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A Model for Fluid Secretion in *Rhodnius* Upper Malpighian Tubules (UMT)

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Abstract. We have measured fluid secretion rate in *Rhodnius prolixus* upper Malpighian tubules (UMT) stimulated to secrete with 5-OH-tryptamine. We used double perfusions in order to have access separately to the basolateral and to the apical cell membranes. Thirteen pharmacological agents were applied: ouabain, Bafilomycin A_1, furosemide, bumetanide, DIOA, Probenecid, SITS, acetazolamide, amiloride, DPC, $BaCl_2$, pCMBS and DTT. These agents are known to block different ion transport functions, namely ATPases, co- and/or counter-transporters and ion and water channels. The basic assumption is that water movement changes reflect changes in ion transport mechanisms, which we localize as follows: (i) At the basolateral cell membrane, fundamental are a Na^+-K^+-$2Cl^-$ cotransporter and a Cl^--HCO_3^- exchanger; of intermediate importance are the Na^+-K^+-ATPase, Cl^- channels and Rp-MIP water channels; K^+ channels play a lesser role: (ii) At the apical cell membrane, most important are a K^+-Cl^- cotransport that is being located for the first time, a V-H^+-ATPase; and a Na^+-H^+ exchanger; a urate-anion exchanger and K^+ channels are less important, while Cl^- channels are not important at all. A tentative model for the function of the UMT cell is presented.

Key words: Secretion in Insects — Ion epithelial transport — Insect ion transport — Fluid secretion — *Rhodnius prolixus* — Malpighian tubules — Secretion

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Symbols and abbreviations: ACTZ, acetazolamide; cAMP, cyclic adenosine-mono-phosphate; DIOA, [(dihydroindenyl)oxy] alkanoic acid; DPC, diphenylamine-2-carboxylate; DTT, dithiothreitol; 5-HT, 5-hydroxy-tryptamine; IR, insect ringer; J_v, secretion rate [nl/cm^2.s]; pCMBS, parachloro-mercuri-benzene-sulphonate; Rp-MIP, *Rhodnius prolixus* water channels; SITS, 4-acetamido-4′-isothiocyanatostilbene -2,2′-disulfonic acid; UMT, upper malpighian tubules.

Introduction

At variance with other insects, *Rhodnius prolixus* is a species adapted to long starvation periods, followed by sporadic large blood meals [52, 63]; after the meals, *Rhodnius* lose most of the ingested volume in a few minutes, because their upper Malpighian tubules (UMT) quickly excrete most of that volume as a "primary" urine quasi isosmotic to their hemolymph [37, 38]. For example, a 20 mg fifth-instar *Rhodnius* can ingest about 300 μl of blood in some 15 minutes, and is able to get rid of half that volume in a comparable time. Hormonal stimuli from the central nervous system regulate secretion. Thus, *Rhodnius* UMT that are in a "resting" or "nonfunctional" state during the starvation period secrete volume at rates of some 50 nl/s.cm^2 of tubule surface area a few minutes after the blood meal [26, 39]. This is due to the influence of diuretic hormones, one of them being 5-hydroxy-tryptamine (5-HT) [2, 41]. Urine is formed by the UMT as the result of the interplay of several cell membrane channels, transporters and intracellular signaling molecules [37, 39, 42, 43]. In *Rhodnius*, and in other insects, the easy access of the basolateral Malpighian tubule cell membrane has led to elucidation in general terms of the cellular basis for such mechanisms [6, 8, 25, 29, 31, 35, 36, 55, 62], but their location at the apical cell membrane remains unclear.

Rhodnius UMT secretion consists usually of some 90 mM of both NaCl and KCl [38, 50]. At the apical cell membrane, ion secretion by Malpighian tubules of *Rhodnius* and of *Aedes* has been proposed to be driven primarily by an apical vacuolar-type

H^+-ATPase. This electrogenic transport of H^+ from cell to lumen is thought to activate an amiloride-sensitive exchange of cytoplasmic K^+ and/or Na^+ for luminal H^+ [6, 42, 62]. At the basolateral cell membrane, a Na^+-K^+-$2Cl^-$ cotransporter has been proposed for *Rhodnius* UMT, based on the effect of bumetanide, Na^+-free saline and Cl^--free saline on fluid secretion and transepithelial potential [29, 48]. This cotransporter and/or a DIOA-sensitive K^+-Cl^- cotransporter have also been related to the basolateral entry of ions into Malpighian tubules of *Aedes* [25], *Drosophila* [36] and *Formica* [35]. In addition, it has been proposed that water passively follows ion movements in insects [40, 51] as in other epithelia [61]. As just mentioned, most of the information has been obtained at the peritubular side of the preparations. Because of the difficult access to the lumen, little is known about the direct effect on secretion of agents that act on mechanisms located at this cell membrane.

In the present paper, we have used *Rhodnius* UMT because their apical and basolateral cell aspects have been made accessible by double perfusion [26]. In addition, *Rhodnius* UMT is a cylindrical monolayered epithelium made up of a single, well characterized cell type [4, 27, 57, 59, 65]. Fifth-instar UMT have a convenient lumen diameter suitable for lumen perfusion, while at the same time they can be perfused from the peritubular side [26]. We have used this double perfusion to investigate the effects on UMT secretion of pharmacological agents known to alter the function of various channels, cotransporters and ATPases, adding these agents either to the peritubular, or to the luminal perfusion fluids, respectively, to explore whether they act either at the basolateral, the luminal or at both cell membranes. In this approach, we follow the two membrane models [33, 34].

