CELLULAR MECHANISMS OF KCl REABSORPTION IN THE LOWER MALPIGHIAN TUBULE OF *RHODNIUS PROLIXUS*

CELLULAR MECHANISMS OF KCl REABSORPTION IN THE LOWER MALPIGHIAN TUBULE OF *RHODNIUS PROLIXUS*

By

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Abstract

Ionoregulation and osmoregulation by insect Malpighian tubules involves both *secretion* of ions (*i.e.* transport from haemolymph to tubule lumen) and *reabsorption* of ions (*i.e.* transport from lumen to haemolymph). Although cellular and molecular aspects of ion secretion and its control have been examined extensively in recent years, the process of ion reabsorption has received relatively little attention. This thesis examines the mechanisms of KCl reabsorption by the lower Malpighian tubule of *Rhodnius*. This tissue is ideal for this type of study because it reabsorbs most of the KCl from the fluid secreted by the upper Malpighian tubule; concentrations of KCl are reduced from *ca.* 80 mmol l^{-1} to less than 10 mmol l^{-1}, so that a hypoosomtic NaCl-rich fluid is passed into the hindgut. Concentrations of K^+ in fluid secreted by isolated whole Malpighian tubules were measured by K^+-selective microelectrodes. Substances which inhibited the process of KCl reabsorption could be detected by the resultant rise in K^+ concentration of the secreted fluid droplets. In addition, cellular and transepithelial electrical gradients were measured by intracellular recording.

The ion porters implicated in the reabsorptive process differ dramatically from those involved in secretion of NaCl and KCl by the upper tubule. Stimulated fluid secretion by the upper tubule involves osmotic coupling of water flow to active transport of ions. Na^+, K^+ and Cl^- enter the upper tubule through a furosemide-sensitive $Na^+/K^+/2Cl^-$ cotransporter in the basolateral membrane. Na^+ and K^+ are transported from cell to lumen through amiloride-sensitive apical antiporters which exchange cellular Na^+ or K^+ for lumenal H^+. The driving force for these exchanges is provided by pumping of H^+ from cell

to lumen by a bafilomycin-sensitive vacuolar-type H^+-ATPase. In contrast, the process of KCl reabsorption is unaffected by high concentrations of drugs known to inhibit Na/K/2Cl^- cotransporters, Na^+/H^+ exchangers and vacuolar H^+-ATPases.

KCl reabsorption is metabolically dependent and inhibitable by the carbonic anhydrase inhibitor acetazolamide, consistent with the presence of transporters requiring H^+ and/or $HCO3^-$. Entry of K^+ from the lumen into the cell involves a mechanism which is sensitive to inhibitors of H^+/K^+-ATPases, including vanadate, omeprazole, SCH 28080, SKF 96767 and SKF 96356. In addition, measurements of secreted fluid pH and the effects of acetazolamide are consistent with Cl^- entry into the cell through a $Cl^-/HCO3^-$-ATPase. Exit of K^+ and Cl^- from cell to haemolymph appears to be mediated by channels. Changes in basolateral membrane potential (V_{bl}) in response to changes in bathing saline [K^+] or [Cl^-] are consistent with the presence of conductive pathways for these ions. Changes in V_{bl} and inhibition of KCl reabsorption by the K^+ channel blockers Ba^{2+} and tetraethylammonium suggest the presence of basolateral but not apical K^+ channels. Similarly, changes in V_{bl} and inhibition of KCl reabsorption by diphenylamine-2-carboxylate, SITS, DIDS and SCN^- are consistent with the presence of basolateral but not apical channels for Cl^-.

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CHAPTER 1

General Introduction

Blood-feeding by *Rhodnius prolixus*

The hemipteran insect *Rhodnius prolixus* is a member of the family Reduviidae, which is a large group consisting mostly of predaceous bugs and a few species of blood-feeders. Reduviids often prey on other insects, and are commonly referred to as Assassin bugs. Blood-feeders in the genera *Triatoma* and *Rhodnius* feed at night on avian or mammalian hosts, including humans. At long range they are attracted by host odours or exhaled carbon dioxide (Schofield, 1979). At short range, heat receptors on the antennae detect the warmth of the host (Wigglesworth and Gillett, 1934). The insect will bite exposed parts, such as the face, of sleeping people, and they are sometimes called 'kissing bugs' because of their tendency to bite people about the mouth. In South and Central America, kissing bugs are vectors of a trypanosome disease known as Chagas' disease.

After locating a suitable host, the insect probes the skin with the tip of the labium, and the elongated mandibles are used to penetrate the surface. The maxillae, which form separate canals for food and saliva, are then inserted in a series of thrusts and withdrawals. During this process, saliva is continuously ejected from the tips of the maxillae.

The saliva contains several different factors which delay clotting, prevent platelet aggregation induced by ADP or arachidonic acid, and induce vasodilation (references in Ribeiro *et al.*, 1990). The vasodilatory factor is a nitrosylhemoprotein (nitrophorin) which

stores and delivers nitric oxide (NO) to the host tissues during probing and feeding. It appears that NO is produced intracellularly, then passed across the luminal microvilli of the salivary glands and taken up by nitrophorins in the salivary gland lumen (Nussenveig *et al.*, 1995).

During probing, the insect samples the host's fluids. Ingestion begins after appropriate phagostimulants have been detected by epipharyngeal sensillae. In *Rhodnius*, di- and triphosphate nucleotides act as phagostimulants - adenosine triphosphate (ATP) is the most potent of the compounds found in the blood (Friend and Smith, 1977). Feeding stops when abdominal stretch receptors detect that a critical abdominal volume has been reached (Maddrell, 1964). The receptors are probably located in the vertical tergosternal muscles found near the lateral edge of the abdominal segments. These receptors are well-placed for detection of vertical distension of the abdomen produced by filling of the anterior part of the mid-gut during feeding (*ibid*).

The insect continues to feed until it has ingested a blood meal which may equal 10 - 12 times its unfed weight. The blood is retained in the anterior midgut; distension of this organ stimulates stretch receptors in the dorsal cuticle, thereby inducing the release of two diuretic hormones, a peptide of approximately 3000 Daltons, and 5-hydroxytryptamine (serotonin). Both hormones are found in neurosecretory cells in the mesothoracic ganglion (Maddrell, 1963; Orchard *et al.*, 1989). One effect of these hormones is the stimulation of the transport of NaCl and water from the crop to the haemolymph (Farmer, Maddrell and Spring 1981). In addition, the hormones act synergistically to bring about a 1000-fold stimulation of fluid secretion by the Malpighian tubules. Together, the four Malpighian tubules produce a NaCl-

rich urine at combined rates of up to 600 nl/min, so that much of the plasma fraction of the blood meal is eliminated within 3-4 h (Maddrell *et al.*, 1991a).

Each Malpighian tubule in *Rhodnius* consists of two structurally and functionally distinct segments which are described in detail in the following sections. The upper Malpighian tubule secretes fluid which is isoosmotic with the insect's haemolymph, and consists of an approximately equimolar mixture of NaCl and KCl. As the fluid (primary urine) passes through the lower Malpighian tubule KCl, but not water, is reabsorbed, so that what passes into the hindgut is a hypotonic NaCl-rich urine. Overall, this process of isoosmotic secretion and KCl reabsorption maintains the osmolality of the insect's haemolymph following ingestion of a hypo-osmotic blood meal (Fig. 1.1). The net effect of diuresis is elimination of the plasma fraction of the blood meal, and concentration of the blood cells in the anterior mid-gut.

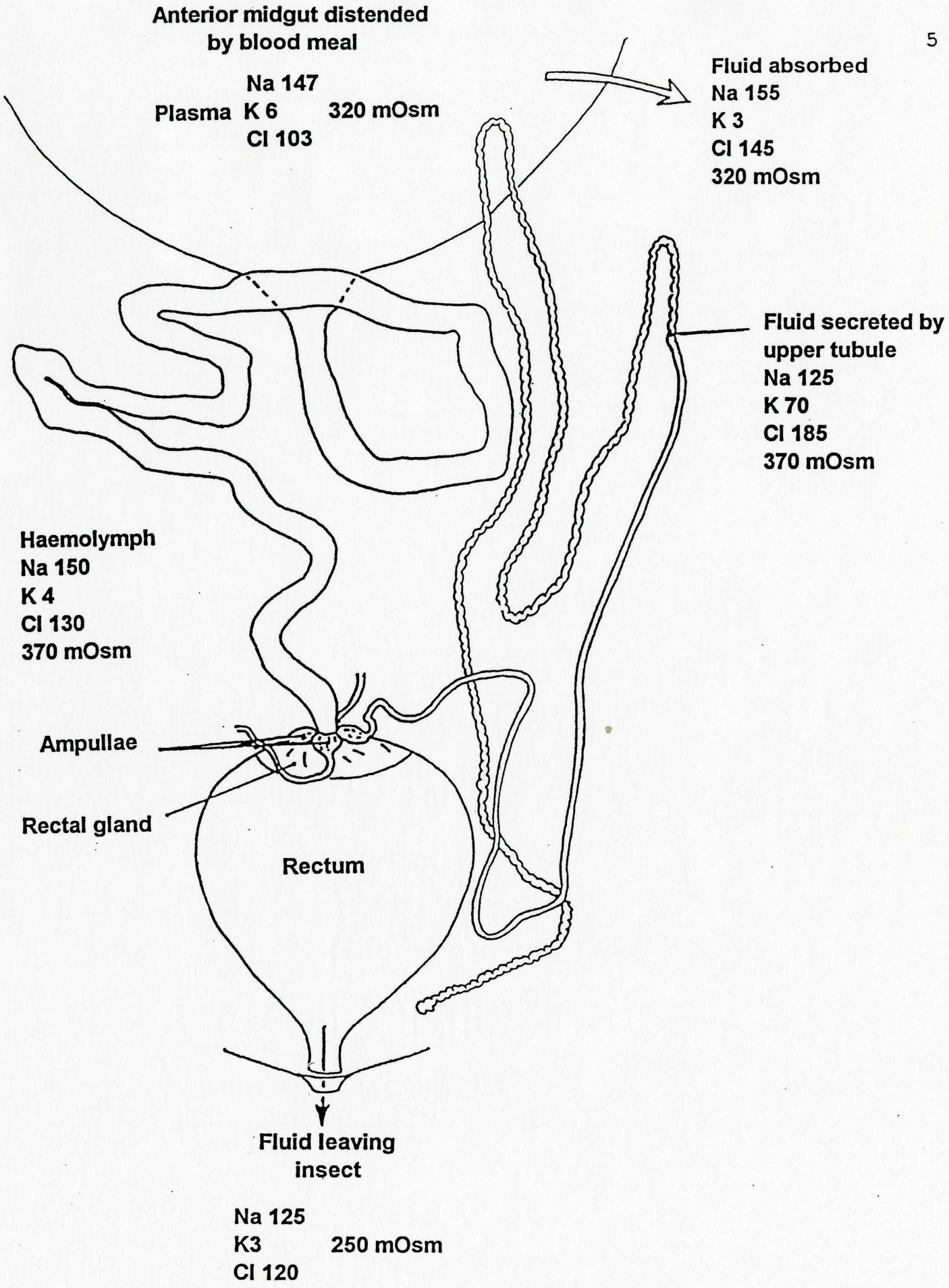
Structure and function of the excretory system:

Organization of the excretory system.

The excretory system in insects consists of the Malpighian tubules and the hindgut. In *Rhodnius*, there are four blind ended tubules which open into the gut at the junction of the midgut and hindgut (Fig. 1.1). Each tubule is connected to the rectum through a bulb-like ampulla. The function of the ampulla is unknown, although extensive ion transport seems unlikely since the osmotic concentration of the fluid leaving the lower tubule is equal to the fluid leaving the rectum. The ampulla may act as a valve, mediating flow of urine from lower

Figure 1.1

Drawing of the gut and excretory system of *Rhodnius prolixus*. Concentrations (in mmol l^{-1}) of the main inorganic ions and osmolality of the fluids in the various compartments are shown. Note that the insect's haemolymph osmolality is hyperosmotic to the blood meal. Osmoregulation is provided by the combined actions of the upper and lower segments of the Malpighian tubules. The upper tubule secretes a fluid which is isoosmotic to its own haemolymph, and consists of NaCl and KCl. The lower tubule reabsorbs KCl but not water, thereby maintaining haemolymph potassium levels and resulting in the production of a dilute final urine. (Redrawn from Maddrell and Phillips, 1975).


Anterior midgut distended by blood meal
Na 147
Plasma K 6 320 mOsm
Cl 103
Fluid absorbed
Na 155
K 3
Cl 145
320 mOsm
Fluid secreted by upper tubule
Na 125
K 70
Cl 185
370 mOsm
Haemolymph
Na 150
K 4
Cl 130
370 mOsm
Ampullae
Rectal gland
Rectum
Fluid leaving insect
Na 125
K3 250 mOsm
Cl 120

tubule to rectum. This possibility is suggested by the presence of small muscle fibres which are attached to the tubule and ampullae (Wigglesworth, 1931). The fibres could pull on the ampulla to enlarge its internal diameter, thereby permitting urine to flow more easily into the rectum.

Each Malpighian tubule in a 5th instar *Rhodnius* is approximately 45mm in length and 100 μm in diameter. The upper fluid secretory tubule is approximately 30 mm in length, and is relatively opaque, due to a large number of Ca-rich intracellular concretions. The lower Malpighian tubule lacks such concretions and is more translucent (Maddrell *et al.*, 1991).

Anatomy and ultrastructure of the upper Malpighian tubule.

The upper tubule is composed of a single layer of squamous epithelial cells, each approximately 100 μm by 20 μm in depth. Each cell resembles a half-cylinder, which is thickened in the perinuclear region and is joined to the adjacent cells by septate junctions to form a tubule. The cells are spaced alternately along the tubule and bulge away from the lumen, giving the upper tubule a zig-zag appearance (Bradley, 1983). The number of cells remains constant at about 770 between 1st - 5th instar larvae and adult, so that increases in tubule dimensions require corresponding increases in cell size (Skaer, Maddrell and Harrison, 1990).

Electron micrographs show the apical and basal membranes of the upper Malpighian tubules to be highly folded. The increases in surface area are presumed to be necessary for the placement of membrane proteins involved in ion transport, osmotic water flow, and excretion. The membrane area of the apical side of the cell is increased 150-fold, relative to a flat surface,

by membrane infoldings. Similarly, the area of the basal side of the cell is 40 times larger than the frontal area. Based on the cell dimensions (50 μm) and the width of the intercellular space (17 nm), the ratio of surface areas of the basal surface of the tubule and the intercellular clefts is calculated to be 3000:1. When the 40-fold amplification of the basal surface is accounted for, the relative surface areas of the basal cell membranes and the intercellular clefts are typically in the ratio of 120,000:1. Transport of ions across an epithelium may involve either the paracellular pathway (*i.e.* the space between the cells) or the transcellular pathway (*i.e.* through the cells). The foregoing calculations based on ultrastructure suggest that the transcellular path is the dominant one in *Rhodnius* Malpighian tubules, and experimental observations support this view (O'Donnell and Maddrell, 1983; O'Donnell *et al.*, 1984)

The apical microvilli typically contain large numbers of mitochondria. Electron micrographs reveal that 60% of the microvilli contain mitochondria (Bradley, 1984). Presumably, mitochondria placement within microvilli minimizes the diffusion distance between sites of ATP production and consumption by mitochondria and ion transporting ATPases, respectively.

Fluid and ion secretion by the upper Malpighian tubule

Fluid and ion secretion rates by the upper Malpighian tubule differ dramatically in diuretic versus non-diuretic insects. Tubules from non-diuretic 5th instar *Rhodnius* secrete a KCl-rich fluid at low rates, *ca.* 0.1 nl min^{-1} tubule^{-1}. During diuresis, fluid and ion secretion rates increase dramatically to about 150 nl min^{-1} tubule^{-1} *in vivo*, and about 100 nl min^{-1} tubule^{-1} for tubules isolated *in vitro*. The latter rate is equivalent to each cell secreting a volume of fluid

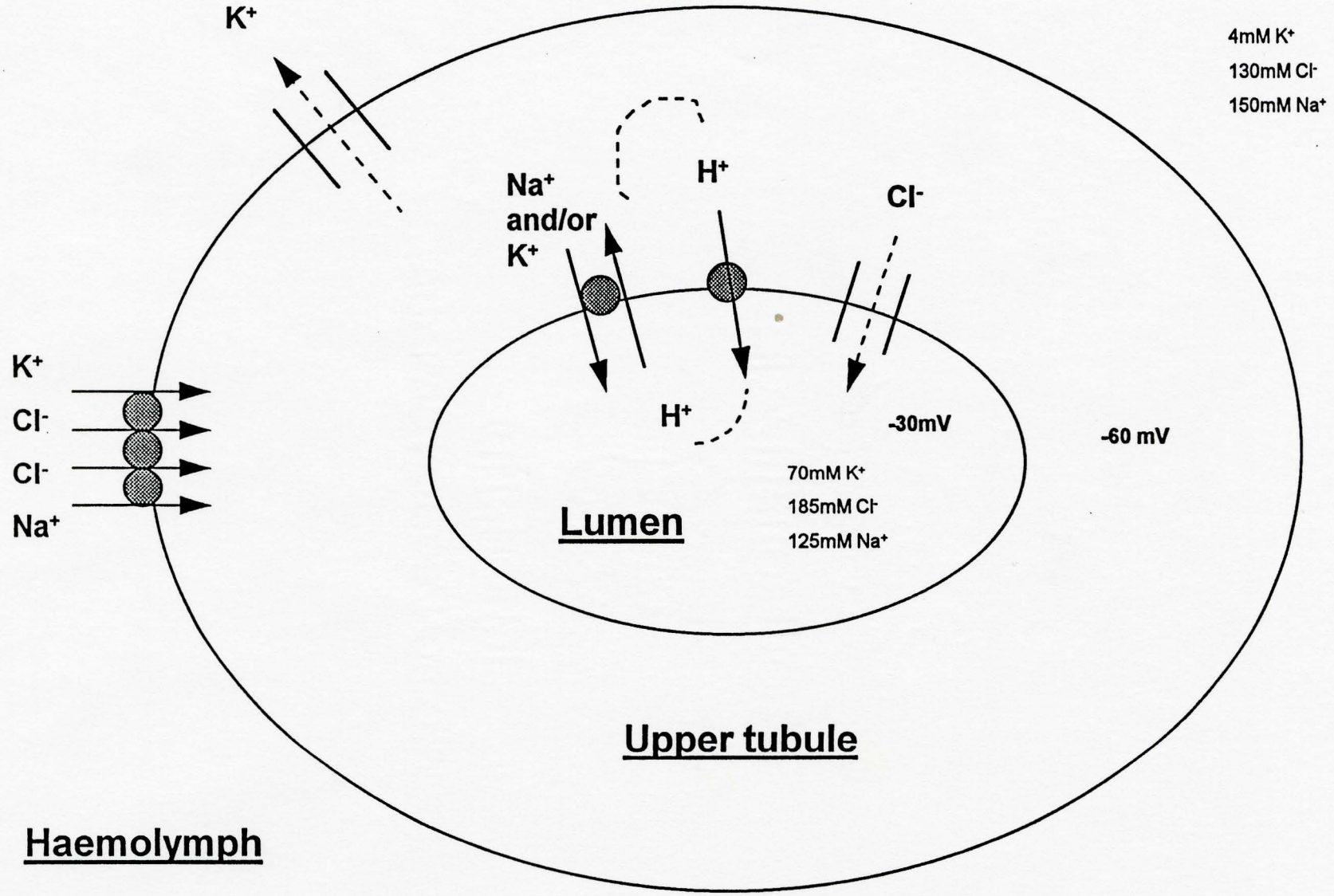
equal to its own volume every 15 s. Fluid secreted by diuretic tubules consists of approximately 125 mmol l^{-1} NaCl and 70-80 mmol l^{-1} KCl (Figure 1.1).

Secreted fluid is only slightly hyper-osmotic (about 1.3%) to the bathing saline, and water transport across the cells in the upper tubule is apparently a passive osmotic consequence of active ion transport of Na^+, K^+ and Cl^- (O'Donnell & Maddrell 1983). Because the frontal area of the intracellular clefts of the upper tubule is small, and the osmotic permeability of the cells is large, the route for transepithelial water movement is predominantly transcellular (O'Donnell and Maddrell, 1983). Calculations based on direct measurements of osmotic permeability indicate that very small osmotic gradients of the order of 0.7 mOsm and 2.6 mOsm across the basal and apical cell surfaces, respectively, are sufficient to account for observed rates of fluid secretion. Given that the secreted fluid is very nearly isoosmotic therefore, rates of ion transport can be readily calculated from fluid secretion rates and fluid composition. For example, a tubule secreting fluid containing 100 mmol l^{-1} NaCl at 100 nl min^{-1} is transporting 10 nmol Na^+ per minute.

The current model of ion transport by the upper tubules is presented in Fig. 1.2. Ion (and fluid) transport is driven by an apical vacuolar-type (V-type) H^+-ATPase, which is insensitive to ouabain and vanadate, but inhibited by bafilomycin A_1 (Bertram *et al.*, 1991; Weltens *et al.*, 1992), N-ethyl maleimide (NEM; Weltens *et al.*, 1992) and 4-chloro-7-nitrobenzo-2-oxa-1,3-diazol (NBD Cl; Maddrell and O'Donnell, 1992). The V-type ATPase pumps protons from cell to lumen, thereby providing the driving force for secondary movement of Na^+ and/or K^+ from cell to lumen through Na^+/H^+ and K^+/H^+ antiporters.

Figure 1.2

Schematic diagram summarizing current proposals for cellular mechanisms of ion transport by the upper Malpighian tubule. The overall process of secretion of Na^+, K^+ and Cl^- into the lumen is driven by electrochemical gradients established by the apical vacuolar-type H^+-ATPase. The pump provides a favourable gradient for movement of H^+ from lumen to cell, thereby energizing Na^+ (or K^+)/H^+ antiporters which transfer Na^+ and K^+ into the lumen. In turn, the antiporters establish a favourable gradient for entry of Na^+ and K^+ into the cell across the basolateral membrane. This gradient drives the coupled entry of Cl^- as well. Some K^+ exits through basolateral K^+ channels, thereby contributing to the cell-negative basolateral membrane potential. Cl^- movements from cell to lumen are presumed to be a passive response to the favourable electrical gradient (lumen positive to cell) across the apical membrane.


K+
4mM K+
130mM Cl-
150mM Na+
Na+
and/or
K+
H+
Cl-
K+
Cl-
Cl-
Na+
H+
-30mV
-60 mV
70mM K+
185mM Cl-
125mM Na+
Lumen
Upper tubule
Haemolymph

Amiloride, which is known to inhibit Na^+/H^+ exchange in many cell types, inhibits tubule fluid secretion, and causes lumen pH to decline about 1 pH unit, from 6.96 to 5.93, consistent with the presence of a lumen-directed proton pump which will acidify the lumen in the absence of Na^+/H^+ and K^+/H^+ exchange (Maddrell and O'Donnell, 1992).

Entry of Na^+, K^+ and Cl^- into the cell involves a basolateral cotransporter which can be inhibited by drugs such as furosemide and bumetanide (O'Donnell and Maddrell, 1984). The suggested stoichiometry is $Na^+/K^+/2Cl^-$. The cotransporter energizes the movement of Cl^- into the cell, against an opposing electrical gradient of -65 mV across the basolateral membrane, by coupling Cl^- entry to that of Na^+ and K^+. Favourable gradients for entry of Na^+ and K^+ are maintained, in turn, by the actions of the apical pump and antiporters. Some K^+ leaks from cell to haemolymph through a basolateral K^+ conductance, which accounts for most of the basolateral membrane potential (O'Donnell and Maddrell, 1984).

This scheme proposes a single form of active transport (*i.e.* transport directly coupled to the hydrolysis of ATP), the pumping H^+ from cell to lumen. Movement of Na^+ and K^+ from cell to lumen are examples of secondary active transport, coupled to the electrochemical gradient for H^+ across the apical membrane. Chloride entry across the basolateral membrane is an example of tertiary active transport, coupled to the electrochemical gradient favouring sodium entry into the cell.

Chloride movements from cell to lumen probably involve apical chloride channels, and are driven by a favourable electrical gradient of about 35 mV.

Ultrastructure of the lower Malpighian tubule.

Although microvilli are found on the apical surface of the lower Malpighian tubule, they are more widely spaced than the microvilli of the upper Malpighian tubule. Individual microvilli of the upper Malpighian tubule are separated by no more than 15 - 20 nm, resulting in a honey-comb type of brush border. Microvilli of the lower Malpighian tubule are separated by spaces equal to or exceeding their own diameter, about 250 nm. In non-diuretic tubules, the mitochondria in the apical portion of the cell are localized to the cell cortex; none are found in the microvilli (Bradley and Satir, 1981). In tubules stimulated with 5-HT, there is a 3-fold increase in the volume of the apical microvilli, consistent with membrane insertion, and 19% of the microvillar volume is occupied by mitochondria. Movement of mitochondria into the microvilli appears to involve an actin-based motile system (Bradley and Satir, 1979). The actin is contained in microfilaments which form a sheath running up the length of the microvillus in stimulated tubules.

In addition to microvilli, structures termed axopods are found on the apical surface of the lower Malpighian tubule, oriented at right angles to the tubule's length (Bradley and Satir, 1979). Axopods are 0.2 - 0.8 μm in diameter, and may exceed 10 μm in length, considerably longer than the 2 - 5 μm length of the microvilli. Axopods viewed in cross-section are seen to contain from 1 to 46 microtubules arranged in an inexact array. Axopods are not ciliary or flagellar derivatives, therefore, since the microtubules do not show a 9 + 2 or related configuration, and do not arise from a basal body. The microtubules arise from within the cell near mitochondria or cell junctions. Axopods are apparently motile, and are probably involved in propelling uric acid crystals towards the hindgut in non-diuretic tubules.

Ion reabsorption by the lower Malpighian tubule.

One of the functions of the lower Malpighian tubule in *Rhodnius* is the modification of fluid secreted by the upper Malpighian tubule during diuresis. Specifically, K^+ and Cl^-, but not water, are rapidly reabsorbed as the fluid passes through the lower Malpighian tubule, so the fluid which enters the hindgut consists of a hypoosmotic urine composed primarily of NaCl. Although salt and water reabsorption occur in the rectum in most insects (Phillips, 1981), the extensive membrane surface presented by the microvilli and the narrow lumen make the lower Malpighian tubule of *Rhodnius* more suited for KCl reabsorption than the rectum (Maddrell and Phillips, 1975). In other words, the surface area of transporting membrane relative to the volume of fluid enclosed is much increased in a narrow tube relative to the sac-like rectum. This permits more rapid modification of the secreted fluid ionic composition.

KCl reabsorption is confined to the 30% of the lower tubule's length closest to the hindgut (Maddrell, 1978). Osmotic permeability is also reduced in this region (O'Donnell *et al.*, 1982), so that water is not reabsorbed along with KCl. The upper 2/3 of the lower tubule's length is osmotically permeable, and is not involved in ion reabsorption. These physiological discontinuities along the lower tubule are surprising because there are no corresponding differences in structure; the ultrastructure of the lower tubules is uniform along its length (Wigglesworth and Salpeter, 1962).

It is worth emphasising the speed and extent of KCl reabsorption by the lower Malpighian tubule. Fluid flows through the lower tubule at about 0.6 mm s^{-1}, and is in contact with the reabsorptive region for less than 10 s. During this time, potassium concentration

plummets from 80 $mmol^{-1}$ to less than 5 $mmol^{-1}$, equivalent to 1 $mmol^{-1}$ every 130 milliseconds. At the same time, osmolality drops from 370 to 250 mOsm, a rate of change of 12 mOsm s^{-1}. Based on estimates of tubule dimensions, K^+ is reabsorbed across the tubule wall at a rate of 0.80 mmol cm^{-2} min^{-1} at 22°C, and as high as 0.95 mmol cm^{-2} min^{-1} at 37°C (Maddrell, 1978). These rates are 2-fold to 7-fold higher than rates of sodium chloride transport achieved in mammalian tissues such as rabbit gall bladder (0.13 mmol cm^{-2} min^{-1}; Diamond, 1964) and rat proximal kidney tubule (0.46 mmol cm^{-2} min^{-1}; Giebisch et al., 1964).

