A PHARMACOLOGICAL STUDY OF SIGNAL TRANSDUCTION MECHANISMS CONTROLLING FLUID REABSORPTION AND ION TRANSPORT IN THE LOCUST RECTUM.

by

LLOYD BRIAN JEFFS

B.Sc., The University of British Columbia, 1990.

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

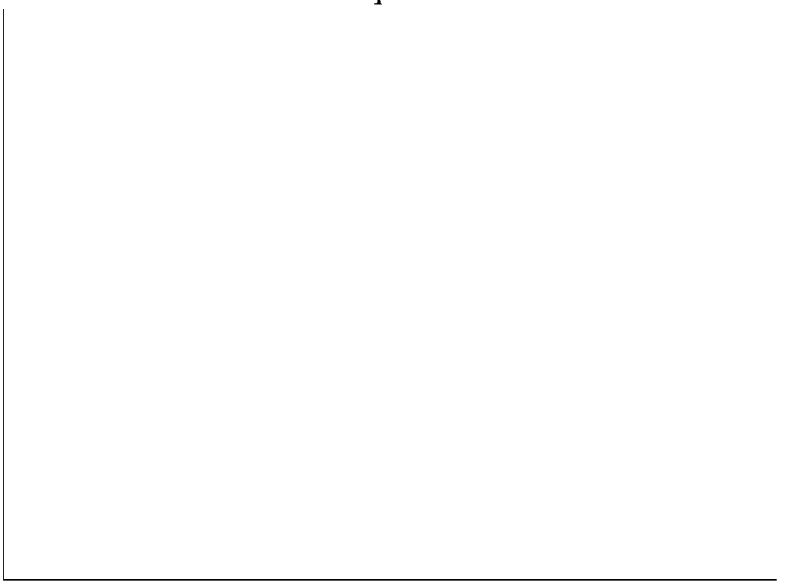
in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF ZOOLOGY

We accept this thesis as conforming

to the required standard



THE UNIVERSITY OF BRITISH COLUMBIA

May 1993

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature) _____

Department of ZOOLOGY

The University of British Columbia
Vancouver, Canada

Date 31st MAY 1993

ABSTRACT

Like most insects, locusts face severe regulatory challenges associated with arid habitats, high metabolic rates and high surface area to volume ratios. Therefore, the conservation of water, essential ions and metabolites is very important. Consequently, locusts regulate their hemolymph composition primarily by controlling epithelial transport in the excretory system. The locust excretory system is typical of many insects and consists of the Malpighian tubules and the hindgut. The Malpighian tubules secrete a primary isosmotic urine rich in KCl and low in Na^+ that contains most small hemolymph solutes, waste products and toxic plant chemicals. The hindgut (ileum and rectum) is responsible for the enormous changes in composition of the urine, by the selective reabsorption of water, ions and metabolites. Both the Malpighian tubules and the hindgut are under endocrine control.

The purpose of this study was to investigate the involvement of second messengers in the control of fluid reabsorption (J_V) and Cl^- transport in the rectum of the desert locust, *Schistocerca gregaria*. Various agents known to block or activate specific signal transduction pathways were added to everted rectal sacs and short-circuited rectal flat-sheet bioassays. Cyclic AMP and its analogs were shown to stimulate rectal J_V and Cl^- transport to the same extent as aqueous extracts of the nervous lobes of the corpora cardiaca, suggesting that activation of the adenylate cyclase pathway is sufficient for maximal stimulation. It also appears that cGMP is involved, since its addition partially stimulated both Cl^- and fluid reabsorption. External Ca^{2+} was not required for the maintenance or stimulation of rectal transport. However, intracellular Ca^{2+} was shown to influence the control of rectal transport. The role of intracellular Ca^{2+} appears to be quite complex and may vary with its relative concentration. Finally, it was found that Protein

kinase C and the phosphotidylinositol cycle do not appear to be involved in the stimulation of rectal Cl^- and fluid transport.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ΔJ_V	- change in fluid reabsorption
Ωcm^2	- ohms centimetre squared
$\mu eq.cm^{-2}.h^{-1}$	- micro equivalents per centimetre squared per hour
μL	- microlitre
$\mu L.h^{-1}$	- microlitres per hour
μM	- micro molar
μm	- micron
°C	- degrees Celsius
5-HT	- 5-hydroxytryptamine
Å	- Ångstrom (0.1 nanometres)
A/D	- analog-to-digital
AA, aa	- amino acid(s)
AD	- antidiuretic
ADH	- antidiuretic hormone
ADP	- adenosine diphosphate
AG	- abdominal ganglion
AP	- *Acheta* peptide
ATP	- adenosine 5' triphosphate
ANOVA	- analysis of variance
AVG	- abdominal ventral ganglion
AVP	- arginine vasopressin
AVP-LDH	- arginine vasopressin-like diuretic hormone
AVT	- arginine vasotocin
CA	- corpus allatum
cAMP	- adenosine 3', 5' -cyclic monophosphoric acid

CC	- corpus cardiacum
cDNA	- complementary DNA
CGA	- colour graphics adapter
cGMP	- guanosine 3', 5' -cyclic monophosphoric acid
cm	- centimetre
cm^2	- squared centimetres
CRF	- corticotropin releasing factor
CTSH	- chloride transport stimulating hormone
D	- diuretic
Da	- Daltons
DAG	- sn-1,2 diacylglycerol
DBcAMP	- di-butyryl 3', 5' -cyclic monophosphoric acid
DH	- diuretic hormone
DMSO	- dimethyl sulfoxide
DP	- diuretic peptide
EGTA	- ethyleneglycol-bis-(B-aminoethyl ether) N,N,N',N'-tetraacetic acid
eq.	- equivalents
g	- gravity units
GCC	- glandular lobe of the corpus cardiacum
GTP	- guanosine triphosphate
H	- hemolymph or hemocoel
h	- hours
HPLC	- high performance liquid chromatography
IBMX	- 3 isobutyrl-1-methylxanthine
Ins	- inositol
Ins P	- inositol phosphate
$InsP_3$	- inositol 1,4,5, trisphosphate

$InsP_4$	- inositol 1,3,4,5 tetrakisphosphate
I_{sc}	- short-circuited current
iu	- international units
IY	- ionomycin
J_{amm}	- rate of luminal ammonia secretion
J_v	- fluid reabsorption
KDa	- kilo Daltons
LCCP	- locust corpus cardiacum peptide
M	- molar
M-NSC	- median neurosecretory cells
MI	- *myo*-inositol
min	- minutes
mL	- millilitre
mm	- millimetre
mM	- millimolar
MT	- Malpighian tubule(s)
MTG	- meta-thoracic ganglion
mV	- millivolts
n	- number
NCC	- nervous lobe of the corpus cardiacum
NpA	- neuroparsin A
NpB	- neuroparsin B
Nps	- neuroparsins
P.E.	- polyethylene (tubing)
PD	- potential difference
PdE	- phosphodiesterase
PI	- phosphotidylinositol

$PtdInsP_2$, PIP_2	- phosphotidylinositol 4,5-bisphosphate
PKA	- protein kinase A
PKC	- protein kinase C
PLC	- phospholipase C
PMA	- phorbol 12-myristate-13-acetate
PMXB	- polymyxin B sulfate
PO	- perisympathetic organs
RAM	- random access memory
RpcAMPS	- adenosine 3', 5' -cyclic monophosphothioate Rp- diastereomer
R_t	- transepithelial resistance
S.E.	- standard error
SAG	- 1-stearoyl-2-arachidonoyl-sn-glycerol
ScgITP	- *Schistocerca* ion transport peptide
SO-NSC	- subocellar neurosecretory cells
SOG	- suboesophageal gnanglion
SpcAMPS	- adenosine 3', 5' -cyclic monophosphothioate Sp- diastereomer
TAG	- thoracic abdominal ganglion
TG	- thoracic ganglion
THAPS	- thapsigargin
TMB-8	- 3,4,5 trimethoxybenzoic acid, 8-(diethylamino)-octyl ester
V_t	- transepthelial voltage

ACKNOWLEDGMENTS

I would like to thank Dr. John Phillips for his support, generosity and guidance throughout this study.

I thank Drs. M. Isman, D. Jones and D. Randall for their helpful comments on the manuscript.

I am grateful to Joan Martin for her technical assistance, advice and support.

I thank Bart Demaerschalk for his help with some of the experiments.

I thank Megumi Fusé for her advice on writing this manuscript.

I thank everyone in Dr Phillips' lab that have made working there enjoyable and productive.

I thank Brian, Eileen and Peter Jeffs for always being there when it matters.

I especially want to thank Joyce Chong for her limitless encouragement, assistance and friendship.

CHAPTER 1: GENERAL INTRODUCTION

Insects inhabit a wide range of diverse and variable habitats, including deserts, freshwater, alkaline lakes and tidal pools. Insects face severe regulatory challenges associated with high metabolic rates (e.g. when flying) and high surface area to volume ratios leading to water loss across the integument. The unusual diets of some insects including toxic substances produced by plants, high ingestion rates, molting and metamorphosis are further examples of the stresses placed on insect homeostatic mechanisms. Like most insects, locusts regulate their hemolymph composition very closely. As well as behavioral and structural adaptations, physiological adaptations contribute to this hemolymph homeostasis. The control of epithelial transport in the excretory system is the principal mechanism for making rapid and major adjustments to hemolymph composition.

The organization of the locust excretory system is typical of many insects, consisting of the Malpighian tubules and hindgut (Fig. 1.1). The Malpighian tubules actively secrete a primary isosmotic urine, rich in KCl and low in Na^+, that contains most small hemolymph solutes (including amino acids), waste products (e.g. urate and ammonium) and toxic plant chemicals (reviewed by Phillips, 1981,1983). The hindgut (ileum and rectum) is then responsible for the enormous changes in composition of the urine by the selective reabsorption of water, ions, amino acids and other metabolites (Phillips et al., 1986). This selective reabsorption can create a hyper- or hypo- osmotic urine, or even a powder dry excreta under various environmental conditions.

Structure of the locust excretory system

There are about 250 Malpighian tubules in an adult *Schistocerca gregaria* that empty into the gut lumen at the junction of the midgut and hindgut. The hindgut (ileum, colon

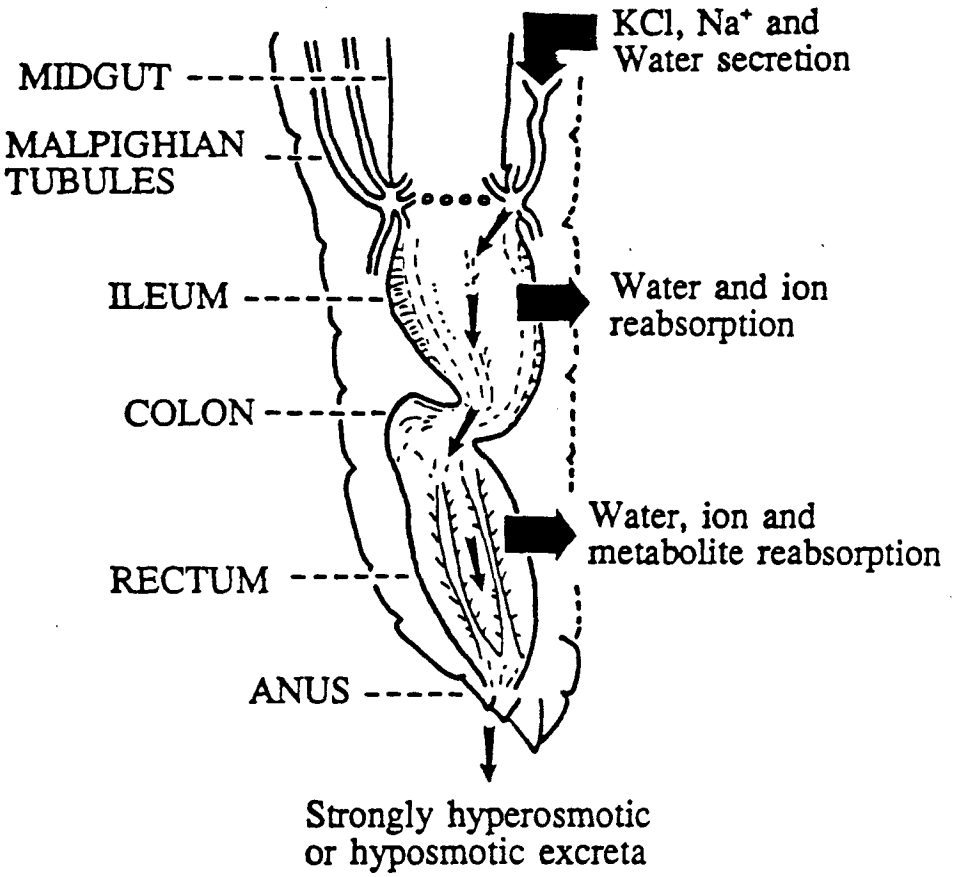


Fig. 1.1. Diagram of the locust excretory system. The flow of urine is shown by the *thin arrows* and transfer across the epithelia is indicated by the *thick arrows* (from Phillips, 1981; modified by Audsley, 1991).

and rectum) is lined on the luminal side with a chitinous cuticle (2-10 μm thick). The cuticle of the ileum and rectum is permeable to small hydrophilic molecules via water-filled 6 Å pores, but blocks the movement of larger toxic substances (Phillips and Dockrill, 1968). These water-filled pores are lined with fixed negative charges (pK ~ 4) that also allow the rapid diffusion of Ca^{2+} and Mg^{2+}, which would otherwise be precluded because of their large hydrated molecular radii (Lewis, 1971).

The ileum is approximately 6 mm long, has a diameter of 2.5 mm and luminal surface area of 0.4 cm^2 (Irvine et al., 1988). The ileum consists of a simple epithelium (40 x 20 μm) and is covered by a firmly attached apical cuticle (Fig. 1.2). The apical membrane has dense infoldings that are associated with mitochondria and apical junctional complexes between adjacent cells. The basal membrane has short narrow infoldings associated with numerous mitochondria and may be analogous to the lateral scalariform complexes of the rectum that are described below (Irvine et al., 1988).

The colon is an "S" shaped tube that is located between the ileum and the rectum (Fig. 1.1). It is narrower than the ileum or rectum and is comprised of small unspecialized epithelial cells. In addition, the colon possesses a cuticle which makes it considerably less permeable than either the rectum or ileum. For these reasons the colon is not thought to play a significant role in absorption (Maddrell and Gardiner, 1980). However, the colon has a structural role in the separation of the gut contents into discrete fecal pellets and in the breaking of the peritrophic membrane that surrounds the fecal material (Goodhue, 1963).