The main conclusions of this paper, based also in previous experimental work on *Rhodnius* and other insects, are that secretion originates in and is maintained by several transport mechanisms, which are localized as follows: (i) At the basolateral cell membrane, the most relevant are a Na^+-K^+–$2Cl^-$ cotransporter and a Cl^--$HCO3^-$ exchanger. The role of the Na^+-K^+-ATPase and of Cl^- channels is significant but intermediate, while K^+ channels seem to be of lesser importance. Rp-MIP water channels are present. We find no evidence for a K^+-Cl^- cotransporter. (ii) At the apical cell membrane, the most relevant transport mechanisms are a K^+-Cl^- cotransporter that we localize for the first time at this level; a V-H^+-ATPase and a Na^+-H^+ exchanger. A urate-anion exchanger and K^+ channels are also present, while Cl^- channels do not seem to participate significantly. The present experiments allow us to build a tentative model for the possible function of the UMT cell, which includes a paracellular pathway for fluid flow.

Materials and Methods

Fifth instar *Rhodnius prolixus* were used 1–4 weeks after molting. The insects were bred and kept in the laboratory at 28°C and 95% humidity in incubators. They were fed (across a rubber membrane) once a month throughout their instars with heparinized (2 units/ml) sheep blood. UMT were dissected out of the insect [26, 27, 59]. These UMT are transparent with few cytoplasmic concretion bodies. UMT of insects fed with hens' blood (which has a high calcium concentration) are opaque, with concretions, which render them cumbersome for some studies [44, 57].

Bathing Solutions

Solutions were used right after being bubbled with 95% O_2-5% CO_2. The basic solution was insect ringer (IR) with composition (in mM), NaCl, 129; KCl, 8.6; NaH_2PO_4, 4.3; $NaHCO_3$, 10.2; $CaCl_2$, 2.0; glucose, 34; alanine, 3; pH, 7.35–7.45; osmolality, 340 mOsm/kg (freezing point determination), i.e., isosmotic to the insects' hemolymph [38]. To stimulate secretion, 5-HT to a concentration of 2 μM was added to the bath [38]. IR was used as bath and as lumen perfusion fluids. Thirteen pharmacological agents were added individually to IR at the concentrations known to have a clear-cut inhibitory effect on the chosen target process, as detailed in Results.

Secretion Rate Measurements

The method has been detailed [26, 27, 59]. Briefly, a UMT was transferred to a drop of 75–100 μl of oxygenated IR thermostated at 25°C, which had been kept under water-saturated and oxygenated liquid paraffin in a specially designed chamber. Some 20 mm of the blind end were left in the IR drop. The cut end was pulled out and secured around a hydrophilic pin 10 mm away to collect secretion. The diameter (d) of the spherical drops of tubule secretion was measured every 5 minutes using a dissecting microscope with a micrometer scale. Secreted volume (V) was calculated from the total spherical volume appearing at the cut end ($V = \pi d^3/6$). Secretion rate (J_v) was expressed per second, per cm^2 of basement membrane surface area (A), calculated from the UMT length and diameter; the UMT being taken as a cylinder [26, 27, 59].

Lumen Perfusion

The method has been described in detail [26, 27] and need only be briefly outlined here: a UMT was transferred to a drop of 75–100 μl of oxygenated IR thermostated at 25°C, which had been kept under water-saturated and oxygenated liquid paraffin in a specially designed chamber. The UMT's blind end was held with specially designed forceps, on top of a Sylgard support, under the liquid paraffin. The tubule wall was cannulated with a sharp beveled pipet containing IR (luminal perfusion fluid), which was then injected by means of a Hampel pump [13], at a rate of 10 nl/min. Lumen perfusate was collected periodically with the added tubule secretion at the tubule's open end. A region of the external surface of the tubule's length was bathed with IR (peritubular bathing fluid), which could be exchanged at a fixed rate, by means of micropipettes aligned perpendicular to the tubule's length. Thus there was appropriate mixing of the external bathing solution as well as of the lumen. When desired, secretion was started by addition to the bath of 5-HT to a concentration of 2-μM [38]. The diameter (d) of the spherical drop made up of perfusate and tubule secretion was measured every 5 minutes, and secreted volume (V) was calculated

Table 1. Effect of pharmacological agents added to the bath (basolateral membrane) at the indicated concentrations, on *Rhodnius* UMT secretory rate (J_v). All after stimulation with5-HT; the Control condition has no further additions.