Other functions of Malpighian tubules

Malpighian tubules have been implicated in active and passive mechanisms for excretion of a variety of potentially toxic organic molecules. Active transport mechanisms have been demonstrated for uric acid (O'Donnell *et al.*, 1983), acylamides (Maddrell *et al.*, 1974), alkaloids (Maddrell and Gardiner, 1976), and cardiac glycosides (Rafaeli-Bernstein and Mordue, 1978). Uric acid is transported by the lower tubule, but not the upper (O'Donnell *et al.*, 1983). Tubules also allow passive diffusive movement of solutes from haemolymph to tubule lumen (O'Donnell *et al.*, 1983). Although high passive permeability to low molecular weight organic solutes necessitates subsequent active reabsorption of useful compounds such as sugars and amino acids, such an arrangement is advantageous in that it ensures automatic and fail-safe excretion of novel toxins that an insect may encounter in its diet (Ramsay, 1952).

Organization and goals of the thesis

This thesis examines the cellular mechanisms of active reabsorption of KCl from the lumen of the lower Malpighian tubule of *Rhodnius prolixus*. It is important to emphasize that no previous studies have examined the nature of the ion porters involved in KCl reabsorption. The contributions of putative ion porters have been evaluated by examining the effects of drugs known to block specific porters in other systems. In chapter 2, the effects of drugs known to affect K^+ transport are reported and interpreted. In chapter 3, the effects of drugs known to inhibit Cl^- transport in other epithelia are examined. Except in cases where a drug was known to permeate the tubule wall easily, drugs were applied to both basolateral (haemolymph-facing) and apical (lumen-facing) surfaces of the tubule. Analysis of the effects of drugs applied from the basolateral surface involved addition of the drug to the saline bathing an isolated tubule. Application of drugs to the apical surface required that the tubule be cannulated and the lumen perfused with saline containing the drug of interest. In chapter 4, the electrical gradients across the epithelium and across apical and basolateral membranes are determined by microelectrode measurement of transepithelial potential, apical membrane potential, and basolateral membrane potential. Chapter 5 integrates the results of Chapters 2 - 4, and proposes a working hypothesis of the mechanism of KCl reabsorption.

In view of the cardinal role of V-type H^+ ATPases in fluid secretion by the Malpighian tubules of *Rhodnius* and other insects, one goal of this thesis is to ascertain the possible role of this type of proton pump in reabsorption of K^+ and/or Cl^-. In addition, the involvement of amiloride-sensitive Na^+/H^+ or K^+/H^+ exchangers and furosemide-sensitive $Na^+/K^+/2Cl^-$ or K^+/Cl^-

cotransporters in fluid secretion has been suggested for tubules of many insects. The possible contributions of these transporters to lower tubule KCl reabsorption has also been examined in detail in the following chapters. The most striking finding of this study has been that the ion transporters involved in reabsorption of K^+ and Cl^- by the lower tubule differ fundamently from the porters involved in secretion of K^+ and Cl^- by the upper tubule of *Rhodnius* or by the Malpighian tubules of other species.

CHAPTER 2

Potassium Transport by the Lower Malpighian Tubule

Introduction

This chapter examines the effects of putative inhibitors of potassium channels, cation antiporters, and cation-transporting ATPases on KCl reabsorption by the lower Malpighian tubule. The compounds were added to the saline bathing the basolateral or apical surface of a lower tubule isolated into a saline droplet under paraffin oil, and inhibition of KCl reabsorption was indicated by a rise in the K^+ concentration of the fluid collected from the ampulla. K^+ concentration in secreted fluid or bathing saline was measured by means of K^+-selective microelectrodes. The presence of a porter sensitive to a particular compound cannot be determined by adding an appropriate inhibitor to only the basolateral surface. The reason for this is that the compounds might not permeate cell membranes sufficiently well to block porters located in the apical membrane. This chapter also reports, therefore, the results of experiments in which isolated tubules were cannulated so that the lumen could be perfused with salines containing the drugs of interest.

Lumenal application of ion transport inhibitors.

Techniques for luminal perfusion of isolated Malpighian tubules involve either the concentric pipette technique first developed for mammalian renal tubules by Burg and co-workers (Burg, M.B. 1972), or the luminal cannulation technique developed by Maddrell and

Phillips (1975). The concentric pipette technique has been used for short tubules, such as those isolated from ants (Leyssens *et al.*, 1992) and mosquitoes (*eg.* Williams and Beyenbach, 1984). For longer (> 1 cm) Malpighian tubules the cannulation technique of Maddrell and Phillips (1975), originally developed for the initial studies of the lower tubule of *Rhodnius*, is a simpler alternative to the concentric pipette technique.

Materials and methods.

Insects

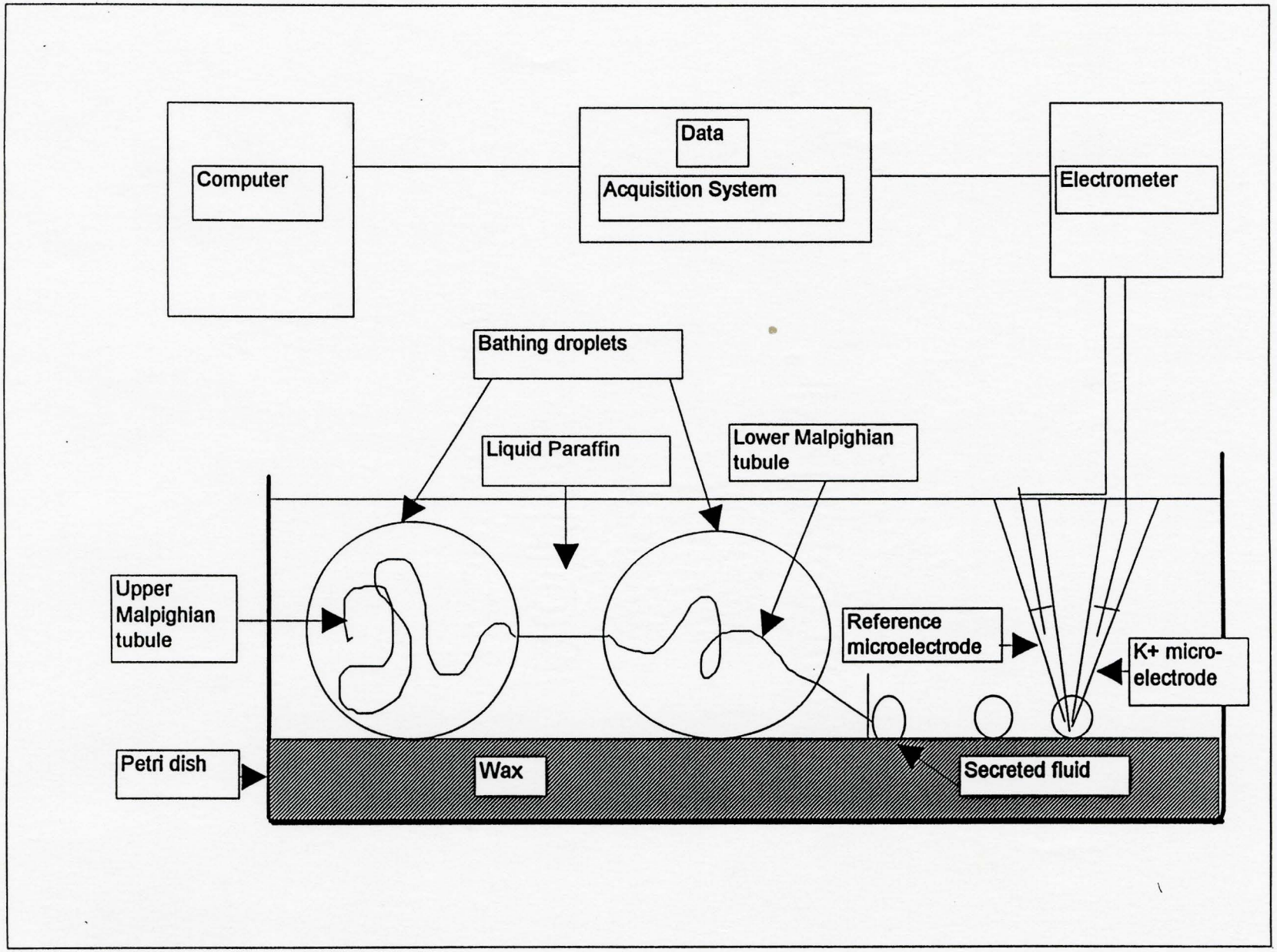
Fifth instar *Rhodnius prolixus* were obtained from a laboratory colony maintained at 25 - 28° C and 60 % relative humidity in the Department of Biology, McMaster University. The colony was maintained by weekly feedings on blood from female New Zealand White rabbits. Rearing techniques have been described in detail by Gardiner and Maddrell (1972). The protocols have been approved by the McMaster University Animal Research Ethics Board. All experiments described in this and subsequent chapters were done at room temperature (20 - 23°C).

Dissection procedures and collection of secreted fluid

Insects were dissected under physiological saline solution which consisted of (in mmol l^{-1}): NaCl (129), KCl (8.6), $MgCl_2$ (8.5), $CaCl_2$ (2.0), glucose (20.0), $NaHCO_3$ (10.2), NaH_2PO_4 (4.3), HEPES (8.6), titrated with NaOH to pH 7.0. Wing buds were clipped away, and a transverse cut was made across the abdomen at the base of the wing buds. Using fine forceps, the dorsal cuticle of the abdomen was peeled back to expose the viscera. Fine probes made by pulling coloured glass rods over a low flame were used to sever tracheal connections between the tubules and the body wall. Each tubule was grasped with fine forceps at the junction of the ampulla and the rectum, and pulled free of the body. Tubules were then transferred on fine glass probes to droplets of saline, usually 100 µl, under paraffin oil in a petri dish lined with wax or Sylgard (Fig. 2.1). The lower tubule was pulled into a second drop of bathing saline,

Figure 2.1

Schematic diagram showing the arrangements for measurement of K^+ concentration in droplets of fluid secreted by whole Malpighian tubules isolated under oil. A 120 mm glass petri dish was lined with either wax or translucent Sylgard to a depth of about 3 mm. Concentrations of drugs or inorganic ions in the saline droplets bathing upper and lower segments of the tubule could be varied independently. Fluid secreted by the upper tubule passed through lower tubule and was collected from the ampulla. The K^+ concentration of secreted fluids was determined by positioning reference and K^+-selective microelectrodes in each droplet. Potentials measured by the electrometer were recorded for subsequent analysis by a computerized data acquisition system.


Computer
Data
Acquisition System
Electrometer
Bathing droplets
Liquid Paraffin
Lower Malpighian tubule
Upper Malpighian tubule
Reference microelectrode
K+ micro-electrode
Petri dish
Wax
Secreted fluid

and the ampulla was then pulled out and wrapped around a glass or steel pin embedded in the Sylgard. Fluid secreted by the upper tubule passed through the lower tubule and emerged from the open end of the ampulla. Droplets of secreted fluid were removed periodically using fine glass probes. At the end of the experiment the tubule was cut at the upper-lower junction and several drops of upper tubule were collected for analysis. Fluid secretion rates (nl min^{-1}) of the spherical drops were calculated by dividing the volume of the droplets by the time over which the droplet was allowed to form. Droplet volume (V) was calculated from the equation: $V = 4\pi/3(d/2)^3$ where d is the droplet diameter measured using an eyepiece micrometer.

After measurement of its diameter, each droplet was placed to one side of the pin securing the ampulla. At the end of the experiment ion concentrations were measured using ion-selective microelectrodes (ISME's). For measurement of secreted fluid pH, droplets were analyzed immediately using pH microelectrodes, to minimize alkaline drift resulting from gradual diffusion of carbon dioxide into the paraffin oil.

Fabrication of pH- and ion-selective microelectrodes.

Ion concentrations and pH in drops of secreted fluid were measured using liquid membrane pH- and ion-selective microelectrodes. Unfilamented 1 mm o.d. borosilicate glass tubing was washed in nitric acid for 5 min, rinsed several times in deionized water and dried on a hot plate at 200°C for 10 - 20 min. Micropipettes with tip diameters *ca.* 0.5 μm were pulled from 7 cm lengths of tubing mounted in a vertical micropipette puller (Narishige, Tokyo, Japan). Micropipettes were further dried on the hot plate for a minimum of 10 min, then exposed to the vapour of dimethyldichlorosilane ('silanization'). The latter process renders the

glass surface hydrophobic and facilitates filling with, and retention of, the ionophore cocktail used to form an ion-selective liquid membrane at the micropipette tip. A drop of dimethyldichlorosilane (Sigma Chemical Corp., St. Louis, Missouri) was placed inside a 150 mm Pyrex Petri dish, which was then inverted over the micropipettes on the hot plate. Micropipettes were removed after a minimum of 20 min exposure to the silane vapour, and could be stored in a desiccator over silica gel for several months before filling.

Micropipette tips were filled with a short length (100 - 400 μm) of a pH- or ion-selective neutral carrier cocktail. The potassium ionophore cocktail was based on valinomycin (potassium ionophore I, cocktail B, Fluka Chemical Corp., Ronkonkoma, NY), and the hydrogen ionophore cocktail was based on tridodecylamine (hydrogen ion ionophore I, cocktail B, Fluka). The cocktail was taken up in the tip of a plastic 1 ml syringe pulled out over a low flame to a fine tip, then injected into the shank of the micropipette (Thomas, 1978). The cocktail ran to the tip by capillarity, and any trapped air bubbles were removed with a fine glass fibre inserted through the back of the micropipette. Micropipette tips were usually broken back to tip diameters of 5 - 10 μm during filling by touching the tip with a smooth glass or steel surface, or by drawing a piece of tissue paper across the tip. Breaking the tips in this manner resulted in lower resistance microelectrodes (*ca* 1 gigaohm) which were less noisy and had faster response times.

Potassium-selective microelectrodes were backfilled with 0.5 mol l^{-1} KCl injected through a second 1 ml syringe pulled to a fine tip. The pH microelectrodes were backfilled with a solution of 100 mmol l^{-1} NaCl and 100 mmol l^{-1} Na acetate, titrated to pH 6. Reference microelectrodes for use with pH and K^+-selective microelectrodes were pulled from 1 mm o.d.

filamented borosilicate glass tubing pulled to tip diameters of about 0.5 μm. The tip and shank were backfilled with 1 mol l^{-1} sodium acetate, and the rest of the barrel was backfilled with 1 mol l^{-1} KCl.

Chlorided silver wires connected to an electrometer of high input impedance (> 10^{14} ohms; Dagan Model 8800, Dagan Corp., Minneapolis, Minnesota) were inserted into the back-filling solution of ion-selective and reference microelectrodes. Calibration solutions for pH microelectrodes were based on standard saline adjusted to two pH values differing by 1 unit, usually 6.5 and 7.5. Potassium microelectrodes were calibrated in solutions containing 150 mmol l^{-1} KCl or 15 mmol l^{-1} KCl:135 mmol l^{-1} NaCl. These concentrations bracketed the range of secreted fluid potassium concentrations in most experiments. Previous studies have shown that the slope of the K^+ electrode response remains constant down to K^+ concentrations as low as 2 mmol l^{-1} (Maddrell, O'Donnell and Caffrey, 1993). The reported detection limit for microelectrodes based on the ionophore cocktail used in this study is less than 0.02 mmol l^{-1} (Amman *et al.*, 1987).

Ion-selective electrodes measure ion activity and not concentration, but data can be expressed in terms of concentration if it is assumed that the activity coefficient is the same in the calibration solutions and the droplets of secreted fluids. Over the range of ionic strengths of the fluids examined in this study (*ca.* 100 - 180 mmol l^{-1}) the errors resulting from this assumption are small. For example, the activity coefficient of NaCl:KCl mixtures with a total concentration of 150 mmol l^{-1} is 0.750, whereas the activity coefficients for 100 mmol l^{-1} and 200 $mmol^{-1}$ NaCl are 0.778 and 0.735, respectively (Robinson and Stokes, 1965). The use of

concentrations in this study simplifies comparisons with previous studies in which ion concentrations were measured by techniques such as flame photometry.

K^+ concentrations or pH in drops of secreted fluid were measured by positioning ion-selective and reference microelectrodes in the drop and measuring the electrical potential relative to that in drops of calibration solutions. K^+ concentrations were then calculated with the equation:

$$[K^+]_f = [K^+]_c \, 10^{(V/S)},$$

where $[K^+]_f$ and $[K^+]_c$ are the potassium concentrations in secreted fluid and a calibration solution, respectively, V is the change in electrical potential (mV) between the secreted fluid drop and the calibration solution, and S is the slope (mV) measured for a 10-fold change in potassium concentration. Control experiments tested whether the drugs used in this study altered the response of the K^+-selective microelectrodes. With the exception of those compounds noted in the results, none of the drugs produced measurable changes in electrode potential.

For pH measurements, the equation used was:

$$pH_f = pH_c - (V/S),$$

where pH_f and pH_c refer to secreted fluids and calibration solutions, respectively, and S is the slope measured for a 1 unit pH difference.

The % inhibition of K^+ reabsorption in response to variations in bathing saline ion composition or the addition of drugs or metabolic inhibitors to the bathing saline was calculated according to the formula:

$$\% \text{ inhibition} = ([K^+]_{L,D} - [K^+]_L)/([K^+]_U - [K^+]_L) \times 100$$

where $[K^+]_{L,D}$ is the potassium concentration in a droplet of tubule fluid collected from the lower tubule after addition of a drug or modification of bathing saline ionic composition, $[K^+]_L$ is the concentration before addition of the drug, and $[K^+]_U$ is the concentration of potassium in fluid collected from the upper Malpighian tubule. The appendix lists the drugs used in this study, their abbreviations, sources of supply, and solvents for preparation of stock solutions.

Application of drugs to the apical surface of the lower Malpighian tubule: Perfusion of the lower Malpighian tubule lumen.

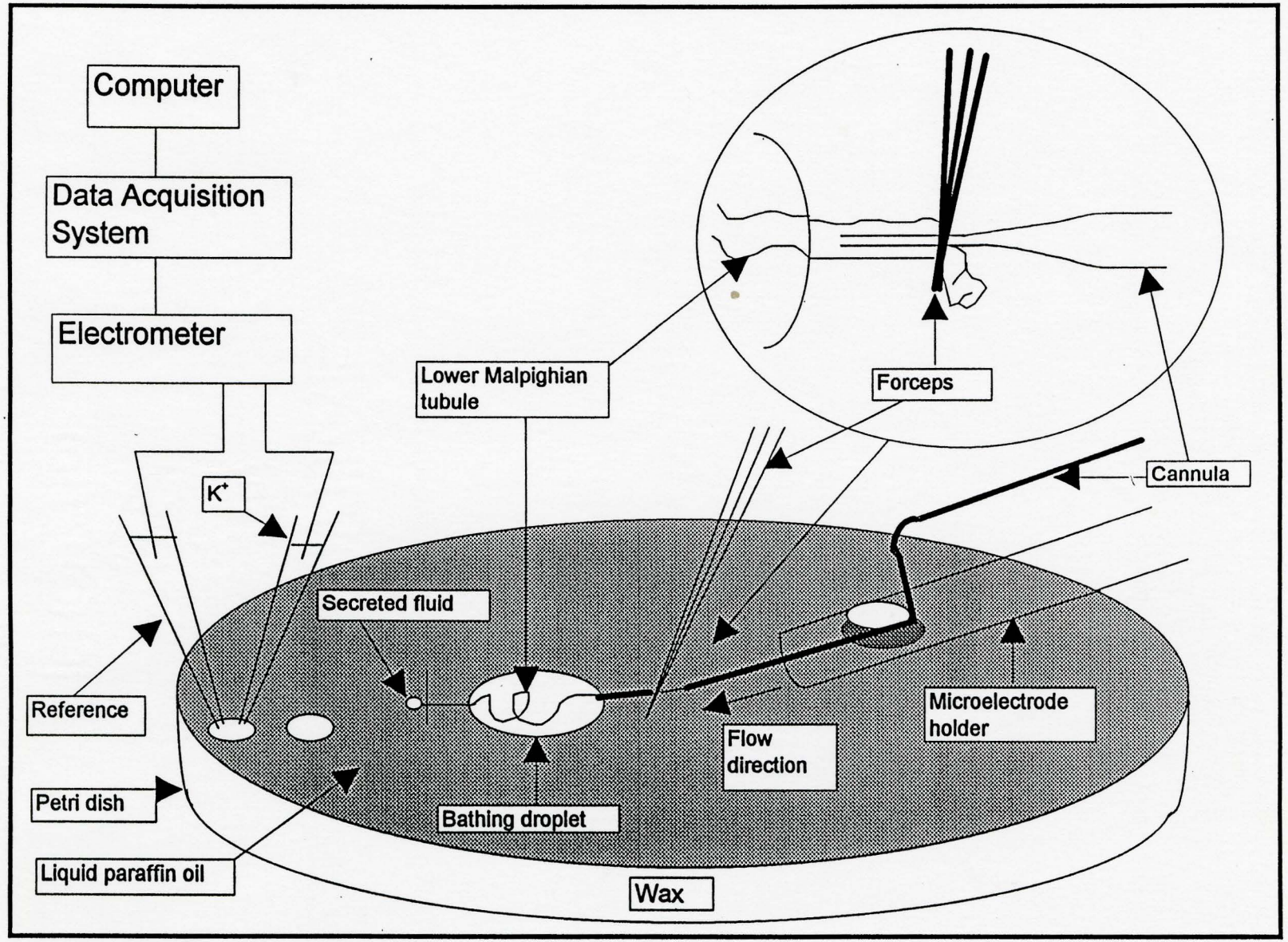
Isolated tubules were cannulated to permit perfusion of the lower tubule lumen with fluid whose ionic composition and drug concentration were known. KCl reabsorption was monitored by K^+-selective microelectrode of K^+ concentration in droplets of fluid which emerged from the ampulla of the lower tubule.

The experimental arrangements for cannulation and lumenal perfusion are shown in figure 2.2. Isolated tubules were cut several mm above the junction of upper and lower tubules and the upper tubule was discarded. A glass probe was used to pull the remaining short length of upper tubule out of the bathing droplet. This length of upper tubule was wrapped around one of the jaws of a pair of #5 forceps, and the forceps were then held closed with a spring clip. The cannula was then aligned with the long axis of the tubule within the bathing drop, and the cannula tip was advanced through the tubule wall and into the lumen.

Cannulae were constructed from 7 cm lengths of unfilamented 1 mm o.d. borosilicate glass tubing pulled to fine tips on a vertical micropipette puller (Narishige, Tokyo). The shaft of each cannula was then bent over a low flame into the dog-leg shape indicated in figure 2.2,

Figure 2.2

Schematic diagram showing the arrangements for lumenal perfusion and measurement of K^+ concentration in droplets of fluid emerging from a lower Malpighian tubule isolated under oil. A glass cannula filled with perfusion saline was mounted on the microelectrode holder. A micromanipulator (not shown) was used to advance the tip of the cannula through the wall of a lower Malpighian tubule held between forceps (inset). PE tubing (not shown) connected the cannula to a motor-driven Hamilton syringe. Tubing and syringe were filled with saline.


Computer
Data Acquisition System
Electrometer
K+
Lower Malpighian tubule
Forceps
Cannula
Secreted fluid
Reference
Petri dish
Liquid paraffin oil
Bathing droplet
Flow direction
Microelectrode holder
Wax

and the tip was broken back to an outside diameter of *ca.* 10 - 20 μm. The micropipette was filled by capillarity with saline (Table 1) and was mounted on the microelectrode holder. The back end of the micropipette was connected with polyethylene tubing (PE 50) to a motor-driven micrometer syringe containing saline. The rotation speed of a DC motor connected by a flexible coupling to the back of a micrometer drive could be adjusted so that the micrometer spindle advanced at a known rate. The spindle depressed the plunger of a 100 μl Hamilton syringe, thereby forcing fluid through the tubing and cannula. The rate of perfusion was determined by removing droplets from the end of the cannula at timed intervals and determining their volume. Fluid perfusion rates were chosen to match the secretion rates of intact whole tubules.

The effects of lumenal application of various drugs on KCl reabsorption were determined as follows. The tubule was first perfused with 75K or 100 K saline (Table 1) and bathed in 4K saline. KCl reabsorption was stimulated by addition of 10^{-6} mol l^{-1} 5HT to the bathing saline droplet. When $[K^+]$ in successive droplets of collected fluid had stabilized, typically at *ca.* 10 mmol l^{-1}, the PE tubing was disconnected from the back of the cannula. The contents of the cannula were then flushed and replaced with the same saline containing the drug of interest at a known concentration. The second solution was injected into the cannula through a 1 ml tuberculin syringe which had been pulled out to a fine tip over a low flame (Thomas, 1978). The PE tubing was then reconnected and the perfusion continued. Because a small volume (*ca.* 200 - 500 nl) of fluid could not be flushed from the shank and tip of the cannula, the second solution did not reach the lower tubule for several minutes after perfusion was re-initiated. Addition of dye to the second solution indicated that there was relatively little

Table 1 Insect salines	2K	4K	8.6K	24K	75K	86K	100K	HCO_3^- Free	PO_4^- Free	PO_4^-/ HCO_3^- Free	Na^+ Free	Cl^- free*	Cl^- Free
pH titrated with NaOH or HCl	7.0 NaOH	7.0 NaOH	7.0 NaOH	7.0 NaOH	7.0 NaOH	7.0 NaOH	7.0 NaOH	7.0 HCl	7.0 NaOH	6.9 HCl	7.0 HCl	7.0 NaOH	7.0 NaOH
Chemicals													
NaCl	135.6	133.6	129	113.6	62.6	51.6	37.6	62.6	133.6	129			
KCl	2	4	8.6	24	75	86	100	75	4	8.6			
$MgCl_2.6H_2O$	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5	8.5		
$CaCl_2.2H_2O$	2	2	2	2	2	2	2	2	2	2	2		
glucose	20	20	20	20	20	20	20	20	20	34	20	20	20
$NaHCO_3$	10.2	10.2	10.2	10.2	10.2	10.2	10.2		10.2			10.2	10.2
NaH_2PO_4	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3				4.3	4.3
HEPES**	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	6.6		8.6	8.6	8.6
BIS-TRIS#										15			
$NaNO_3$													129
KNO_3													8.6
$MgNO_3$													8.5
$CaNO_3$													2
NMDG&											137.6		
K_2SO_4												8.6	
$CaSO_4$												2.0	
$MgSO_4$												8.5	
Na ise$												129	
$KHCO_3$											4 & 8		

* Insect saline replacing NO3 with SO4 and Na isethionate replacing NaCl.
** (N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
# (bis[2-Hydroxyethyl]iminotris-[hydroxymethyl]methane)
& N-methyl-D-glucamine
$ isethionic acid

mixing of the first and second solutions within the cannula. The appearance of dye in the lumen was used to confirm the time required for the drug to reach the lower tubule. Control experiments showed that the dye used for this purpose (amaranth) had no effect on KCl reabsorption.

Statistics

Values are reported as mean ± 1 S.E. Calculations and graphing of experimental results were performed using spreadsheets (Quattro Pro, Excel). Significance of differences between means were calculated by Student's t-tests.

Results

Potassium-selective microelectrode measurements of [K^+] in secreted fluid droplets.

Results of an experiment in which [K^+] was measured in droplets of fluid secreted by a single Malpighian tubule before and after addition of 5-hydroxytryptamine (5HT) and 1% dimethylsulphoxide are shown in Figure 2.3A,B. The electrical potential recorded by the K^+-selective microelectrode is shown in Figure 2.3A. In figure 2.3B, the potassium concentration of each droplet has been calculated and plotted against time of droplet collection after the start of the experiment. Stimulation of potassium reabsorption is indicated by the decline in secreted fluid [K^+]. In this experiment, K^+ reabsorption was initiated within 5 min of the addition of 5HT, was fully stimulated in less than 10 minutes, and [K^+] levels were stable thereafter.

Figure 2.3B also shows that addition of 1% dimethylsulphoxide (DMSO) to the saline bathing the lower tubule did not alter the extent of K^+ reabsorption. Many of the compounds used in this study were poorly soluble in aqueous solutions, and stock solutions were therefore made up in either DMSO or ethanol (EtOH). Results in other control experiments with 1% DMSO ($n = 3$) or 1% EtOH ($n = 2$) were similar. In subsequent experiments, therefore, drugs which were poorly soluble in saline were made up as stock solutions in DMSO or EtOH, and then added to bathing saline drops so that the concentration of the solvent did not exceed 1 %.