The rectum is immediately posterior to the colon, consists of six longitudinally arranged thickened pads, and has a luminal surface area of 0.64 cm^2 (Irvine et al., 1988). The rectal cuticle is attached at narrow junctional complexes between each rectal pad, but is free from the pad epithelium creating a subcuticular space above the apical border. The rectal pads are almost exclusively composed of large columnar principal cells (17 x 100 μm) with some small B cells. These B cells have few mitochondria and contact the

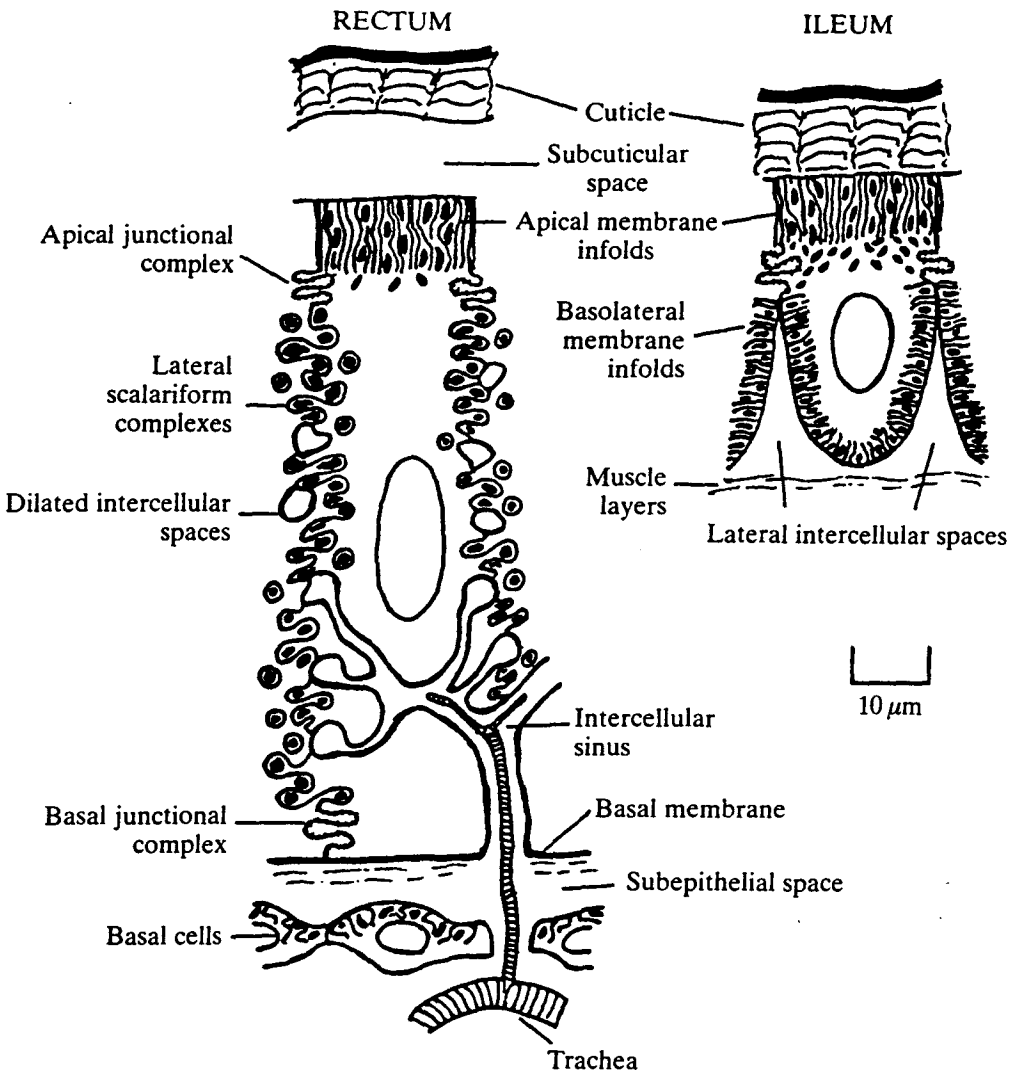


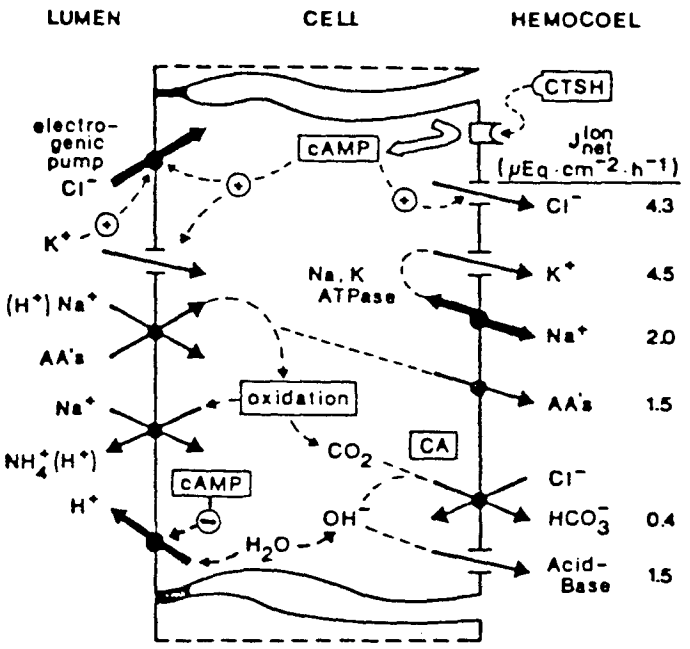
Fig. 1.2. Comparison of ultrastructural organization and gross dimensions of locust rectal pad and ileal epithelial cells (from Irvine et al., 1988).

luminal side only. The principal cells have highly folded apical and lateral membranes with closely associated mitochondria. The lateral membranes have finger-like projections that are intimately associated with the lateral membranes of adjacent cells. Extensive electrical coupling between these principal cells has been demonstrated by dye injection and cable analysis (Hanrahan and Phillips, 1984b). The lateral membrane infolds form complex intercellular channels with three distinct regions (lateral scalariform complexes, dilated intercellular spaces, larger intracellular sinuses with trachea) where ion recycling associated with the formation of hyperosmotic urine is thought to occur (Wall and Oschman, 1970). The basal membrane is unfolded and devoid of mitochondria which occur at the apical and lateral membranes, but is externally covered by a thick basal lamina. Below the basal lamina is a muscle layer, a subepithelial space and a smaller secondary epithelial cell layer. These secondary cells show some degree of membrane complexity and possess many mitochondria, suggesting a transport function (e.g. further ion recycling). Finally the whole structure is encased within a muscle layer through which fluid exits at the points where trachea penetrate into the organ.

Mechanisms of solute and water transport in the locust rectum

Wigglesworth (1931) was the first to report that insect gut contents can become very dry as they pass through the rectum and suggested that the rectum is a major site of fluid reabsorption. Active transport of ions and fluid in an insect rectum was first directly shown by measuring changes in volume and composition of fluid injected in ligated recta of locusts (Phillips, 1964 a-c,). It is now known that Na^+, K^+, Cl^-, water and metabolites are all reabsorbed by locust recta. Figure 1.3 shows the major membrane transport mechanisms demonstrated in locust rectal epithelial cells. The ileum has since been shown to have equal or greater transport capacities for ions and water than the rectum. The differences between rectal and ileal epithelial transport mechanisms will be discussed later.

Chloride (Cl^-) is the major anion that is actively reabsorbed by the locust rectum from



Ion Activities (mM) and PD (mV)			
Na^+	75	9	75
K^+	7.2	70	7.2
Cl^-	82	47	82
Proline	13	66	13
pH	7	7.36	7
mV	+64	0	+34

	Net Electrochemical PD (mV) (Apical)	(Basolateral)
Na^+	127 (favoring)	127 (opposing)
K^+	12 (favoring)	20 (favoring)
Cl^-	50 (opposing)	20 (favoring)
H^+	86 (opposing)	56 (favoring)

Fig. 1.3. Model of transport mechanisms identified in the locust rectal epithelium. The neuropeptide, CTSH, acts via cAMP to stimulate or inhibit four mechanisms. Major pumps are represented by *thick arrows*, carrier mediated co- or counter-transport by *thin arrows through solid circles* and ion channels by *arrows through gaps*. Steady-state values given for net transepithelial flux and electrochemical potential differences across the two borders are for stimulated recta in Ussing chambers and bathed bilaterally in control saline under open circuit conditions, except values for Na^+ and amino acids (AA) which were quantified under short-circuited conditions. From Phillips et al. (1988).

the urine. It can be seen from Figure 1.3 that there is a large net electrochemical difference opposing Cl^- entry at the apical (mucosal) surface of the rectal pad cells. This indicates that the active step for Cl^- transport is located at the apical membrane. The model proposes that there is a primary electrogenic Cl^- pump that is stimulated by cAMP and luminal K^+. There are also basolateral Cl^- channels that allow the passive exit of Cl^- across the basal (serosal) membrane. In support of this model, Hanrahan and Phillips (1983, 1984a,b) showed that addition of 1 mM cAMP to the serosal side of recta caused a 10-fold increase in net Cl^- transport toward the hemocoel side and that the removal of external K^+ reduced net Cl^- flux by 84%. They also provided evidence that Cl^- transport was not coupled to apical Na^+, K^+, HCO_3^- or H^+ transport. In addition, Cl^- exit through the basolateral membrane can be completely inhibited by Cl^- channel blockers applied to the hemocoel side of recta (Phillips et al., 1986).

Potassium (K^+) is the major cation in the primary urine from the Malpighian tubules and is absorbed passively through K^+ channels at both membranes (Hanrahan and Phillips, 1984a). In unstimulated open-circuit recta, there are small favourable electrochemical gradients for entry of K^+ across the apical membrane (3.3 mV) and for exit across the basolateral membrane (3.9 mV; Hanrahan and Phillips, 1983, 1984c). Addition of cAMP stimulates Cl^- transport and subsequently increases apical potential (Va) by 12 mV and decreases basolateral potential (Vb) by 13 mV. These membrane potential changes increase the favourable apical and basolateral electrochemical gradients to 12 mV and 20 mV respectively, resulting in increased net K^+ movement to the hemocoel.

Sodium (Na^+) is usually present at low concentrations in the fluid entering the rectum (20-40 mM), and is almost completely reabsorbed. There is a large favourable electrochemical gradient (127 mV) at the apical membrane for passive Na^+ entry by several mechanisms. These demonstated mechanisms include Na^+/NH_4^+ and Na^+/H^+ counter exchange, Na^+/glycine cotransport, and direct entry through a Na^+ channel (Phillips et al., 1986; Black et al. 1987). Na^+ is actively transported into the hemocoel

against a large electrochemical gradient (127 mV) by Na^+/K^+ ATPase in the lateral membranes (reviewed by Phillips et al., 1986; Lechleitner and Phillips, 1988). Rectal Na^+ transport is independent of Cl^- transport and is not affected by cAMP or any neuroendocrine tissue extracts tested to date (Black et al., 1987).

Berridge and Gupta (1967), and Wall and Oschman (1970), have proposed a model to explain how insect recta can absorb hypo-osmotic fluid from the lumen. The model proposes that ions and other solutes (amino acids) are actively secreted into the intercellular spaces of the lateral scalariform complexes (Fig. 1.2), creating areas of high osmotic concentration relative to the lumen. Water then flows from the gut lumen into these hyper-osmotic spaces (i.e. local osmosis), thereby producing a hydrostatic pressure which causes fluid to flow through the intercellular sinuses towards the hemocoel side. Active solute reabsorption is believed to occur in the dilated intercellular spaces and intercellular sinuses. This reabsorption of solute into the the cells results in the formation of a hypo-osmotic absorbate and concentrated excreta. Goh and Phillips (1978) have shown that water transport in the locust rectum is indeed coupled to ion transport, and Proux et al. (1984) have shown that cAMP-stimulated fluid reabsorption (J_V) is dependent upon Cl^- transport. Recently Lechleitner and Phillips (1989) observed an increase in J_V when recta were exposed to 80 mM proline compared to incubation with 1 mM proline. It was also shown that a low level of J_V (2.3 $\mu L.h^{-1}$) could be maintained for 5 hours with bathing saline containing 80 mM proline and no K^+, Cl^- or Na^+. Combined with the knowledge that proline is actively transported apically at high rates (Lechleitner, 1988) and that 80 mM proline far exceeds the cell's metabolic needs, it has been concluded that proline is directly involved with fluid transport in the rectum.

Five neutral amino acids (proline, glycine, serine, alanine and threonine) have been shown to be actively absorbed by locust recta when J_V was prevented by making the lumen hyper-osmotic with sucrose (Balshin and Phillips, 1971; Balshin, 1973). Meredith and Phillips (1988) found that the rectum has a very high capacity for proline transport at

the apical membrane. This proline transport system operates in the absence of luminal Na^+, Cl^- or K^+. The system also has an electrogenic component, probably due to H^+ cotransport. In addition, proline is the major respiratory substrate in the locust rectum and is the source of the secreted ammonia (Chamberlin and Phillips, 1983; Thomson et al., 1988).

Ammonia is a toxic waste product of amino acid oxidation and must be eliminated or detoxified. Ammonia is secreted into the rectal lumen via an apical $Na^+/NH4^+$ exchanger (Thomson et al., 1988). Acid (H^+) secretion by the rectum is accompanied by an equal movement of base (OH-, $HCO3^-$) equivalents to the hemocoel side (Thomson and Phillips, 1985, Phillips et al., 1988). The bulk (80-90%) of rectal acid secretion is actively driven by an apical electrogenic process (i.e. a H^+ATPase), with Na^+/H^+ exchange playing a minor role (Thomson and Phillips, 1992). The addition of cAMP to rectal preparations reduces active rectal H^+ secretion by 66% but does not change ammonia secretion (Phillips et al., 1988; Thomson et al, 1988).