Agent	J_v (Control)	Paired Difference J_v (Exp)–J_V (Control)	p	Change %
Ouabain (0.2 mM)	26.9 ± 2.1	7.7 ± 1.3 (5)	0.004	+29
Bafilomycin (1 μM)	40.7 ± 7.7	−20.0 ± 5.8 (8)	0.01	−49
Furosemide (10 μM)	23.9 ± 2.5	−7.9 ± 1.7 (9)	< 0.001	−33
Furosemide (20 μM)	22.5	11.6 (1)	—	—
Furosemide (0.1 mM)	19.2 ± 5.5	−8.3 ± 4.8 (7)	< 0.001	−43
Furosemide (0.5 μM)	26.6 ± 5.3	−21.0 ± 3.3 (4)	< 0.001	−79
Bumetanide (50 μM)	17.6 ± 2.7	−14.0 ± 2.3 (5)	< 0.004	−80
DIOA (10 μM)	40.8 ± 4.2	0.2 ± 4.0 (10)	NS	—
Probenecid (1 mM)	26.3 ± 2.5	−6.0 ± 3.4 (7)	NS	—
SITS (1 mM)	16.4 ± 3.1	−14.8 ± 3.0 (9)	< 0.0012	−90
ACTZ (1 mM)	30.5 ± 3.9	4.3 ± 2.7 (7)	NS	—
Amiloride (0.4 mM)	32.8 ± 6.9	−6.0 ± 6.5 (5)	NS	—
DPC (100 μM)	16.3 ± 2.9	6.4 ± 1.5 (6)	< 0.007	+39
$BaCl_2$ (2 mM) (K^+ 9 mM)	26.6 ± 11.0	5.7 ± 12.3 (6)	NS	—
$BaCl_2$ (20 mM)(K^+ 9 mM)	18.0 ± 3.9	2.1 ± 4.5 (8)	NS	—
$BaCl_2$ (6 mM)(K^+ 152 mM)	21.5 ± 8.0 (5)	−8.2 ± 8.2 (5)	NS	—
$BaCl_2$ (20 mM)(K^+ 152 mM)	27.7 ± 5.2 (4)	−3.3 ± 5.7 (4)	NS	—
pCMBS (500 μM)*	41.0 ± 5.7	−22.1 ± 5.2 (8)	0.004	−54
DTT (5 mM)*	41.7 ± 4.8	4.3 ± 4.7 (16)	NS	—

J_v (nl/cm^2.s), is given as mean ± SEM. The different columns show control data; paired difference between control and experimental (*Exp*) data; the number of paired experiments is given in parenthesis. p is the probability that the difference between control and experimental data is significant. NS, non-significant. *From Reference 27.

from the total spherical volume appearing at the cut end (after subtraction of the perfusion volume). J_v (nl/cm^2.s), the secretion rate, was calculated as described above [26, 27]. All chemicals were from Sigma Chemical (St. Louis, MO). DIOA was kindly donated by Dr. J. C. Ellory of Oxford.

Results

Dose Response to 5-HT

The 5-HT concentration that maximally stimulates *Rhodnius* UMT secretion is well known [26, 27, 37, 38, 59]. However, since our experimental design involved use of 5-HT to stimulate fluid secretion and of agents to after it, we re-examined UMT secretory response to various 5-HT doses in the batch of animals used in the present work. Figure 1 shows half-stimulation of J_v at 30 nM 5-HT. The maximal effect (J_v^{max}) was obtained with 100 nM or higher 5-HT concentrations. In the experiments that follow, 2 μM 5-HT was used to insure maximal stimulation.

Effect on J_v of Pharmacological Agents Added to the Bath

There was variation in J_v values among different groups of animals but, as expected, the spread was smaller within a given group. In the present studies each tubule was its own control, as follows: a UMT was set up in the chamber and 5-HT stimulated (*see* Methods). Secreted volume was measured every 5 minutes for an initial 20 minutes control period. Then the chosen agent was added to the bath while secreted volume was measured for a further 30–40 minutes experimental period. J_v values calculated for the two periods were analyzed as paired data. Results with the 13 pharmacological agents used at the basolateral cell aspect are shown in Table 1. They were used at the concentrations known to have a clear-cut inhibitory effect on the chosen target process.

Ouabain, a known inhibitor of the Na^+-K^+-ATPase at a concentration of 200 μM [16], stimulated secretion in 29%. The *Drosophila* Malpighian tubule V-H^+-ATPase inhibitor Bafilomycin A_1 [5] at a concentration of 1 μM inhibited J_v by 49%. The mammalian renal Na^+-K^+-2Cl^- cotransport inhibitor furosemide [58] was used at concentrations ranging from 10 μM to 1 mM. It curtailed J_v with a half-maximal effect at ~20 μM; at 0.5 mM it inhibited J_v by 79%. 1 mM Furosemide did not further inhibit J_v (*data not shown*). Bumetanide (50 μM), the mammalian renal Na^+-K^+-2Cl^- cotransport inhibitor [58], decreased J_v by 80%. Higher bumetanide concentrations showed no further effect (*data not shown*). The red cell K^+-Cl^- cotransport inhibitor DIOA [18] at a concentration of 10 μM did not show any effect on J_v. No effect of the renal urate-anion exchange inhibitor Probenecid at a concentration of 1 mM [53] was observed. The mammalian renal Cl^--HCO_3^- exchange inhibitor SITS [11] at a concentration of 1 mM curtailed J_v by 90%. No effect of the mammalian kidney and *Rhodnius* carbonic anhidrase inhibitor ACTZ [22, 45] at a concentration of 1 mM was observed. The Na^+H^+-exchange inhibitor amiloride at

Table 2. Effect of pharmacological agents added by lumen perfusion to the apical membrane at the indicated concentrations, on *Rhodnius* UMT secretory rate (J_v). All after stimulation with 5-HT; the control condition has no further additions.