Figure 2.3C shows that KCl reabsorption is sustained for prolonged periods (120 min). Similar results were obtained in 4 other tubules over 40-60 minutes. Small increases in secreted fluid [K^+] with time may be related to the increase in bathing saline [K^+] with time as

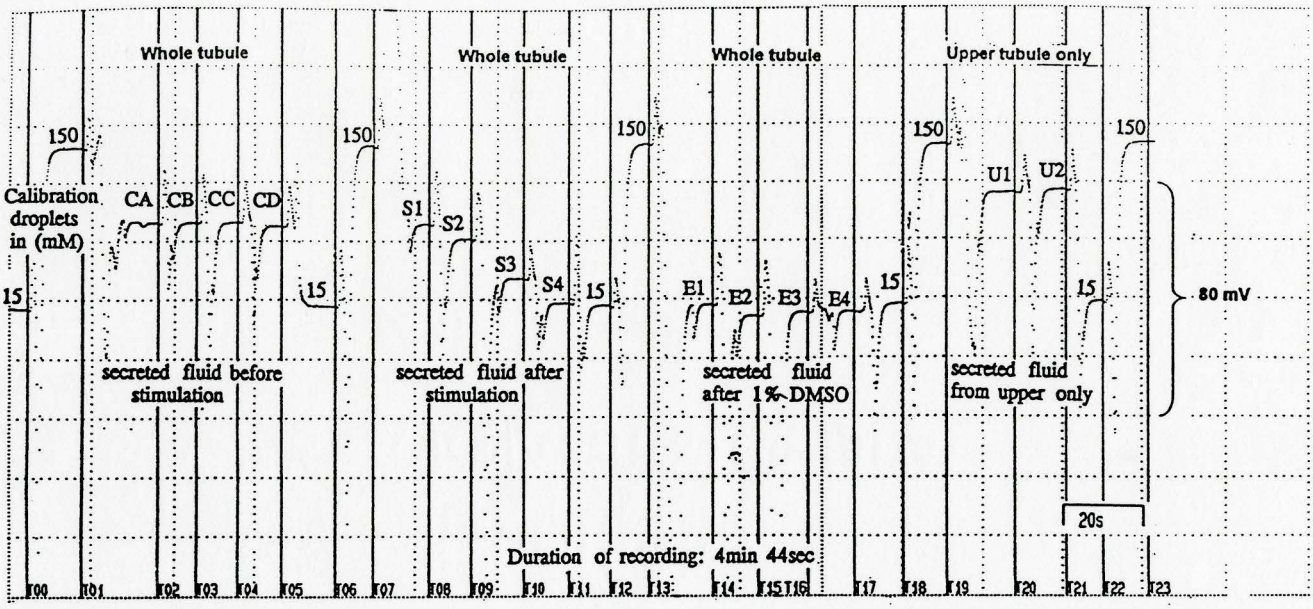
Figure 2.3

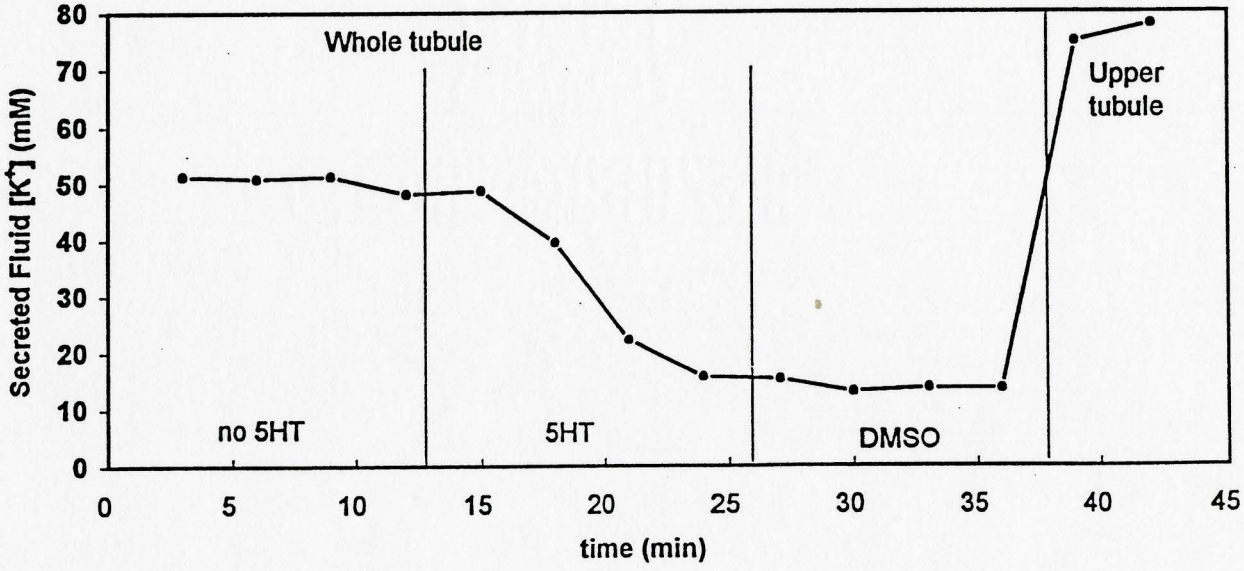
Sample recordings of K^+ concentration in fluid secreted by upper or whole Malpighian tubules.

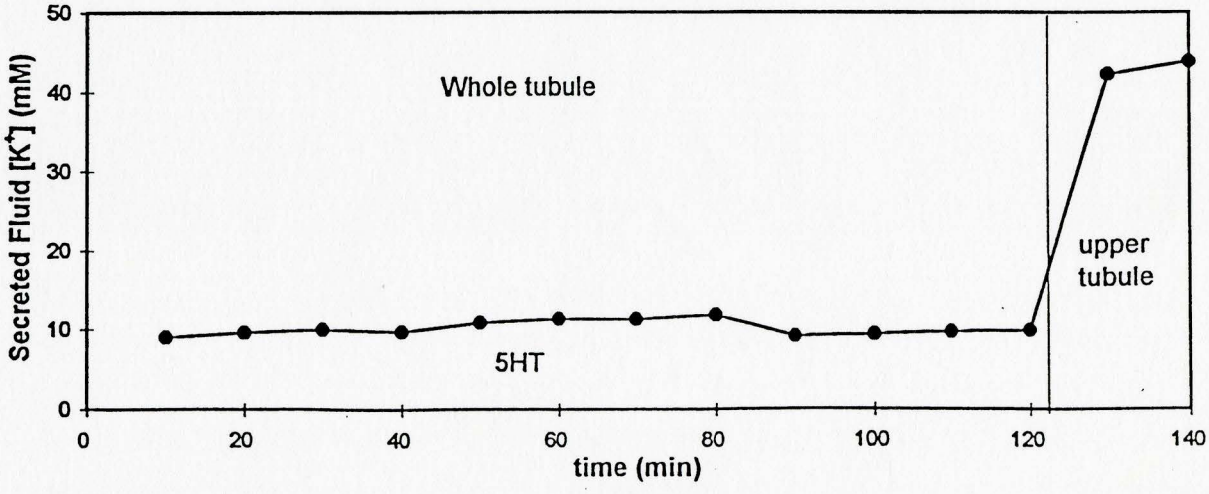
A) Recordings of the electrical potential of a K^+ selective-microelectrode positioned in droplets of secreted fluids or calibration solutions. The labels 150 and 15 refer to calibration solutions containing 150 $mmol^{-1}$ KCl and 15 mmol l^{-1}KCl/135 mmol l^{-1} NaCl, respectively. CA - CD and S1 - S4 refer to secreted fluid droplets collected before and after, respectively, the addition of 5-HT (10^{-6} mol l^{-1}) to the saline droplet bathing the lower tubule. E1 - E4 refer to droplets collected after addition of DMSO (1%) to the droplet bathing the lower tubule, and U1, U2 refer to droplets of fluid collected from the upper tubule after cutting the tubule at the upper/lower junction.

B) K^+ concentrations calculated from the data in A (see text for details). The 14 points correspond in order to CA - CD, S1 - S4, E1 - E4 and U1 - U2 in panel (A) above. The x-axis refers to the time of droplet collection after the start of the experiment.

C) Calculated K^+ concentrations in droplets collected from a second tubule at intervals over 120 minutes. The tubule was bathed in 4K saline containing 5-HT throughout the experiment. Note the stability of K^+ concentration over time.


Whole tubule
Whole tubule
Whole tubule
Upper tubule only
150
Calibration droplets in (mM)
15
CA
CB
CC
CD
S1
S2
S3
S4
E1
E2
E3
E4
U1
U2
20s
80 mV
secreted fluid before stimulation
secreted fluid after stimulation
secreted fluid after 1% DMSO
secreted fluid from upper only
Duration of recording: 4min 44sec


Secreted Fluid [K+] (mM)
time (min)
Whole tubule
Upper tubule
no 5HT
5HT
DMSO


Secreted Fluid [K+] (mM)
time (min)
Whole tubule
upper tubule
5HT

K^+ is transported from the lower tubule lumen to the saline bathing the lower tubule.

Measurement of K^+ concentration in fluid collected from perfused tubules.

Figure 2.4 shows the result of a control experiment in which 100K saline was perfused through a tubule bathed in 4K saline containing 5HT. The K^+ concentration of the perfusate was reduced from 100 mmol l^{-1} to less than 10 mmol l^{-1} as the fluid passed through the lower Malpighian tubule. At the 11 minute mark on the record, the cannula was disconnected from the PE tubing and motor-driven syringe, and the contents of the cannula were flushed and replaced with the same solution (100K saline). The cannula was then reconnected to the PE tubing and motor-driven syringe, and perfusion was restarted. By the 20 minute mark, the K^+ concentration in the droplets of fluid emerging from the ampulla was within 1 - 2 mmol l^{-1} of the value recorded prior to the solution change. The higher K^+ concentration in the droplet collected at the 18 minute mark is probably the result of a transient increase in lumenal perfusion rate. This results from a small increase in pressure which occurs when the PE tubing is reconnected to the back of the cannula. In experiments requiring addition of drugs to the lumenal perfusate, the first 2 - 3 droplets collected in the 5 - 10 minutes after a solution change were disregarded.

Effects of bathing saline $[K^+]$ on $[K^+]_{sf}$

Previous studies of isolated tubules of *Rhodnius* have used a bathing saline containing 8.6 mmol l^{-1} K^+ (eg. O'Donnell and Maddrell, 1984). Figure 2.5 shows the K^+ concentrations in fluid secreted by the upper tubule and after passage through the lower tubule. A higher concentration (24 mmol l^{-1}) of bathing saline K^+ was chosen for upper tubules for most of the

Figure 2.4

K^+ concentration in droplets of fluid collected from a tubule which was cannulated and perfused with 100K saline. The tubule was bathed in 4K saline containing 10^{-6} mol l^{-1} 5-HT throughout the duration of the experiment. K^+ reabsorption by the lower tubule reduced the K^+ concentration of the prefusate from 100 to less than 10 mmol l^{-1}. At the 11 minute mark, the PE tubing connecting the cannula to the motor-driven syringe was removed from the back of the cannula, and the contents of the cannula were flushed with fresh 100K saline. The PE tubing was then re-connected and lumenal perfusion continued. Increases in K^+ concentration in fluid collected during the first few minutes after the change were probably the result of increased pressure and a resultant transient increase in lumenal perfusion rate when the PE tubing was re-connected. Note that K^+ reabsorption by the tubule had reduced droplet K^+ concentration to the level before the flushing of the cannula by the 20 minute mark *i.e* by 10 minutes after the flushing of the cannula.

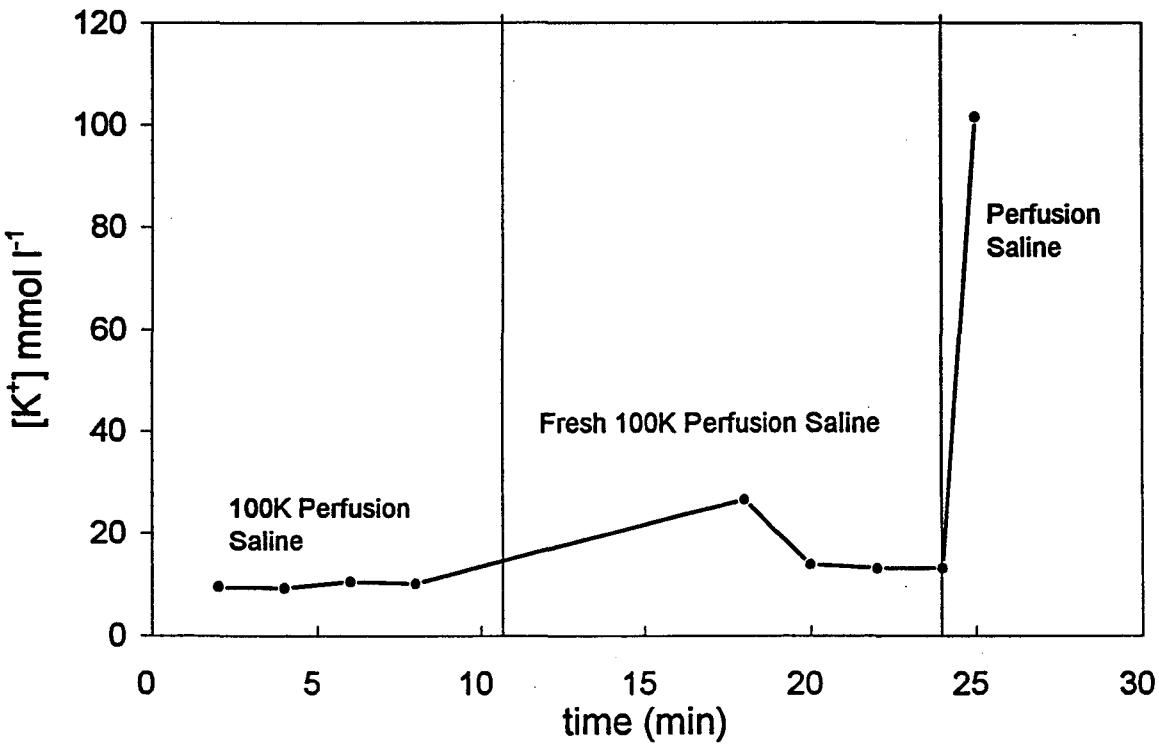
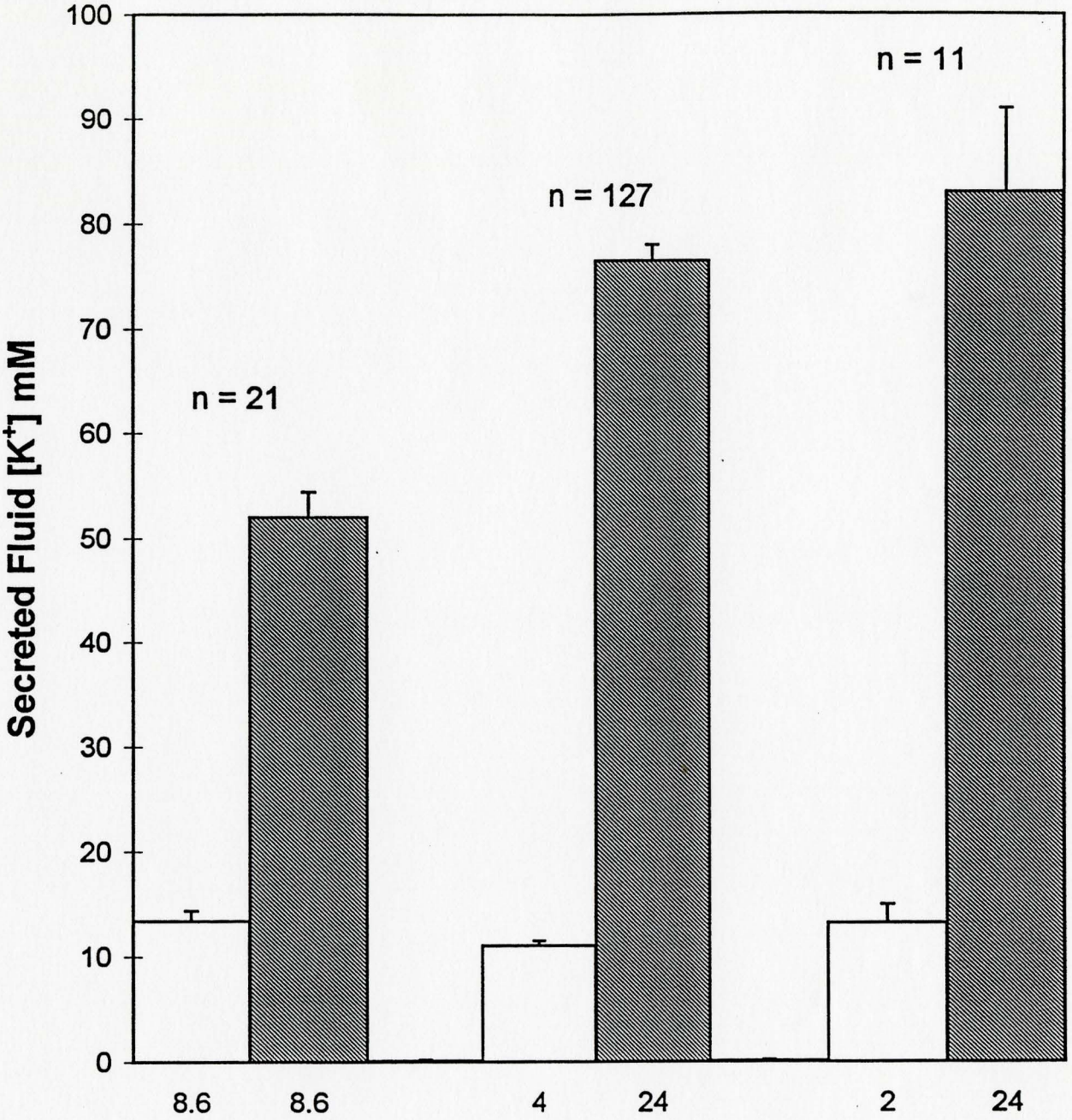

120
100
80
60
40
20
0
[K+] mmol l-1
0
5
10
15
20
25
30
time (min)
100K Perfusion Saline
Fresh 100K Perfusion Saline
Perfusion Saline

Figure 2.5

K^+ concentrations of fluid collected from whole tubules (open bars) or from upper segments (cross-hatched bars) of the same tubules bathed in salines containing the indicated concentration (mmol l^{-1}) of K^+. The height of each bar shows the mean K^+ concentration (+ 1 S.E.) for the indicated number of tubules.


Secreted Fluid [K+] mM
100
90
80
70
60
50
40
30
20
10
0
n = 21
n = 127
n = 11
8.6
8.6
4
24
2
24

subsequent experiments because the upper tubules sustained a steady rate of secretion over longer periods of time, and because the K^+ level in the secreted fluid was higher (*ca.* 70 mmol l^{-1}) and less variable. This permitted easier detection of inhibition of KCl reabsorption, since the scope for increases in the K^+ concentration (*i.e.* the difference between K^+ concentration in upper and lower tubule fluid) was larger. Lower tubules were bathed in 4 mmol l^{-1} K^+ because this value approximates that in the haemolymph (3.6 mmol l^{-1}; Maddrell *et al.*, 1993). A further reduction form 4 to 2 mmol l^{-1} K^+ in the saline bathing the lower tubule was not associated with further decline in the potassium concentration in the fluid *i.e.* there was no further increase in the effectiveness of KCl reabsorption. For most experiments involving application of putative transport inhibitors to the basolateral surface of the Malpighian tubules, therefore, the salines bathing the upper and lower tubules were 24K and 4K, respectively. In some experiments, noted below, the lower tubules were bathed in 8.6K or 2K saline, or the upper tubule was bathed in 8.6K saline

Secreted fluid pH

The pH of fluid collected from lower tubules was 8.48 ± 0.07 (n = 18). For the same tubules, the pH of fluid secreted by the upper segment was 7.26 ± 0.10. The bathing saline pH was 6.9 - 7.0, and K^+ concentration was 8.6 or 24 mmol l^{-1} in the upper tubule droplet, and 8.6 or 4 mmol l^{-1} in the lower tubule droplet. There was no significant difference in secreted fluid pH over these ranges of bathing saline K^+ concentrations, and the data were therefore pooled. These data indicate that the upper tubule fluid is alkalinized by approximately 1.4 pH units as it

passes through the lower tubule, and that lumen of the lower tubule is alkaline with respect to the bath.

Effects of Inhibition of Metabolism and Ion-transporting ATPases on K^+ Reabsorption

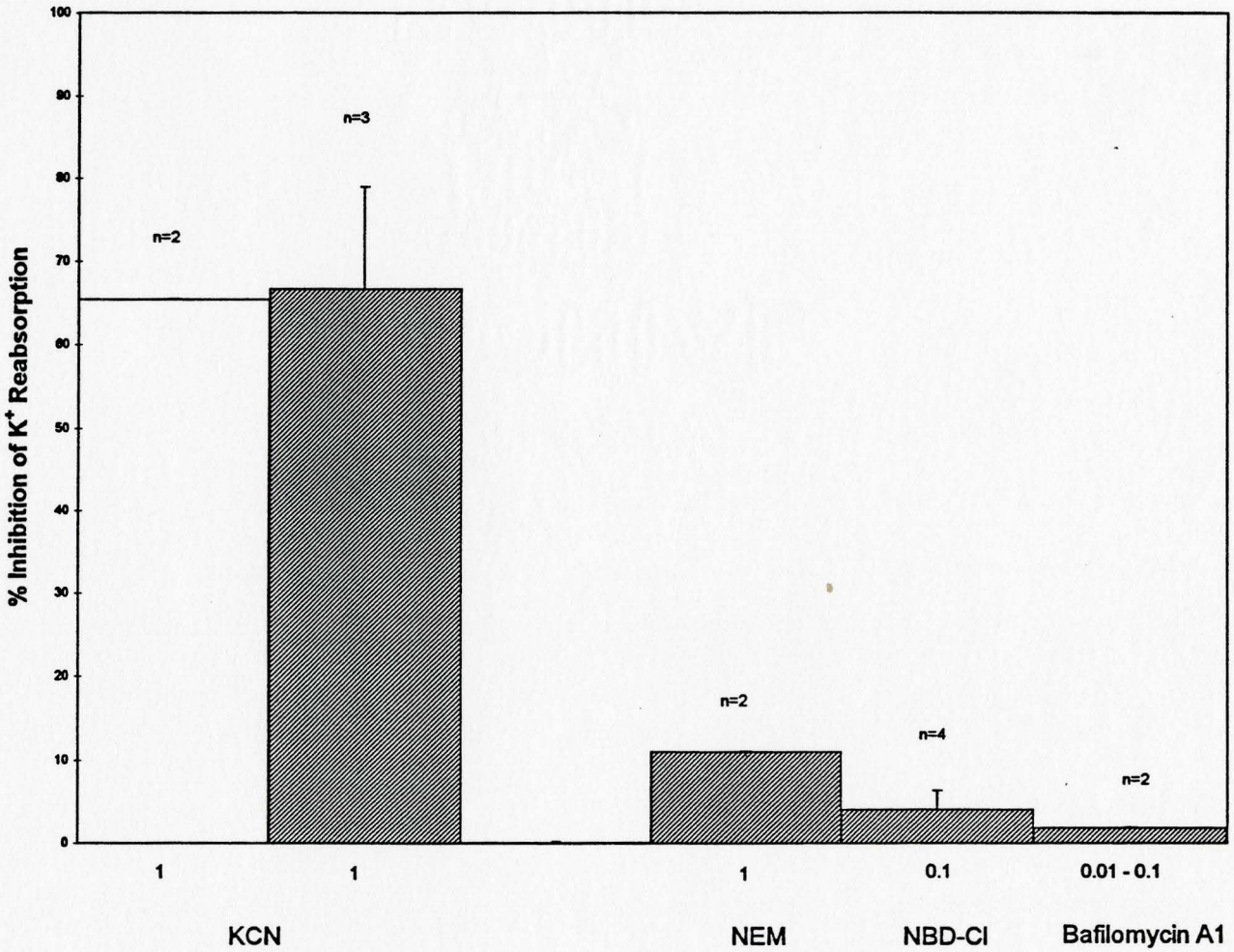
Figure 2.6 shows that KCl reabsorption was inhibited 65% - 70% by basolateral or apical application of 0.02 - 1 mmol l^{-1} KCN, which inhibits ATP synthesis by blocking mitochondrial electron transport (Lehninger, 1970).

The effects of three inhibitors of V-type H^+-ATPases on KCl reabsorption were also examined. Both N-ethylmaleimide (NEM) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) are alkylating reagents; NBD-Cl reacts with tyrosine or cysteine residues, whereas NEM is relatively selective for sulphhydryl groups (Forgac, 1989). Fluid secretion by *Formica* Malpighian tubules is inhibited 93% by 0.05 mmol l^{-1} NEM. Bafilomycin A_1 is a macrolide antibiotic which is a highly specific inhibitor of V-ATPases (Bowman *et al.*, 1988). Fluid secretion in *Formica* and *Drosophila* tubules is inhibited by 5 μmol l^{-1} (Weltens *et al.*, 1992) and 1 μmol l^{-1} (Bertram *et al.*, 1991) bafilomycin A_1, respectively.

Basolateral application of high concentrations of V-ATPase inhibitors had almost no effect on KCl reabsorption by the lower tubule (Fig. 2.6), in marked contrast to the dramatic effects of basolateral application of the same drugs on ion transport by the upper Malpighian tubule. During collection of secreted fluid droplets from the lower tubule, the upper tubule was bathed in saline containing 5HT but no inhibitors. At the end of the experiment, the upper segment was pulled into the droplet containing the lower tubule and the drug. For each drug, fluid secretion by the upper segment stopped within a few minutes of tranfer, confirming the

Figure 2.6

Effects of KCN or inhibitors of V-type H^+ ATPases on K^+ reabsorption. The height of each bar shows the mean % inhibition (+ 1 S.E.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are shown above each drug listed on the abscissa. In this and subsequent figures, apical and basolateral application of the drugs are indicated by open and cross-hatched bars, respectively. Apical application required cannulation of the tubule and addition of the drug to the 75K lumenal perfusion saline. For basolateral application the drugs were dissolved in 4K saline bathing the lower tubule, and the upper tubules were bathed in 24K saline.


