, short-circuit current; I_{amil}, amiloride-sensitive Na^+ current.

An asterisk indicates a significant difference from the control value ($P<0.05$).

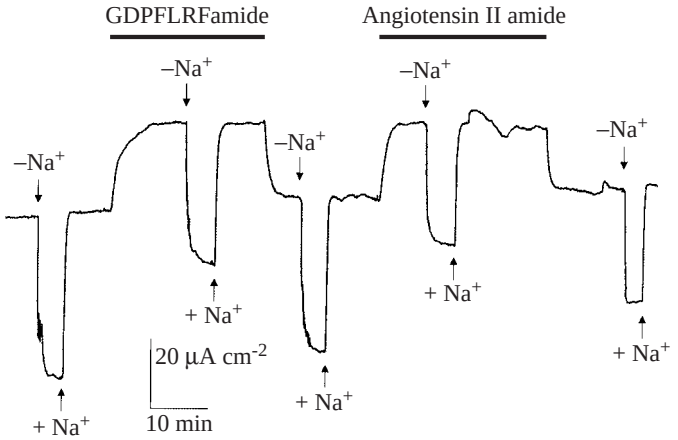

Fig. 1. Effects of GDPFLRFamide and angiotensin II amide, applied as shown by the black bars, on short-circuit current (I_{sc}) in leech caecal epithelium. Addition of GDPFLRFamide (5 µmol l^{-1}) to the serosal solution caused an immediate increase in I_{sc}, which could be reversed after a short wash. Downward deflections mirror the substitution of Na^+ by the impermeant ion tetramethylammonium in order to quantify Na^+-mediated current. The trace is representative of four similar experiments.

the effects of GDPFLRFamide and angiotensin II amide on the electrical variables of leech caecum by measuring transepithelial short-circuit current (I_{sc}), potential (V_t) and resistance (R_t). In this tissue, the majority of ion transport is mediated by an electrogenic non-selective cation channel (NSCC) (as reported previously; Milde et al., 1996) which could be further stimulated by cAMP. Serosal application of GDPFLRFamide (5 µmol l^{-1}) resulted in a large increase in I_{sc}, which could be reversed after a wash-out period (Fig. 1). Prior to application of GDPFLRFamide, apical Na^+ was withdrawn by equimolar replacement with TMA^+ to measure the Na^+-mediated current (left part of the trace). In the presence of GDPFLRFamide, the Na^+-mediated portion was unchanged, arguing that the increase in I_{sc} was mediated by an activation of Cl^- secretion. Similar results were obtained with angiotensin II amide (5 µmol l^{-1}; Fig. 1): I_{sc} and I_{amil} were reduced by 28.6±5.8 % and 44.8±11.5 %, respectively (N=3). Again, after serosal application of the peptide hormone, I_{sc} increased while the Na^+-mediated current remained stable, indicating the activation of Cl^- secretion. Since the secretion of Cl^- is always passively followed by water, both peptide hormones could cause a potential water loss across leech caecal epithelium.

Oxytocin/vasopressin peptide family

Lysine-conopressin, which differs from the vertebrate peptide vasopressin in just one amino acid residue (Table 2), caused very complex changes in ion transport across the leech integument. After a short initial decrease, I_{sc} increased temporarily until it finally stabilized at a level that was 27.7±7.6 % lower than the control value (N=3). The amiloride-sensitive Na^+ transport was inhibited by 26.8±9.6 % (Fig. 2; Table 3).

It has been found in several studies that vasopressin

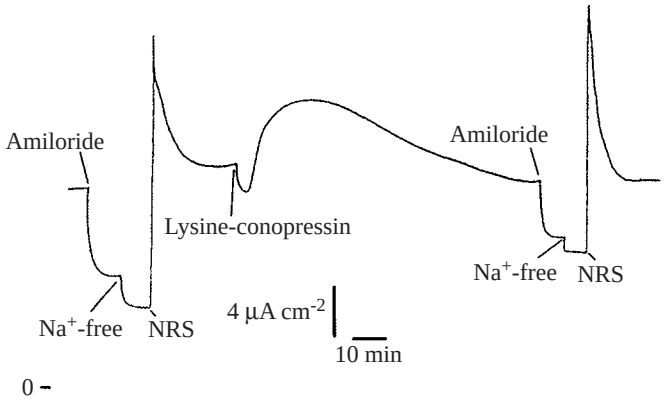

Fig. 2. The time course of the short-circuit current (I_{sc}) of leech skin. The Na^+ current was measured in the presence of 100 µmol l^{-1} amiloride and in Na^+-free solution, before and after treatment with lysine-conopressin (5 µmol l^{-1}). NRS, Na^+ Ringer's solution.