Agent	J_v (Control)	Paired Difference J_v (Exp) — J_v (Control)	p	Change %
Ouabain (1 mM)	15.8 ± 1.2	7.2 ± 2.0 (3)	< 0.001	+46
Bafilomycin (10 μM)	40.3 ± 3.9	−9.2 ± 4.7 (5)	NS	—
Furosemide (0.1 mM)	17.0 ± 2.7	−13.5 ± 3.1 (4)	< 0.02	−79
Bumetanide (50 μM)	36.1 ± 7.8	−29.5 ± 8.0 (5)	0.03	−82
DIOA (10 μM)	33.3 ± 9.6	−31.9 ± 9.9 (3)	< 0.005	−96
Probenecid (1 mM)	43.9 ± 11.0	−26.1 ± 7.6 (6)	< 0.02	−59
SITS (1 mM)	39.0 ± 3.6	1.4 ± 4.7 (11)	NS	
ACTZ (1 mM)	32.0 ± 1.9	12.0 ± 1.3 (8)	< 0.001	+38
Amiloride (0.4 mM)	32.7 ± 6.9	−25.1 ± 6.1 (5)	< 0.015	−77
DPC (0.1 mM)	44.2 ± 3.7	0.3 ± 3.4 (5)	NS	—
DPC (0.5 mM)	40.2 ± 3.7	0.0 ± 3.4 (5)	NS	—
$BaCl_2$ (2-20 mM)(K^+ 9 mM)	18.4 ± 3.8	12.9 ± 3.9 (10)	< 0.004	+70
pCMBS(2mM)*				

Luminal perfusion rate was 10 nl/min/tubule. J_v (nl/cm^2.s), is given as mean ± SEM. The different columns show control data; paired difference between control and experimental (Exp) data; the number of paired experiments is given in parenthesis. p is the probability that the difference between control and experimental data is significant. NS, non-significant. *Experiment not possible due to cell desquamation into the lumen. DTT had no effect.

a concentration of 0.4 mM, known to inhibit this exchanger at the apical membrane of the proximal kidney tubule cells [12], did not affect J_v. DPC (0.1 mM), the mammalian renal Cl^- channel blocker [20], increased J_v by 39%. The mammalian kidney K^+ channel blocker barium [3] was also tried at concentrations of 2 and 20 mM added to IR with its normal [K^+] of 9 mM and to IR with a high [K^+] of 152 mM, respectively. The controls contained no $BaCl_2$. No significant effect of barium was observed at either concentration of [K^+] and of barium. Addition of 500 μM pCMBS, the mammalian renal proximal tubule aquaporin water channel blocker [60], inhibited secretion by 54%. This effect was reverted by 5 mM DTT [27]. It is known that DTT counteracts the inhibitory effect of pCMBS on aquaporins [60]. DTT alone did not alter J_v [27].

Lumen Perfusion Does not Alter J_v

Figure 2 shows secreted volume as a function of time of two series of 8 experiments each. In the resting condition (*filled circles*, no 5-HT) experiments started without lumen perfusion. Notice that lumen perfusion, at a rate of 10 nl/min, which begun at minute 17.5 (↓), did not alter secretion. Secretion accumulated to values of less than 50 nl during the time of the experiment (40 minutes). These values are within the error of our measurements in the perfused condition. In the stimulated series, 5-HT was added to the bath at time 0 (*empty circles*) without lumen perfusion. Lumen perfusion started (↓) at minute 17.5. On average, secretory rates from minute 20 onwards were not statistically different from those before minute 15. Comparisons were made between data obtained with the same tubule before and after perfusion (paired data). In this series of experiments, mean J_v was 55.6 ± 6.2 nl/cm^2 of basement membrane area per second. In another set of experiments (not shown) there was no statistical difference in J_v between early and late perfusions (i.e., perfusions carried out after minute 17.5, and after minute 30, respectively). Therefore, we conclude that lumen perfusion does not alter UMT secretory rate, in contrast with results of others [46, 47] at rather high perfusion rates.

Effect on J_v of Pharmacological Agents Added to the Lumen

These studies were performed as follows: a tubule was set up in the perfusion chamber and stimulated with 5-HT (*see* Methods). Secreted volume was measured every 5 minutes for an initial period of 20 minutes (control period). Then lumen perfusion with IR begun (at the rate of 10 nl/min) with the chosen experimental agent and measurements of secretion rate continued for another 40 minutes (experimental period). J_v was calculated for the two periods. The concentrations of the pharmacological agents used and the results obtained are shown in Table 2. They were analyzed as paired data. The fact that lumen perfusions are difficult limited the number of experiments and the test of a broader range of concentrations, which would have been ideal for some of the pharmacological agents that were tried.

Ouabain (1 mM) increased secretion in 46%. Bafilomycin A_1 (10 μM) did not show any action from the lumen, in contrast with its effect from the bath. Furosemide (100 μM) inhibited J_v by 79%, and 50 μM bumetanide inhibited fluid secretion by 82%. DIOA (10 μM) inhibited J_v by 96%, in sharp contrast with its lack of action from the bath. 1 mM Probenecid

inhibited 59% of J_v. SITS (1 mM) did not alter secretion, while it had a clear effect from the bath side. ACTZ (1 mM) increased secretion by 38%. Amiloride (0.4 mM) inhibited secretion by 77%. No effect was noticed with either 0.1 or 0.5 mM DPC, showing a difference with its clear effect (0.1 mM) from the bath side. To investigate the possible role of K^+ channels at the UMT apical cell membrane, $BaCl_2$ was added at concentrations from 2 to 20 mM to the apical perfusion fluid. It was found that at the barium concentrations tested, $BaCl_2$ significantly stimulated secretion by 70% ($p < 0.004$).