% Inhibition of K+ Reabsorption
100
90
80
70
60
50
40
30
20
10
0
n=2
n=3
n=2
n=4
n=2
1
1
1
0.1
0.01 - 0.1
KCN
NEM
NBD-Cl
Bafilomycin A1

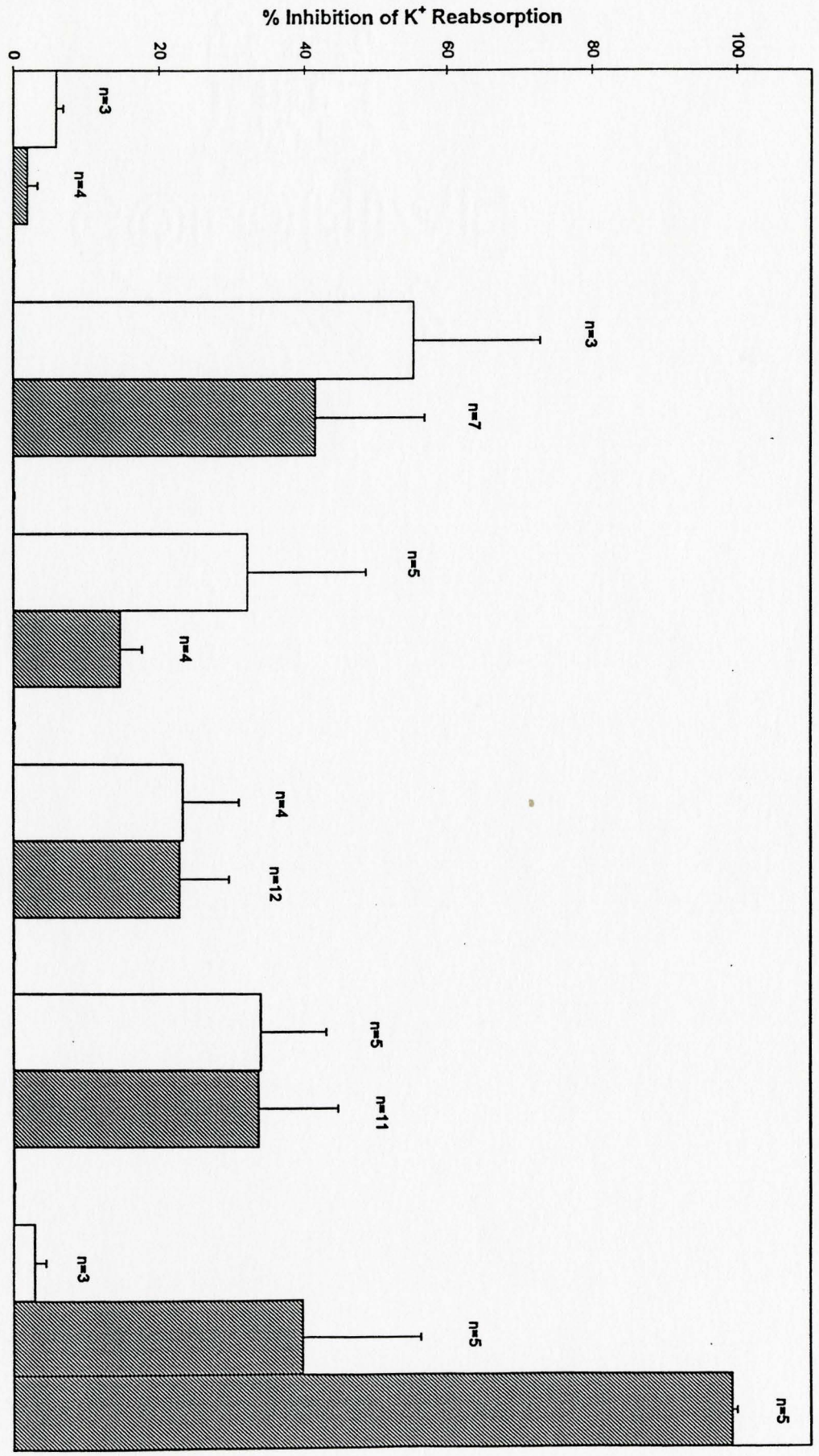
inhibition of the apical V-type H+-ATPase of the upper tubule by addition of the drugs to the saline bathing the basolateral surface.

Previous studies have provided evidence for the presence of Na^+,K^+-ATPase activity in upper Malpighian tubules of *Rhodnius* (Maddrell and Overton, 1988) and in the upper portion of the lower Malpighian tubule (O'Donnell and Mandelzys, 1988). Ouabain (1 mmol l^{-1}) does not inhibit hormonally-stimulated fluid secretion (Maddrell, 1969). Surprisingly, ouabain actually increases fluid secretion by unstimulated tubules (Maddrell and Overton, 1988). The evidence suggests that a basolateral Na^+,K^+-ATPase normally transports sodium in a direction opposite to the flow of water and ions involved in fluid transport, thereby reducing the net flow of Na^+ across the epithelium and slowing fluid secretion. Ouabain poisons the sodium pump, and removes this brake, so that the rates of both sodium transport and fluid secretion increase. This effect is not seen in stimulated tubules, since the rate of Na^+ movement through the basolateral $Na^+/K^+/2Cl^-$cotransporter and the combination of the apical H^+-ATPase, Na^+/H^+ antiporter is so much larger than through the basolateral Na^+/K^+-ATPase. When the Na^+/K^+-ATPase inhibitors ouabain (0.1 - 0.22 mmol l^{-1}) or dihydro-ouabain (0.1 mmol l^{-1}) were added to the saline bathing the lower Malpighian tubule, KCl reabsorption was unaffected (n = 5 tubules for ouabain, n = 2 tubules for dihydro-ouabain).

Vanadate is a general inhibitor of P-type (E1-E2) ATPases, which form a phosphorylated intermediate. The Na^+/K^+-ATPase, the plasma membrane Ca_2^+-ATPase and the gastric mucosa H^+/K^+-ATPase are all of this type. Vanadate (5 mmol l^{-1}) inhibited KCl reabsorption by 40% - 55% when applied basolaterally or apically (Fig. 2.7). The effects of

Figure 2.7

Effects of vanadate and H^+/K^+ ATPase inhibitors on K^+ reabsorption.The height of each bar shows the mean % inhibition (+ 1 S.E.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are listed above each drug listed on the abscissa. Apical and basolateral application of the drugs are indicated by open and cross-hatched bars, respectively.


% Inhibition of K+ Reabsorption
0
20
40
60
80
100
1 - 3
1 - 3
5
5
0.05
0.05
0.1
0.1
0.1
0.1
0.4
0.1
0.2 - 0.4
n=3
n=4
n=3
n=7
n=5
n=4
n=4
n=12
n=5
n=11
n=3
n=5
n=5
Vanadate
Vanadate
SKF 96067
SKF 96356
SCH 28080
Omeprazole

vanadate were reversible (figure 2.8) and 5 mmol l^{-1} was much more effective than 3 mmol l^{-1}. Taken together, the effects of vanadate and the absence of significant inhibition of KCl reabsorption by ouabain suggested the possible involvement of a P-type ATPase other than the Na^+/K^+-ATPase during KCl reabsorption by the lower Malpighian tubule. The effects of specific inhibitors of the H^+/K^+-ATPase were therefore examined.

In gastric mucosa the imidazopyridine SCH 28080 interacts competitively with the lumenal K^+ binding site of the H^+/K^+-ATPase. Two other compounds, the acylquinoline SKF 96067 and the pyrroloquinoline SKF 96356 lack obvious structural similarity to SCH 28080, but appear nonetheless to inhibit the H^+/K^+ ATPase by a similar mechanism (Pope and Sachs, 1992). All three of these compounds at concentrations of 50 - 100 μmol l^{-1} appeared to inhibit partially KCl reabsorption by the lower tubule (Fig. 2.7). Figure 2.9 shows the progressive inhibition of K^+ reabsorption over time for a single Malpighian tubule in which 100 μmol l^{-1} SCH 28080 was added to the saline bathing the lower tubule. Levels of inhibition were similar when the drugs were applied to either the basolateral or apical surfaces of the lower Malpighian tubule. Both SCH 28080 and SKF 96356 partially inhibited fluid secretion by the upper Malpighian tubule, whereas SKF 96067 did not. Possible explanations for the effects of these compounds on the upper tubule are discussed below.

One feature of all drugs which inhibited KCl reabsorption (Figure 2.10) was the high degree of variability in response. Figure 2.10 shows the effects of 100 μmol l^{-1} SKF 96356 on tubules from the same insect. Maximum inhibition observed for the other 3 tubules was 83%, 31% and 18%, respectively; there was no significant change in K^+ secreted by a control tubule over the same time period. These data suggest that the variability is not just caused by genetic

Figure 2.8

K^+ concentration in emergent fluid collected from a cannulated lower tubule perfused with 75K saline. Vanadate at 3 and 5 mmol l^{-1} was added to the 75K perfusing saline at 9 and 24 minutes. Partial inhibition of KCl reabsorption is indicated by the increase in the K^+ concentration of the collected fluid. At 41 minutes the perfusing saline was changed back to 75K saline and the level of K^+ in the emergent fluid returned to that measured prior to addition of vanadate. At 57 minutes the tubule was removed from the cannula and two droplets of the perfusion saline were collected.

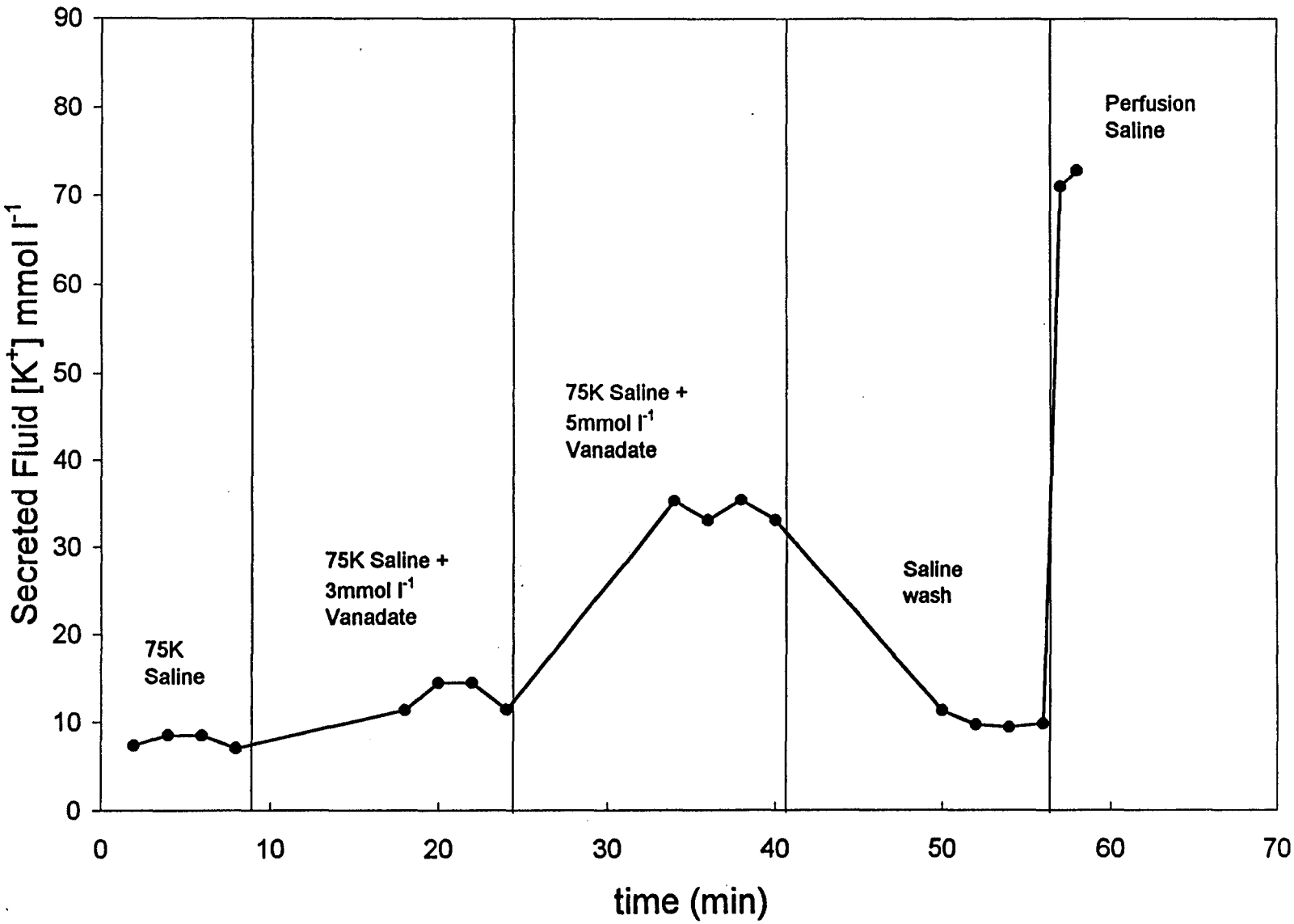

Secreted Fluid [K+] mmol l-1
time (min)
0
10
20
30
40
50
60
70
80
90
75K Saline
75K Saline + 3mmol l-1 Vanadate
75K Saline + 5mmol l-1 Vanadate
Saline wash
Perfusion Saline

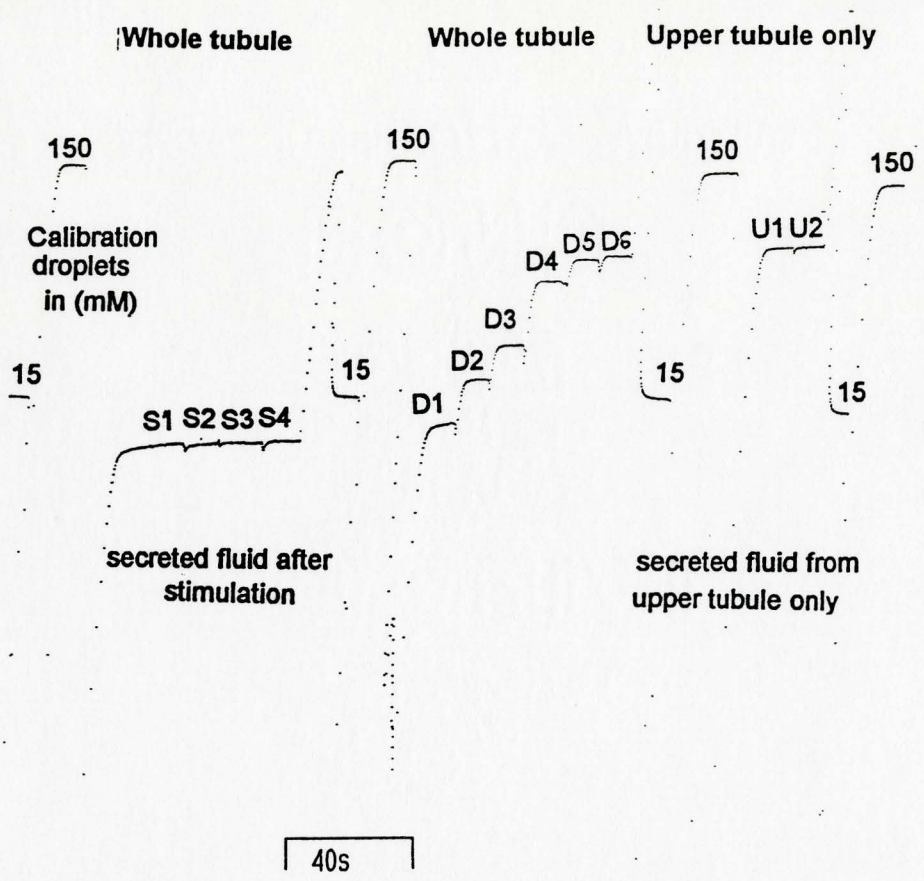
Figure 2.9

Sample recording showing the effects of SCH 28080 on K^+ concentration in fluid secreted by a single Malpighian tubule.

A) Recordings of the electrical potential of a K^+ selective-microelectrode positioned in droplets of secreted fluids or calibration solutions. The labels 150 and 15 refer to calibration solutions containing 150 $mmol^{-1}$ KCl and 15 mmol l^{-1}KCl/135 mmol l^{-1} NaCl, respectively. S1 - S4 refer to secreted fluid droplets collected after addition of 5-HT (10^{-6} mol l^{-1}) to the saline droplet bathing the lower tubule. D1 - D6 refer to droplets collected after addition of 0.1 mmol l^{-1} SCH28080 (in 1% DMSO) to the droplet bathing the lower tubule, and U1, U2 refer to droplets of fluid collected from the upper tubule after cutting the tubule at the upper/lower junction.

B) K^+ concentrations calculated from the data in panel A, above. The 12 points correspond in order to S1 - S4, D1 - D6 and U1 - U2 in panel (A) above. The x-axis refers to the time of droplet collection after the start of the experiment.

A


Whole tubule
Whole tubule
Upper tubule only
150
150
150
150
Calibration
droplets
in (mM)
15
15
15
15
S1 S2 S3 S4
D1
D2
D3
D4
D5 D6
U1 U2
secreted fluid after
stimulation
secreted fluid from
upper tubule only
40s

B

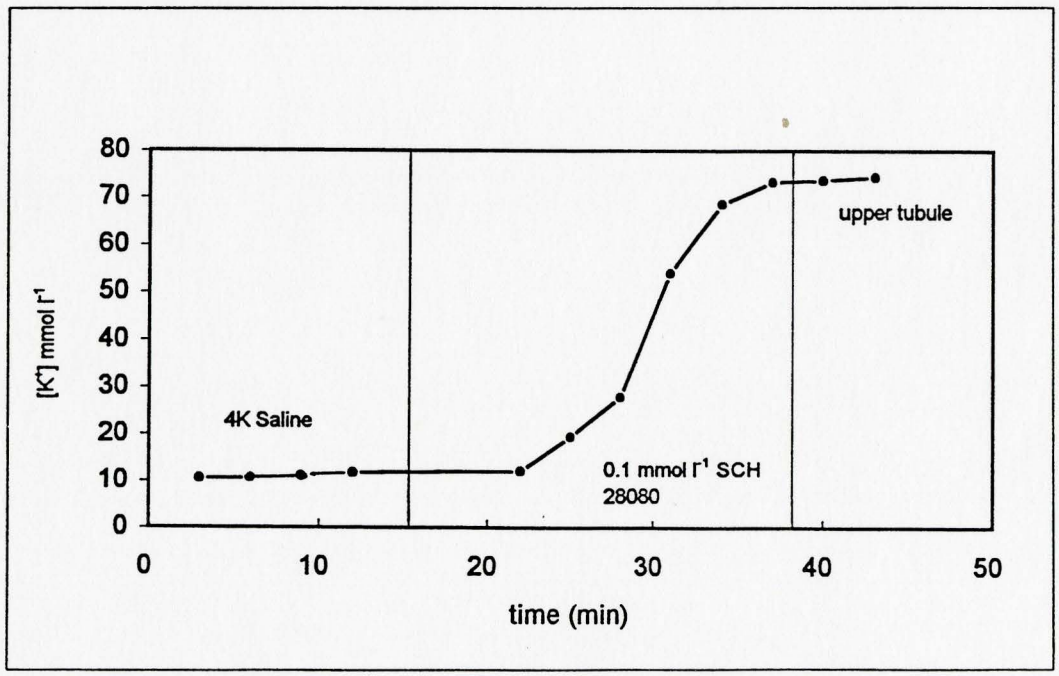
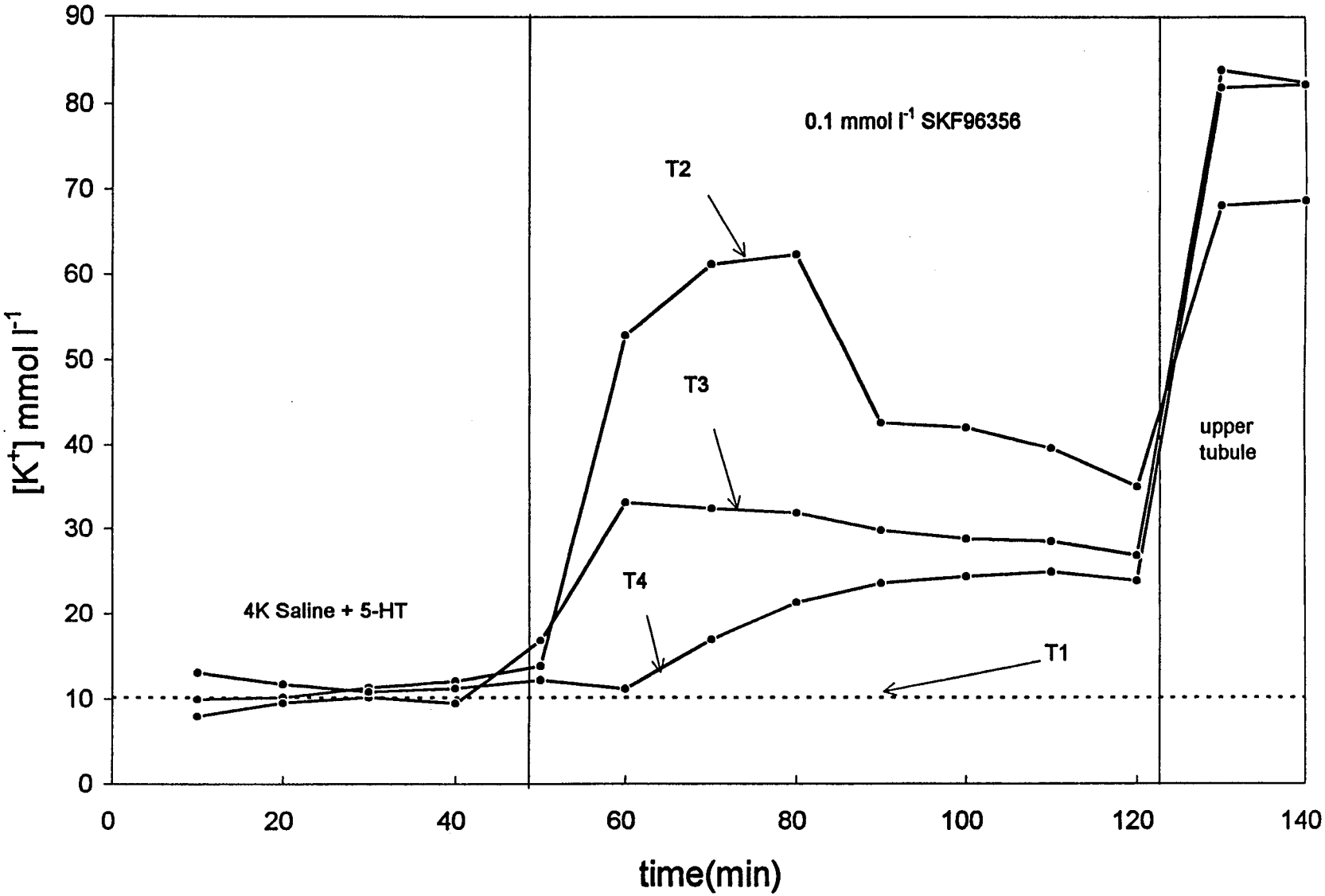

80
70
60
50
40
30
20
10
0
[K+] mmol l-1
4K Saline
0.1 mmol l-1 SCH
28080
upper tubule
0
10
20
30
40
50
time (min)

Figure 2.10

Effects of SKF 96356 on K^+ concentration in fluid secreted by 3 Malpighian tubules from the same insect. Upper segments of each tubule were bathed in 24K saline containing 10^{-6} mol l^{-1} 5-HT. At 50 minutes, 0.1 mmol l^{-1} SKF 96356 was added to the droplets of 4K saline bathing the lower segment of three of the tubules (T2 - T4). The mean K^+ concentration in fluid secreted by a fourth tubule (T1) which was not exposed to the drug is shown by the dashed line. At 120 minutes, the tubules were transected at the upper/lower junction and droplets of upper tubule fluid were collected. Note the variability in the time course and extent of inhibition of KCl reabsorption, indicated by the increase in K^+ concentration of the secreted fluid droplets.


90
80
70
60
50
40
30
20
10
0
[K+] mmol l-1
0
20
40
60
80
100
120
140
time(min)
0.1 mmol l-1 SKF96356
T2
T3
T4
T1
4K Saline + 5-HT
upper
tubule

differences among insects, but is a function of physiological differences in tubules from the same individual.

SKF 96067, SKF 96356 and SCH 28080 are reversible inhibitors which compete with K^+ for a binding site on the H^+/K^+-ATPase, whereas the irreversible inhibitor omeprazole is itself ineffective but is converted at acidic pH into a sulphenamide which reacts with cysteines in the pump molecule (Pope and Sachs, 1992; Wallmark et al., 1984). Omeprazole inhibited K^+ reabsorption virtually completely when applied to the basolateral membrane at concentrations of 0.2 - 0.4 mmol l^{-1}, but resulted in only a trace level of inhibition when applied to the lumenal surface at 0.4 mmol l^{-1} (Fig. 2.7). Two possible explanations for the latter difference are: 1) the site of action of omeprazole (*i.e.* the putative H^+/K^+-ATPase) is on the basolateral membrane, or 2) the site of action is on the apical membrane but access of the active form of the drug to the cytoplasmic surface of the apical membrane is limited by bath-lumen pH differences or reduced permeability of the apical membrane. These possibilities are discussed further below. Omeprazole did not inhibit fluid secretion by the upper Malpighian tubule.

Effects of amiloride.

Amiloride inhibits epithelial Na^+ channels at micromolar concentrations, and Na^+/H^+ exchange at concentrations above 100 μmol l^{-1} (Kleyman and Cragoe, 1988). Amiloride is an effective inhibitor of fluid secretion by Malpighian tubules of *Locusta* (Fathpour and Dahlman, 1994), *Rhodnius* (Maddrell and O'Donnell, 1992) and *Drosophila hydei* (Bertram, 1989). In both *D. hydei* (*ibid*) and *Rhodnius* (Maddrell and O'Donnell, 1992) amiloride also results in acidification of luminal fluid, and this acidification is abolished in the presence of the V-ATPase

inhibitor bafilomycin A_1. These data suggest that Na^+ and K^+ movement across the apical membrane is mediated by amiloride-sensitive Na^+/H^+ or K^+/H^+ exchange, and that the exchange is driven by the active secretion of H^+ from cell to lumen by the apical V-ATPase.

In marked contrast to the effect of amiloride on fluid secretion by the upper tubule, 0.5 mmol l^{-1} amiloride did not significantly reduce KCl reabsorption by the lower tubule (n = 8 tubules). Effects of amiloride analogues such as hexamethylene amiloride, methylisopropylamiloride and ethylisopropyl amiloride were not examined in detail because these drugs interfered with K^+ selective microelectrodes.

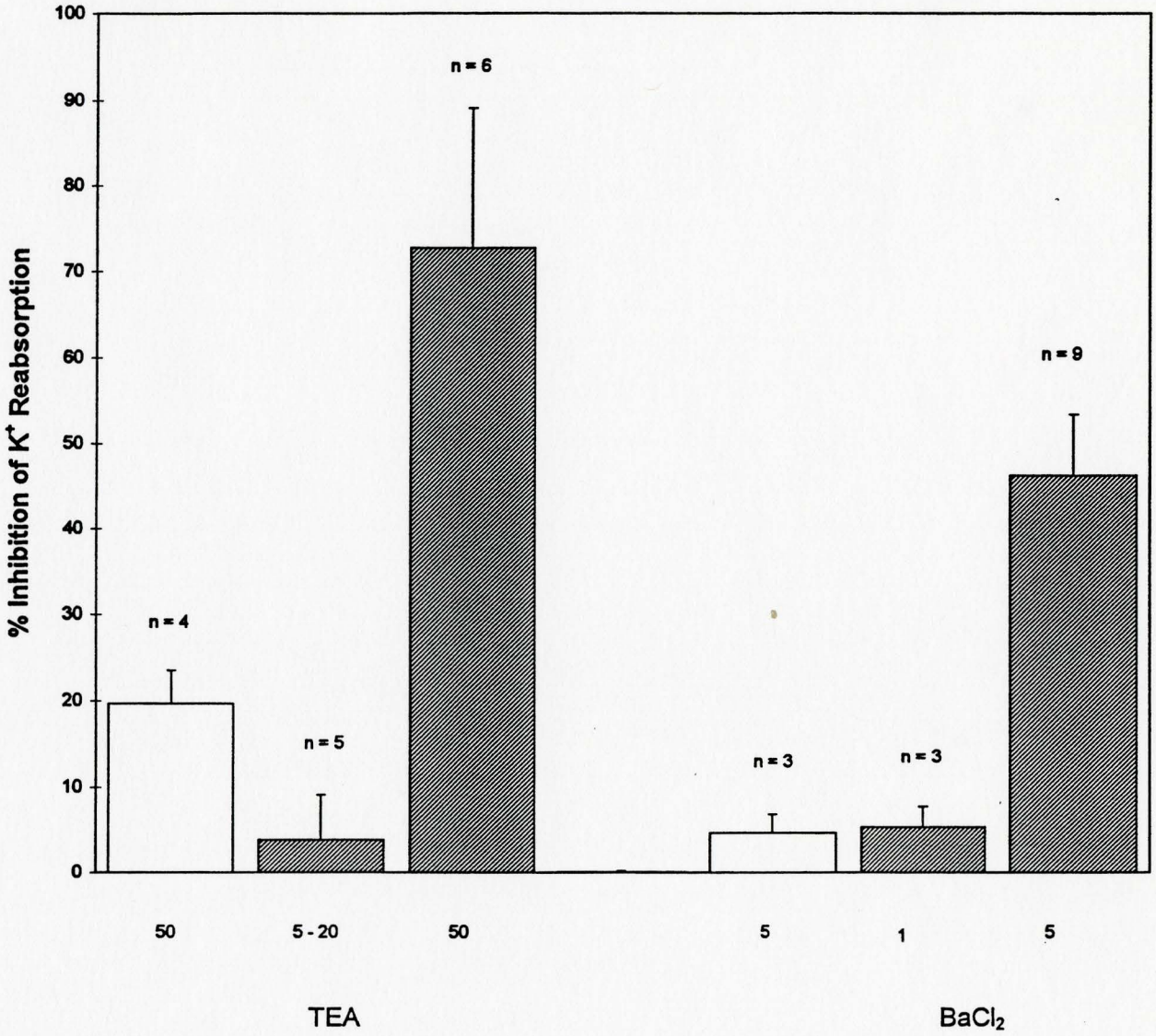
Effects of potassium channel blockers

Barium ions are known to block many types of potassium channels (Hille, 1984). The diameter of Ba^{2+} (0.135 nm) is similar to that of K^+, so that although Ba^{2+} ions apparently pass through K^+ channels, their passage is so slow as to effectively block the pore. Since ions must be stripped of some of their hydration shell as they pass through a channel, Ba^{2+} blockade is due in part to the slower exchange of water molecules around divalent cations, relative to monovalent cations. Concentrations of external Ba^{2+} required for effective blockade of K^+ channels ranges from 0.8 mmol l^{-1} for the delayed rectifier of frog muscle (Standen and Stanfield, 1978) to 6 mmol l^{-1} for Malpighian tubules (Leyssens *et al.*, 1994), 10 mmol l^{-1} for insect follicles (O'Donnell, 1988).