increases Na^+ transport in vertebrate epithelia. For instance, biphasic stimulation was observed in monolayers of A6 cells, an amphibian kidney cell line (Chalfant et al., 1993). To the best of our knowledge, there are no reports of the effects of vasopressin on invertebrate epithelia. Therefore, we tested whether vasopressin evoked any changes in ion transport across the model invertebrate epithelium of leech skin. We used both 8-arginine-vasopressin (Fig. 3), which is found in humans, and 8-lysine-vasopressin (Fig. 4), which occurs, for example, in pigs. The final concentration in the serosal Ringer's solution was 20 munits ml^{-1}.

8-Arginine-vasopressin caused a sequence of current changes that showed striking similarity to the reaction induced by application of lysine-conopressin. A short initial decrease in I_{sc} was followed by a large transient increase and a long-lasting subsequent decrease. Finally, I_{sc} stabilized at a value that was 24.0±9.6 % higher than under control conditions (N=4). Amiloride-sensitive Na^+ transport was inhibited by 44.7±10.4 % (Fig. 3; Table 3). In contrast to the above findings, 8-lysine-vasopressin (Fig. 4) caused a monophasic decrease in I_{sc} that lasted approximately 30 min. I_{sc} was then reduced by 31.1±7.6 % and I_{amil} was inhibited by 49.5±10.1 % (N=5).

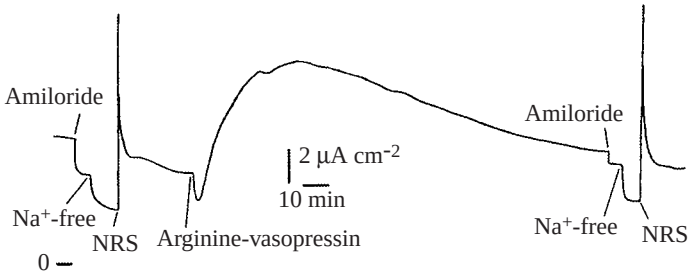

Fig. 3. The time course of the short-circuit current (I_{sc}) of leech skin. The Na^+ current was measured in the presence of 100 µmol l^{-1} amiloride and in Na^+-free solution, before and after treatment with arginine-vasopressin (20 munits ml^{-1}). NRS, Na^+ Ringer's solution.

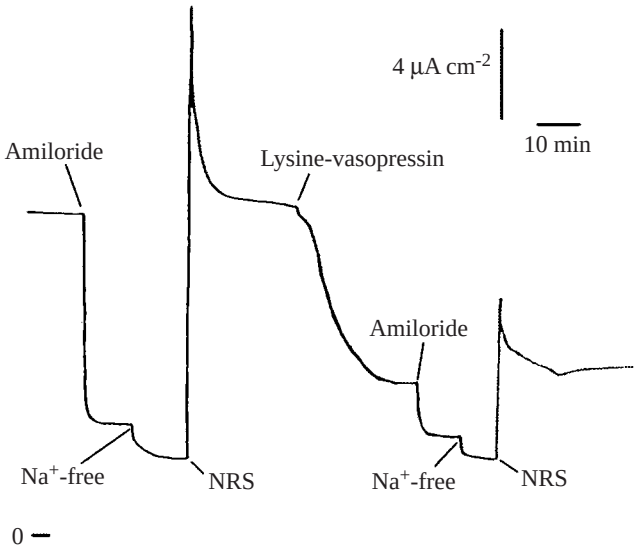

Fig. 4. The time course of the short-circuit current (I_{sc}) of leech skin. The Na^+ current was measured in the presence of 100 μmol l^{-1} amiloride and in Na^+-free solution, before and after treatment with lysine-vasopressin (20 munits ml^{-1}). NRS, Na^+ Ringer's solution.