Solute and water transport in the ileum

Many of the ileal transport mechanisms are similar to those previously described in the rectum, but there are some distinct differences. The primary difference is that the ileum can only produce an iso-osmotic absorbate, because it lacks the complex lateral membrane system for solute re-cycling found in the rectum (see Fig. 1.2.; Irvine et al., 1988). Moreover, ileal J_V is driven by both NaCl and KCl transport, while rectal J_V is driven largely by proline and KCl (Lechleitner and Phillips, 1989). Unlike the rectum, Na^+ is transported at high rates in the ileum and its transport is stimulated by cAMP (Lechleitner et al., 1989b). Acid (H^+) secretion in the rectum is inhibited by cAMP, while ileal H^+ secretion is inhibited by the neuropeptide ScgITP but not cAMP (Audsley, 1991). Ileal $NH4^+$ secretion rate (Jamm) is 2.5 times greater than in the rectum and is stimulated by cAMP (Lechleitner, 1988; Peach and Phillips, 1991). As previously mentioned, luminal

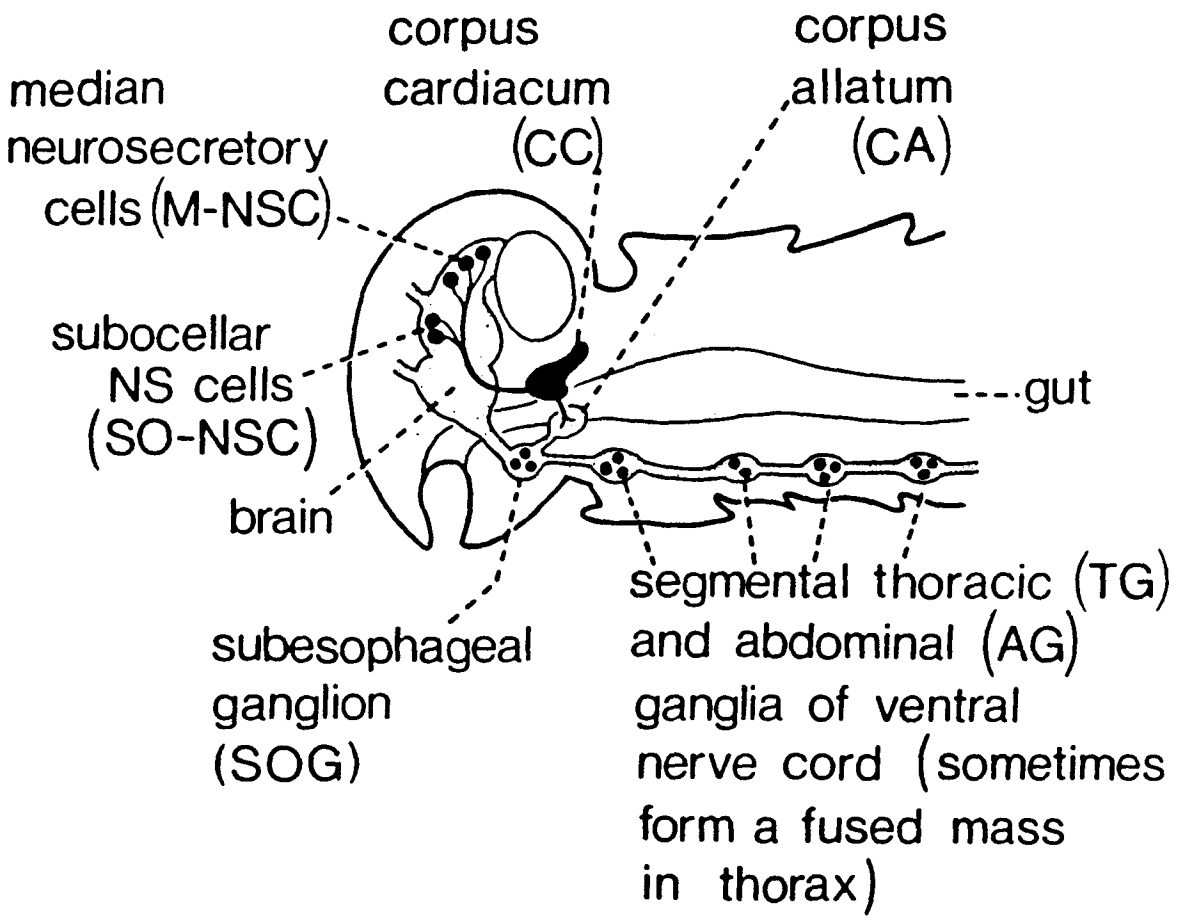


Fig. 1.4. Side view of an insect showing the neuroendocrine organs implicated in the control of fluid balance and excretion (from Phillips 1982).

proline is the major respiratory substrate and ammonia source in the rectum, while the ileum uses luminal alanine, asparagine, glutamine, serine and bilateral proline (Peach and Phillips, 1991). There is negligible proline transport in the ileum (Lechleitner and Phillips, 1989).

Endocrine control of excretion: Malpighian tubule secretion

Malpighian tubule secretion and hindgut reabsorption are both under endocrine control. The insect neuroendocrine organs thought to be involved in the control of fluid balance and excretion are shown in Fig.1.4. The first demonstration of a diuretic factor that stimulated Malpighian tubule secretion was in the blood feeder, *Rhodnius prolixus* (Maddrell 1963,1964a-b). After feeding, the body volume of *Rhodnius* increases ten-fold, necessitating the excretion of excess fluid. Maddrell showed that feeding triggered the release of a proteinacious factor from the thoracic-abdominal nerve mass that immediately stimulated the secretion of large amounts of urine. Various methods have been used to study Malpighian tubule (MT) secretion: Ramsay's (1954) *in vitro* fluid secretion method with isolated tubules and its variants; measuring changes in MT transepithelial voltage (Maddrell and Klunsuwan, 1973; Williams and Bayenbach, 1983,1984); measuring changes in tissue cAMP levels (Morgan and Mordue, 1985; Kay et al., 1991a); and by measuring water loss from the whole insect (Kataoka et al., 1989). Diuretic factors have been reported for many types of insects, and these factors appear to be present in various locations throughout the neuroendocrine system (reviewed by Spring, 1990; Raabe, 1991). However, only a few of these factors have been purified and their amino acid sequence determined (see Table 1.1.).

High performance liquid chromatography (HPLC) has been used extensively to isolate insect diuretic and antidiuretic factors. HPLC can rapidly separate small amounts of material with high resolution and recovery, and is far superior to older chromatographic techniques that were slower, less sensitive and required more material (Schram, 1980).

Table 1.1. A list of fully and partially characterized insect diuretic peptides

SPECIES	PEPTIDE	SOURCE	DESCRIPTION
Locusta migratoria (migratory locust)	DP-1	CC	only partial amino acid sequence (Morgan et al., 1987)
	AVP-LDH	SOG	18 aa anti parallel homodimer, 67% and 78% homology with AVP and AVT respectively (Proux et al., 1987)
	Locusta-DP	head	46 aa amidated peptide, chemically similar to DP-1, 48% and 49% homology with *Acheta*-DP and *Manduca*-DH respectively (Kay et al., 1991b)
Manduca sexta (tobacco hornworm)	*Manduca*-DH	head	41 aa amidated peptide (Kataoka et al., 1989)
Acheta domesticus (house cricket)	AP-1	CC	18 aa peptide (Coast et al., 1990)
	Acheta-DP	head	46 aa amidated peptide, 41% homology to *Manduca*-DH (Kay et al, 1991a)

Abbreviations:

aa, amino acid; CC, corpus cardiacum; SOG, suboesophageal ganglion; DP/DH, diuretic peptide/diuretic hormone; AP, *Acheta* peptide; AVP, arginine vasopressin; AVT, arginine vasotocin; AVP-LDH, arginine vasopressin-like diuretic hormone.

Improvements to mass spectrometry, peptide sequence analysis, and the development of automated gas-sequencers have greatly increased the speed of peptide sequencing, and have reduced the amount of material required by up to 1000 fold (Holman et al., 1991).

Arginine vasopressin-like diuretic hormone (AVP-LDH) was the first putative insect diuretic factor to be fully characterized, and was isolated using HPLC and immunological techniques (Proux et al., 1987). Although AVP-LDH closely resembles mammalian AVP, its structure is unlike other purified diuretic factors. Many of the recently characterized diuretic peptides such as *Locusta*-DP, *Manduca*-DH, *Acheta*-DP (see table 1.1.), *Leucophaea*-DP and *Periplaneta*-DP (Coast, personal communication), share homologous regions in their amino acid sequences. In addition, this family of diuretic peptides has sequence homology with some vertebrate hormones, namely corticotropin releasing factor (CRF), sauvagine and suckerfish urotensin 1 (Coast et al.,1992). Coast et al. (1992) demonstrated that CRF, sauvagine and urotensin 1 all increased intracellular cAMP levels in *Acheta* Malpighian tubules, mimicking the action of *Acheta*-DP. They have proposed that these insect diuretic peptides belong to a superfamily of peptides that includes CRF, sauvagine and urotensin 1. The proposed members of this superfamily are known to be involved in the regulation of fluid and solute transport, and act by elevating cAMP and perhaps by increasing intracellular Ca^{2+} levels.

Secretion by insect Malpighian tubules is also stimulated by members of a family of myotropic peptides (myokinins), that have been isolated from *Leucophaea maderae* (Leucokinins) and *Acheta domesticus* (Achetakinins) (Hayes et al., 1989; Coast et al., 1990). Myokinins are known to stimulate contractile activity in the insect hindgut (Holman et al., 1991). These myokinins all possess a highly conserved pentapeptide carboxy terminal sequence, which is responsible for activity (Holman et al., 1990; Nachman et al., 1990). The myokinins are thought to work on Malpighian tubules via a cAMP independent mechanism, perhaps by elevating intracellular Ca^{2+}.

Table 1.2. Insects shown to possess antidiuretic factors that increase rectal fluid reabsorption.

SPECIES	SOURCE	REFERENCE
Carausius morosus (stick insect)	brain	Vietinghoff (1966)
Acheta domesticus (house cricket)	GCC CC	De Bessé and Cazal (1968) Albarwani (1988), Spring et al. (1988a)
Locusta migratoria (migratory locust)	CC PO NCC, GCC	Cazal and Girardie (1968) De Bessé and Cazal (1968) Hérault et al. (1985)
Schistocerca gregaria (desert locust)	GCC GCC, NCC AVG 5	Mordue (1969, 1970, 1972) Proux et al. (1984) Lechleitner and Phillips (1989)
Periplaneta americana (American cockroach)	brain, MTG, CA TAG	Wall (1967) Wall (1967), Goldbard et al. (1970)
Blaberus craniifer (cockroach)	CC	Bourême et al. (1989)
Leucophaea maderae (cockroach)	CC	Bourême et al. (1989)

Abbreviations:

GCC/NCC, glandular/nervous lobes of corpus cardiacum; CA, corpus allatum; PO, perisympathetic organs; AVG 5, fifth abdominal ventral ganglion; MTG, meta-thoracic ganglion; TAG, thoracic abdominal ganglion.

Spring et al. (1988b) isolated an antidiuretic hormone (ADH) from *Acheta domesticus* CC that inhibited MT fluid secretion by 70%, but had no effect upon the hindgut. It was also shown that the release of this ADH into the hemolymph was triggered by dehydration. There is indirect evidence that this ADH may act by elevating intracellular Ca^{2+}, since the divalent ionophore A23187 also reduced tubule secretion by 85% (Spring and Clark, 1990). The structure of this ADH has not yet been determined.

Endocrine control of excretion: Hindgut reabsorption

Vietinghoff (1966) was the first to show that insect hindgut reabsorption is under endocrine control. There have been several subsequent reports of antidiuretic (AD) and diuretic (D) factors that control hindgut fluid reabsorption (J_V); see Table 1.2. for AD factors (reviewed by Phillips, 1982,1983; Phillips et al., 1986; Spring, 1990). However, the validity of some of these earlier reports have been questioned (Phillips et al., 1986). Most of the earlier studies were performed on uncharacterized *in vitro* hindgut preparations during an initial transitory absorptive period, when the hindgut tissue was adjusting to the new osmotic environment (causing tissue swelling) and was possibly still responding to factors that were present *in vivo*. In addition, the earlier studies often used salines that were lacking essential metabolic substrates (e.g. proline for the locust rectum) and were not sufficiently oxygenated (see Phillips et al., 1986).

Phillips et al. (1980) partially purified a neuropeptide from *Schistocerca gregaria* CC that stimulated electrogenic chloride transport and named it chloride transport stimulating hormone (CTSH). Proux et al. (1984) later showed that CTSH also stimulates rectal fluid reabsorption. CTSH activity is present in both the glandular (GCC) and storage lobes (NCC) of the CC but 80% of the activity is found the NCC. It appears that CTSH is released into the hemolymph, presumably upon feeding (Spring et al., 1978; Spring and Phillips, 1980a,b,c; Proux et al., 1984; Hanrahan and Phillips, 1985). CTSH is thought to act by elevating intracellular cAMP levels, because exogenous cAMP stimulates both

rectal Cl^- transport and water reabsorption (Phillips et al., 1980; Proux et al., 1984)). In addition, Chamberlin and Phillips (1988) found that rectal tissue levels of cAMP increase between five and ten minutes after exposure to CC extracts. It has also been shown that Cl^- transport and intracellular cAMP levels increased when pharmacological agents (theophylline and forskolin) known to elevate cAMP levels in other systems, were applied to isolated recta (Spring et al., 1978; Hanrahan et al., 1985; Chamberlin and Phillips, 1988).

Hérault et al. (1985) have isolated two AD factors from *Locusta migratoria* that increase rectal J_V over a five hour period. One factor is reported to be localized in the GCC while the other factor is exclusive to the NCC. Hérault and Proux (1987) have shown that the GCC factor elevates intracellular cAMP levels over a similar time course as CTSH. The GCC factor is an unstable peptide (Hérault et al., 1985) and has not yet been purified.

Girardie et al. (1987b) have purified and characterized an AD factor from *Locusta* NCC, which they have named neuroparsins (Nps), and have shown that it is produced by the A1 type protocerebral median neurosecretory cells (M-NSC; Girardie et al., 1987a). As well as increasing rectal J_V, Nps inhibits the effects of juvenile hormone (Girardie et al., 1987b) and increases hemolymph trehalose and lipid levels (Moreau et al., 1988). Girardie et al. (1989,1990) used anion exchange chromatography and HPLC to isolate two forms of neuroparsins, neuroparsin A (NpA) and neuroparsin B (NpB). Both forms have molecular weights of ~14 KDa, and are composed of two polypeptide chains (~7 KDa) linked by disulfide bonds. The NpA monomer has 83 amino acid residues while the NpB monomer consists of 78 residues (i.e. identical to residues 6-83 of NpA). Since the NpB monomer sequence is identical to a major part of NpA, Girardie et al. (1989) have postulated that NpB is a post-translational product of NpA. Recently, Hietter et al. (1991) used HPLC to characterize three neuroparsin-like 8-9 KDa monomeric peptides from *Locusta* CC. Two of the peptides correspond to NpA and NpB, while the third peptide

possesses 81 residues (3-83 of NpA). All three peptides have been shown by mass spectrometry to exist as monomers containing six disulfide bridges. Hietter et al. (1991) also noticed that part of the Nps amino acid sequence is almost identical to the partially sequenced 4.5 KDa, 46 residue *Locusta* Corpus Cardiacum peptide (LCCP) purified by Mordue et al. (1985). Hietter et al. (1991) used a different approach for extraction, isolation and characterization of these peptides to that used by Girardie et al. (1989,1990), which may account for their differing results. Lagueux et al. (1992) have cloned a *Locusta* cDNA that encodes NpA. They have proposed that NpA is processed from a 107 residue precursor in two steps; cleavage of a 22 residue signal peptide and removal of a Glu-Arg dipeptide. It has also been shown that transcripts encoding NpA occur in both adult and larval brains.