KCl reabsorption was inhibited less than 5% and more than 45% by basolateral application of 1 mmol l^{-1} and 5 mmol l^{-1} Ba^{2+}, respectively (Figure 2.11). KCl reabsorption was inhibited to much smaller extent when applied apically.

Figure 2.11

Effects of Ba^{2+} and TEA on K^+ reabsorption.The height of each bar shows the mean % inhibition (+ 1 S.E.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are listed above each drug listed on the abscissa. Apical and basolateral application of the drugs are indicated by open and cross-hatched bars, respectively.


% Inhibition of K+ Reabsorption
100
90
80
70
60
50
40
30
20
10
0
n = 4
n = 5
n = 6
n = 3
n = 3
n = 9
50
5 - 20
50
5
1
5
TEA
BaCl2

High external concentrations of quaternary ammonium compounds such as tetraethylammonium (TEA) block most types of K^+ channel (Hille, 1984). K^+ channels in insect motoneurones and follicles are blocked by 50 mmol l^{-1} TEA (Pitman, 1978) and 10 - 80 mmol l^{-1} TEA (O'Donnell, 1988), respectively. KCl reabsorption by the lower tubule was inhibited only slightly by basolateral application of 5 - 20 mmol l^{-1} TEA, whereas a concentration of 50 mmol l^{-1} TEA resulted in an inhibition of more than 70% (fig. 2.11). As noted above for Ba^{2+}, TEA was much more effective when applied to the basolateral surface of the tubule.

Effects of Na^+-free saline on K^+ reabsorption.

Previous studies have shown that KCl reabsorption does not require Na^+ in the lumen (Maddrell and Phillips, 1975). However, for 7 tubules bathed in Na^+-free salines (Table 1), KCl reabsorption was inhibited 49.7 ± 13.5 %.

Discussion

Comparison of NaCl/KCl secretion by the upper Malpighian tubule and KCl reabsorption by the lower Malpighian tubule

Inhibition of KCl reabsorption by KCN indicates the metabolic dependence of the process, and suggests the involvement of an ATP-dependent pump. However, the effects of a variety of ion transport inhibitors highlight dramatic differences in the mechanisms by which ions are secreted by the upper tubule and reabsorbed by the lower tubule. Foremost among these is the absence of any significant inhibition of KCl reabsorption by V-ATPase inhibitors (NEM, NBD-Cl, bafilomycin A_1) at concentrations equal to or exceeding those which inhibit ion secretion (and hence fluid secretion) by the Malpighian tubules of *Rhodnius* and other species. In theory, a V-ATPase could energize KCl reabsorption; protons pumped into the lumen could provide the electrochemical gradient driving cellular Na^+ into the lumen through an Na^+/H^+ exchanger. The sodium gradient established in this manner could then energize lumen to cell movements of Na^+, K^+ and Cl^- through a furosemide or bumetanide-sensitive cotransporter. Such a mechanism would cycle Na^+ and H^+ across the apical membrane and would lead to net transfer of K^+ and Cl^- from the lumen to the cell.

KCl reabsorption was also unaffected by basolateral application of inhibitors of the Na^+/K^+ ATPase (ouabain, dihydro-ouabain), suggesting that the process is not directly or indirectly dependent upon the actions of the sodium pump. Insects such as the Monarch butterfly feed on plants rich in cardiac glycosides and are insensitive to ouabain and other cardiac glycosides, possibly because of a single amino acid substitution in the α-subunit of the

Na^+,K^+-ATPase (Holzinger *et al.*, 1992). Such insensitivity is not apparent in *Rhodnius*, however; physiological effects consistent with sodium pump inhibition by ouabain have been found in both the upper (Maddrell and Overton, 1988) and lower (O'Donnell and Mandelzys, 1988) segments of *Rhodnius* Malpighian tubules. The results do not rule out the possibility of an apical Na^+/K^+-ATPase; although this enzyme is generally believed to be restricted to the basolateral cell membrane, an apical location has been proposed for the larval salt gland of the brine shrimp *Artemia* (Conte, 1984). However, exchange of Na^+ for K^+ across the apical membrane of the lower tubule would maintain or increase secreted fluid osmolality, whereas previous studies have clearly shown that fluid osmolality and conductivity decline as the fluid passes through the lower tubule (Maddrell and Phillps, 1975).

Possible roles for K^+ channels in KCl reabsorption.

Inhibition of KCl reabsorption by basolateral application of the potassium channel blockers TEA and Ba^{2+} is consistent with a role for K^+ channels in the reabsorptive mechanism. The concentrations of Ba^{2+} used in this study (1 - 5 mmol l^{-1}) are in the same range or below those used in studies of *Formica* tubules (6 mmol l^{-1}; Leyssens *et al.*, 1994) or insect hindgut (< 10 mmol l^{-1}; Hanrahan *et al.*, 1986).

Possible roles for H^+/K^+ ATPase in KCl reabsorption.

Inhibition of KCl reabsorption by SCH 28080, SKF 96067 and SKF 96356 is consistent with the presence of a H^+/K^+-ATPase in the lower tubule. The H^+/K^+-ATPase is a member of the phosphorylating class (P-type) of ion transporting ATPases, which form a

covalent aspartyl phosphate intermediate as part of the reaction transport cycle. P-type ATPases are sensitive to vanadate, which blocks a conformational change of the unphosphorylated form (eg. Karlish *et al.*, 1979).

Concentrations of omeprazole which effectively inhibited KCl reabsorption by the lower tubule are similar to those used in studies of tissues other than the gastric mucosa, but much higher than the concentrations which inhibit gastric acid secretion in mammals. Formation of $CaCO_3$ spicules by sea urchin embryos involves an H^+/K^+ ATPase and is inhibitable by 0.1 - 0.4 mmol l^{-1} omeprazole at alkaline pH in sea water (Fujino *et al.*, 1987). H^+/K^+ ATPase-mediated secretion of H^+ into the gastric lumen of frogs during intracellular acidosis is inhibited by 0.3 mmol l^{-1} omeprazole (Yanaka *et al.*, 1991), and the H^+/K^+ ATPase in the terminal segments of the amphibian nephron is inhibited by 0.1 mmol l^{-1} omeprazole (Planelles *et al.*, 1991). In contrast, acid secretion in stimulated mammalian gastric glands *in vitro* is inhibited approximately 70% by 1 μmol l^{-1} omeprazole (Wallmark *et al.*, 1983). Unstimulated gastric glands require higher concentrations of the drug: 0.01 mmol l^{-1} and 0.1 mmol l^{-1} omeprazole inhibit approximately 50% and 75% of basal acid secretion (Wallmark *et al.*, 1983). The explanation for these differences presumably reflects the lower pH, and therefore more effective activation of omeprazole, in stimulated gastric glands relative to unstimulated glands or tissues which function in more alkaline conditions. The pKa of omeprazole is 4, and its ability to block acid secretion in the vertebrate gut is related to the activation of the drug by the low pH (2) in the lumen of stimulated gastric glands.

The concentrations of SCH 28080 used in this study are similar to those used to block H^+/K^+ ATPases implicated in K^+ reabsorption and lumenal acidification in human airway

epithelia (30 μmol l^{-1}; Smith and Welsh, 1992) and rat distal kidney tubules (50 - 100 μmol^{-1}; Cheval *et al.*, 1990; Okusa *et al*, 1992; Fernandes *et al.*, 1994). The H^+/K^+ ATPase which contributes to intracellular pH regulation in a colon carcinoma cell line is inhibitable by 100 μmol l^{-1} SCH 28080 (Abrahamse *et al.*, 1992).

Recent studies of nongastric tissues have revealed that 100 μmol l^{-1} omeprazole and SCH 28080 may also inhibit some V-type H^+-ATPases (Sabolic *et al.*, 1994). The inhibition of fluid secretion by upper tubules bathed in 100 μmol^{-1} SCH 28080 is consistent with V-ATPase inhibition. However, fluid secretion was not blocked by 0.2 - 0.4 mmol l^{-1} omeprazole, suggesting that the V-type H^+-ATPase of *Rhodnius* Malpighian tubules is not sensitive to omeprazole. The contribution of a V-type H^+-ATPase to ion transport by the lower tubule appears unlikely because high concentrations of the more specific V-type ATPase inhibitors (bafilomycin A_1, NEM and NBD-Cl) did not inhibit KCl reabsorption. Parallel studies showed that these drugs completely blocked fluid secretion by the upper tubule.

The much greater effectiveness of basolateral versus lumenal application of omeprazole is unexpected. The electrophysiological studies described in Chapter 4, along with effects of Ba^{2+} and TEA described in this chapter, are consistent with the presence of basolateral channels for K^+. A basolateral K^+/H^+ ATPase is unlikely, therefore, since the presence of K^+ channels would tend to short-circuit the actions of the pump in driving K^+ from cell to haemolymph. An alternative explanation for the greater effectiveness of omeprazole when basolaterally is that the drug is more effective in acidic conditions. The lumenal fluids are approximately 1.4 pH units alkaline to the bathing saline in the experiments described above. One possible explanation therefore is that the drug is activated within the cytosol or an acidic subcellular organelle, and

that it then crosses the apical membrane to react with SH groups on the lumenal surface of the molecule. The drug is known to react exclusively with the external surface of intact cells (Sachs *et al*, 1989).

At pH of 7, the proportion of the activated form of omeprazole will be reduced dramatically, and much higher concentrations may be required, therefore, to inhibit H^+/K^+ATPases. Similarly, the reversible inhibitors of H^+/K^+ ATPases (SKF 96067, SKF 96356 and SCH 28080) are weak bases which tend to accumulate in acidic compartments (Pope and Sachs, 1992), and this may enhance their effectiveness in gastric cells as opposed to the lower Malpighian tubule. More importantly, these drugs work by competing with K^+ for its binding site on the enzyme; in the high K^+ of the Malpighian tubule lumen (50 - 100 mmol l^{-1}), much higher concentrations may be required than in the low K^+ fluids of the gastric lumen.

Inhibition by vanadate is consistent with involvement of a P-type ATPase in KCl reabsorption by the lower Malpighian tubule. However, it is worth noting that the Cl^-/HCO_3^- ATPase in the intestinal mucosa of *Aplysia* is also a P-type ATPase which is sensitive to vanadate (Gerencser and Lee, 1985; Gerencser and Zelezna, 1993). The possible contribution of a Cl^-/HCO_3^- ATPase to KCl reabsorption is discussed further in Chapter 3. The effects of vanadate on KCl reabsorption are consistent, therefore, with the presence of H^+/K^+ ATPase activity, Cl^-/HCO_3^- ATPase activity, or both.

Effects of Na^+-free media on KCl reabsorption

Although lumenal Na^+ is not required for KCl reabsorption, removal of Na^+ from the bathing saline was found to be inhibitory. Previous studies have demonstrated the requirement

of Na^+ in the bathing saline for cell volume regulation by the lower tubule (O'Donnell and Mandelzys, 1988). The interference with KCl reabsorption in Na^+ free media may represent an indirect effect of cell swelling and concomitant disruptions of metabolism and intracellular homeostasis, rather than a direct effect on the ion transporters involved in KCl reabsorption.

Summary of Chapter 2.

The results of this chapter indicate that active K^+ transport by the lower Malpighian tubule does not involve amiloride-sensitive K^+/H^+ exchange or V-type H^+-ATPases. The effects of vanadate and specific inhibitors of H^+/K^+-ATPases suggest that a pump similar to the H^+/K^+-ATPase of the gastric mucosa is involved in KCl reabsorption. Inhibition of KCl reabsorption by basolateral but not apical application of the K^+ channel blockers TEA and Ba^{2+} suggests the presence of K^+ channels in the basolateral membrane in the lower Malpighian tubule. It is proposed, therefore, that K^+ is pumped from lumen to cell by an ATP-dependent pump resembling the H^+/K^+-ATPase of the gastric mucosa, and that K^+ leaks from cell to bathing saline (haemolymnph) via an electrodiffusive pathway (*ie*. K^+ channels).

CHAPTER 3

Chloride Transport by the Lower Malpighian Tubule

Introduction

This chapter examines the effects of putative inhibitors of Cl^- channels, $Na^+/K^+/2Cl^-$ symporters, Cl^-/HCO_3^- antiporters, and chloride-transporting ATPases on KCl reabsorption by the lower Malpighian tubule. The methods used are the same as those described in Chapter 2. Although the results describe the effects of drugs which affect Cl^- transport, the parameter measured in these experiments was the K^+ concentration of fluid collected after passage through the lower tubule. Although the upper tubule secretes two cations (K^+, Na^+), Cl^- is the only anion which has been detected in significant quantities in fluid secreted by upper tubules (Ramsay, 1952; Maddrell, 1969). Reabsorption of K^+, therefore, is accompanied by reabsorption of equimolar quantities of Cl^-. Correspondingly, drugs which interfere with Cl^- transport by the lower tubule can be detected by an increase in K^+ concentration of the lower tubule fluid. The alternative method is the measurement of secreted fluid Cl^- concentration. This approach was not chosen because the drop in Cl^- concentration is less than two-fold as fluid passes through the lower tubule, whereas the drop in K^+ concentration may exceed ten-fold. For example, a typical droplet of fluid collected from an upper tubule will contain 100 mmol l^{-1} Na^+, 80 mmol l^{-1} K^+ and 180 mmol l^{-1} Cl^-. A droplet of fluid collected after passage through a 5-HT-stimulated lower tubule will contain 100 mmol l^{-1} Na^+, 10 mmol l^{-1} K^+ and 110 mmol l^{-1} Cl^-. The potential of a K^+-selective microelectrode may change, therefore, by 60 mV or more when moved

between droplets of fluid collected from upper tubules and lower tubules, whereas a Cl^--selective electrode would change by less than 19 mV when moved between the same two droplets. The use of K^+-selective microelectrodes, therefore, is the method with the higher resolution for analysis of KCl reabsorption by the lower tubule.

Materials and methods

The techniques used in the experiments described below were identical to those described in Chapter 2. The drugs used, sources of supply, and the solvents used for preparation of stock solutions are listed in Appendix 1.

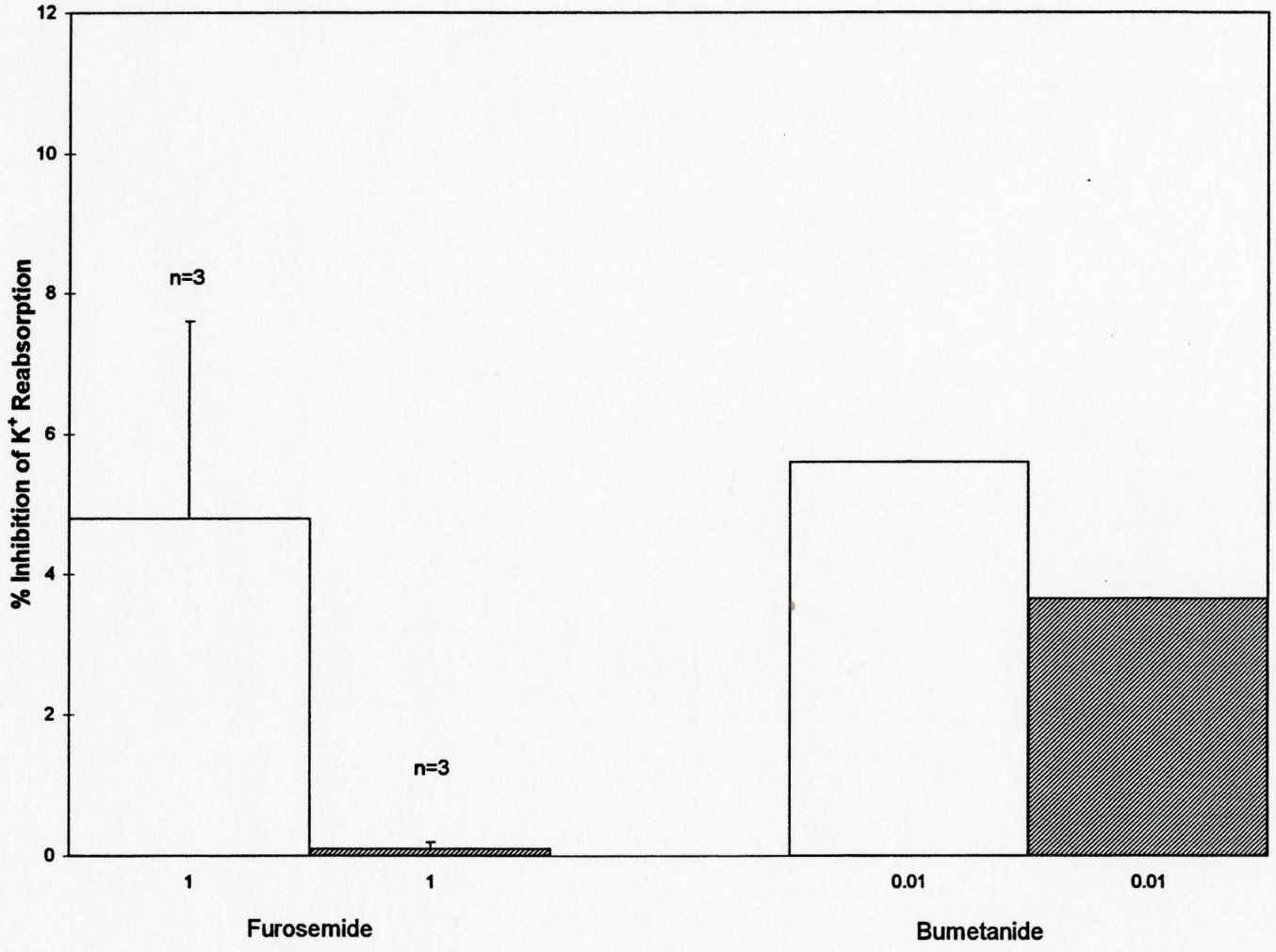
Results

Effects of furosemide and bumetanide

Furosemide and analogous compounds such as bumetanide inhibit Na^+/Cl^-, $Na^+/K^+/2Cl^-$ and K^+/Cl^- cotransporters in many epithelia, including Malpighian tubules (Palfrey *et al.*, 1980; O'Donnell and Maddrell, 1984; Leyssens *et al.*, 1994). KCl cotransport mechanisms are inhibitable by 10^{-4} - 10^{-3} mol l^{-1} bumetanide or 10^{-3} mol l^{-1} furosemide (Ellory and Hall, 1988; Kaji, 1986; Leyssens *et al.*, 1994). Fluid secretion by the upper Malpighian tubule of *Rhodnius* is reduced 79 - 80% by 10^{-4} mol l^{-1} furosemide or 10^{-5} mol l^{-1} bumetanide (O'Donnell and Maddrell, 1984). Electrophysiological effects of these drugs are consistent with inhibition of Cl^- entry into the upper tubule cells. In contrast, KCl reabsorption by the lower tubule was relatively unaffected by addition of

Figure 3.1

Effects of furosemide and bumetanide on K^+ reabsorption. The height of each bar shows the mean % inhibition (+ 1 S.E.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are listed above each drug listed on the abscissa. Apical and basolateral application of the drugs are indicated by open and cross-hatched bars, respectively. Note that the maximum inhibition produced by either drug is less than 5%.


12
10
8
6
4
2
0
% Inhibition of K+ Reabsorption
n=3
n=3
1
1
0.01
0.01
Furosemide
Bumetanide

bumetanide or furosemide to either the apical or basolateral surface of the lower tubule (Figure 3.1).

Effects of thiocyanate.

Thiocyanate at concentrations around 10 mmol l^{-1} inhibits chloride transport by epithelia of vertebrates (references in Epstein *et al*, 1973) and invertebrates. Replacement of Cl^- (110 mmol l^{-1}) by SCN^- inhibits fluid secretion by *Calliphora* Malpighian tubules by 25% (Berridge, 1968) and 10 mmol l^{-1} SCN^- inhibits secretion by the Malpighian tubules of *Locusta* migratoria by 44% (Fathpour and Dahlman, 1994). In some tissues the site of action of thiocyanate appears to be inhibition of Cl^-/HCO_3^- ATPases (Gassner and Komnick, 1982; Gerencser and Lee, 1985; Lechleitner and Phillips, 1987). Thiocyanate at high concentrations (>25 mmol l^{-1})may also act as a competitive inhibitor of epithelial Cl^- channels (Mandel et al., 1986). Addition of 50 mmol l^{-1} NaSCN (NaCl replacement) to saline bathing the lower tubule inhibited K^+ reabsorption by 60% (Figure 3.2), whereas there was negligible inhibition of KCl reabsorption in response to apical SCN^-.

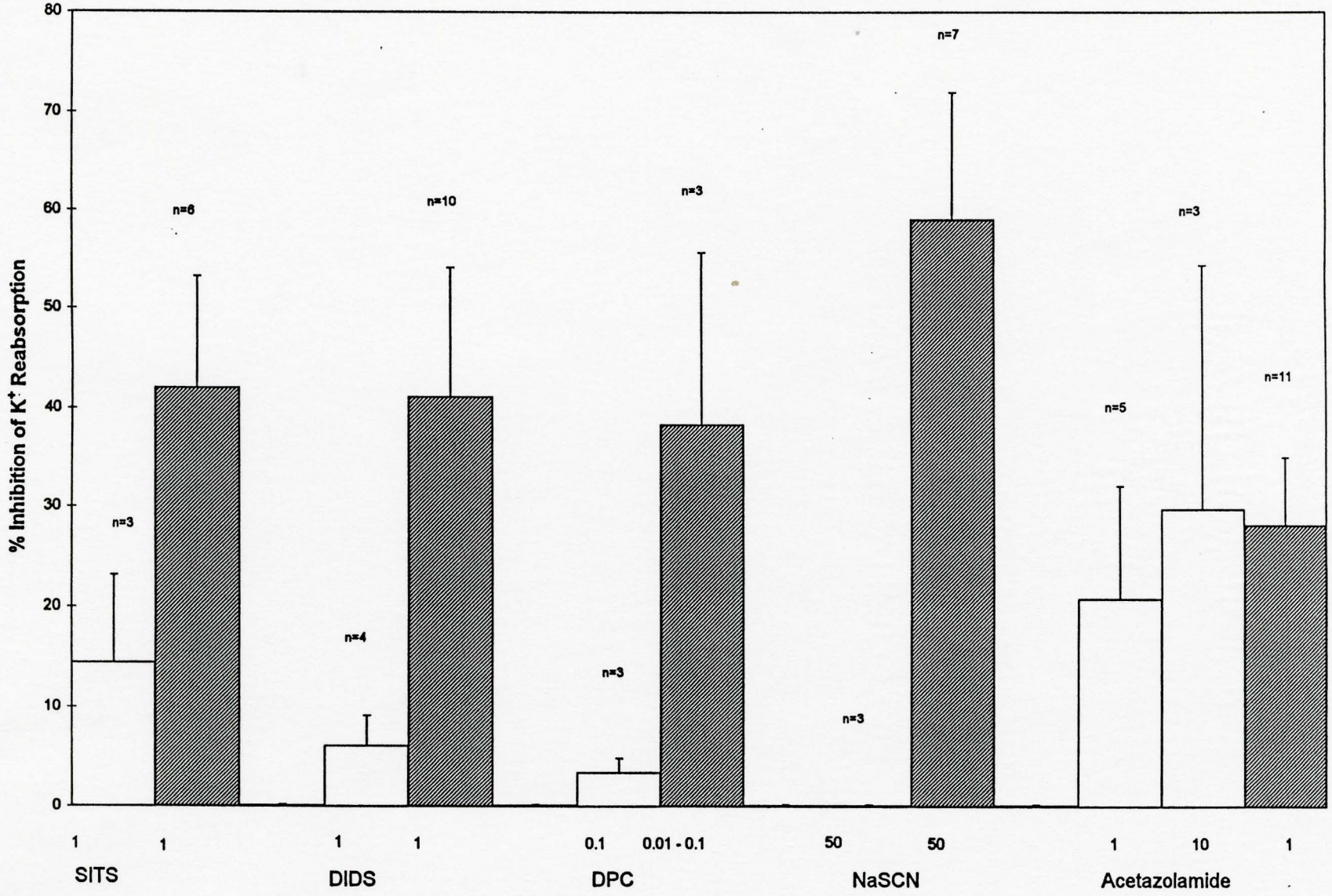
The effects of SCN^- on secreted fluid pH were also examined. The rationale behind these measurements was that partial blockadge of Cl- transport might reveal whether the H^+/K^+-ATPase proposed in Chapter 2 was capable of altering secreted fluid pH. Addition of 50 mmol l^{-1} SCN^- to the saline bathing the lower tubule resulted in a lowering of secreted fluid pH by more than 0.6 pH units, from 8.43 ± 0.08 to 7.71 ± 0.02 (n = 6).

Effects of chloride channel blockers.

Diphenylamine-2-carboxylate (DPC) is also referred to as N-phenylanthranilic acid in the literature (eg. Mandel et al., 1986). Concentrations required for half-maximal inhibition of

Figure 3.2

Effects of SITS, DIDS, DPC, SCN^- and acetazolamide on K^+ reabsorption.The height of each bar shows the mean % inhibition (+ 1 S.E.) of K^+ reabsorption for the indicated number of tubules. The concentrations (in mmol l^{-1}) are shown above each drug listed on the abscissa. Apical and basolateral application of the drugs are indicated by open and cross-hatched bars, respectively.


% Inhibition of K+ Reabsorption
80
70
60
50
40
30
20
10
0
n=3
n=6
n=4
n=10
n=3
n=3
n=7
n=5
n=3
n=11
1
1
1
1
0.1
0.01 - 0.1
50
50
1
10
1
SITS
DIDS
DPC
NaSCN
Acetazolamide

epithelial chloride channels range from 2.5 x 10^{-5} mol l^{-1} to 10^{-3} mol l^{-1}, depending on the epithelium (Wangemann *et al*, 1986; Mandel *et al.*, 1986). KCl reabsorption by the lower tubule was inhibited 40% by basolateral application of 0.1 mmol l^{-1} DPC, whereas the drug resulted in less than 5% inhibition when applied apically (Figure 3.2).

Disulphonic stilbenes such as diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS) and 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulphonic acid (SITS) are known to inhibit Cl^-/HCO_3^- exchange processes (Boron, 1983; Schlue and Deitmer, 1988) and also Cl^- channels (Tilmann *et al.*, 1991). SITS and DIDS inhibited K^+ reabsorption by 37 - 40% when applied basolaterally. As for DPC, the percentage inhibition was much lower when SITS or DIDS were applied to the lumenal surface (Figure 3.2). There was no effect of HCO_3^--free saline on KCl reabsorption (n = 3), so the effects of SITS and DIDS do not suggest the presence of a basolateral Cl^-/HCO_3^- exchanger dependent upon external HCO_3^-.

Effects of acetazolamide

The primary action of acetazolamide is the inhibition of carbonic anhydrase (CA) which catalyses the reversible hydration/dehydration of carbon dioxide (Maren, 1967). In crustacean gills, CA functions in the hydration of CO_2 to form H^+ and HCO_3^-, which can then act as counterions for membrane associated ion transport mechanisms (Na^+/H^+ exchange, Cl^-/HCO_3^- exchange; Henry, 1988). Acetazolamide inhibits fluid secretion by Malpighian tubules of *Locusta* at 0.1-10 mmol l^{-1} (Fathpour and Dahlman, 1994) but does not affect tubules of *Rhodnius* (Maddrell, 1969) or *Calliphora* (Berridge, 1968). HCO_3^- transport in *Aedes* rectal glands is inhibited by 1 mmol l^{-1} acetazolamide (Strange and Phillips, 1984).