Table 3 presents a summary of the ion transport changes in leech dorsal integument evoked by the two forms of vasopressin compared with the effects of the leech peptides tested in our study. The effects of both forms of vasopressin were fully reversible by wash-out with normal NaCl Ringer's solution.

Neurotransmitters

Dopamine is the most prominent catecholamine in *H. medicinalis* (McCaman et al., 1973) and has been shown to increase the Cl^- permeability of Retzius neurons (Sunderland et al., 1979). Dopamine, like its precursor L-Dopa, caused an inhibition of I_{sc} that was preceded by a transient increase. However, the inhibitory effect of dopamine was larger than that of L-Dopa (see below). Finally, I_{sc} and I_{amil} were reduced by 43.3±7.6 % (Fig. 5) and 67.8±5.2 % (N=3, Fig. 6), respectively, by dopamine. After application of the dopamine precursor L-Dopa, we observed a short initial increase in I_{sc} followed by a sustained decrease, which levelled off at

Table 4. *Percentage changes in* I_{sc} *and amiloride-sensitive* Na^+ *current induced by the catecholamines* L-*Dopa and dopamine*

	Percentage changes		
Neurotransmitter	I_{sc}	Amiloride-sensitive Na^+ current	N
L-Dopa	–35.0±6.3*	–44.5±14.3*	4
Dopamine	–43.3±7.6*	–67.8±5.2*	3

Values are means ± S.E.M.

An asterisk indicates a significant difference from the control value (P<0.05).

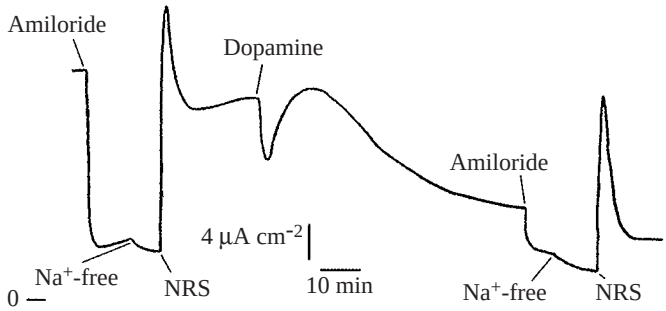

Fig. 5. The time course of the short-circuit current (I_{sc}) of leech skin. The Na^+ current was measured in the presence of 100 μmol l^{-1} amiloride and in Na^+-free solution, before and after treatment with dopamine (100 μmol l^{-1}). NRS, Na^+ Ringer's solution.

35.0±6.3 % below the control value (N=4). Amiloride-sensitive Na^+ transport was, on average, inhibited by 44.5±14.3 %. The reactions to L-Dopa and dopamine could not be reversed by wash-out with serosal Ringer's solution. Percentage changes in I_{sc} and amiloride-sensitive Na^+ current are summarized in Table 4.

Two other neurotransmitters reportedly present in leeches, serotonin (5-hydroxytryptamine) and GABA, had no effect on the ion transport properties of leech skin (data not shown).

Discussion

Active and regulated ion transport across the integument is an important feature for the survival of blood-sucking leeches in freshwater habitats. Although mechanisms for electrolyte