Neuroparsin-like peptides have also been identified in the cockroaches *Blaberus craniifer* and *Leucophaea maderae* (Bourême et al., 1989). Both of these peptides increase *Locusta* rectal J_V, and NpA stimulated rectal J_V in both cockroaches, suggesting that these peptides are related and may belong to an intraphyletic neuroprotein family. Immunological techniques have also been used to show that neuroparsin-like factors exist in brain neurosecretory cells of three other insect orders (Isoptera, Odonata and Dictyoptera; Tamarelle and Girardie, 1989). In addition, NpA has 37% and 27% amino acid sequence homology with *Manduca sexta* and *Bombyx mori* eclosion hormones respectively (Girardie et al., 1990). Furthermore, NpA shows some sequence identity (~30%) with some vertebrate hormones, such as hypothalamic pituitary hormones and pre-proinsulin (Girardie et al., 1990).

There has also been interest in determining the second messenger systems whereby Nps stimulates rectal J_V. Fournier and Dubar (1989) have shown that Nps has no effect upon intracellular cAMP levels, while slightly increasing cGMP levels. They concluded that both cAMP and cGMP are not second messengers of Nps. They suggest that the slight increase in cGMP levels is a result of Nps-stimulated elevation of cGMP in a different cell

type, such as rectal muscle cells, resulting in a small diuretic effect! Moreover, Fournier (1990b) has used a pharmacological approach to provide indirect evidence that Nps induces phosphoinositide turnover in *Locusta* rectal cells. Diacylglycerol (DAG) and inositol trisphosphate ($InsP_3$), products of phosphotidylinositol bis-phosphate ($PtdInsP_2$) breakdown, have both been implicated as possible second messengers of Nps. DAG is known to stimulate protein kinase C (PKC; Nishizuka, 1984), while $InsP_3$ triggers the release of intracellular calcium from internal stores and the entry of extracellular Ca^{2+} (Berridge and Irvine, 1989). Fournier (1991) has since directly shown that Nps stimulates phospholipase C (PLC) mediated hydrolysis, resulting in an increase in intracellular $InsP_3$ levels.

Audsley et al. (1992) have used reverse phase (RP)-HPLC to isolate and purify a 7.7 KDa factor from *Schistocera gregaria* CC, called *Schistocerca gregaria* ion transport peptide (ScgITP). At the moment only 50 of the estimated 65 amino acid residues of ScgITP have been identified (Audsley, 1991). ScgITP is completely different from any other known vertebrate or insect hormones, but its first 34 residues have approximately 50% sequence identity with crustacean hyperglycemic and moult inhibitory hormones (Audsley et al., 1992). ScgITP has quantitatively similar effects upon ileal transport as does crude CC homogenates: increasing ileal J_V; stimulating Cl^-, K^+ and Na^+ reabsorption; and inhibiting H^+ secretion (Audsley et al., 1992). ScgITP also sub-maximally stimulates rectal I_{SC} at very high doses, but has no effect upon rectal J_V or K^+ permeability. This evidence indicates that there are at least two unique factors (CTSH and ScgITP) that control *Schistocerca* hindgut solute and fluid reabsorption. ScgITP is thought to act via cAMP, since exogenous cAMP mimicks most of the effects of ScgITP and of crude CC extracts. However, ScgITP mediated inhibition of ileal H^+ secretion is not mimicked by cAMP, suggesting that another second messenger may be involved.

Objectives of this study

The purpose of this study was to investigate what second messengers are involved in controlling rectal fluid reabsorption (J_V) and Cl^- transport in the desert locust (*Schistocerca gregaria*). To determine which second messengers are important in controlling these rectal transport processes, various agents known to block or activate specific signal transduction pathways were added to everted rectal sac and short-circuited rectal flat-sheet bioassays. Chapter 2 examines and reviews the roles of the adenylate and guanylate cyclase pathways in rectal reabsorption. In chapter 3, the following questions are addressed: 1) Is extracellular Ca^{2+} necessary for rectal J_V and ion transport? 2) Does modulating cytosolic Ca^{2+} levels have any effect upon rectal transport properties?. Chapter 4 studies the role of protein kinase C (PKC) and the involvement of the phosphatidylinositol (PI) cycle in controlling rectal transport. In addition, *Locusta* rectal sacs and short-circuited rectal flat sheets were used to investigate the role of the PI cycle in *Locusta* recta. Finally, the effects of *Locusta* Nps, which is thought to act by stimulating the PI cycle in *Locusta* (Fournier, 1990b,1991), was tested on *Schistocerca* rectal J_V and I_{SC} to see if there was any cross-reactivity.

CHAPTER 2: EFFECTS OF NCC, cAMP AND RELATED COMPOUNDS ON RECTAL FLUID AND ION TRANSPORT

INTRODUCTION

The cyclic nucleotides cAMP and cGMP are thought to be the second messengers of many hormonally controlled processes in both vertebrate and insect cells (reviewed by Bodnaryk, 1983). In insects, cAMP has an important role in controlling Malpighian tubule secretion and hindgut reabsorption (Bodnaryk, 1983; Raabe, 1989; Spring, 1990). Exogenous cAMP and pharmacological agents known to elevate intracellular cAMP (e.g. theophylline and forskolin), have been shown to increase Malpighian tubule fluid secretion in various insect species (reviewed by Coast et al., 1991). These agents also stimulate fluid and ion reabsorption *in vitro* by *S. gregaria* rectum (Spring and Phillips, 1979; Hanrahan, 1982) and ileum (Audsley and Phillips, 1990); as well as stimulating fluid reabsorption by the recta of *Locusta migratoria* (Hérault and Proux, 1987), *Leucophaea maderae* and *Blaberus craniifer* (Fournier, 1990a). Measurements of increases in intracellular cAMP in response to stimulants, have provided direct evidence that cAMP is a second messenger of insect excretory processes. Elevation of intracellular cAMP is associated with the stimulation of Malpighian tubule fluid secretion by CRF-related diuretic peptides (Coast et al., 1992). In addition, extracts of CC have been shown to simultaneously elevate intracellular cAMP levels and stimulate fluid and ion reabsorption in locust and cockroach recta (Spring and Phillips, 1979; Chamberlin and Phillips, 1988; Hérault and Proux, 1987; Fournier, 1990a). Chamberlin and Phillips (1988) have also shown that locust rectal cGMP levels increase to a maximum value after 60 minutes of exposure to CC extract. This increase in rectal cGMP was much slower than the increase in cAMP that peaked between five and ten minutes after the addition of CC. Furthermore, this slow increase in

rectal intracellular cGMP corresponds quite well with the slow and gradual stimulatory effect of exogenous cGMP upon rectal Cl^- transport (Chamberlin and Phillips, 1988).

The elevation of cAMP is brought about by hormonal activation of the adenylate cyclase signal transduction pathway (Fig. 2.1.). Signal transduction mechanisms translate and amplify external signals, enabling cells to respond quickly and precisely to specific stimulants/inhibitors at low concentrations. The first step in the Adenylate cyclase pathway is the binding of a neuropeptide to its target receptor. This hormone-receptor complex then catalyzes the binding of GTP to the α subunit of a stimulatory G protein (G_S). The GTP-$G_{S\alpha}$ complex dissociates from the remaining β and γ G_S subunits and binds to adenylate cyclase, which is also located on the plasma membrane (reviewed by Stryer, 1988). A single hormone-receptor complex catalyzes the formation of many GTP-$G_{S\alpha}$ molecules, thus amplifying the original signal. The activated adenylate cyclase converts ATP to cAMP, resulting in another amplification of the signal. The increased amounts of cAMP then stimulate cAMP specific protein kinases (PKA). These activated protein kinases can now phosphorylate target proteins (e.g. enzymes, ion channels), activating or inhibiting specific cellular processes (Cohen, 1989). This protein modification results in an additional amplification of the original, external signal. In the case of the locust hindgut, it is likely that proteins involved in Cl^- transport are modified by PKA, since exogenous cAMP can stimulate Cl^--dependent I_{SC}.

Increased cGMP levels are brought about by the activation of the guanylate cyclase signal transduction pathway. The guanylate cyclase pathway is comprised of similar functional components as the adenylate cyclase pathway. The major differences between the two pathways are that cGMP levels in animal cells are usually more than ten times lower than cAMP levels (Bodnaryk, 1983), and there are multiple isozymes of guanylate cyclase that are found in both the cytosol and plasma membrane (Shultz et al., 1989).

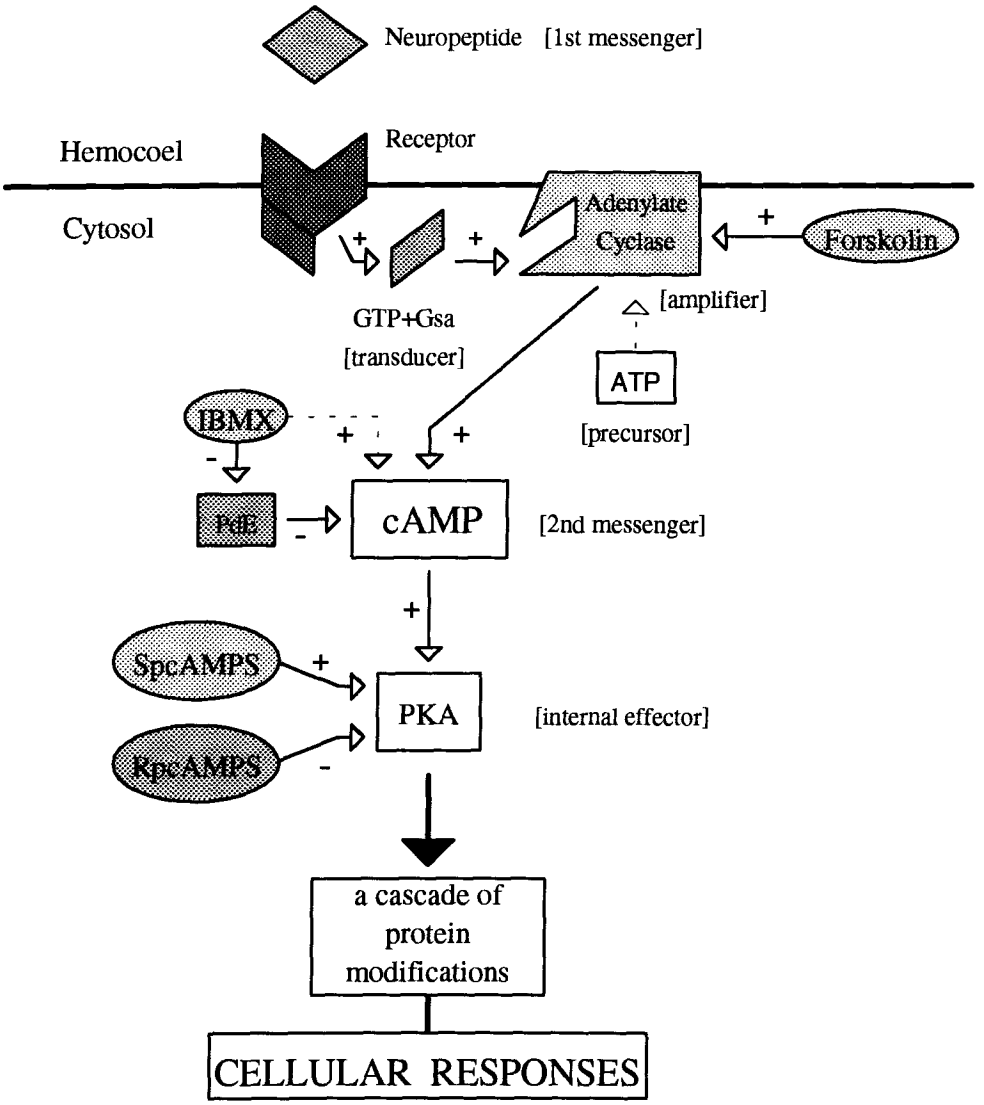


Fig. 2.1. Diagram of the adenylate cyclase signal transduction pathway. Shaded circles indicate agents that are added exogenously to affect the pathway.

In this chapter, the roles of the adenylate and guanylate cyclase signal transduction pathways in the control of rectal J_V and I_{SC} were investigated. Initially, the effects of NCC homogenate, exogenous cAMP and dibutyrl cAMP upon rectal J_V and I_{SC} were tested and compared. Then the involvement of protein kinase A was tested by adding the diastereo-isomers SpcAMPS and RpcAMPS, which specifically stimulate and inhibit PKA, respectively (Rothermel and Parker-Botelho, 1988). The effects of ATP and adenosine were tested to ensure that cAMP was not acting by stimulating purinergic cell surface receptors (Olsson and Pearson, 1990). The effect of inhibiting cAMP-dependant phosphodiesterase (PdE) with IBMX was also examined. The stimulatory actions of micromolar amounts of forskolin, which directly activates adenylate cyclase, on rectal I_{SC} was previously reported (Spring et al., 1978; Hanrahan and Phillips, 1985). Finally, the effects of cGMP upon rectal J_V and Isc were tested and compared to those of cAMP.

MATERIALS AND METHODS

Insects

Adult female *Schistocerca gregaria*, 2-4 weeks past their final molt were used for all experiments. The locusts were maintained on a 12 h light: 12 h dark cycle at 28°C and 60% relative humidity, under crowded conditions. Animals were fed a mixture of dried grass, bran, powdered milk and yeast; with fresh lettuce supplied daily. The nervous lobes of the corpus cardiacum (NCC) were excised from adult male and female locusts 4-6 weeks past their final molt.

Chemicals

Most chemicals, including those used to make the complex saline, were supplied by the Sigma Chemical Company (St. Louis MO, USA). SpcAMPS and RpcAMPS were

supplied by BIOLOG Life Science Institute (La Jolla, CA, USA). Cyclic AMP, DbcAMP, SpcAMPS, RpcAMPS, ATP and cGMP were all dissolved in complex saline. IBMX and adenosine were initially dissolved in DMSO and these stock solutions were then diluted with complex saline (final DMSO concentration $\leq 1\%$).