Inhibition of K^+ reabsorption by 1 mmol l^{-1} acetazolamide was similar when the drug was applied to either the lumenal or basolateral surface of the lower tubule (Figure 3.2). A ten-fold increase in the concentration of acetazolamide resulted in only a small further increase in inhibition.

Discussion

Reabsorption of KCl is unaffected by furosemide or bumetanide, which block coupled movements of Na^+, K^+ and Cl^- across the basolateral membrane of the upper tubule (O'Donnell and Maddrell, 1984). In other epithelia, these drugs inhibit either *secretion* of NaCl across the basolateral membrane (*eg.* shark rectal gland, Epstein *et al.*, 1983) or *reabsorption* of NaCl across the apical membrane (*eg.* thick ascending loop of Henle; Greger and Schlatter, 1981). The drugs do not readily cross epithelia, so application to the appropriate surface of the epithelium is necessary. In canine tracheal epithelium, for example, furosemide inhibits Na^+-coupled Cl^--transport across the basolateral cell surface when applied to the basolateral side of the epithelium, but has no effect when applied on the mucosal (apical) side (Welsh, 1983). The presence of a basolateral $Na^+/K^+/2Cl^-$ cotransporter in Malpighian tubules of *Rhodnius* (O'Donnell and Maddrell, 1984), *Aedes* (Hegarty *et al.*, 1991) and *Formica* (Leyssens *et al.*, 1994) has been proposed in part on the basis of the inhibition of fluid secretion by 10^{-5} - 10^{-4} mol l^{-1} bumetanide or 10^{-3} mol l^{-1} furosemide. The absence of significant inhibition of KCl reabsorption by application of furosemide or bumetanide to apical and basolateral surfaces of the lower tubule argues against involvement of a KCl cotransporter.

Inhibition of KCl reabsorption by basolateral application of DPC is consistent with a role for Cl^- channels. This proposal is supported by the results of Chapter 4, which describes changes in basolateral membrane potential in response to changes in bathing saline Cl^- concentration or the addition of Cl^- channel blockers The concentration of DPC used in this

chapter (100 μmol l^{-1}) is the same as that shown to be effective for inhibition of Cl^- channels in the apical membrane of the shark rectal gland and the basolateral membrane of the rabbit thick ascending loop of Henle (Di Stefano *et al.*, 1985), and well below the concentrations used to inhibit chloride channels in mouse pituitary tumour cells (500 μmol l^{-1}; Heisler, 1991), pulmonary endothelial cells (2.5 mmol l^{-1}; Ueda *et al.*, 1989) or a colonic carcinoma cell line (3 mmol l^{-1}; Mandel *et al.*, 1986).

The stilbene derivatives SITS and DIDS are known to affect both Cl^-/HCO_3^- exchange processes (Boron, 1983) and Cl^- channels (White and Miller, 1979; Nelson *et al.*, 1984; Hanrahan *et al.*, 1985; Tilmann *et al.*, 1991). Relatively high concentrations are required to inhibit Cl^-/HCO_3^- exchange in invertebrate preparations. For example, Cl^-/HCO_3^- exchange in the gills of land crabs is inhibited by 3 mmol l^{-1} SITS (Varley and Greenaway, 1994), 1 mmol l^{-1} SITS reduces fluid secretion rates of mosquito Malpighian tubules more than 20% (Hegarty *et al.*, 1991), and 0.5 mmol l^{-1} DIDS inhibits Cl^-/HCO_3^- exchange of mosquito rectal salts glands by 40% (Strange and Phillips, 1984). The Cl^-/HCO_3^- exchanger of leech neurons is inhibitable by 0.5 mmol l^{-1} DIDS and 1 mmol l^{-1} SITS (Schlue and Deitmer, 1988). The absence of significant inhibition of KCl reabsorption by HCO_3^--free bathing saline is inconsistent with the presence of a basolateral Cl^-/HCO_3^- exchanger dependent upon external HCO_3^-. Similarly, the absence of inhibition of KCl reabsorption by apical SITS, DIDS and DPC argue against the presence of apical Cl^- channels or Cl^-/HCO_3^- exchangers sensitive to these drugs. The data can be explained however, by the presence of DPC-, SITS- and DIDS-sensitive Cl^- channels in the basolateral membrane. The concentrations of SITS or DIDS used in this chapter are similar to those used for inhibition of Cl^- channels in cultured epithelial cells (1 mmol l^{-1}; Nelson *et al.*,

1984), rabbit urinary bladder (0.1 mmol l^{-1}; Hanrahan *et al.*, 1985), and *Torpedo* electric organ (0.1 - 0.7 mmol l^{-1}; Miller and White, 1979). The colon carcinoma cell line is inhibited 50% by 0.1 mmol l^{-1} SITS or DIDS and more than 95% by 1 mmol l^{-1} SITS or DIDS (Tilmann *et al.*, 1991).

Thiocyanate is known to inhibit both Cl^- channels (eg. Mandel *et al.*, 1986) and Cl^-/HCO_3^- ATPases. Anion-stimulated ATPase activity in blue crab gill (Lee, 1982) is inhibited 50% by 4.8 mmol l^{-1} SCN^-, and anion-stimulated ATPase activity in locust rectal epithelium is inhibited 90% by 10 mmol l^{-1} SCN^- (Lechleitner and Phillips, 1987). Studies of fluid secretion by Malpighian tubules indicate that thiocyanate can be inhibitory or stimulatory. Thiocyanate can partially substitute for Cl^- in supporting fluid secretion by tubules of *Calliphora*; fluid secretion rate is reduced only 25% when all Cl^- in the bathing saline (110 mmol l^{-1}) is replaced by SCN^- (Berridge, 1968). Secretion rates of *Musca domestica* tubules increase in response to 10 mmol l^{-1} SCN^- (Dalton and Windmill, 1980), and the secreted fluid becomes more hyperosmotic to the bathing solution. One possible explanation is that *Musca* tubules secrete fluid rich in NaCl and KCl and that some of the NaCl and water is reabsorbed. Thiocyanate is known to inhibit Na^+-dependent Cl^--transport in fish gills (Epstein *et al.*, 1973), possibly through inhibition of Cl^-/HCO_3^- stimulated ATPase activity (Bornancin *et al.*, 1980), and if this occurs in *Musca* tubules then blockage of NaCl reabsorption by thiocyanate would lead to increases in both rates of fluid secretion and secreted fluid osmolality, as observed.

As noted above, the effects of apical application of SITS, DIDS, DPC and SCN^- are inconsistent with the presence of Cl^- channels or Cl^-/HCO_3^- exchangers in the apical membrane of the lower tubule. An alternative mechanism for Cl^- movement from lumen to cell would be

via a Cl^-/HCO_3^- ATPase. In *Aplysia* midgut, the P-type Cl^-/HCO_3^- ATPase identified by Gerenscer and co-workers has been shown to be insensitive to SITS (25 μmol l^{-1}; Gerenscer and Lee, 1985) and furosemide and inhibitable by SCN^- and vanadate. In contrast, ATP-dependent Cl- transport in the gastric mucosa accepts SCN- as readily as Cl-, and is insensitive to vanadate and SITS (Soumarmon *et al*, 1980). In the lower tubule, cellular HCO_3^- produced by the actions of CA on metabolic CO_2 could provide the driving force for Cl^- entry into the cell. The HCO_3^- might recombine with H^+ from an apical K^+/H^+ ATPase (chapter 2) to prevent acidification of the lumen. Efflux of Cl^- from cell to bathing saline through basolateral Cl^- channels could be driven in part by the basolateral membrane potential. Inhibition of KCl reabsorption by vanadate and acetazolamide and insensitivity to apical application of SITS are consistent with the suggestion of a Cl^-/HCO_3^- ATPase similar to that of the *Aplysia* gut (Gerenscer and Lee, 1985). Most Cl^-/HCO_3^- ATPases are sensitive to SCN^-, however, whereas this compound blocked KCl reabsorption only from the basolateral and not the apical side of the lower tubule. One explanation for this result is that the effects of SCN^- are exclusively on the basolateral Cl^- channels proposed above, and that an apical Cl^-/HCO_3^- ATPase, if present, is insensitive to SCN^-. At present, research on Cl^-/HCO_3^- ATPases is hampered by the absence of highly specific inhibitors, whereas studies of K^+/H^+ ATPases have been facilitated by the availability of relatively specific inhibitors such as omeprazole and SCH 28080; the latter compounds were developed because of the medical importance of K^+/H^+ ATPase inhibitors in the treatment of stomach ulcers.

Acetazolamide may inhibit the process of KCl reabsorption at more than one stage. By inhibiting the catalysis of CO_2 dehydration/rehydration by carbonic anhydrase (CA),

acetazolamide may secondarily inhibit transporters dependent upon adequate supplies of H^+ and HCO_3^-. In other words, V-type ATPases, H^+/K^+-ATPases and Cl^-/HCO_3^- exchangers or pumps may depend upon the H^+ and HCO_3^- provided by the actions of CA. In addition, it may directly inhibit Cl^-/HCO_3^--ATPase activity (Gerencser and Lee, 1985).

Summary of Chapter 3

Reabsorption of KCl by the lower tubule does not appear to involve coupled transport of Na^+(and/or K^+) and Cl^-. Instead, the effects of a Cl^- channel blockers suggests the presence of Cl^- channels in movement of Cl^- from cell to haemolymph. Movement of Cl^- does not appear to involve Cl^-/HCO_3^- exchange processes sensitive to SITS or DIDS. It is suggested that a Cl^-/HCO_3^--ATPase may be involved. This pump would be insensitive to SITS, DIDS, SCN^- and furosemide, but may be inhibited by vanadate, and directly or indirectly by acetazolamide.

Chapter 4

Transepithelial and Cellular Electrical Potentials in the Lower Malpighian Tubule

Introduction

Discussion of mechanisms of ion transport across epithelia requires analysis of the chemical and electrical gradients which will influence the movement of each ion. Epithelia consist of basolateral (haemolymph-facing) and apical (lumen-facing) membranes in series. Ions which cross an epithelium by a transcellular route will therefore be influenced by both the apical membrane potential (V_{ap}) and the basolateral membrane potential (V_{bl}). The transepithelial potential (TEP) is equal to the sum of these potentials in series: $TEP = V_{bl} + V_{ap}$.

Chemical gradients influencing movement of K^+ and Cl^- from the lower tubule lumen to the haemolymph can be assessed from calculated or measured concentrations of these ions in the lumen and haemolymph or bathing saline. *In vivo*, urine K^+ concentrations are 2.3 mmol l^{-1}, and haemolymph K^+ concentration is 3.6 mmol l^{-1} (Maddrell *et al.*, 1993). Fluid secreted by the upper tubule contains 75 - 80 mmol l^{-1} K^+ (Chapter 2). These values indicate that whereas a chemical gradient favours K^+ movement from lumen to haemolymph in the early stages of K^+ reabsorption, the progressive reduction of $[K^+]$ as fluid passes through the lower tubule means that K^+ is moved against a transepithelial chemical gradient in the latter stages. Although intracellular K^+ concentrations have not been measured in the lower tubule, all eukaryotic cells studied to date maintain a K^+ concentration higher than that in surrounding blood, typically of

the order of 100 mmol l^{-1} (Yancey *et al.*, 1982). For Cl^-, the haemolymph or bathing saline concentration *in vitro* is around 180 mmol l^{-1}, and secreted fluid Cl^- concentration decreases from approximately 180 $mmol^{-1}$ to 100 mmol l^{-1} by KCl reabsorption in the lower tubule. Although intracellular Cl^- concentrations have not been measured in the lower tubule, typical values for $[Cl]_i$ average 20 mmol l^{-1} (Spring and Kimura, 1978), so movement of Cl^- from lumen to cell may be favoured by a chemical gradient, whereas movement from cell to bathing saline is opposed by the chemical gradient.

The electrical potentials across the lower Malpighian tubule have not previously been examined in detail. Preliminary measurements in unstimulated tubules *in vitro* indicate that the lower tubule lumen is approximately 17 mV negative to the bath, and that the basolateral membrane potential is approximately 14 - 32 mV negative to the bath (O'Donnell *et al.*, 1983). However, there are no published measurements of TEP's or V_{bl} in 5-HT-stimulated lower tubules.

This chapter reports measurements of V_{bl} and TEP in unstimulated and stimulated lower Malpighian tubules. The apical membrane potential can be calculated from these data using the equation $V_{ap} = TEP - V_{bl}$. This chapter also reports the changes in V_{bl} in response to changes in bathing saline ion concentration (K^+, Cl^-) or the addition of drugs known to block epithelial K^+ or Cl^- channels. These measurements provide further evidence for the presence of basolateral channels for K^+ and Cl^- in the lower tubule.

Materials and methods

Tubules were dissected as described in Chapter 2, and the sources of drugs used in this study are reported in Appendix 1.

Saline irrigation of lower Malpighian tubules.

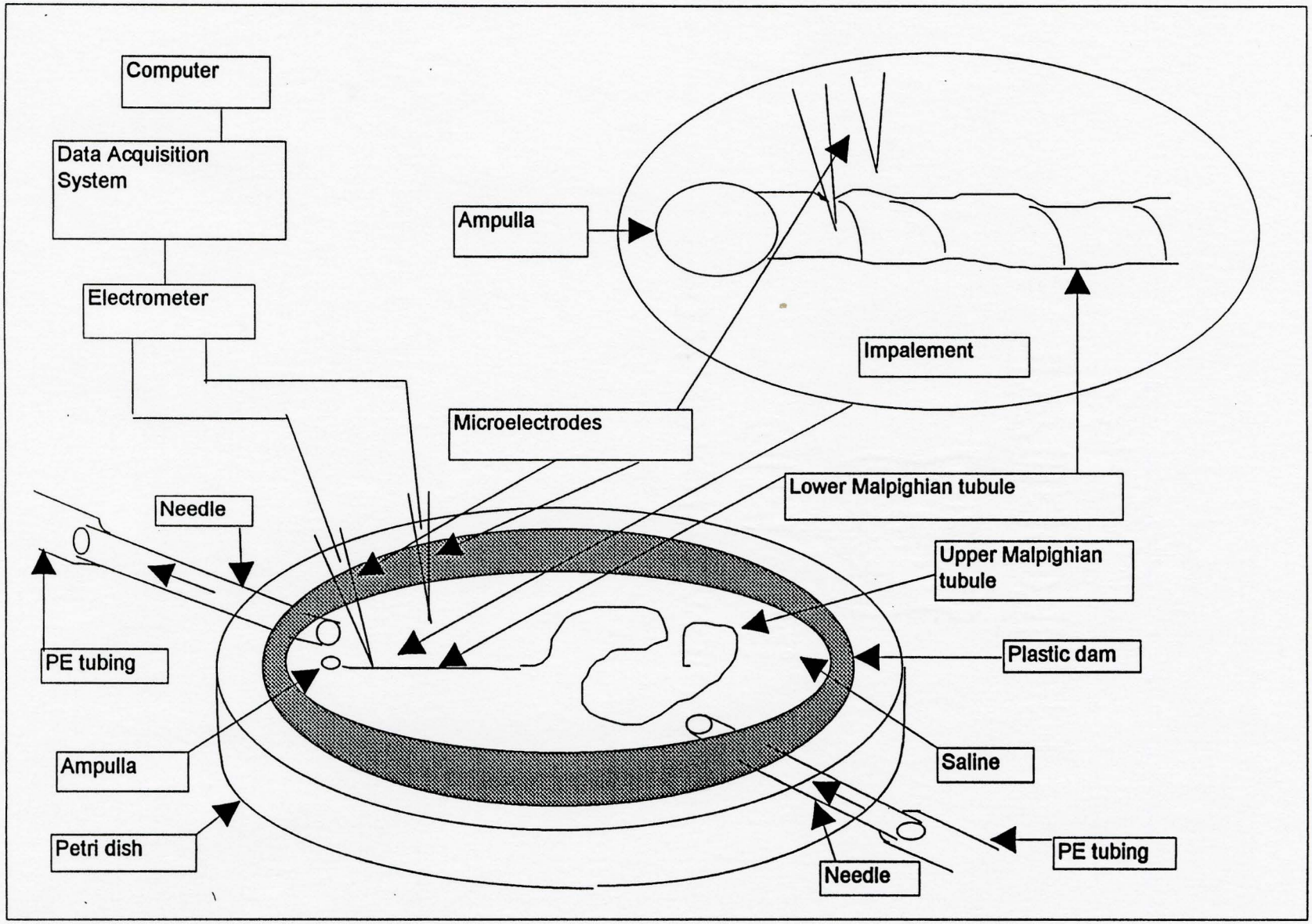
Changes in ionic composition of the bathing saline or the addition or washing off of drugs were accomplished by irrigation of an isolated tubule (Figure 4.1). Low melting point plastic was applied from a glue gun to form a ring approximately 3 mm high and 1 cm in diameter on the bottom of the petri dish. Two syringe needles were removed from their plastic Luer fittings, and the tip of each needle was forced through opposite sides of the plastic dam. Polyethylene tubing (PE 50) was fitted to each needle and connected to 25 ml syringes. One syringe acted as a saline reservoir, and the other was used to remove saline. Co-ordinated advancement and withdrawal of the syringe plungers permitted the volume of fluid enclosed in the dam to be maintained at a constant depth and exchanged within a few seconds.

Measurement of basolateral membrane potential

Standard techniques for intracellular recording were used to measure V_{bl}. Glass microelectrodes were pulled from 1 mm filamented borosilicate glass tubing and were filed with 3 mol l^{-1} KCl. Resistances of the microelectrodes were approximately 20 megaohms. Microelectrodes were connected through AgCl-coated silver wires to a high impedance (> 10^{11} ohms) electrometer (WPI model 707; Sarasota, Fl).

Figure 4.1

Schematic diagram showing the arrangements for measurement of V_{bl} and exchange of bathing saline. A plastic dam enclosed a small volume (*ca.* 0.4 ml) of saline in a 15 mm petri dish mounted on the stage of an inverted microscope. Two 25 gauge syringe needles were inserted through the plastic dam (*stippling*) and were connected through PE tubing to two syringes for supply and removal of saline. The tubules adhered readily to the bottom of the petri dish, which had been coated with poly-L-lysine. The basolateral membrane of the lower tubule was impaled with one microelectrode, and V_{bl} was measured with respect to a second microelectrode positioned in the bathing saline.


Computer
Data Acquisition System
Electrometer
Ampulla
Impalement
Microelectrodes
Lower Malpighian tubule
Needle
Upper Malpighian tubule
PE tubing
Plastic dam
Ampulla
Saline
Petri dish
PE tubing
Needle

Microelectrodes were mounted on 1-axis hydraulic micromanipulator (Narishige) which was itself mounted on an 3-axis Marzhauser HS-6 micromanipulator (Fine Science Tools, Vancouver). The microelectrode tip was positioned over a lower tubule under saline using the HS-6, and was then advanced at an oblique angle (*ca.* 30 degrees from the bottom of the petri dish) towards the surface of the tubule by means of the hydraulic micromanipulator. Petri dishes were pre-coated with poly-L-lysine to promote adhesion of the tubule to the bottom of the dish, thereby preventing movement of the tubule and facilitating impalement (Fig. 4.1). The petri dish was positioned on the stage of a Nikon inverted microscope.

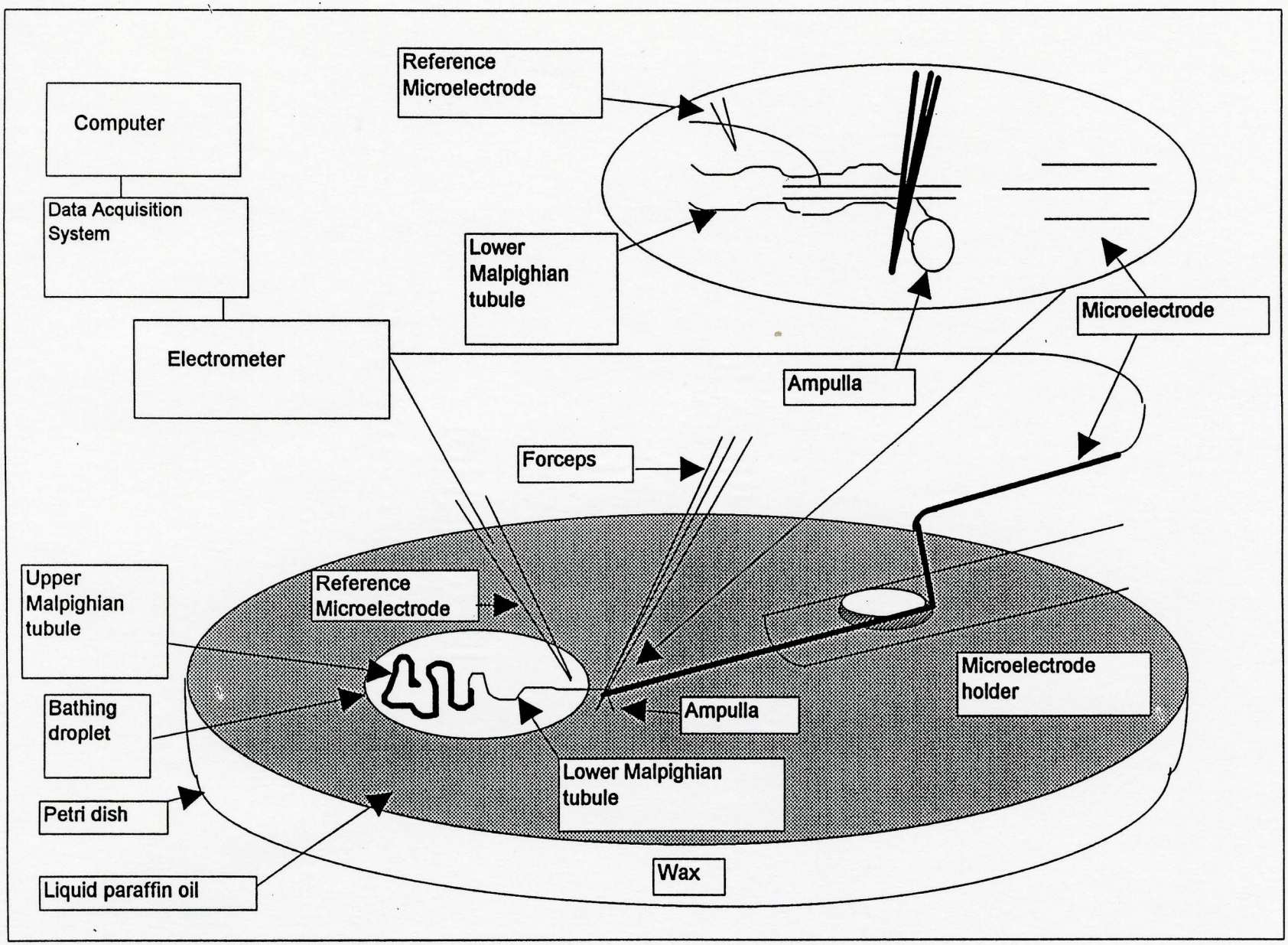
Contact of the microelectrode with the surface of the tubule was indicated by a change in potential of a few mV and an increase in electrical noise. Impalement was then accomplished by gently tapping the baseplate of the micromanipulator. Basolateral membrane potential was measured with respect to a reference microelectrode positioned in the bathing saline. Acceptable recordings met the following criteria: an abrupt change in potential upon impalement, maintenance of a stable potential for > 30 s, and a return to within 2 mV of the pre-impalement potential when the microelectrode was withdrawn from the cell. Electrical potentials were recorded using a computerized data acquisition and analysis system (AXOTAPE, Axon Instruments, Burlingame, CA).

Measurement of transepithelial potential.

For measurement of TEP, isolated tubules were transferred to saline droplets under oil. TEP's were measured using procedures similar to those for cannulation and lumenal perfusion (Fig. 4.2). A length of tubule was pulled out of the drop of bathing saline and held by

Figure 4.2

Schematic diagram showing the arrangements for measurement of TEP. An isolated tubule was positioned in a droplet of saline under paraffin oil. A glass microelectrode was mounted on the microelectrode holder, and a micromanipulator (not shown) was used to advance the tip of the electrode through the wall of a lower Malpighian tubule held between forceps (inset).


Reference Microelectrode
Computer
Data Acquisition System
Lower Malpighian tubule
Microelectrode
Electrometer
Ampulla
Forceps
Upper Malpighian tubule
Reference Microelectrode
Microelectrode holder
Bathing droplet
Ampulla
Lower Malpighian tubule
Petri dish
Wax
Liquid paraffin oil

microforceps. A microelectrode prepared as described above was mounted on a micromanipulator (MM33) and the tip was broken back to an outside diameter of *ca.* 10 μm. The broken tip was too coarse to permit intracellular recordings, which require tip diameters < 0.5 μm. The microelectrode was then inserted into the tubule lumen and advanced axially several hundred μm until its tip was inside a segment of the tubule within the bathing saline. The TEP was measured with respect to a reference microelectrode positioned in the bathing drop, using the electrometer and data acquisition system described in Chapter 2.

Results

Cellular and transepithelial electrical potentials.

Figure 4.3 shows a representative recording of V_{bl} in a lower Malpighian tubule cell. For unstimulated tubules bathed in 8.6K saline, the mean value of V_{bl} was -46.2 ± 2.8 mV (n = 9), cell negative. This potential did not differ significantly from the value of -47.3 ± 3.4 mV (N = 15) recorded in tubules stimulated with 5HT. The mean transepithelial potential recorded in 5-HT-stimulated tubules was -13.4 ± 2.2 mV (n = 9). This value is similar to the value of -17 mV previously reported for unstimulated tubules (O'Donnell *et al.*, 1983). The electrical potential profile across the lower Malpighian tubule is shown in Figure 4.4. The apical membrane potential calculated from the measured values of TEP and V_{bl} is 33.9 mV, lumen positive. These data indicate that the electrical potential across the apical membrane will favour cation movements from lumen to cell and will oppose the entry of anions. Conversely, the basolateral membrane potential will favour the movement of anions from cell to bath, and will oppose the movement of cations in this direction.

Effects of bathing saline potassium concentration ($[K^+]_o$) on V_{bl}

Figure 4.5 is a typical recording showing the effects of a ten-fold change in $[K^+]_o$ on V_{bl}. For 14 tubules, the mean change in potential when $[K^+]_o$ was changed from 8.6 to 86 mmol l^{-1} was 16.9 ± 2.7 mV. A membrane potential which was determined exclusively by K^+ diffusion

Figure 4.3

Typical recording of V_{bl} in a cell of the lower Malpighian tubule. Contact with the basolateral surface is indicated by the short downward arrow. Impalement was achieved by tapping the baseplate of the micromanipulator at the point marked by the longer downward arrow. Withdrawal of the microelectrode is indicated by the upward arrow. The tubule was bathed in 2K saline.