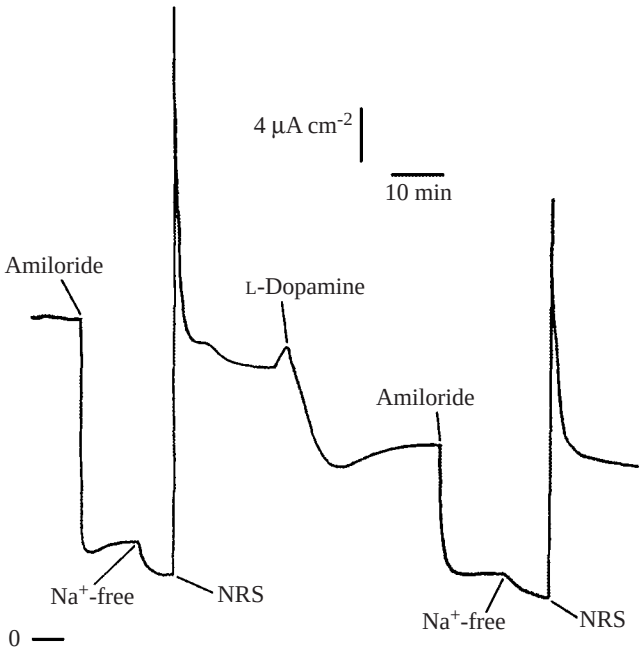

Fig. 6. The time course of the short-circuit current (I_{sc}) of leech skin. The Na^+ current was measured in the presence of 100 μmol l^{-1} amiloride and in Na^+-free solution, before and after treatment with L-dopamine (100 μmol l^{-1}). NRS, Na^+ Ringer's solution.

retention exist in the nephridia (Zerbst-Boroffka and Wenning, 1986), salt loss *via* the urine cannot be completely prevented. This loss has to be compensated by active uptake of ions through the integument against immense concentration gradients. Leeches can be found in temporary ponds and streams (Sawyer, 1986), so they are faced with changing environmental ion concentrations caused, for example, by seasonal alterations in evaporation and rainfall. The animals must therefore be able to control their salt uptake through the integument very closely to maintain constant internal electrolyte concentrations.

In vertebrates, water and electrolyte homeostasis depend largely on fast-acting peptide hormones such as the octapeptide angiotensin II and the cyclic nonapeptides of the oxytocin/vasopressin superfamily. Therefore, these or related molecules are likely candidates for regulators of ion transport across leech skin.

A wide range of peptide hormones has been identified in invertebrates (Al-Yousuf, 1990; Salzet, 2001). The existence of vasopressin/oxytocin-like substances has been demonstrated in numerous species from different invertebrate phyla (Cruz et al., 1987; Van Kesteren et al., 1992; Van Kesteren et al., 1996; McMaster et al., 1992; Oumi et al., 1994; Oumi et al., 1996; Salzet et al., 1994; Satake et al., 1999; Fujino et al., 1999; Salzet, 2001). In several species, such as earthworms (Kinoshita and Kawashima, 1985; Oumi et al., 1994; Oumi et al., 1996; Satake et al., 1999; Fujino et al., 1999), insects (Strambi et al., 1978), molluscs (Cruz et al., 1987; Van Kesteren et al., 1992; Van Kesteren et al., 1996; McMaster et al., 1992) and the rhynchobdellid leech *Theromyzon tessulatum* (Salzet et al., 1994; Salzet, 2001), it has been suggested that peptides belonging to the oxytocin/vasopressin superfamily are involved in osmoregulation.

In the insect *Locusta migratoria*, Proux et al. (Proux et al., 1987) demonstrated that the diuretic effect of a vasopressin-like substance was mediated by intracellular cAMP. In annelids, Oumi et al. (Oumi et al., 1996) confirmed the existence of an oxytocin-related peptide in oligochaete annelids by the purification of annetocin (a peptide with seven residues of nine in common with leech lysine-conopressin). Its cDNA has been cloned from a lumbricid earthworm *Eisenia foetida* (Satake et al., 1999). This peptide, as in vertebrates, is associated with a neurophysin, sharing approximately 40 % sequence identity to vertebrate neurophysin (Satake et al., 1999). In annelids, both annetocin and lysine-conopressin may be involved in osmoregulation *via* pulsatory contractions and bladder-shaking movements of the nephridia (Salzet et al., 1993; Oumi et al., 1996).