Salines

The complex bathing saline used was based on the measured composition of locust hemolymph (Hanrahan et al. 1984) and contained (mM): 100 NaCl, 5 K_2SO_4, 10.9 $MgSO_4$, 10 $NaHCO_3$, 5 $CaCl_2$, 10 glucose, 100 sucrose, 2.9 alanine, 1.3 asparagine, 1.0 arginine, 5 glutamine, 11.4 glycine, 1.4 histidine, 1.4 lysine, 13.1 proline, 6.5 serine, 1.0 tyrosine, 1.8 valine. The saline was adjusted to pH 7.1 and continuously bubbled with a 95% O_2 : 5% CO_2 gas mixture that ensured rapid mixing. Complex saline has been shown to sustain transport activities of locust hindgut at near constant values for many hours (>8h.; reviewed by Phillips et al.,1986).

Flat sheet rectal bioassay

Electrogenic ion transport was measured by mounting recta between two Ussing chambers, as described by Hanrahan et al. (1984) and Audsley (1990). Recta were removed from animals, cut longitudinally to produce a flat sheet and immediately secured on tungsten pins over a $0.196cm^2$ opening using a neoprene O-ring to form a seal (Fig. 2.2.). The chambers were held together with elastic bands and placed in a vice-like frame. Each chamber contained 2mL of complex saline that was continuously stirred by vigorous bubbling with 95% O_2 : 5% CO_2 gas mixture, at $23^{\circ}C \pm 2^{\circ}C$. Transepithelial potential (V_t) was measured by placing 3M KCl agar bridges (size P.E. 90) near the tissue through ports on the side of the chambers with leads connected to a high impedance differential amplifier (4253, Teledyne Philbrick, Dedham, Mass. USA) which continually monitored

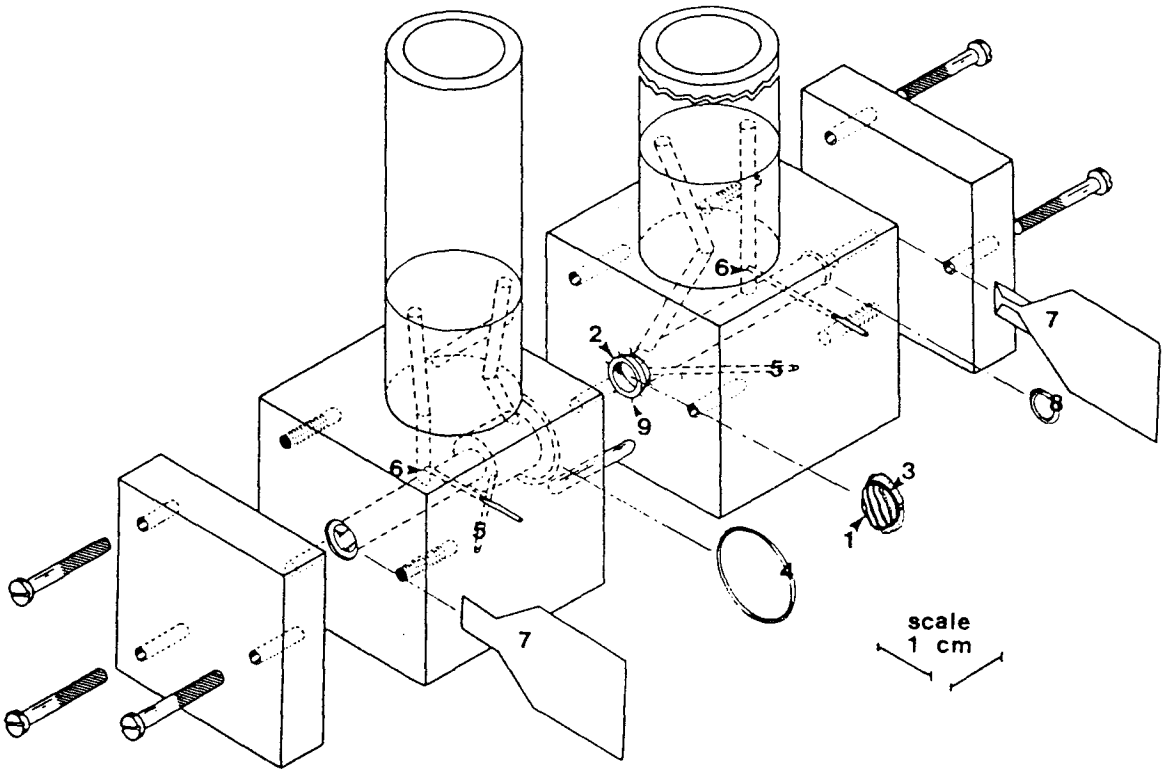


Fig. 2.2. Standard Ussing chamber assembly used to measure Cl^- dependent rectal short-circuit current (I_{SC}). 1) rectal flat-sheet preparation, 2) Plexiglas collar that rectum is mounted over, 3) Neoprene O-ring for securing rectal attachment to collar, 4) neoprene chamber seal, 5) agar bridge port for measurement of transepithelial potential (V_t), 6) gas islet for saline aeration and mixing, 7) current sending electrodes, 8) rear chamber seal, 9) tungsten pins for attachment of rectum to collar (taken from Hanrahan et al., 1984).

V_t. Short-circuit current (I_{SC}), a direct and continuous measure of electrogenic ion transport, was measured by maintaining V_t , at 0 mV by a second amplifier (725, National Semiconductor Corp., Santa Clara, CA. USA) which passed current (I_{SC}) between two Ag-AgCl electrodes at either end of the chamber. A third amplifier (308, Fairchild, Mountain View CA. USA) was then used to measure I_{SC}. Williams et al. (1978) reported that locust rectal I_{SC} is Cl^--dependent and is therefore a direct measure of electrogenic Cl^- transport. Flux studies by Hanrahan (1982) have confirmed this report. Both I_{SC} and V_t were monitored by connecting the respective amplifier to an analog-to-digital (A/D) converter on a IBM® XT bus card (supplied by Brynhyfryd Consulting, Vancouver B.C.). A microcomputer logging program (supplied by David M. Jones Department of Oceanography, U.B.C.) collected and displayed the outputs from these amplifiers and all signals were recorded at one second or greater intervals (see Jones et al., 1991). The card and program were used with an IBM® compatible personal computer equipped with a CGA video output and 640K RAM. Corrections were made for series resistance of the external saline and asymmetries between voltage-sensing electrodes as described by Hanrahan et al. (1984). While the tissue was under short-circuit conditions, V_t was monitored at intervals by stopping the voltage clamp for 30-60 seconds and using an alternative circuit to measure voltage difference. Transrectal resistance (R_t) was calculated from I_{SC} and V_t using Ohm's law.

The I_{SC} and V_t decline rapidly over the first 1-2 hours after excision of the recta, but thereafter these variables of ion transport activity decline very little (if at all) over the next several hours (i.e. steady-state phase: Williams et al. 1978; Spring and Phillips, 1980a). After the rectal tissue had reached steady-state, fresh saline was added to the chambers and the tissue was exposed to various substances to study their effect on rectal I_{SC}, V_t and R_t.

Everted rectal sac bioassay

Everted rectal sacs were prepared as described by Goh and Phillips (1978) and Hanrahan et al. (1984). A 3 cm length of PE 90 tubing with a slightly flared end was inserted through the anus of the locust until its flared end reached the anterior boundary of the rectum. The hindgut was raised slightly and the anterior boundary of the rectum was ligated with surgical silk on to the flared end of the tubing. The colon and connecting trachea were cut away and the rectum was slowly everted by pulling the PE tubing through the anus. The everted rectum was then cut from the animal and rinsed with 1 mL of complex saline to remove any hemolymph or fecal material and the posterior was ligated. Any remaining internal fluid was withdrawn completely with a 100 µL. Hamilton syringe and the empty sac was weighed to an accuracy of ± 0.1 mg on an August Sauter balance. Rectal sacs were filled hourly with 10µL of fresh saline, incubated at 30°C in 25 mL of complex saline, and bubbled with a 95% O_2: 5% CO_2 gas mixture. The weight gain and the tissue volume changes were determined at hourly intervals by weighing sacs before and after removal of internal (Hemocoel side) fluid. The true rate of transepithelial fluid movement (J_V) was determined by correcting for tissue volume changes.

The rectal sacs were allowed to equilibrate for two hours and putative stimulators of fluid transport were added to the hemocoel (H) side of the sac at the start of the third hour. The change in rate of rectal fluid reabsorption (ΔJ_V) was calculated by subtracting the J_V for the second hour from the third hour value. These ΔJ_V values were then compared to the average control values for unstimulated sacs over the same time period.

Preparation of NCC extracts

NCC were excised from adult locusts and immediately frozen on dry ice and stored at -70°C. NCC were then homogenized in complex saline using a Tissue Tearer Homogenizer (Bartlesville OK, USA) for 2-4 minutes. The homogenate was then centrifuged at 12,000g

and 4°C for 10 minutes. The pellet and the floating lipid layer were discarded and the supernatant was kept at -20°C until use.

Statistics

The significance of differences between two means was determined by paired and independent t-tests. Analysis of variance (ANOVA) with the Tukey test was employed to determine differences between multiple means.

RESULTS

Effect of NCC and cAMP

The effects of NCC and cAMP upon rectal I_{sc}, V_t, and R_t are shown in Table 2.1 and Fig. 2.3. Large increases in I_{sc} occcured within 2 minutes of addition of 5 mM cAMP, and within 5 minutes of adding 2NCC. (Fig. 2.3). Cyclic AMP maintained I_{sc} at a constant high level for over an hour after initial stimulation, and NCC caused I_{sc} to peak after 30 minutes then decline slowly. NCC and cAMP caused similar (not significantly different) maximal increases in I_{sc} and V_t, and both significantly reduced R_t (Table 2.1). These electrical variables for steady-state unstimulated recta (controls) and the values after NCC stimulation were similar to those reported by Hanrahan and Phillips (1985), using extracts of whole corpora cardiacum (CC).

Table 2.1. Effect of NCC and cAMP upon rectal electrical variables

	I_{sc} ($\mu eq.cm^{-2}.h^{-1}$)	V_t (mV)	R_t ($\Omega.cm^2$)	n
C	1.44 ± 0.22a	8.48 ± 0.80a	265.41 ± 48.82a	8
+ 2NCC	7.27 ± 0.92b	20.89 ± 1.77b	120.88 ± 18.85bc	
C	1.45 ± 0.24a	3.98 ± 0.47a	118.22 ± 18.66b	10
+ 5mM cAMP	9.30 ± 1.01b	19.18 ± 2.68b	79.27 ± 7.57c	

Values sharing the same letter are not significantly different from each other (Tukey test, $p > 0.05$).

V_t values are lumen side positive

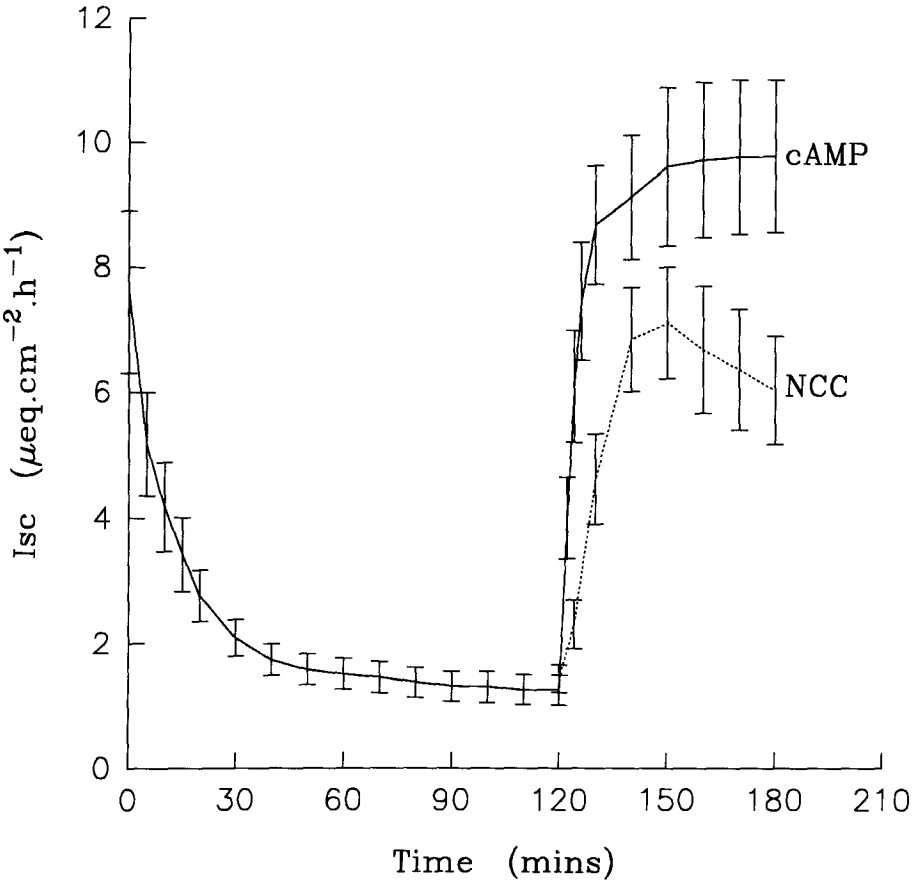


Fig. 2.3. Effect of NCC and cAMP on rectal I_{SC}: 5mM cAMP and 2 NCC equivalents were added to the hemocoel side of recta that had been allowed to equilibrate for 2h. Mean ± S.E. (n=6-8).