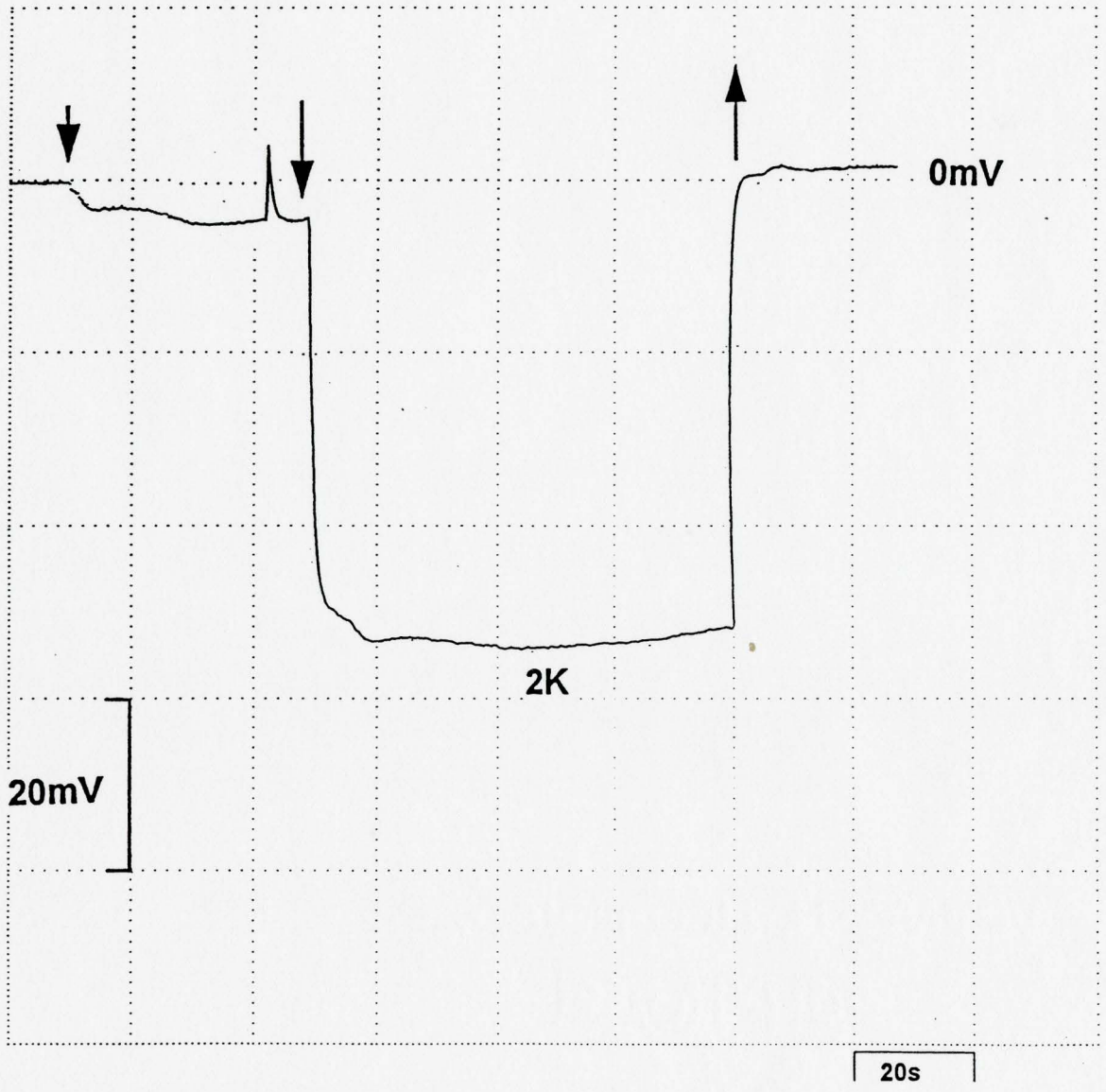

0mV
2K
20mV
20s

Figure 4.4

Electrical potential profile across the lower Malpighian tubule. V_{bl} and TEP were measured in separate tubules bathed in 8.6K saline, and V_{ap} was calculated from the difference in their mean values.

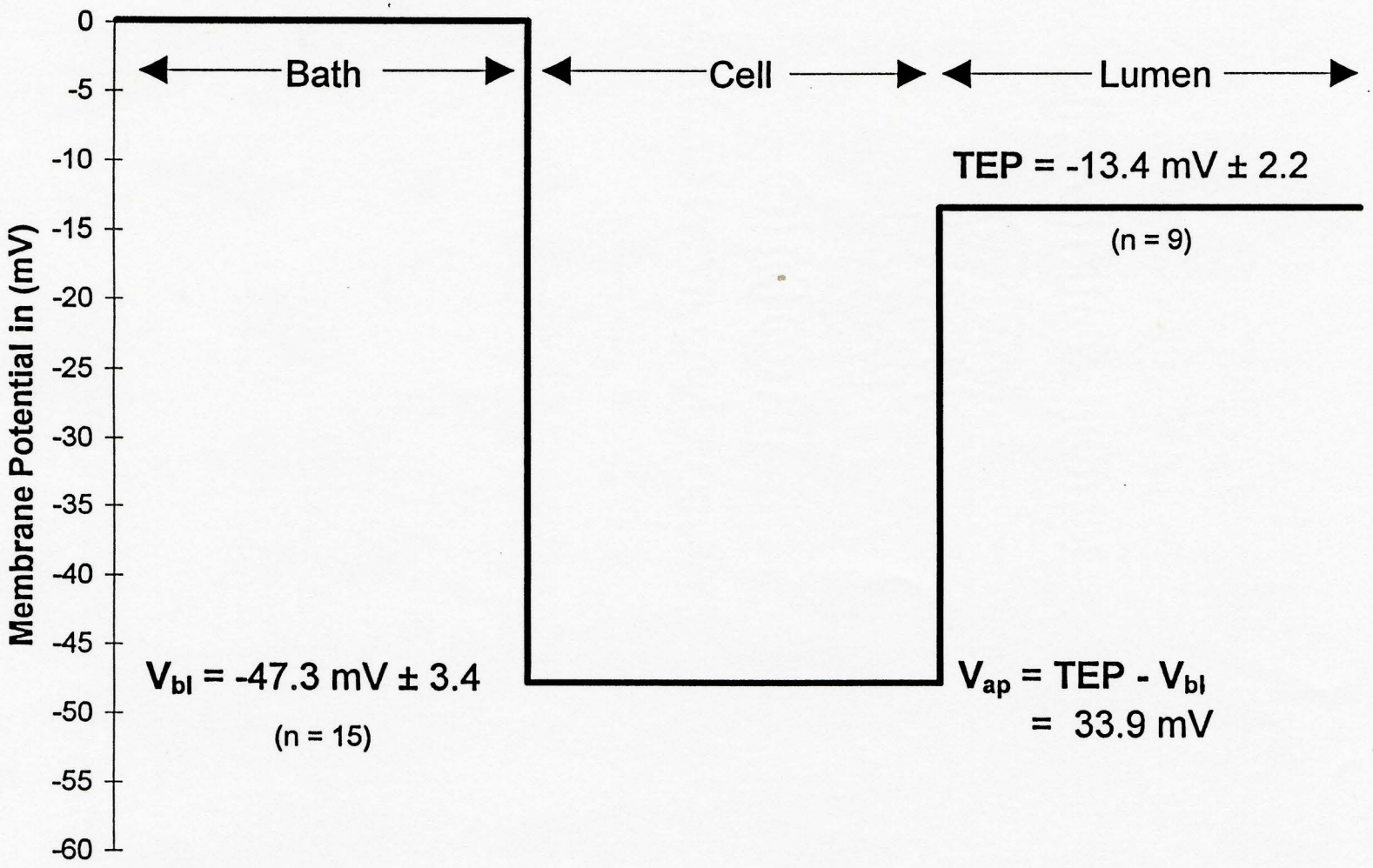
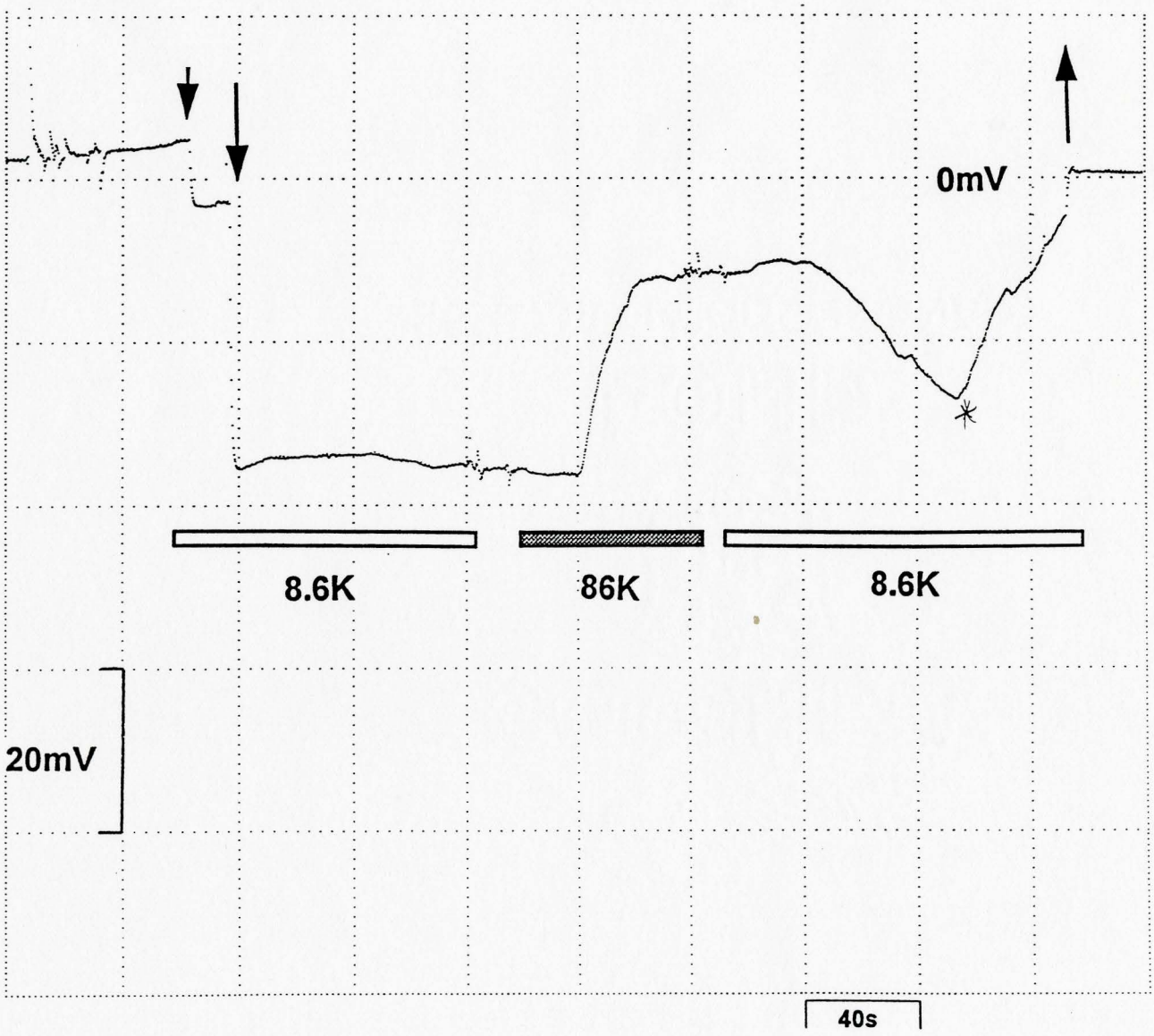

Bath
Cell
Lumen
Membrane Potential in (mV)
0
-5
-10
-15
-20
-25
-30
-35
-40
-45
-50
-55
-60
TEP = -13.4 mV ± 2.2
(n = 9)
V_{bl} = -47.3 mV ± 3.4
(n = 15)
V_{ap} = TEP - V_{bl}
= 33.9 mV

Figure 4.5

Typical recording showing effects of $[K^+]_o$ on V_{bl} in a cell of the lower Malpighian tubule. Contact with the basolateral surface is indicated by the short downward arrow. Impalement was achieved by tapping the baseplate of the micromanipulator at the point marked by the longer downward arrow. For the period indicated by the stippled horizontal bar the bathing saline was changed from 8.6K to 86K.Upon the return to 8.6K saline, the potential declined steadily, but the electrode was dislodged by mechanical disturbances associated with perfusion at the point marked by the asterisk, and the electrode was withdrawn at the upward arrow.


0mV
8.6K
86K
8.6K
20mV
40s

would change by 58 mV for a ten-fold change in $[K^+]_o$. The data above suggest that approximately 29% (= 100 * 16.9/58) of V_{bl} is attributable to K^+ diffusion. In a separate experiment, V_{bl} changed by 23 mV, from -59 mV to -36 mV when $[K^+]_o$ was changed from 2 to 20 mmol l^{-1}.

Effects of bathing saline chloride concentration on V_{bl}

The depolarizing effect of a 10-fold reduction in bathing saline chloride concentration ($[Cl^-]_o$) on V_{bl} is shown in figure 4.6. For 5 tubules bathed in saline containing 2 mmol l^{-1} K^+, V_{bl} *depolarized* by 25.9 ± 4.0 mV. In control experiments with the microelectrode placed in the bathing saline, the microelectrode potential *hyperpolarized* by 4 - 7 mV when $[Cl^-]$ was reduced 10-fold. This change presumably results from an increased liquid junction potential in low Cl^- salines, and indicates that the true change in V_{bl} was of the order of 30 - 33 mV when $[Cl^-]_o$ was reduced 10-fold. In other experiments in which $[Cl^-]_o$ was reduced 10-fold, tubules bathed in saline containing 4 mmol l^{-1} K^+ depolarized by 17 ± 3.7 mV (n = 3) and tubules bathed in 8.6 mmol l^{-1} K^+ depolarized by 8.8 ± 2.2 mV (n = 3).

A membrane potential which was determined exclusively by Cl^- diffusion would change by 58 mV for a ten-fold change in $[Cl^-]_o$. The data above suggest that in salines containing 2 mmol l^{-1} K^+ that approximately 45% (= 100 * 25.9/58) of V_{bl} is attributable to Cl^- diffusion. Potassium and chloride together account for approximately 74% (= 29% + 45%) of V_{bl}. This figure may underestimate the contributions of the two ions to V_{bl}, as discussed below.

Effects of potassium channel blockers on V_{bl}

Figure 4.7 shows the depolarizing effect of 5 mmol l^{-1} Ba^{2+} on V_{bl}. For 7 tubules in 4K

Figure 4.6

Typical recording showing effects of $[Cl^-]_o$ on V_{bl} in a cell of the lower Malpighian tubule. Impalement and withdrawal of the microelectrode are indicated by downward and upward arrows, respectively. For the period indicated by the stippled horizontal bar the bathing saline $[Cl^-]$ was reduced to 10% of the normal level, from 159 mmol l^{-1} to 15.9 mmol l^{-1}. Small transient changes in V_{bl} in this and subsequent figures(< 4 mV) were electrical and mechanical artefacts of the perfusion process; stable potentials were recorded after the exchange of the perfusion chamber was complete.

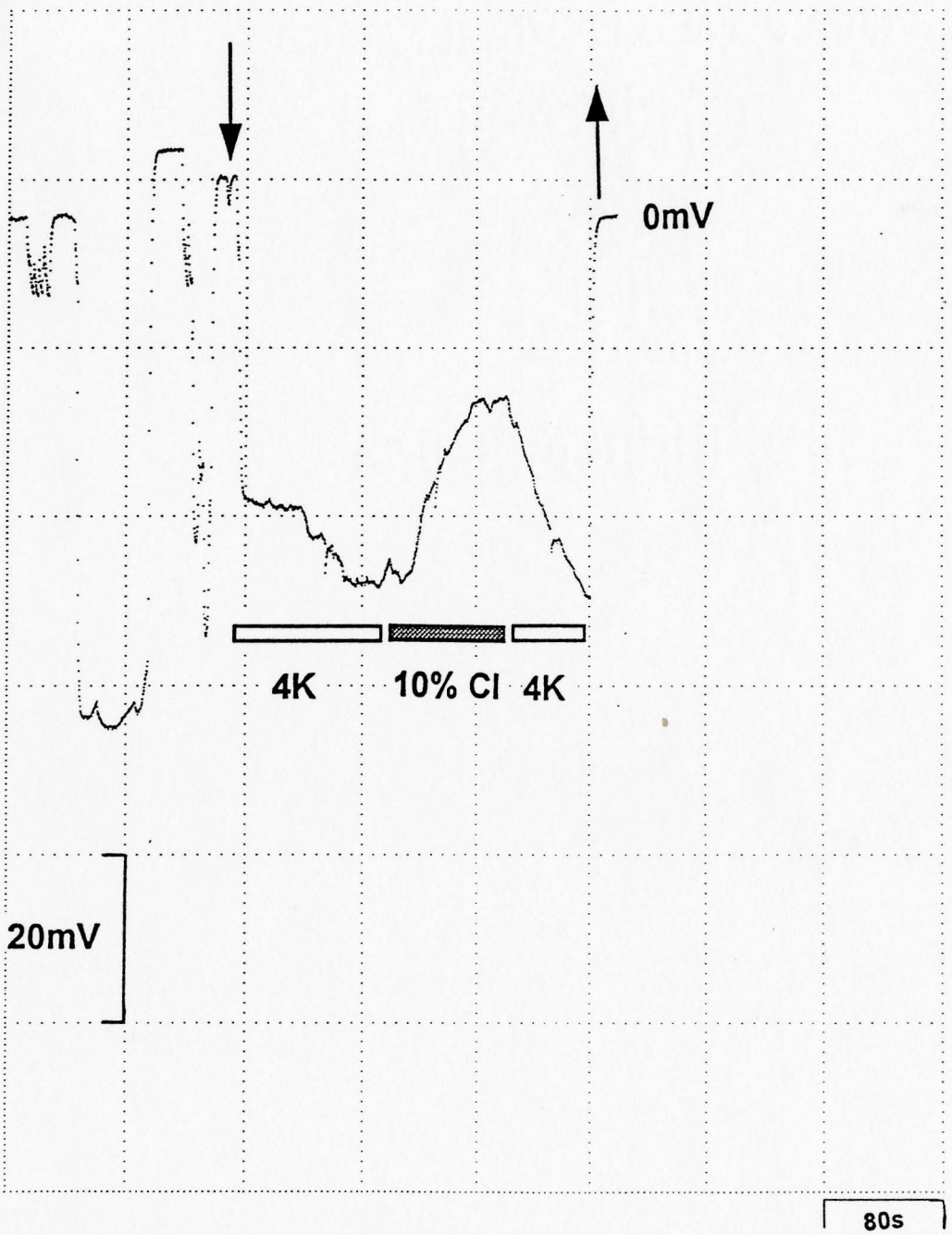
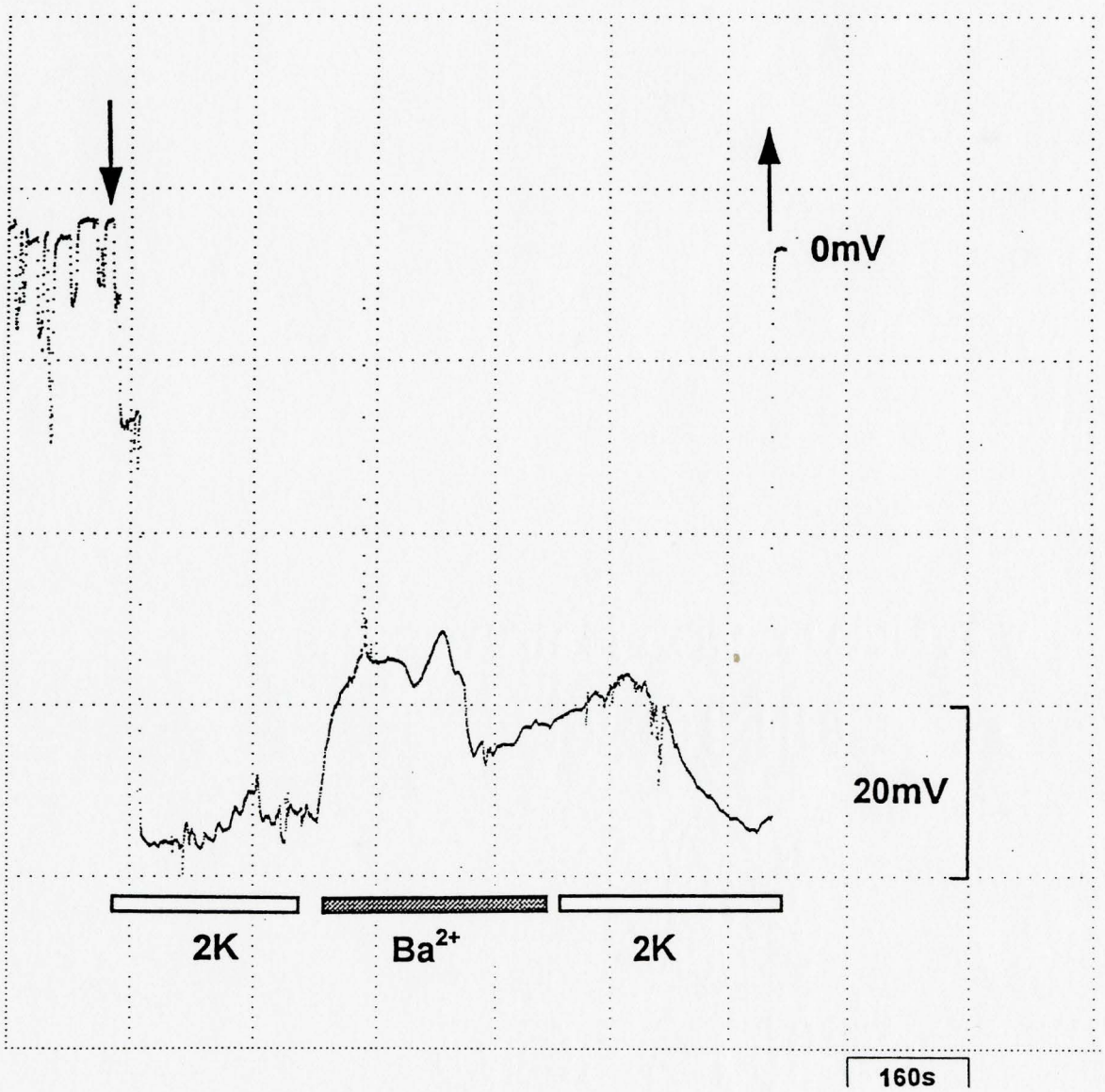

0mV
4K
10% Cl
4K
20mV
80s

Figure 4.7

Typical recording showing effects of Ba^{2+} on V_{bl} in a cell of the lower Malpighian tubule. Impalement was achieved by tapping the baseplate of the micromanipulator at the point marked by the downward arrow. Withdrawal of the microelectrode is indicated by the upward arrow. For the period indicated by the stippled horizontal bar Ba^{2+} (5 mmol l^{-1}) was added to the 2K bathing saline.


0mV
20mV
2K
Ba2+
2K
160s

the mean change in potential in 5 mmol l^{-1} Ba^{2+} was a 16.8 ± 3.7 mV. Addition of TEA, the other potassium channel blocker shown to inhibit KCl reabsorption in Chapter 2, also altered V_{bl} significantly, but the response was quite variable (Figure 4.8). V_{bl} depolarized by 2 - 34 mV in 4 tubules, and hyperpolarized by 7 mV in the fifth tubule. The average magnitude (*i.e.* absolute value) of the change in potential was 10.5 ± 6.1 mV. Sources of this variability will include changes in intracellular ion activities in response to the inhibition of KCl reabsorption by channel blockers, and the relative values of the equilibrium potentials and conductances for K^+, Cl^- and other ions along the length of the lower tubule. These factors are examined in detail in the discussion.

Effects of Cl^- channel blockers on V_{bl} and TEP.

Figure 4.9 shows the effect of DPC on basolateral membrane potential. Addition of 100 µmol l^{-1} DPC to 4K saline changed V_{bl} in each of 6 tubules; 4 tubules depolarized by 5 - 36 mV, and 2 hyperpolarized by 9 - 21 mV. The average magnitude of change was 21.1 ± 5.2 mV. A similar pattern was apparent in the responses to SITS and DIDS. Basolateral membrane potential depolarized as much as 11 mV or hyperpolarized as much as 5 mV in 3 tubules exposed to 1 mmol l^{-1} SITS, and TEP hyperpolarized by 2 to 9 mV. The average magnitude of change in TEP in response to 1 mmol l^{-1} DIDS was 9.8 ± 2.4 mV, but the changes ranged from a depolarization of 11 mV to hyperpolarizations as large as 16 mV. The TEP equals the sum of the basolateral and apical potentials, and since SITS and DIDS have been shown to have little effect on KCl reabsorption when applied apically, the changes in TEP in response to SITS and DIDS are likely to reflect changes primarily in V_{bl}.

Figure 4.8

Typical recording showing effects of TEA on V_{bl} in a cell of the lower Malpighian tubule. Impalement and withdrawal of the microelectrode are indicated by downward and upward arrows, resepectively. For the period indicated by the horizontal bar TEA (50 mmol l^{-1}) was added to the 2K bathing saline.

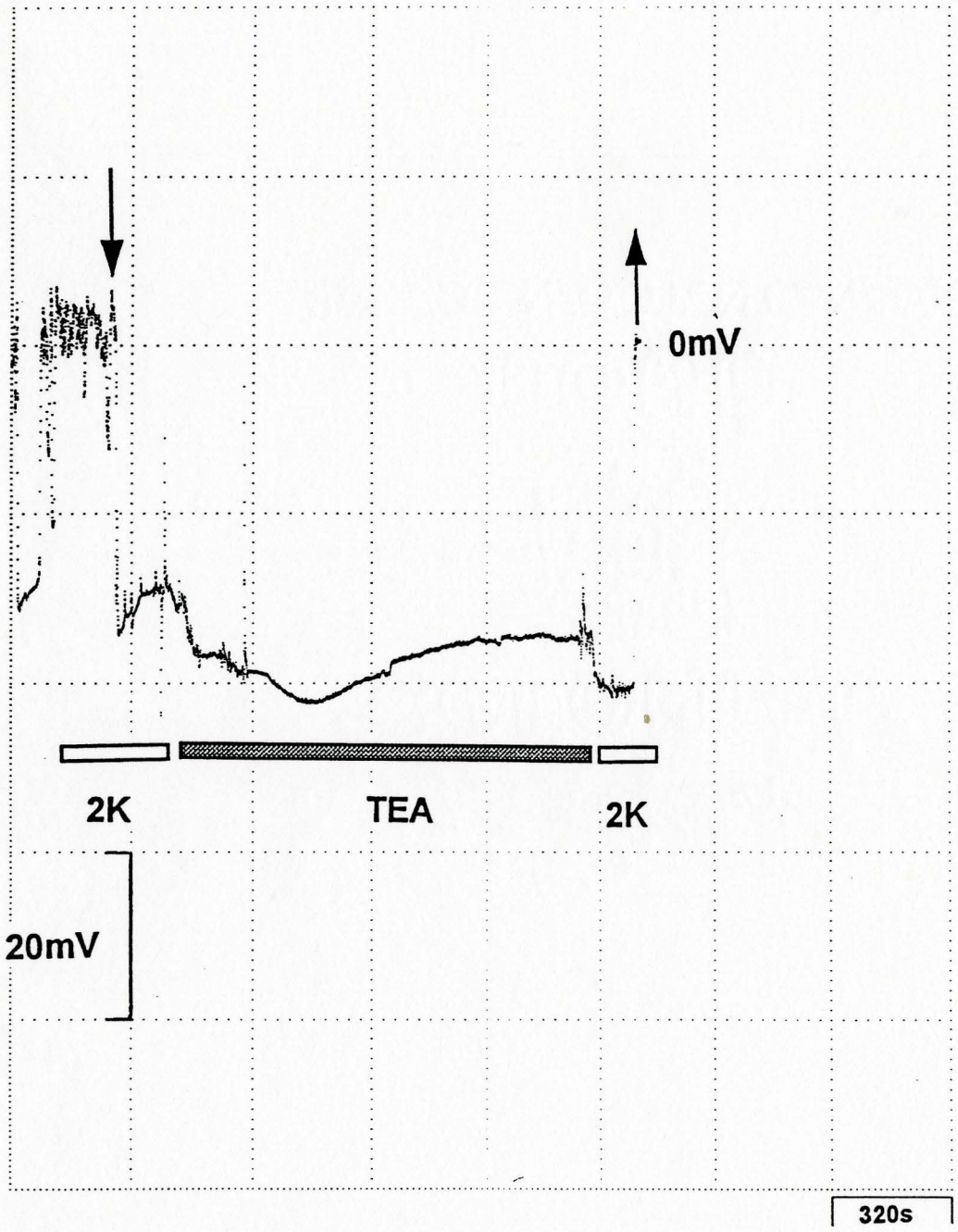
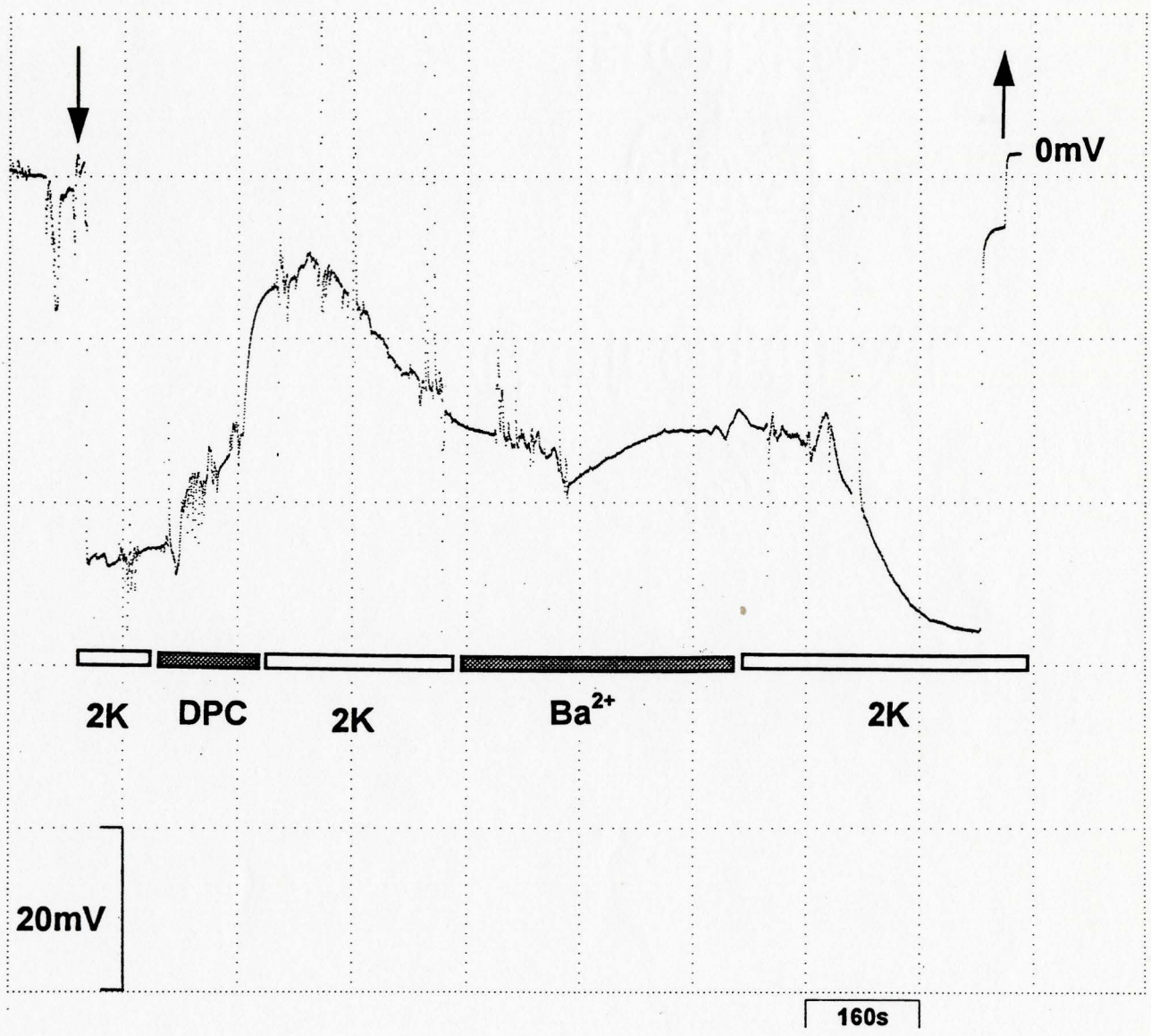

0mV
2K
TEA
2K
20mV
320s

Figure 4.9

Typical recording showing effects of DPC and Ba^{2+} on V_{bl} in a cell of the lower Malpighian tubule. Impalement and withdrawal of the microelectrode are indicated by downward and upward arrows, resepectively. For the periods indicated by the stippled horizontal bars DPC (100 μmol l^{-1}) or Ba^{2+} (5 mmol l^{-1}) was added to the 2K bathing saline.