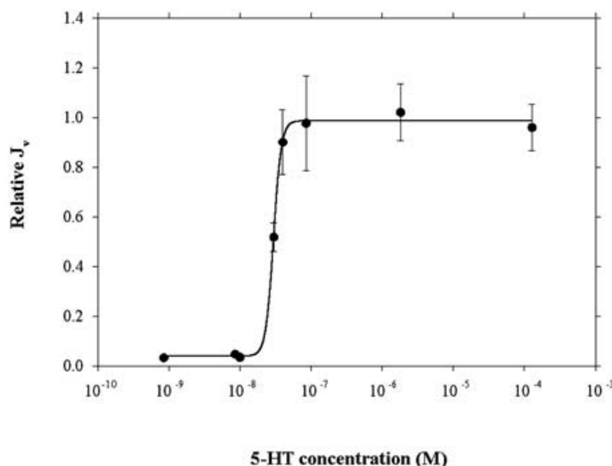

Fig. 1. Dose response curve. J_v values, expressed as a fraction of the maximal J_v values obtained, plotted as a function of the bath 5-HT concentration in moles. Half maximal and maximal stimulation were observed with 30 nM and with 100 nM or higher 5-HT concentrations, respectively.

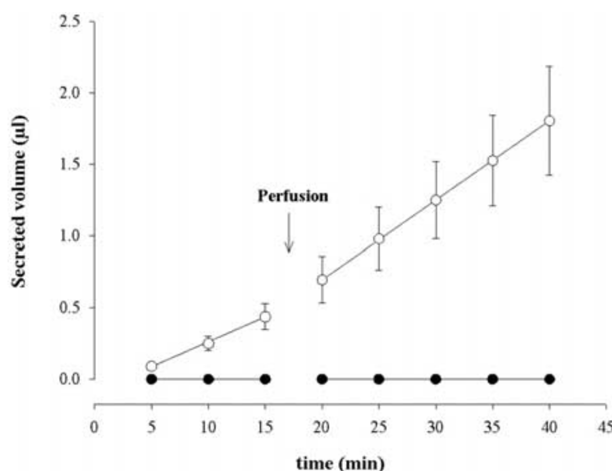

Fig. 2. Effect of lumen perfusion on UMT secreted volume. Secreted volume is plotted as a function of time. Experiments begun without perfusion. Perfusions started at minute 17.5 (↓). In the first series of experiments (8 experiments) in resting condition (●), no peritubular 5-HT was added. In the second series (8 experiments) in stimulated condition (o), 5-HT had been added at time 0.

Discussion

As mentioned in the introduction, *Rhodnius* withstand long starvation periods. Under those circumstances, their UMT barely secrete spontaneously. After a meal, some hormones (one of them being 5-HT) and other factors are released into the hemolymph to stimulate UMT secretion. 5-HT is known to act by elevating the intracellular cAMP concentration [37, 38], thus activating several mechanisms that lead to the large secretion rates observed.

Previous models for ion and fluid transport in *Rhodnius* UMT depict at the basolateral cell membrane channels for K^+ and a Na^+-K^+-$2Cl^-$ cotransporter and at the apical cell membrane, a V-H^+-ATPase, a cation (Na^+ and/or K^+) vs H^+ exchanger, and a Cl^- channel. As already mentioned, these models are based on basolateral cell membrane solution changes [30, 42]. In the present experiments, we compare the action of several pharmacological agents on J_v in stimulated UMT. An underlying assumption of this paper is that net ion movements are followed by water flow across the bilayer, through water channels or through both [28, 61]. Therefore, the observation of a given alteration in J_v reflects a corresponding change of the net ionic movement the pharmacological agent is affecting. Ions would move following their electrochemical potential difference if moving independently. The direction of transport will vary according to each specific mechanism involved [66]. It should be pointed out that since we measure steady secretion rates, the observed changes may already be compensated by other mechanisms. It will remain for the future to study these preparations under conditions where the kinetics of the inhibited functions can be ascertained.

Two groups of experiments were needed to set up an experimental framework before the pharmacological agents were tried. First, a dose-response curve (*see* Results and Fig. 1) led to the use of a 2 µM 5-HT concentration to obtain maximal J_v stimulation. This confirms previous experimental results [38]. Second, our studies led to the conclusion that the rate of lumen perfusion used did not affect J_v (Fig. 2).

The agents were added separately at the basolateral and at the luminal cell membrane (*see* Methods), to determine whether they act at one or at both cell membranes. Some of the agents have effects at both cell membranes, while others at only one. UMT are known to transport substances from basolateral to apical cell membranes and vice versa [64]. Therefore, localization of the agents used at a given cell membrane cannot be absolute, except for those agents that show a clear-cut unilateral action.

The results are given in Tables 1 and 2, and are summarized in Table 3 to facilitate the description of the working model shown in Fig. 3 that follows.

Table 3. Summary of the effect of the pharmacological agents added to either the bath (basolateral membrane) or to the lumen perfusion (apical membrane), in percent change (%).