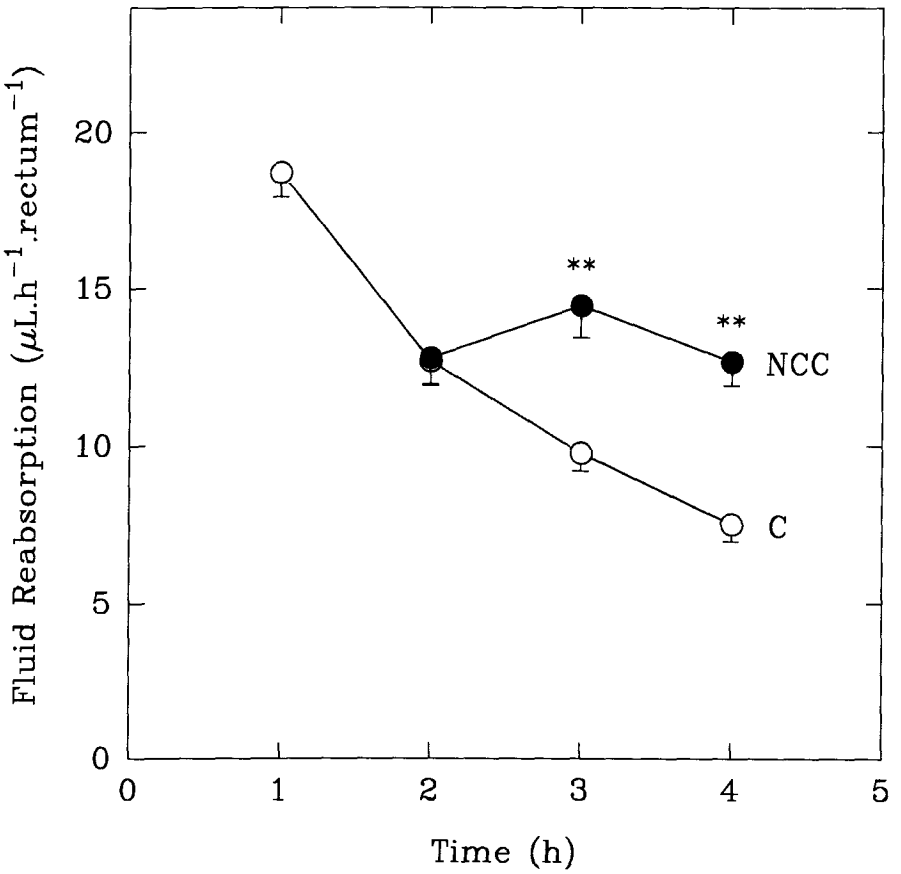


Fig. 2.4. Fluid reabsorption by everted rectal sacs with time. Complex saline controls hours 1-4 (C), stimulation with 2 NCC equivalents during hours 3+4 (NCC). Mean ± S.E. (n=8-11). ** values are significantly different from same hour controls (C) (independent t-test, $p < 0.01$).

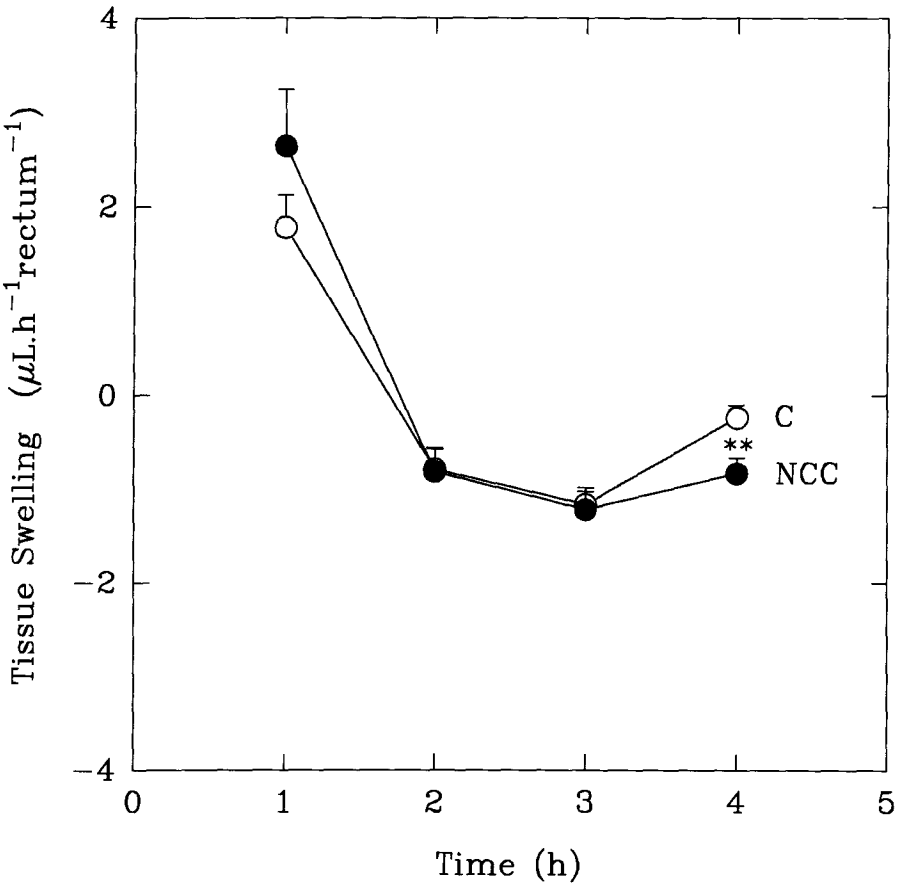


Fig. 2.5. Tissue swelling by the everted rectal sacs from Fig. 2.4. Complex saline controls hours 1-4 (C), stimulation with 2 NCC equivalents during hours 3+4 (NCC). Mean ± S.E. (n=8-11). ** value is significantly different from same hour control (C), (independent t-test, $p < 0.01$).

Unstimulated rectal sacs had a mean J_V of 18.7 ± 0.8 $\mu L.h^{-1}$ during the first hour after excision (Fig. 2.4). J_V fell steadily to 12.7 ± 0.7 $\mu L.h^{-1}$ during the second hour, but decreased more slowly during the third and fourth hours. Although steady J_V was not observed, these J_V values are much higher than those reported in earlier studies (Proux et al., 1984). Tissue swelling of 1.8 ± 0.3 $\mu L.h^{-1}$ was observed during the first hour, but tissue volume fell slowly over the next three hours to near the initial value (Fig. 2.5). The addition of 2 NCC to the hemocoel side of rectal sacs during the third hour increased J_V by 4.4 ± 0.5 $\mu L.h^{-1}$ compared to the control values for the same period (Fig. 2.4 and 2.6). This increase in J_V was similar to maximal values obtained in previous experiments (Proux et al., 1984). When rectal sacs were also exposed to NCC during the fourth hour, J_V remained elevated compared to the control value (Fig. 2.4) and there was a small but significant reduction in tissue swelling compared to control values (Fig. 2.5). DiButyryl cAMP (DBcAMP, 1 mM) and cAMP (5 mM) were also shown to increase J_V during the third hour to a similar extent as NCC (Fig. 2.6). DBcAMP and cAMP caused no differences in tissue swelling compared to control values.

Effect of chemicals acting on the cAMP mediated pathway

SpcAMPS and RpcAMPS are respectively specific stimulators and inhibitors of protein kinase A (PKA; see Fig. 2.1). SpcAMPS (5 mM) maximally stimulated J_V, while 1-10 mM RpcAMPS had no effect upon J_V when added during the third hour (Fig. 2.7). However when rectal sacs were pre-incubated with 10 mM RpcAMPS during the second hour and then stimulated with NCC on the third hour, there was a significant reduction in ΔJ_V compared to the NCC control (1.8 ± 1.0 $\mu L.h^{-1}$ compared to 4.4 ± .05 $\mu L.h^{-1}$; Fig. 2.8).

Although the stimulatory effect of cAMP upon J_V and I_{SC} had already been demonstrated, it was also necessary to show that cAMP was acting intracellularly and not via cell surface purigenic receptors. The involvement of purigenic surface receptors in

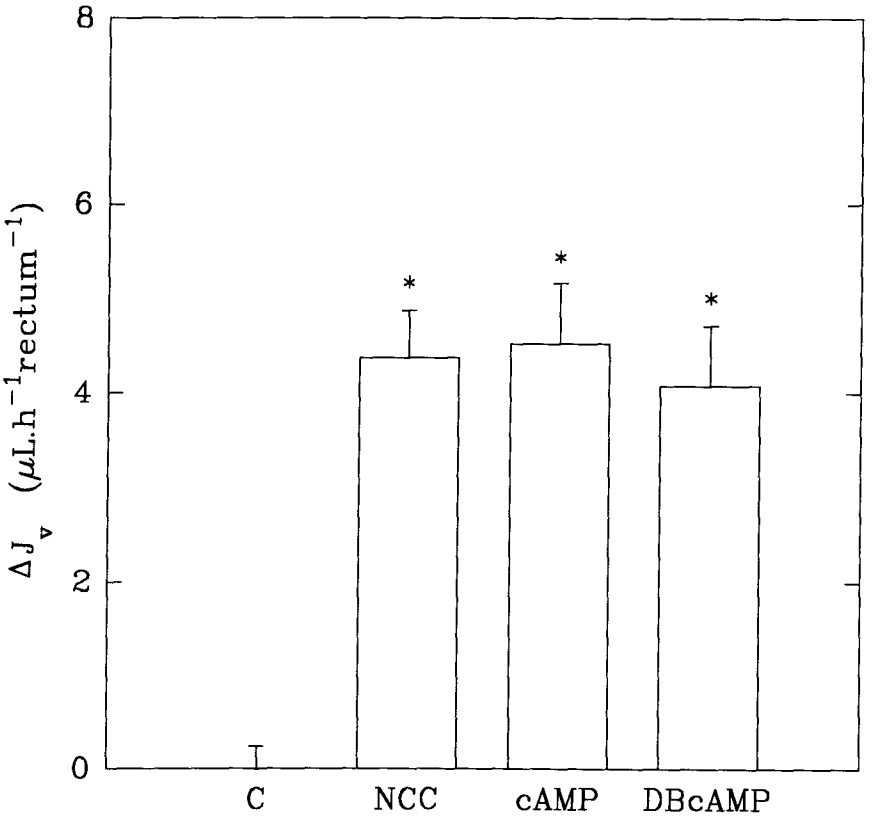


Fig. 2.6. Effect of 2 NCC, 5 mM cAMP and 1 mM DBcAMP upon rectal J_V. Mean ± S.E. (n=6-11). * values are significantly different from control (C), (Tukey test, $p< 0.05$).

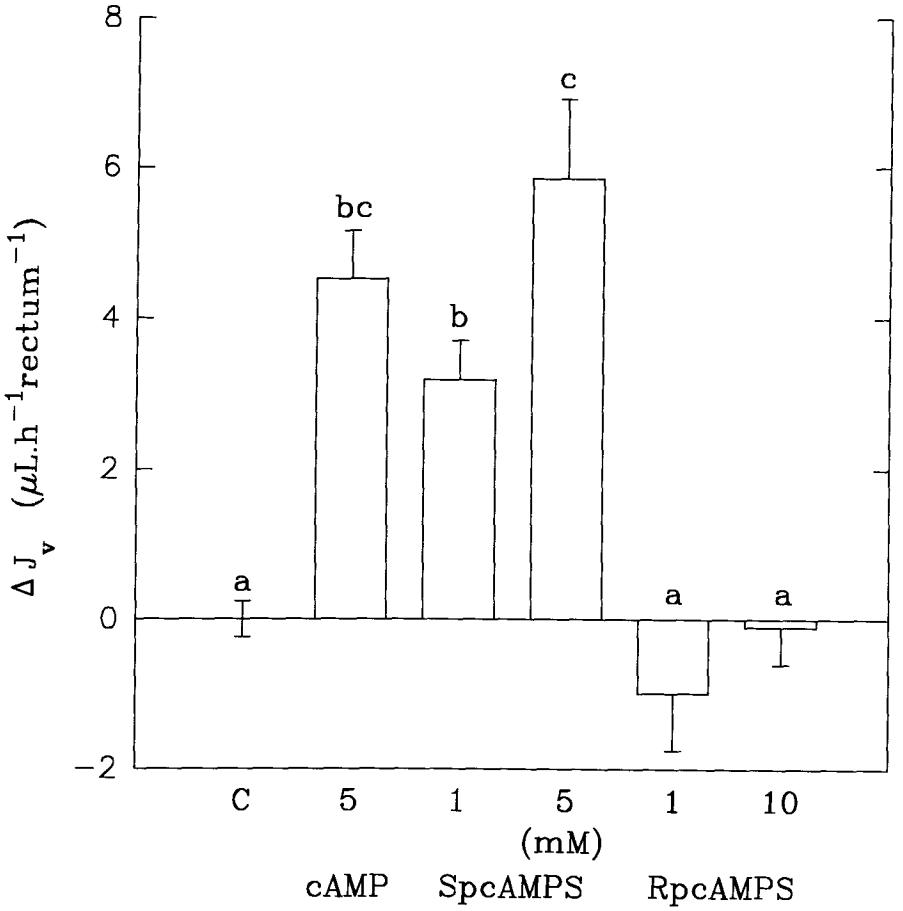


Fig. 2.7. Effect of cAMP, SpcAMPS and RpcAMPS upon rectal J_V. Mean ± S.E. (n=6-8). Values sharing the same letter are not significantly different from each other (Tukey test, $p > 0.05$).

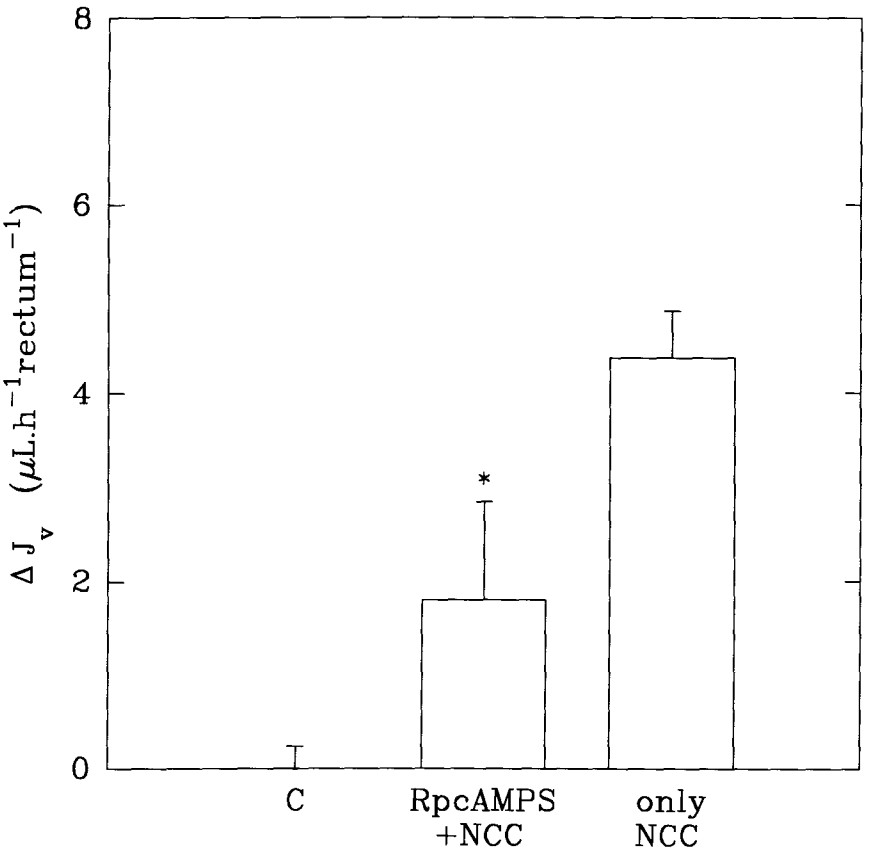


Fig. 2.8. Effect of a 1h pre-incubation with 10 mM RpcAMPS on stimulation of rectal J_V by 2 NCC. Mean ± S.E. (n=11-12). * RpcAMPS+NCC is significantly different from both control (C) and NCC values (Tukey test, $p< 0.05$).