Lysine-conopressin inhibits the amiloride-dependent transient Na^+ current before greatly stimulating it in *Hirudo medicinalis* stomach or integument preparations (Table 3). Moreover, lysine-conopressin and annetocin induce egg-laying in the earthworm, and this is accompanied by secretion of mucus from the clitellum (Fujino et al., 1999). This confirms that, over the course of evolution, the function of the oxytocin/vasopressin peptide family for both osmoregulation and reproduction has been conserved. Moreover, we have demonstrated that, in leech skin, both LORF (Salzet et al., 1996) and lysine-conopressin decreased I_{sc} and amiloride-sensitive Na^+ transport. Lysine-conopressin, in contrast to LORF, induced a transient increase in I_{sc}, which may correspond to the increase in current observed after intracellular cAMP levels are increased.

During continuous perfusion with Ringer's solutions containing membrane-permeable cAMP analogues, the intracellular cAMP concentration is permanently elevated. When a hormonal stimulus is applied, intracellular cAMP levels might, after an initial increase caused by an increase in production, be reduced to normal as a result of the desensitization of receptors and the degradation of cAMP. Such a time course for the cellular cAMP concentration might explain the short-term increase in cellular cAMP content after conopressin application observed in our experiments. However, the inhibition of transepithelial Na^+ transport caused by both substances cannot be mediated by intracellular cAMP, so which second messenger is responsible for this reaction? Cytosolic Ca^{2+} is a possible candidate, since it is involved in Na^+ feedback inhibition (Chase, 1984). In this regulatory process, a rise in intracellular Na^+ concentration caused by an increased uptake of Na^+ through apical amiloride-sensitive Na^+ channels reduces Ca^{2+} extrusion *via* basolateral Na^+/Ca^{2+} exchangers, thereby leading to an elevated Ca^{2+} concentration inside the cells. Ca^{2+} blocks apical Na^+ channels either directly (Garty and Asher, 1986) or indirectly *via* protein kinase C (Smith and Benos, 1991). The proposed dual action of lysine-conopressin through two different second-messenger systems would automatically require the presence of two receptor types for this peptide.

For the third leech peptide hormone we tested, angiotensin II amide, a diuretic effect has been demonstrated in the leech *Theromyzon tessulatum* (Salzet et al., 1995; Salzet, 2001). In leech skin, angiotensin II amide caused a monophasic decrease in I_{sc} and amiloride-sensitive Na^+ current. The time course of current changes closely resembled that after application of LORF (Salzet et al., 1996); however, the degree of inhibition was apparently greater. Because of its inhibitory effect on Na^+ transport across leech integument, angiotensin II amide, like LORF, must be ruled out as the first messenger of the well-known transport stimulation induced by cytosolic cAMP (Wenning et al., 1993).

In addition to the leech peptides, we tested two types of vertebrate vasopressin: 8-arginine-vasopressin, which is active in humans, and 8-lysine-vasopressin, which is found, for instance, in pigs. Vasopressin is well-known for its cAMP-mediated stimulatory effect on Na^+ transport through amiloride-sensitive Na^+ channels in epithelia such as frog skin (Kamemoto and Oyama, 1985) and urinary bladder (De With et al., 1988). In leech skin, 8-lysine-vasopressin induced a monophasic inhibition of I_{sc} and amiloride-inhibitable Na^+ currents. This reaction was similar to the effects seen after application of LORF (Salzet et al.,1996) or angiotensin II amide. 8-Arginine-vasopressin, like lysine-conopressin,

evoked biphasic current changes: a transient increase in I_{sc} was followed by an eventual decrease. The degree of inhibition of Na^+ transport in our experiments was similar for leech and vertebrate peptide hormones; however, a direct comparison was not feasible because the biological activity of the leech peptides was unknown. In contrast to our results in leech skin, Zerbst-Boroffka and Wenning (Zerbst-Boroffka and Wenning, 1986) found no influence of arginine-vasopressin or lysine-vasopressin on urine flow in the same species.