0mV
2K
DPC
2K
Ba2+
2K
20mV
160s

Discussion

The effects of changes in bathing saline ion concentration and ion channel blockers on basolateral membrane potential are consistent with the presence of significant conductances for Cl^- and K^+. The changes in V_{bl} in response to changes in $[Cl^-]_o$ or $[K^+]_o$ indicate that approximately 74% of V_{bl} can be accounted for by these two ions. The fractional contribution (%) of each ion to membrane electrogenesis was obtained by expressing the mean change (mV) in V_{bl}/10-fold change in the extracellular concentration of each ion as a fraction of 58 mV, the theoretical maximum (at 20°C) for a membrane selectively permeable to that ion (*cf.* Dawson *et al.*, 1989).

The figure of 74% may be an underestimate, because the time required to exchange the fluids in the perfusion chamber was of the order of 30 s, which is a long period relative to the time required for a rapidly transporting epithelium such as the lower tubule to alter its internal ionic concentrations. As noted in the introduction, each upper Malpighian tubule cell secretes a volume of near isoosmotic fluid equal to its own volume every 15 s. Rates of ion transport by cells of the lower tubule are even higher, since approximately 40% of the ions secreted by the upper tubule are reabsorbed by the lower 1/3 of the lower tubule. Rapid changes in intracellular K^+ activities in response to changes in bathing saline K^+ concentration have been inferred from studies of *Formica* Malpighian tubules (Leyssens *et al.*, 1992). Decreases in bathing saline K^+ concentration are associated with a decrease in intracellular concentration, so that the ratio $[K^+]_i/[K^+]_o$ declines, and the membrane potential gradually depolarizes. For the *Rhodnius* lower tubule, an increase in $[K^+]_o$ may be followed rapidly by a increase in $[K^+]_i$, so that the ratio $[K^+]_i/[K^+]_o$ will increase and V_{bl} will become more negative, *i.e.* the depolarization produced by

an increase in $[K^+]_o$ will be underestimated, since the change in $[K^+]_i$ may occur as rapidly as the contents of the perfusion chamber are exchanged. For the same reasons, the estimate for the contribution of Cl^- to V_{bl} may be an underestimate.

The permeability of the basolateral membrane of the lower Malpighian tubule to both Cl^- and K^+ is in marked contrast to the permeability properties of the upper Malpighian tubule. Complete replacement of bathing saline Cl^- with impermeant anions (isethionate and sulphate) has a negligible effect on V_{bl} of the upper tubule, whereas a ten-fold change in bathing saline $[K^+]$ changes V_{bl} by 54.8 mV (O'Donnell and Maddrell, 1984). Chloride entry into the upper tubule, therefore, is mediated not by channels but by a furosemide-sensitive $Na^+/K^+/2Cl^-$ cotransporter which is driven by the sodium gradient established by the combined action of the apical H^+ ATPase and Na^+/H^+ antiporter (Chapter 1). As noted in Chapter 3, the insensitivity of KCl reabsorption by the lower tubule to drugs such as furosemide and bumetanide highlights the differences in the ion porters involved in Cl^- transport across basolateral membranes of upper versus lower Malpighian tubules.

Although all of the channel blockers examined in this chapter produced substantial changes in V_{bl} and/or TEP, the direction of change was quite variable; some tubules depolarized in response to DPC for example, whereas others hyperpolarized. There are several factors which contribute to this variability. If V_{bl} is strongly influenced by basolateral K^+ and Cl^- conductances, then blockage of Cl^- channels will tend to shift V_{bl} towards the equilibrium potential for K^+ (E_K). In the short term, the response to DPC will be a hyperpolarization or depolarization, depending on whether E_K is more or less negative, respectively, than E_{Cl}. The values of E_K and E_{Cl} may vary along the lower 1/3 of the lower tubule, since lumenal K^+

concentration is reduced from *ca.* 80 mmol l^{-1} to less than 10 mmol l^{-1} *in vitro*, and Cl^- concentration is reduced from 180 mmol l^{-1} to 100 mmol l^{-1}.

In the longer term, blockage of Cl^- channels will presumably alter intracellular Cl^- concentrations. Moreover, there will be a secondary effect of Cl^- channel blockage on intracellular K^+ concentrations, since K^+ reabsorption requires an anion to maintain charge balance. Similarly, blockage of K^+ channels will have short term and long term effects on intracellular concentrations of K^+ and Cl^-.

The potential change produced by Cl^- channel blockers may also have been variable because of the concentration of the drug used. In a study of Cl^- channels in the mammalian thick ascending loop of Henle, Wangemann *et al.*, (1986) found that at low concentrations (10^{-6} -10^{-5} mol^{-1}) compounds related to DPC uniformly hyperpolarized V_{bl}, whereas at 10^{-4} mol l^{-1}, 3 of the 6 drugs tested depolarized V_{bl}. This depolarization occurred within 20 - 60 s, and the authors therefore interpreted it as a secondary phenomenon.

Variability in response notwithstanding, V_{bl} is significantly altered by K^+ channel blockers (TEA and Ba^{2+}) and Cl^- channel blockers (DPC, SITS, DIDS). In conjunction with the changes in V_{bl} in response to changes in bathing saline [K^+] or [Cl^-], these findings provide direct evidence for basolateral K^+ channels and Cl^- channels in the lower Malpighian tubule. The involvement of these channels in KCl reabsorption was suggested by the results of Chapter 2 and 3. In the following chapter, the results of chapters 2 - 4 are integrated and a working hypothesis for the mechanism of KCl reabsorption is proposed.

Chapter 5

A Working Hypothesis for the Mechanism of KCl Reabsorption by the Lower Malpighian Tubule

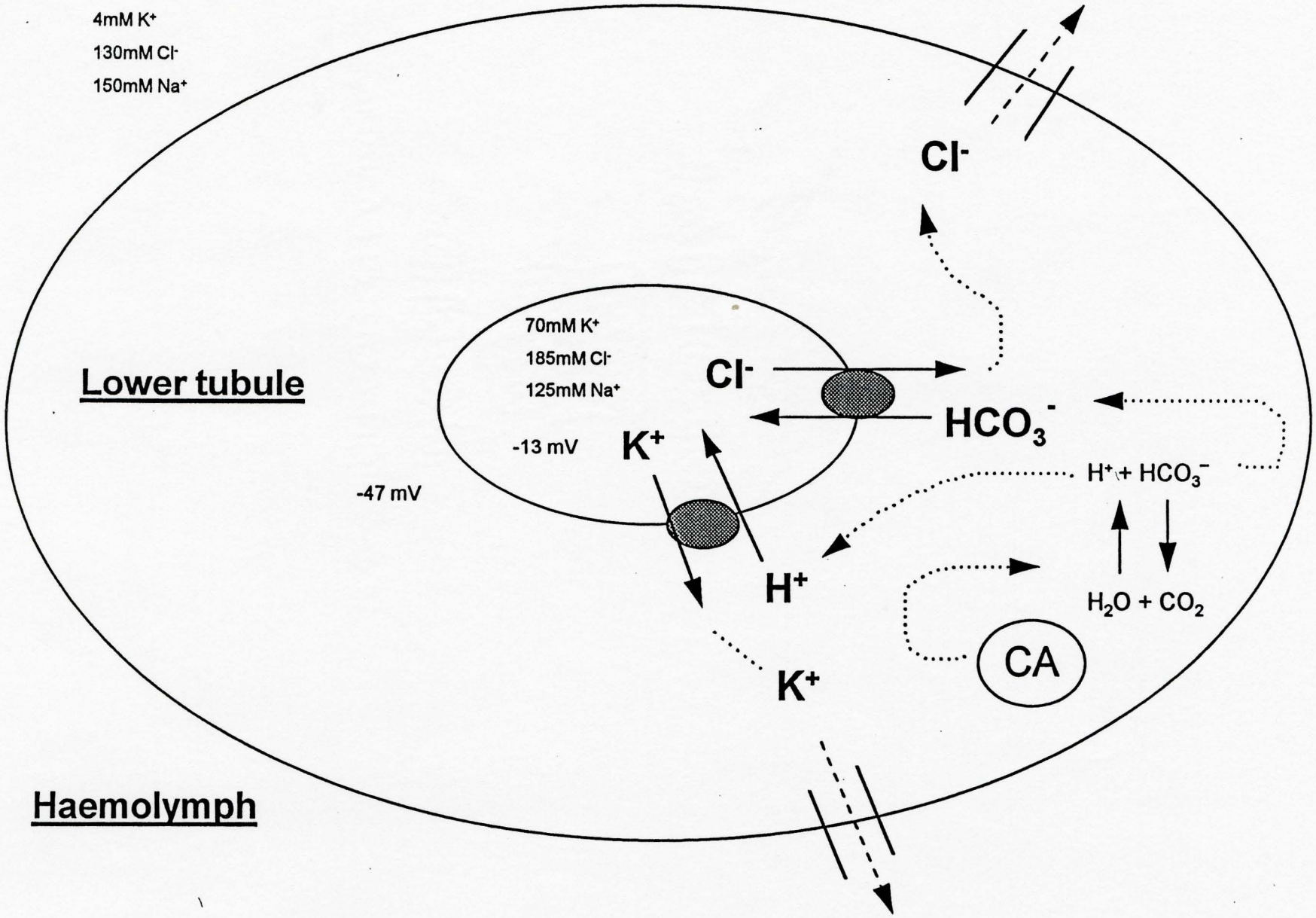
Figure 5.1 provides in schematic form a working hypothesis for the mechanism of KCl reabsorption by the lower Malpighian tubule of *Rhodnius prolixus*. The model is based on the results of chapters 2- 4, and consists of two ATPases which exchange cellular H^+ and HCO_3^- for lumenal K^+ and Cl^- respectively, and two basolateral ion channels for movement of K^+ and Cl^- from cell to haemolymph. Counterions (H^+, HCO_3^-) for the apical transporters are supplied by the actions of carbonic anhydrase. Conversion of H^+ and HCO_3^- back to CO_2 may be accelerated by the actions of apical or lumenal CA. It is worth noting that the apical microvilli of the lepidopteran midgut contains CA activity which is greatly stimulated by KCl (Johnston and Jungreis, 1979; Ridgway and Moffett, 1986). The evidence for each of the four proposed transporters is summarized as follows:

Basolateral K^+ channels

Changes in V_{bl} in response to the K^+ channel blockers TEA and Ba_2^+ and to changes in bathing saline [K^+] are consistent with the presence of K^+ channels in the basolateral membrane of the lower tubule. Similarly, KCl reabsorption was blocked by TEA and Ba_2^+ more effectively when the blockers were applied to the basolateral, as opposed to lumenal, surface of the tubule. Electroneutral mechanisms of K^+ transport across the basolateral membrane seem unlikely since high concentrations of furosemide and bumetanide, which block these cotransporters in

Figure 5.1

Schematic diagram summarizing the proposed working hypothesis for the cellular mechanisms of KCl reabsorption by the lower Malpighian tubule. The overall process of reabsorption of K^+ and Cl^- into the cell from the lumen is driven by an apical P-type H^+/K^+-ATPase and possibly by a Cl^-/HCO_3^--ATPase as well. These pumps raise the cellular levels of both ions so that channel-mediated electrodiffusive movements of K^+ and Cl- from cell to haemolymph are favoured.


4mM K+
130mM Cl-
150mM Na+
Cl-
70mM K+
185mM Cl-
125mM Na+
Cl-
Lower tubule
HCO3-
-13 mV
K+
H+ + HCO3-
-47 mV
H+
H2O + CO2
CA
K+
Haemolymph

the upper tubule of *Rhodnius* and in Malpighian tubules of other insects, do not block KCl reabsorption by the lower tubule.

Apical H^+/K^+-ATPase

Inhibition of KCl reabsorption by KCN indicates that active (*i.e* ATP-dependent) transport is involved. The H^+/K^+-ATPase of the gastric mucosa is inhibited irreversibly by omeprazole and reversibly by the compounds SCH 28080, SKF 96356 and SKF 96067. The latter 3 compounds inhibited KCl reabsorption effectively when applied from either apical or basolateral surface. Although omeprazole is more effective when applied from the basolateral surface of the cell, it is suggested that this is due to the pH difference between the lower tubule lumen and the bathing saline. Omeprazole is activated by exposure to low pH, and this activation is presumably more effective in the bathing saline at pH 6.9, in the cytosol or in acidic intracellular compartments than in the relatively alkaline lumen. Although concentrations of 100 $\mu mol\ l^{-1}$ omeprazole are known to inhibit vacuolar type H^+-ATPases as well (Sabolic *et al.*, 1994), this possibility was ruled out on the basis of the lower tubule's insensitivity to NEM, NBD-Cl and bafilomycin A_1, which are more specific inhibitors of V-type H^+-ATPases.

Nonetheless, it is important to point out that inhibition of KCl reabsorption by vanadate, SCH28080, SKF 96067, SKF 96356 and omeprazole means simply that the transporter shares some similarities with the gastric H^+/K^+ ATPase. In the rat distal colon, for example a vanadate-sensitive, ouabain-insensitive K^+ ATPase has been suggested on the basis of blockage of K^+ transport by some but not all types of monoclonal antibodies to the H^+/K^+ ATPase (Tabuchi *et al.*, 1992). However, the finding that basolateral SCN^- treatment results in

a decline in lumen pH in the *Rhodnius* lower tubule suggests that when Cl^- transport is blocked, the remaining transporters tend to acidify the lumen. This finding, therefore, suggests that SCH 28080 blocks an H^+/K^+ ATPase rather than a K^+ ATPase. The finding that TEP's in stimulated and unstimulated tubules are similar is also more consistent with an electroneutral mechanism (eg. H^+/K^+ ATPase) than with a K^+ ATPase which would presumably be electrogenic and drive the lumen potential more negative when stimulated.

Possible contributions of passive K^+/H^+ exchange to KCl reabsorption were precluded on the basis of insensitivity to amiloride, which effectively blocks fluid secretion by the upper tubule of *Rhodnius* and by Malpighian tubules of other species such as *Drosophila hydei* (Bertram *et al.*, 1991). Similarly, the Na^+/K^+ ATPase was ruled out since ouabain and dihydro-ouabain did not block KCl reabsorption. Moreover, a basolateral Na^+/K^+ ATPase would drive K^+ in a direction opposing K^+ reabsorption (*i.e* from haemolymph into the cell). Although an apical Na^+/K^+ ATPase has been suggested for the brine shrimp Artemia (Conte, 1984), direct evidence for an apical site is lacking. An apical Na^+/K^+ ATPase is also unlikely since the process of KCl reabsorption occurs at highest rates when lumen solutions contain just KCl *i.e* are effectively Na^+-free (Maddrell and Phillips, 1975).

Basolateral Cl^- channels

Changes in V_{bl} in response to the Cl^- channel blockers DPC, SITS and DIDS, and to changes in bathing saline [Cl^-] suggest the presence of Cl^- channels in the basolateral membrane. KCl reabsorption was blocked by application of DPC, SITS, DIDS and SCN^- to a

much greater extent when these compounds were applied to the basolateral, as opposed to the apical, side of the lower Malpighian tubule.

Chloride transport from lumen to cell

A major stumbling block in the analysis of ATP-dependent Cl^- transport is the absence of highly specific inhibitors of Cl^--transporting ATPases. An apical Cl^-/HCO_3^- ATPase has been included in the model proposed in figure 5.1 not on the basis of direct experimental evidence, but to be consistent with the other transporters proposed. A transcellular path for Cl^- transport seems likely since Cl^- channel blockers effectively inhibit KCl reabsorption when applied basolaterally. Entry of HCO_3^- into the lumen through this ATPase provides a means of compensating for the lumen acidification resulting from the actions of the proposed H^+/K^+ ATPase. Alternatively, the need for HCO_3^- transport from cell to lumen could be avoided if H^+ were cycled from lumen to cell through H^+ channels. In this context, it is important to point out that K^+ is cycled across the apical membrane of the gastric parietal cells by the combined actions of the H^+/K^+ ATPase and a K^+ channel (Rabon and Reuben, 1990).

By analogy, cycling of H+ would eliminate the need for compensatory transfer of basic equivalent (HCO_3^-) into the lumen. As noted above, a K^+ ATPase similar to the gastric H^+/K^+ ATPase would not tend to acidify the lumen of the lower tubule. In the absence of H^+ entry into the lumen, Cl^- might be moved by a second ATPase, or through channels. However, the channels would be unusual in being relatively insensitive to compounds which appear to block the basolateral Cl^- channels (*i.e.* DPC, SITS, DIDS and SCN^-). An alternative possibility has emerged from studies of the gastric mucosa. Vesicles prepared from gastric mucosa show

ATP-dependent Cl^- transport that accepts SCN^- as readily as Cl^-, and is insensitive to both vanadate and SITS (Soumarmon *et al.*, 1980). In contrast, the Cl^- ATPase of the *Aplysia* gut is sensitive to vanadate and SCN^-, but not to SITS (Gerenscer, 1988). Lastly, it has also been suggested that the Cl^- channel in hog gastric vesicles is in fact part of the function of the H^+/K^+ ATPase (Asano *et al.*, 1987). This proposal is based on the finding that SCH 28080 inhibits the opening of Cl^- channels by S-S crosslinking, and that a monoclonal antibody that inhibits the H^+/K^+ ATPase also inhibits Cl^- channels. However, other groups have developed a monoclonal antibody which inhbits H^+/K^+ ATPase activity but not the Cl^- conductance of hog gastric membranes (Benkouka *et al.*, 1989). The latter authors suggest that the H^+/K^+ ATPase and the Cl^- channel of the gastric mucosa apical membrane are distinct but very closely related proteins.

In sum, the proposal for a Cl^-/HCO_3^- ATPase is a consequence of the acidifiying tendency of the proposed H^+/K^+-ATPase and the evidence against apical Cl^- channels.

Implications of thesis results for studies of ion reabsorption by Malpighian tubules of other insect species

Studies by many groups over the last decade have greatly enhanced understanding of the major ion transporters involved in *secretion* of ions by Malpighian tubules. Recently,there have been several reports describing ion *reabsorption* by Malpighian tubules of crickets (Spring and Hazelton, 1987; Marshall *et al.*, 1993), and *Drosophila melanogaster* (O'Donnell and Maddrell, 1995). The cellular mechanisms involved in ion reabsorption by these tubules have not been examined, and the results of this thesis therefore provide a useful starting point. For example, in *Drosophila melanogaster* both KCl and water are reabsorbed in a segment of

Malpighian tubule downstream from the main segment which secretes a KCl-rich fluid. During passage through the reabsorptive segment, K^+ content of the fluid is reduced from 133 mmol l^{-1} to 113 mmol l^{-1}, and approximately 15 - 20% of the fluid is reabsorbed. In contrast to the lower tubule of *Rhodnius*, the reabsorptive segment of *Drosophila* tubules acidifies the fluid passing through it. Nonetheless, it would be of interest to examine the effects of inhibitors of H^+/K^+-ATPases (*eg.* omeprazole) on the pH and K^+ concentration of fluid secreted by the reabsorptive segments of tubules from *Drosophila*.

Further analysis of the mechanisms of KCl reabsorption by the lower Malpighian tubule of Rhodnius prolixus.

Many aspects of the working hypothesis summarized in Figure 5.1 can be tested experimentally. In particular, the model proposes active transport of K^+ and Cl^- from lumen to cell, and passive diffusion of these ions from cell to haemolymph, down their respective electrochemical gradients. Measurement of intracellular K^+ and Cl^- activities by ion-selective microelectrodes would permit calculation of the electrochemical gradients across the apical and basolateral membranes. The model predicts that the electrochemical gradients across the apical membrane should be positive with respect to the lumen *i.e.* the gradients should indicate active accumulation of K^+ and Cl^-. Gradients across the basolateral membrane should be near equilibrium.

The proposed basolateral channels for K^+ and Cl^- warrant further investigation. For example, changes in V_{bl} in response to variations in bathing saline $[K^+]$ or $[Cl^-]$ should be measured in the presence of compounds identified as putative blockers of K^+ channels and Cl^-

channels. For example, if DPC acts by blocking basolateral Cl^- channels, then one would expect the depolarization in response to low $[Cl^-]_o$ to be reduced or abolished in the presence of the drug. Further studies of contributions to V_{bl} using intracellular microelectrodes would also permit the assessment of contributions of other ions (*eg.* H^+) or electrogenic ion pumps to V_{bl}. For example, in many tissues the basolateral Na^+/K^+-ATPase contributes to cell negativity because of its stoichiometry of $3Na^+/2K^+$ (*eg.* O'Donnell and Sharda, 1994).

Ion channels on the basolateral membrane might also be amenable to further analysis using patch clamp techniques. There are only a few patch clamp studies of Malpighian tubules to date. Potassium channels have been examined in the basolateral membrane of tubules of a Tenebrionid beetle (Nicolson and Isaacson, 1990) and Cl^- channels have been suggested on the basis of preliminary studies of *Aedes* (Wright and Beyenbach, 1987) and *Drosophila* (Dow and Harvey, pers. comm.). Several techniques for removal of the basement membranes of insect Malpighian tubules have been developed (Satmary and Bradley, 1984; Nicolson and Isaacson, 1990). and could be used, if necessary, to expose the basolateral membrane of the lower tubule and facilitate attachment of patch pipettes.

A very different approach to the analysis of membrane ion transport involves application of biochemical and immumological techniques. Micro-methods for analysis of ATPase activities in insect epithelia have been useful in characterizing the ATP-dependent pumps present in Malpighian tubules (*eg.* Fogg *et al.*, 1992), hindgut (Lechleitner and Phillips, 1987) and midgut (Wieczorek *et al.*, 1986). If sufficient quantities of lower tubule could be harvested for analysis, the presence of ATPase activity sensitive to omeprazole and SCH28080 could be used to assess the presence of H^+/K^+-ATPase activity, for example. An alternative

approach involves the production of antibodies to particular membrane proteins. Immunofluorescence studies using monoclonal antibodies to the midgut V-ATPase and fluorescein isothiocyanate (FITC) -conjugated secondary antibodies have demonstrated the apical localization of the V-ATPase in secretory Malpighian tubules and midgut (Klein, 1992). Monoclonal antibodies to gastric H^+/K^+-ATPase are available (Tabuchi *et al.*, 1992; Swenson *et al.*, 1994) and FITC-conjugates of secondary antibodies could be used to determine if proteins resembling the H^+/K^+-ATPase are present in the lower tubule. The presence and localization of carbonic anhydrase activity might also be assessed by histochemical (Ridgway and Moffett, 1989) or immunohistochemical (eg. Holthofer *et al.*, 1991) techniques. Immunohistochemical assays for chloride/bicarbonate exchangers are also available (eg. Holthofer *et al.*, 1991). However, most of these antibodies have been raised against the band III protein of the vertebrate erythrocytes. Since Cl^-/HCO_3^- exchange in vertebrate erythrocytes is SITS-sensitive, whereas the putative Cl^-/HCO_3^--ATPase of the lower tubule is SITS-insensitive, the antibodies might not react with the latter protein.

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Appendix

Name of Chemical	Supplier	Solvent for Stock Solution	Name of Chemical	Supplier	Solvent for Stock Solution
Acetazolamide	Sigma	CS	7-Chloro-4-nitrobenz 2-oxa-1,3-diazole (**NBD-Cl**)	Sigma	EtOH
Amiloride Hydrochloride	Sigma	CS & MeOH	N-ethylmaleimide (**NEM**)	Sigma	EtOH
5-(N,N-hexamethyl-ene)-amiloride	Sigma	DMSO	Omeprazole	Molndal	EtOH
5-(N,N-dimethyl)-amiloride	Sigma	DMSO	Ouabain	Sigma	CS
5-(N-methyl-N-iso-butyl)-amiloride (**MIBA**)	RBI	DMSO	Potassium cyanide (KCN)		CS
5-(N-ethyl-N-iso-propyl)-amiloride (**EIPA**)	RBI	DMSO	SCH 28080	SPRI	DMSO
Bafilomycin		DMSO	4-acetamido-4'-isothiocyanayo-stilbene-2,2'-disulfonic acid (**SITS**)	Sigma	CS
Barium chloride	BDH	CS	SKF 96067 & 96356	SKF	DMSO
Bumetanide	Sigma	CS	Sodium orthovanadate	Sigma	CS/10% H_2O
Diisothiocyanato-stilbene-2,2'-disulfonic acid (**DIDS**)	Sigma	CS	Sodium thiocyanate (NaSCN)	Sigma	CS
N-phenylanthranilic acid (**DPC**)	RBI	EtOH	Tetraethylammonium chloride (**TEA**)	Sigma	CS
Furosemide	Sigma	CS			