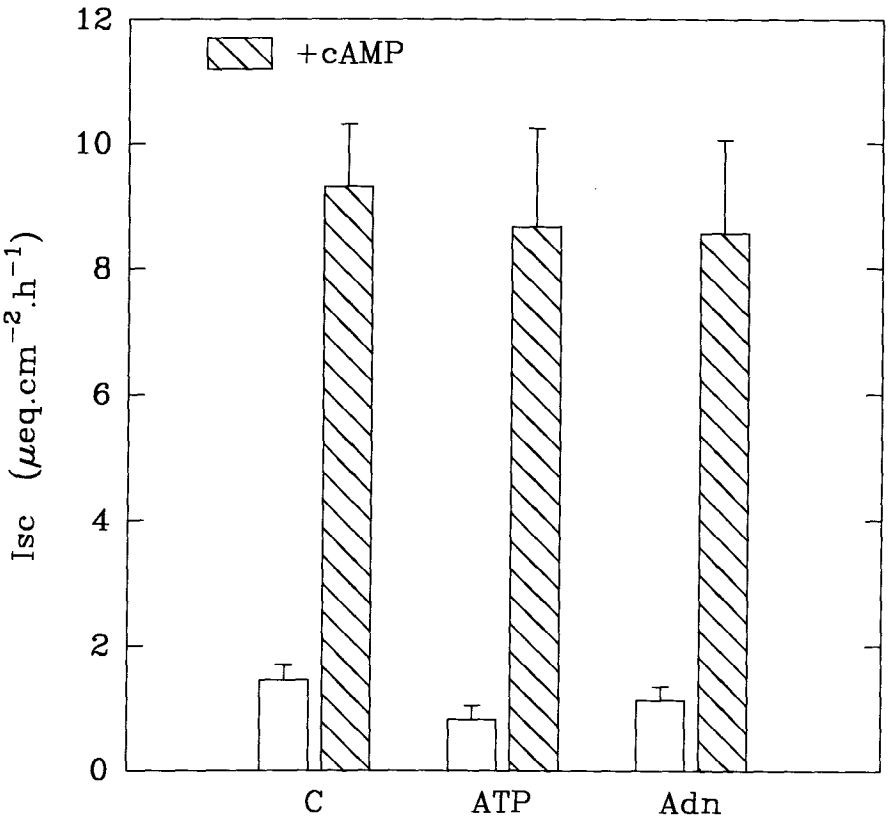


Fig. 2.9. Effect of ATP and Adenosine (Adn) upon rectal I_{sc} (*open bars*). 5 mM ATP and 5mM Adn were added to hemocoel side at steady-state: 30 min. after testing these agents, 5mM cAMP was added to hemocoel side of the same preparations (*hatched bars*). Mean ± S.E. (n=4-10).

stimulation of I_{sc} was tested by adding 5 mM adenosine (preferred by P_1 purigenic receptors) and 5 mM ATP (preferred by P_2 purigenic receptors) to the hemocoel side of recta at Steady-state. Neither adenosine nor ATP had any effect upon rectal I_{sc} or on the response of recta to stimulation with cAMP (Fig. 2.9). The effects of these agents on rectal J_v were not tested.

The effect of IBMX (a phosphodiesterase inhibitor) upon rectal I_{sc} is shown in Fig. 2.10. IBMX alone significantly increased I_{sc} from 1.44 ± 0.29 $\mu eq.cm^{-2}.h^{-1}$ to 4.96 ± 1.33 $\mu eq.cm^{-2}.h^{-1}$. When NCC was added I_{sc} further increased significantly but this IBMX + NCC value was not significantly different (Tukey test, $p > 0.05$) from the value for NCC alone.

Effect of cGMP

Cyclic GMP (5 mM) significantly increased ΔJ_v but not to the extent of 5 mM cAMP (2.3 ± 0.6 $\mu L.h^{-1}$ compared to 4.5 ± 0.6 $\mu L.h^{-1}$, see Fig. 2.11). Cyclic GMP (5 mM) also slowly increased I_{sc} (Fig 2.12), increased V_t and lowered R_t (Table 2.2). Subsequent addition of 5 mM cAMP in the presence of cGMP further increased V_t and further reduced R_t, and caused I_{sc} to rise four times more rapidly (Fig 2.12 and Table 2.2).

Table 2.2. Effect of cGMP upon rectal electrical variables and the subsequent addition of cAMP 30 minutes later (n=8).

t (mins)		I_{sc} ($\mu eq.cm^{-2}.h^{-1}$)	V_t (mV)	R_t ($\Omega.cm^2$)
120	**C**	1.41 ± 0.12	6.12 ± 0.81	167.63 ± 24.06
150	**5mM cGMP**	3.69 ± 0.26*	13.80 ± 1.77*	141.55 ± 17.42*
180	**5mM cAMP**	7.95 ± 0.57*	20.67 ± 2.31*	97.49 ± 9.23*

* values are significantly different from preceding values (paired t-test, $p< 0.05$).

t is time after mounting recta

V_t is lumen side positive

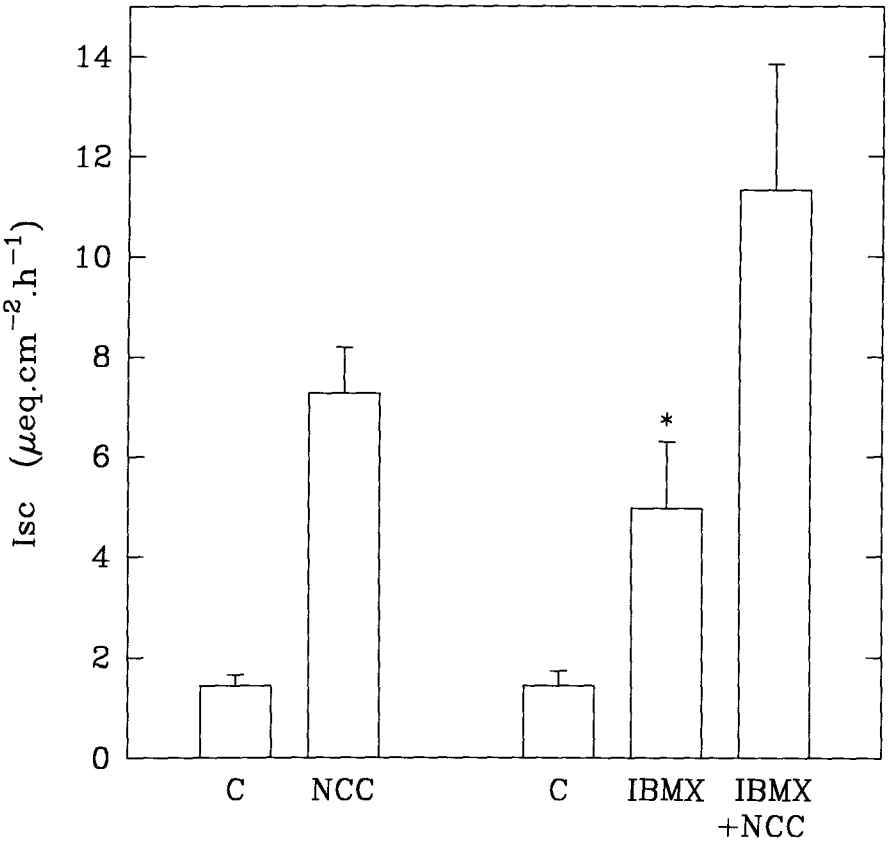
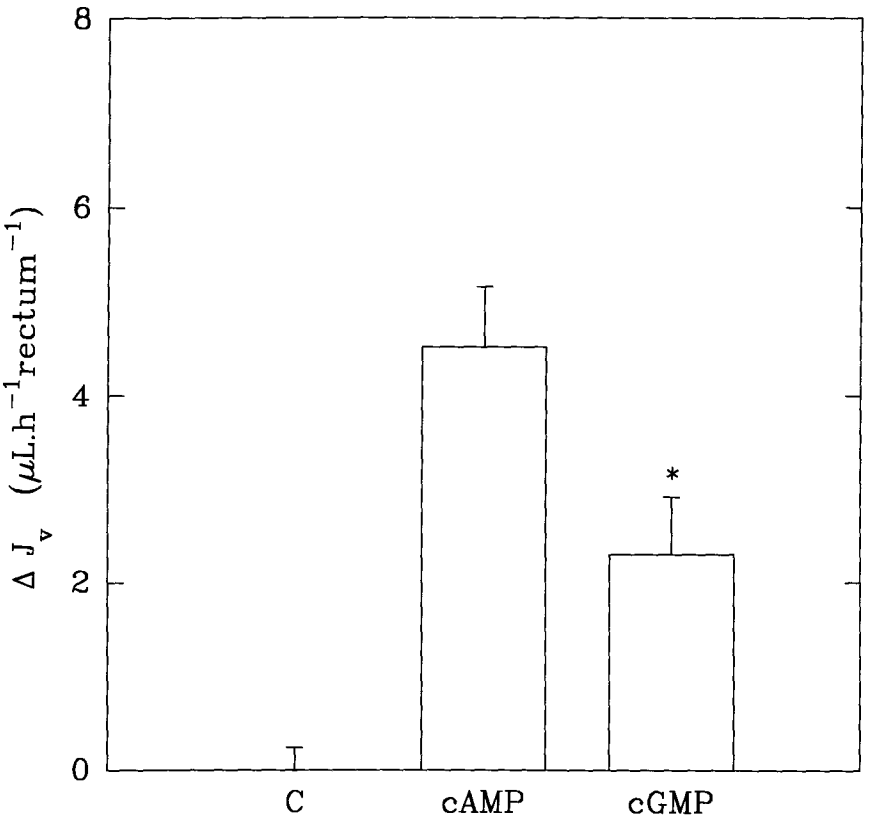


Fig. 2.10. Effect of IBMX upon rectal I_{SC}. IBMX (0.1 mM) was added to hemocoel side at Steady-state: 30 min. later 2 NCC equivalents were added to hemocoel side. Mean ± S.E. (n=8). * IBMX is significantly different from its control (C), (paired t-test, $p < 0.05$).



Fig, 2.11. Effect of 5mM cGMP and 5 mM cAMP upon rectal J_V . Mean ± S.E. (n=6).

* cGMP is significantly different from both the Control (C) and cAMP values (Tukey test, $p< 0.05$).

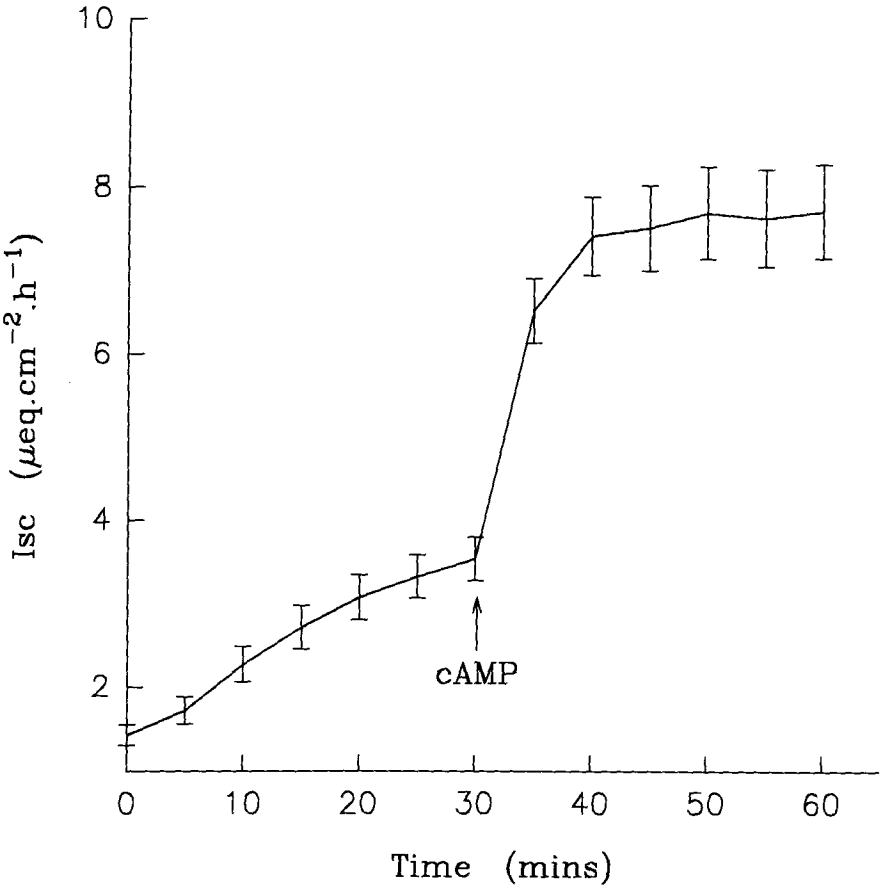


Fig. 2.12. Effect of cGMP upon rectal I_{SC}. 5 mM cGMP was added to hemocoel side at steady-state, 30 min. later 5mM cAMP was added to hemocoel side. Mean ± S.E. (n=8).

DISCUSSION

In this chapter, the effects of agents that influence the adenylate cyclase pathway on rectal J_V and I_{SC} were found to be quantitatively similar, as expected if active transport of Cl^- (I_{SC}) drives secondary transport of fluid (J_V). Cyclic AMP and its analogs (Dibutyryl cAMP, SpcAMPS) were shown to stimulate rectal J_V and I_{SC} to the same extent as NCC homogenate. This suggests that activation of the adenylate cyclase signal transduction pathway is sufficient for the maximal stimulation of rectal J_V and I_{SC}. In support of this, Chamberlin and Phillips (1988) showed that forskolin, an adenylate cyclase activator, also maximally stimulated rectal I_{SC}. While the cAMP analog SpcAMPS maximally stimulated J_V, its diastereoisomer RpcAMPS partially inhibited the stimulation of J_V by NCC. Since RpcAMPS is known to inhibit cAMP dependant protein kinases (i.e. PKA; Rothermel and Parker-Botelho, 1988), this partial inhibition of J_V could indicate that NCC factors may utilize other signal transduction pathways to stimulate rectal reabsorption. Another possibility is that RpcAMPS was unable to fully inhibit the activation of PKA by elevated intracellular cAMP levels. It seems unlikely that cAMP and its analogs act by stimulating cell surface purinergic receptors since addition of ATP and adenosine had no effect on rectal Cl^- transport. The inhibition of rectal cAMP phoshodiesterase activity by IBMX, presumably increased cAMP levels to cause a half maximal increase in rectal Cl^- transport. Chamberlin and Phillips (1988) have shown that theophylline, another PdE inhibitor, did cause an increase in rectal cAMP levels. The effects of IBMX and NCC were not additive, since addition of both only stimulated I_{SC} to the same extent as addition of NCC alone.