Numerous neurotransmitters and some of their receptors have been identified in leeches (Salzet et al., 1998). In addition to the monoamines serotonin, dopamine and octopamine, acetylcholine and the amino acids GABA, L-glutamic acid and glycine have been detected (Sawyer, 1986). The catecholamine dopamine has been shown to influence ion transport in different epithelia: in the gills of the crustacean *Callinectes sapidus*, it increases Na^+ influx (Kamemoto and Oyama, 1985), while it inhibits the Na^+/H^+ exchanger in the renal proximal tubule (Gesek and Schoolwerth, 1990). In *Necturus maculosus* gallbladder, dopamine activates the apical Cl^- conductance and inhibits fluid absorption (Subramanyam et al., 1995). Since all these effects are brought about by cAMP, it seemed quite reasonable to assume that dopamine might also be the physiological trigger for cAMP-mediated stimulation of Na^+ transport across leech skin. However, both dopamine and its precursor L-Dopa inhibited ion transport across leech skin, a reaction that cannot be mediated cAMP. So, in addition to the peptide hormones we tested, one could speculate, for instance, that intracellular Ca^{2+}-dependent signalling pathways might transfer the inhibition of Na^+ transport induced by dopamine binding to an extracellular receptor. However, since the concentrations used in our experiments were considerably higher than those found in living organisms, non-specific effects are quite likely. This assumption is substantiated by the fact that the dopamine precursor L-Dopa produced an almost similar level of inhibition to that caused by dopamine itself. In addition, the effects of both dopamine and L-Dopa on Na^+ transport across leech integument were irreversible. Therefore, a physiological role for these catecholamines in the regulation of Na^+ transport across leech skin seems very unlikely. This is corroborated by the observation that lower and, hence, more physiological concentrations of dopamine had no effect on urine flow in *Hirudo medicinalis* (Zerbst-Boroffka and Wenning, 1986). Moreover, it has been shown that dopamine does not alter the bioelectric properties of leech caecum (Milde et al., 1996).

In leeches, serotonin is known to play an important role in the control of swimming and feeding behaviour (Lent and Dickinson, 1988), and it seems to be involved in non-associative learning (Catarsi et al., 1990). In the tactile sensory neurones (T-cells) of *H. medicinalis*, serotonin blocks the Na^+/K^+-ATPase *via* intracellular cAMP (Catarsi et al., 1993). Cyclic AMP also mediates the serotonin-induced stimulation of current flow across the body wall of the freshwater snail *Lymnaea stagnalis* (De With et al., 1988). Ion transport across leech integument, however, was not affected by serotonin, although the concentrations in our experiments were higher than those applied to snail skin by De With et al. (De With et al., 1988). Our results are in good agreement with the observations that serotonin neither affects cation transport across leech caecum (Milde et al., 1996) nor alters urine flow in *Hirudo medicinalis* (Zerbst-Boroffka and Wenning, 1986). Cline (Cline, 1986) has presented convincing evidence that GABA acts as an inhibitory transmitter in leech body wall muscles. Local application to muscle fibres induced an increase in conductance and a depolarization. However, the transport properties of leech skin were insensitive to GABA, as is the case for leech caecum (Milde et al., 1996).

In conclusion, our study revealed that several peptide hormones and neurotransmitters are capable of affecting ion transport across leech integument. Only conopressin, a peptide hormone isolated from the leech *Theromyzon tessulatum*, induced a transient increase in current. All the other substances we tested inhibited Na^+ transport across the skin of *Hirudo medicinalis*. The observed similarities in the actions of the peptide hormones from leeches and vertebrates on the bioelectrical activity of leech skin suggest that both transmitter and receptor molecules have been strongly conserved during evolution. However, the effects of vasopressin on the skin of *Hirudo medicinalis* differ from those observed in vertebrate epithelia. This disparity may be due to changes in the effectors molecules, i.e. ion channels, or to coupling of the receptors to different second-messenger systems. The observed effects of neuroactive substances on epithelial cells were unexpected; however, such reactions may not be as unique. In a recent study Blank et al. (Blank et al., 1996) found that nicotine, which is generally thought to act exclusively on excitable cells, inhibited Na^+ transport in cultured nasal epithelial cells.

This study again emphasizes the virtue of leech dorsal integument as a model for studying epithelial Na^+ transport. However, conclusions from investigations in invertebrate models cannot always be transferred directly to vertebrate epithelia. Clearly, much additional work on vertebrate epithelia and on invertebrate models is required before a complete understanding of transepithelial Na^+ transport and its regulation is accomplished.

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