Agent	Target	Bath (%)	Lumen (%)
Ouabain (0.2 mM)	Na^+-K^+-ATPase	+29	—
Ouabain (1 mM)	Na^+-K^+-ATPase	—	+46
Bafilomycin (1 μM)	V-H^+-ATPase	−49	—
Bafilomycin (10 μM)	V-H^+-ATPase	—	NS
Furosemide (0.1 mM)	Na^+-K^+−$2Cl^-$ cotransport	−43	−79
Furosemide (0.5 mM)	Na^+-K^+-$2Cl^-$ cotransport	−79	—
Bumetanide (50 μM)	Na^+-K^+-$2Cl^-$ cotransport	−80	−82
DIOA (10 μM)	K^+-Cl^- cotransport	NS	−96
Probenezid (1 mM)	$Urate^-$-Anion exchange	NS	−59
SITS (1 mM)	Cl^--HCO_3^- exchange	−90	NS
ACTZ (1 mM)	Carbonic Anhydrase	NS	+38
Amiloride (0.4 mM)	Na^+-H^+-exchange	NS	−77
DPC (0.1 mM)	Cl^- channel	+39	NS
DPC (0.5 mM)	Cl^- channel	—	NS
$BaCl_2$	K^+ channel	NS	+70
PCMBS (500 μM)	Water channel	−54	—
DTT (5 mM)	Water channel	NS	—

ATPases

The Na^+-K^+-ATPase is located at the basolateral cell membrane of most epithelia, including *Rhodnius* UMT [43]. Its known inhibitor ouabain, when applied at the basolateral side, significantly stimulated J_v by 29%. Since there is no luminal radioactive ouabain-labeling [43], ouabain's action from the lumen may be attributed to a crossing over of this agent towards the basolateral side. The Na^+-K^+-ATPase is known to move 3 Na^+ from cytosol to peritubular side (i.e., backwards as related to secretory flow), while it takes up 2 K^+ from the peritubular side into the cell (i.e., forwards) [56]. Therefore it produces the net movement "backwards" of one ion per turn, i.e., the return of Na^+ from cell to peritubular space. The later tends to "decrease" secretion. If this "backward" ion movement is blocked by ouabain, the Na^+ concentration difference across the basolateral cell membrane would be reduced, facilitating Na^+ secretion and increasing J_v [43].

Secretion in *Rhodnius* UMT has been described as energized by an apical V-H^+-ATPase [42], which is blocked by an oxidative environment and activated by low pH [24]. Mitochondria-rich cells have an important density of plasma membrane V-H^+-ATPase [9, 62], as would be the case for *Rhodnius* UMT where numerous gigantic mitochondria penetrate the apical membrane microvilli [4, 65]. This would create an environment consistent with reducing conditions, which would stimulate this H^+-ATPase activity. Although direct phosphorylation has not been detected [62], cAMP stimulates apical V-H^+-ATPase in *Drosophila* Malpighian tubules [14]; in the tobacco hornworm, after metabolism is resumed, intracellular pH decreases stimulating V-H^+-ATPase activity [62]. We used the V-H^+-ATPase inhibitor Bafilomycin A_1. In the bath it inhibited J_v by 49%, indicating an important role of this ATPase in UMT secretion. This ATPase is absent in *Rhodnius* UMT basolateral cell membrane [42]. The action of Bafilomycin A_1 from the bathside, thus, can only be explained if this agent crosses the basolateral cell membrane to act on the apical V-H^+-ATPase [5, 42]. Since 5-HT leads to cAMP elevation [34] and increased metabolism, it should create conditions similar to those just mentioned for *Drosophila* and the tobacco hornworm. Bafilomycin A_1 had no effect when added to the lumen. This would indicate that this inhibitor does not cross the apical membrane.

We found that SITS, which is known to block the Cl^--HCO_3^- exchanger [11], inhibits J_v (by 90%) only from the basolateral, but not from the lumen side. This effect could not be due to SITS acting on basolateral Cl^- entry, since Cl^- enters mainly through the Na^+-K^+-$2Cl^-$ cotransporter. SITS rather would lead to intracellular HCO_3^- accumulation, which in turn would hinder the apical V-H^+-ATPase. ACTZ from the lumen increased J_v (by 39%). This is the expected opposite effect. ACTZ should reduce intracellular $HCO3^-$ concentration by inhibiting the formation of bicarbonate from CO_2 [22, 45]. ACTZ showed no action from the bath. It probably does not penetrate the basolateral cell membrane.

Cotransporters

If either the Na^+-K^+-$2Cl^-$ and/or the K^+-Cl^- cotransporters were present at the basolateral cell membrane, its inhibition would significantly curtail water

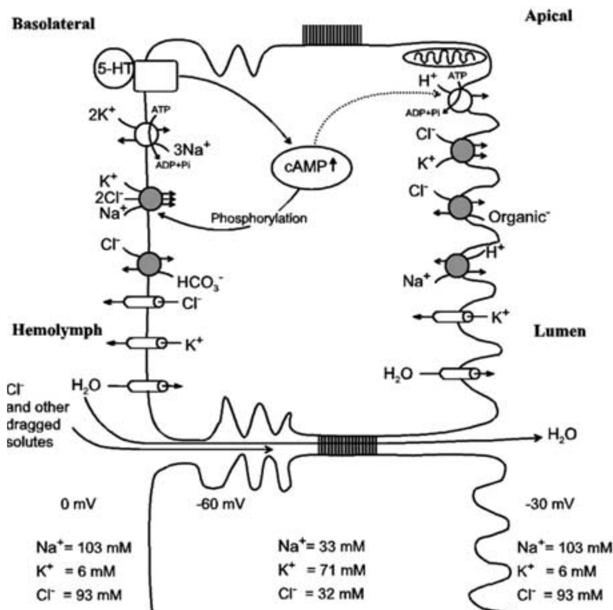

Fig. 3. Working model for secretion in a *Rhodnius* UMT cell. Secretion proceeds from left (basolateral membrane, peritubular side, hemolymph) to right (apical membrane, lumen). Pumps are shown as empty circles, cotransporters or exchangers as shaded circles, and channels as cylinders. Arrows show the proposed flow directions. The UMT electrical potential profile and ionic activities are shown at the bottom. They are taken from references [29, 30, 54] *Basolateral membrane*. 5-HT molecules acting on receptors through a cAMP cascade activate several mechanisms, like the Na^+-K^+-$2Cl^-$ cotransporter. Cl^- must leak from cell to peritubular side. Then Cl^- is taken back into the cell by the Na^+-K^+-$2Cl^-$ cotransport, and by a Cl^--HCO_3^- exchange. The ouabain-sensitive basolateral Na^+-K^+ ATPase is shown. K^+ channels seem to intervene only in the generation of the cell membrane electrical potential. There does not seem to be a K^+-Cl^- cotransport, or an organic anion transport mechanism. RpMip water channels are also shown. *Apical membrane*. Although Bafilomycin A_1 acts from the peritubular side, there must exist at the apical membrane level a V-H^+-ATPase. An apical mitochondrion is shown close to it. There must be a K^+ -Cl^- cotransport mechanism, as well as an urate-anion exchange mechanism. An amiloride Na^+-H^+ exchange mechanism is shown. K^+ must leak from lumen to cell. RpMIP water channels are also shown. *The paracellular pathway*. Through this pathway, the drag by water of large extracellular markers can be accounted for if the paracellular pathway is 11.8 Å wide [26, 59]. There is general agreement as to the movement of Cl^- through this pathway. Other solutes and ions may also move [7, 8].

movement, as it would move several ions (and therefore water) from bath to cell (Tables 1, 2 and 3). Basolateral use of the Na^+-K^+-$2Cl^-$ cotransport inhibitors furosemide and bumetanide [58] curtailed J_v by ~80%. In secretory epithelia, a basolateral Na^+-K^+-$2Cl^-$ cotransport is activated by phosphorylation in response to an increase of cAMP [21]. This leads to ion uptake from bath to cell (followed by water influx), leading to cell salt loading (and cell volume increase), as shown by the direction of arrows in Fig. 3, which are in agreement with the electrochemical differences. Figure 3 shows the electrical potential differences and ionic activities. We conclude that a Na^+-K^+-$2Cl^-$ cotransporter acts in the direction from bath to cell at the basolateral cell membrane in agreement with published findings for *Rhodnius* UMT [42] and *Drosophila* Malpighian tubules [5]. It is known that furosemide and bumetanide also inhibit a K^+-Cl^- cotransport [19]. To sort out whether we are in the presence of a Na^+-K^+-$2Cl^-$, a K^+-Cl^- cotransport system or both, we resorted to the more specific inhibitor of the K^+-Cl^- cotransport, DIOA [18, 29]. It had no effect on J_v from the basolateral side. Therefore, only the Na^+-K^+-$2Cl^-$ cotransporter is present at the basolateral side and it is not inhibited by DIOA under our experimental conditions.

From the lumen, furosemide and bumetanide also inhibited J_v. This could have two explanations: (a) these agents cross the cells from the lumen towards the basolateral cell membrane to inhibit there the basolateral Na^+-K^+-$2Cl^-$ cotransport or (b) either a Na^+-K^+-$2Cl^-$ or a K^+-Cl^- cotransporter is also present at the apical membrane. To distinguish between these alternatives, DIOA was lumen-perfused. We observed that luminal DIOA (which did not act basolaterally) brought J_v down to 4%, indicating that a K^+ -Cl^- cotransport system acts at the apical membrane. One of the major physiological regulators of this cotransporter is cell volume [17, 23]. Therefore, the cell volume increase produced by the activity of the basolateral Na^+-K^+-2Cl cotransporter would activate the apical K^+-Cl^- cotransporter. This would be the major route for K^+ and probably for Cl^- excretion (*arrows* in Fig. 3). This cotransporter seems fundamental in fluid secretion in view of the magnitude of the effect shown by DIOA on J_v. The cotransporter would be driven by the apical H^+ gradient, producing an electrical gradient that drives Cl^- into the lumen. Definitive proof for the presence of this apical K^+-Cl^- cotransporter requires future experiments.

Ion Exchangers

As mentioned above, an apical Na^+ and/or K^+ vs H^+ exchanger has been proposed for *Rhodnius* [30, 42] and for *Aedes* [55]. We have just presented evidence for an apical K^+-Cl^- cotransporter. We are left to explore the possible function of a Na^+ for H^+ exchanger. Amiloride is its known inhibitor but it also interacts with many other proteins, among them other Na^+ transporters and channels. Although amiloride discriminates poorly between different H^+-Na^+ exchanger isoforms as compared with its analogs [12, 32], we chose it since we do not know which isoform is most common in *Rhodnius* UMT. We found that amiloride in the lumen, but not in the bath, strongly inhibited J_v. Electrical measurements have produced no evidence for the presence of Na^+

channels at the apical membrane level [54]. Therefore, our results agree with the view that an apical amiloride-sensitive H^+-Na^+ exchanger is an important route for Na^+ excretion. Other mechanisms for Na^+ extrusion into the tubule lumen cannot be ruled out, for example the Ouabain-insensitive Na^+ pump already described *Rhodnius* UMT [10], which at present waits for a more specific inhibitor.

Urate transport is crucial in *Rhodnius* Malpighian tubule physiology [49, 63, 64]. Therefore, we tried the Cl^--organic anion exchange inhibitor Probenecid [53]. We found that it did not affect J_v from the basolateral side, but it curtailed 59 % of J_v from the lumen, suggesting that this exchanger (which may absorb urate in exchange for Cl^-) could play a role in urate secretion at the UMT apical membrane level.

Ion and Water Channels

Microelectrode studies in stimulated UMT showed that ~25% of the total basolateral electrical permeability was due to Cl^-, which was blocked by the Cl^- channel blocker DPC [54]. In the present experiments, bath DPC increased J_v by 39%, suggesting the presence of a net return of Cl^- from cell to hemolymph (Fig. 3), which would be inhibited by DPC. This inhibition would tend to increase Cl^- within the cell, thus favoring net transepithelial Cl^- secretion. Cl^- channels seem to be unimportant at the apical membrane, since DPC did not show any effect from the lumen.

Rhodnius UMT basolateral membrane potential was shown to be largely a K^+ diffusion potential (outward-pointing arrow in Fig. 3), which was blocked by 2 mM $BaCl_2$ from the bath [54]. In the present experiments, $BaCl_2$ had no action on J_v from the bath, indicating that a K^+ permeability does not directly contribute to *Rhodnius* UMT secretion at this membrane level [c.f. ref. 29]. A small apical permeability to K^+ has been reported [54]. We found that apical $BaCl_2$ stimulated secretion in 70%, suggesting that during secretion there might exist a backflux of K^+ from lumen to cell (Fig. 3) which would tend to reduce net K^+ flux into the lumen.

So far we have approached ion movements and their inhibition. We now come to consider the role of cell membrane water channels in UMT secretion. Rp-MIP water channels are expressed in *Rhodnius* UMT [15], where cells volume-regulate [1]. Rp-MIP water channels are blocked by mercurial reagents, like pCMBS, in an action that is reversed by DTT [15, 60]. Bath pCMBS inhibited 54% of J_v. This effect was reversed by 5 mM of the sulfhydryl reducing agent DTT [27] (Tables 1 and 3), indicating that Rp-MIP water channels are indeed present in the basolateral cell membranes. These results are in agreement with the assumption mentioned above that water follows ion movements [28, 61]. No conclusion can be obtained from experiments where lumen was perfused with pCMBS, because cell desquamation into the lumen was observed.

UMT Secretion Model

The present experimental results and those previously published [6, 8, 25, 29, 31, 35, 36, 55, 62] lead us to propose for UMT secretion in *Rhodnius* the model shown in Figure 3. The directions of the proposed flows (arrows) are in agreement with the electrical potential differences and ionic activities shown in the figure [29, 30, 54]. The basolateral location of the Na^+-K^+-ATPase, of the Na^+-K^+-$2Cl^-$ and the apical localization of the V-H^+-ATPase agree with previous propositions for *Rhodnius* and insects like *Drosophila* [31], *Aedes* [8] and *Formica* [35], Which were based on the effect of changes in the solutions bathing the basolateral cell aspect. Luminal perfusions have produced the present model, which keeps the concept that the V-H^+-ATPase provides the driving force for apical ion transport. However, it separates Na^+ from K^+ flows at the apical cell membrane. In our model, Na^+ is exchanged for H^+, and K^+ is cotransported with Cl^-. Previous models suggested that Na^+ and K^+ moved in exchange for H^+.

Our working hypothesis puts together the observed facts about secretion in *Rhodnius prolixus* UMT. 5-HT triggers the cAMP cascade, activating several cell functions. Increase in metabolism by lowering cell pH would activate the apical V-H^+-ATPase and protein kinases would activate the basolateral Na^+-K^+-2Cl cotransporter. This in principle would initially tend to increase cell volume, stimulating the apical K^+-Cl^- cotransporter which would, by its function, compensate for the cell volume increase. As has been already mentioned, at the apical membrane, secretion is supported by a lumen-to-cell H^+ gradient, which maintains the function of the Na^+-H^+ exchanger and the other transport functions. Once a steady rate of secretion is established, the apical transport rate would tend to be higher than the basolateral one, leading to the presence of a zone of slight hyperosmolality. In fact, there are reports of cell volume decrease [27], as well as the presence of a slightly hyperosmotic fluid during secretion as compared to that of the hemolymph [38]. Evidence has been provided for the existence of paracellular water flow (Fig. 3). This is based in the observation that during secretion, water drags extracellular markers as a monotonic function [7, 26, 59]. Recently a fast regulation of tight junction ion permeability has been put forward [7]. The ratio of transcellular to paracellular flows is still controversial [61].

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