This study also provides evidence that the guanylate cyclase signal transduction pathway is involved with the stimulation of rectal fluid and ion reabsorption. Addition of exogenous cGMP caused partial stimulation of rectal I_{SC} and J_V (compared with NCC and cAMP). This cGMP-stimulated increase in rectal I_{SC} is similar in magnitude to a cGMP-

stimulated increase in I_{SC} previously reported by Chamberlin and Phillips (1988), and the rise in I_{SC} correlates with the delayed rise in rectal tissue cGMP levels observed by these workers. Fournier and Dubar (1989) have reported that nitroprusside, which is an activator of soluble guanylate cyclase, and cGMP both had a diuretic effect upon *Locusta migratoria* recta. They also showed that neuroparsins, an antidiuretic factor isolated from NCC of *L. migratoria*, does not increase cellular cAMP levels but does slightly elevate cellular cGMP. These findings suggest that *S. gregaria* and *L. migratoria* which are closely related orthopterans, possess different antidiuretic factors that utilize different signal transduction mechanisms. However these differences may be of result of the unusual conditions used to measure *Locusta* rectal J_V. In the Locusta experiments, Cl^- was omitted from the bathing saline on the first hour and added to the saline at the same time as exposure to the treatment (i.e. neuroparsins, pharmacological agents) at the onset of the second hour (see Fournier et al., 1987). Restoration of Cl^- to the tissue might be expected to trigger tissue swelling and hence second messenger events to restore cell volume.

The involvement of both the adenylate and guanylate cyclase signal transduction pathways in controlling *S. gregaria* rectal reabsorption, does not rule out the possibility that other alternative control pathways are also involved. Rectal transport mechanisms may be controlled and fine-tuned by the interaction of multiple intracellular mechanisms (e.g. elevated intracellular cAMP might increase cellular Ca^{2+} levels etc.) . The following two chapters will investigate the role of other signal transduction mechanisms in the control of rectal transport.

CHAPTER 3: THE INVOLVEMENT OF CALCIUM IN CONTROLLING RECTAL TRANSPORT

INTRODUCTION

Heilbrunn and Wiercenski (1947) were the first to demonstrate that Ca^{2+} is involved in cellular control when they injected Ca^{2+} into frog muscle cells and caused them to contract. Subsequently, intracellular Ca^{2+} has been shown to regulate many cellular processes in vertebrates, but less is known about the role of Ca^{2+} in controlling insect cellular events (see: Berridge, 1983; Berridge and Irvine, 1984). A rise in cytosolic Ca^{2+} concentration elicits a plethora of protein modifications that allows the cell to respond specifically and quickly to numerous stimuli. Low resting cytosolic Ca^{2+} levels are crucial for cellular control and homeostasis. Free cytosolic Ca^{2+} is usually less than 10^{-7} M, while concentrations of external Ca^{2+} and the Ca^{2+} sequestered in intracellular compartments are normally in the millimolar range (Berridge, 1983). Therefore there is a large gradient for the movement of Ca^{2+} into the cytosol, although the total Ca^{2+} concentration in the cell is roughly equal to the external concentration. This huge gradient is maintained by numerous mechanisms that include: the active pumping of Ca^{2+} by Ca^{2+}/ATPases into intracellular stores or outside of the cell, the expulsion of Ca^{2+} via plasma membrane Na^{+}/ Ca^{2+} ion exchangers, the entry of Ca^{2+} across the favourable cation gradient of the mitochondria, and binding of Ca^{2+} to molecules in the cytosol (see Fig. 3.1; reviewed by Alberts et al., 1989). It is important to note that entry of Ca^{2+} into the cytosol is primarily across the membranes of the internal Ca^{2+} sequestering compartments, since the surface area of the plasma membrane is 10-100 times less than the total surface area of the Ca^{2+} containing organelles (Alberts et al., 1989).

Calcium binding proteins, such as troponin C and calmodulin, are responsible for translating these Ca^{2+} increases into cellular responses (Berridge, 1983). These proteins

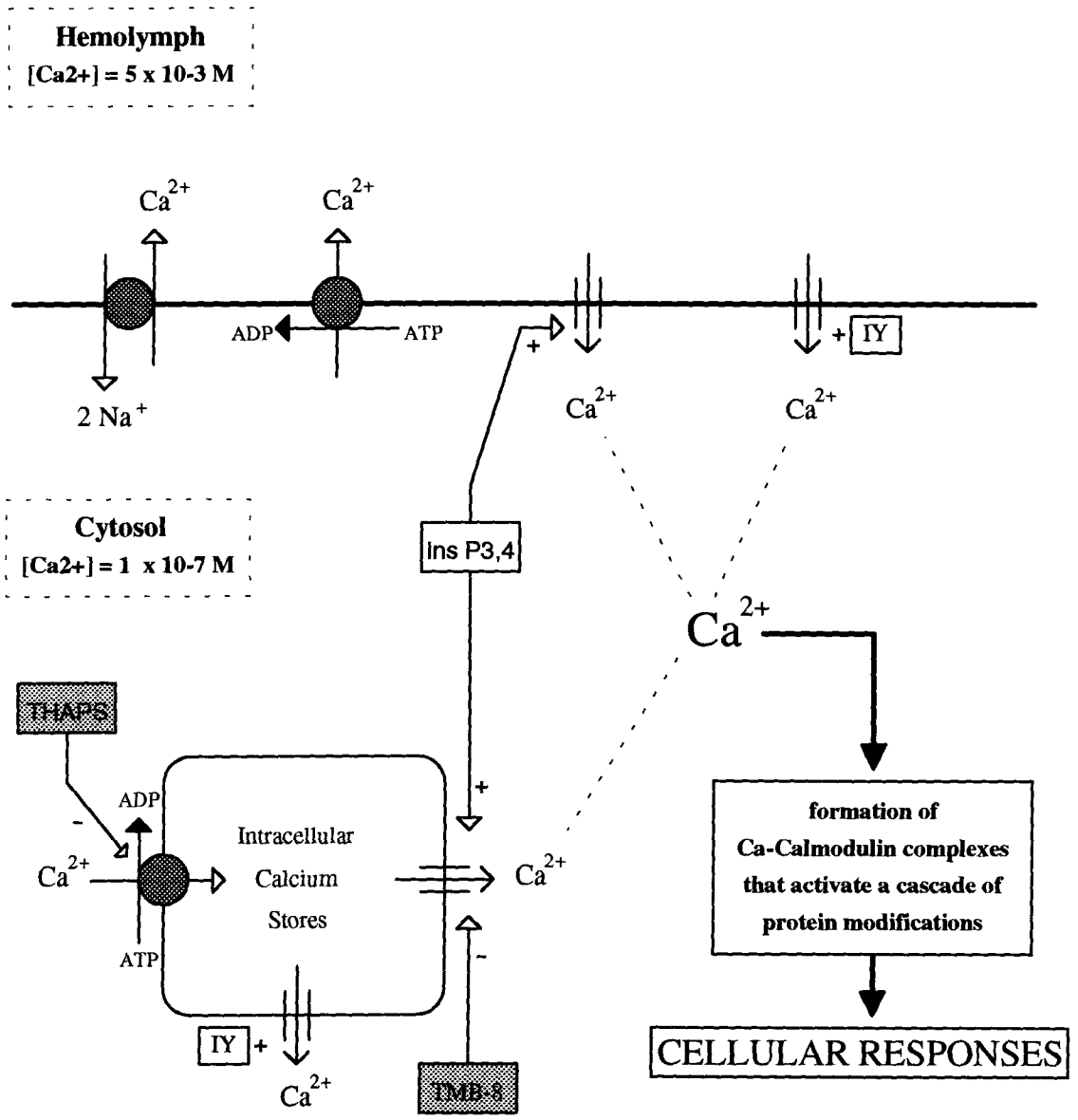


Fig.3.1. Schematic diagram of the cellular processes involved with the regulation of intracellular Ca^{2+}. Calcium pumps and ion exchangers are represented by *shaded circles* at the plasma membrane and the membrane of the intracellular stores. Ion channels and ionophores are represented by an *arrow passing between parallel lines*, while agents that decrease or increase cytosolic Ca^{2+} appear in *solid boxes with associated -/+ signs*.

possess high-affinity Ca^{2+} binding sites that are very specific for Ca^{2+} since cytosolic Mg^{2+} levels are typically high (in the millimolar range, Alberts et al., 1989). The binding of Ca^{2+} to these high-affinity sites alters the conformation of these Ca^{2+} binding proteins, allowing them to associate with and activate enzymes involved with various cellular processes. Cellular effects that are mediated through Ca^{2+} binding proteins include: Ca^{2+} induced contraction of vertebrate skeletal muscle, pre-synaptic release of neurotransmitters by nerve cells, and the activation of Ca^{2+} pumps that lower cytosolic Ca^{2+} to create a negative feedback loop (Berridge, 1983). It must be kept in mind that intracellular Ca^{2+} and cAMP can interact to control various cellular processes (reviewed by Cheung, 1982). The first of these interactions is that Ca^{2+} and cAMP levels can modulate each other. For example Ca^{2+} levels can regulate enzymes that breakdown (e.g. phosphodiesterases) or synthesize cAMP. In addition cAMP-dependent protein kinases (e.g. protein kinase A) can phosphorylate Ca^{2+} channels or pumps, resulting in increases or decreases in intracellular Ca^{2+}. In the second type of interaction, both Ca^{2+} and cAMP can regulate the same protein. An example of this is phosphorylase kinase, which is involved with glycogen breakdown and can be stimulated by a cAMP-dependent kinase as well as by Ca^{2+} binding to calmodulin.

There is some evidence that Ca^{2+} is involved with the control of insect excretion. Spring et al. (1988b) have isolated an antidiuretic factor from the cricket (*Acheta domesticus*) that was shown to inhibit Malpighian tubule secretion by 70%. Spring and Clark (1990) later showed that the calcium ionophore A23187 also reduced Malpighian tubule secretion by 85%, and proposed that this antidiuretic factor inhibited fluid secretion by elevating intracellular Ca^{2+} (see also Kim and Spring, 1992). Since many workers have reported that insect diuretic factors stimulate Malpighian tubule secretion via cAMP (Aston, 1975; Morgan and Mordue, 1985; Beyenbach and Petzel, 1987; Spring and Clark, 1990; Coast et al., 1992), it would appear as though cAMP and Ca^{2+} may interact in an antagonistic manner. Fogg et al. (1990) showed that when *L. migratoria* Malpighian

secretion was stimulated with CC homogenate, both cAMP and inositol trisphosphate ($InsP_3$) levels rose. Since $InsP_3$ stimulates the release of sequestered Ca^{2+}, it would appear that both Ca^{2+} and cAMP act cooperatively in the stimulation of fluid secretion. Coast (personal communication, 1992) has used the calcium ionophore ionomycin to increase Ca^{2+} levels and has also seen an increase in Malpighian tubule secretion. Apparently Ca^{2+} is important for the control of Malpighian tubule secretion, but there is conflicting evidence concerning the role Ca^{2+} plays in this control.

Calcium may also be involved with the control of rectal transport mechanisms, even though Hanrahan and Phillips (1985) saw no effect upon *S. gregaria* rectal Cl^- transport when a Ca^{2+} ionophore was added to the preparation. Fournier (1990b) presented evidence to show that *L. migratoria* rectal fluid transport (J_V) increased when Ca^{2+} ionophores were added. He has also shown that *L. migratoria* neuroparsins (a potential insect antidiuretic peptide) increased $InsP_3$ levels suggesting that Ca^{2+} is involved with the stimulation of rectal J_V. Fournier (1990b) claims that rectal J_V is dependant upon extracellular Ca^{2+} levels, since removal of Ca^{2+} from incubating saline significantly reduced rectal J_V.

This chapter deals with the role of calcium in the control of rectal J_V and I_{SC} in *S. gregaria*. The influence of external Ca^{2+} upon rectal transport was investigated by removing Ca^{2+} from the bathing medium. In addition, the ability of NCC homogenate and cAMP to stimulate rectal transport under Ca^{2+} free conditions was studied. The role of intracellular Ca^{2+} in controlling rectal transport was explored by using agents known to modulate cytosolic Ca^{2+} levels (see Fig.3.1). The calcium ionophore ionomycin (IY) in known to increase cytosolic Ca^{2+} levels by inserting into both the plasma membrane and the membranes of the intracellular organelles (Fournier, 1990b). Thapsigargin (THAPS) is also known to increase cytosolic Ca^{2+} by inhibiting the Ca^{2+}/ATPase pump that is responsible for removing Ca^{2+} from the cytosol (Takemura et al, 1989; Thastrup et al., 1990). Trimethoxybenzoate hydochloride (TMB-8) is thought to lower cytosolic Ca^{2+} by

preventing its release from intracellular stores (Smith and Iden, 1979). The phosholipase C (PLC) mediated signal transduction pathway, via inositol 4,5-trisphosphate ($InsP_3$), is also known to increase intracellular Ca^{2+} levels. The involvement of this PLC pathway in the control of rectal transport will be addressed in the next chapter.

MATERIALS AND METHODS

The methods used in this study were similar to those described in Chapter 2, unless otherwise indicated. The locust colony was maintained under similar conditions and the NCC homogenates were prepared using the methods described in Chapter 2.

Most of the chemicals were supplied by the Sigma Chemical Company (St. Louis MO, USA). Thapsigargin was supplied by CALBIOCHEM Corporation (San Diego CA, USA). Most of the tested substances were dissolved in complex saline or calcium-free complex saline. However, Ionomycin and Thapsigargin were initially dissolved in 100% DMSO and these stock solutions were then diluted with complex saline (final DMSO concentration $\leq$ 1%). DMSO (1%) alone had no effect upon rectal J_V or I_{SC} compared to complex saline controls (e.g. 3rd hour rectal J_V: 10.3 $\pm$ 0.4 $\mu L.h^{-1}$, n=7, for the DMSO control; compared to the complex saline control of 9.8 $\pm$ 0.6 $\mu L.h^{-1}$, n=8).

The composition and maintenance of the complex saline were described in Chapter 2. The Ca^{2+} free saline had a similar composition to the complex saline except that it did not have 5 mM $CaCl_2$ and contained 1 mM EGTA to ensure that any trace amounts of Ca^{2+} would be chelated.

RESULTS

Effect of Ca^{2+} free saline

Incubation of rectal sacs in calcium-free saline (Caf) for four hours did not cause any decrease in J_V compared to control values over the same time course (Fig.3.2). EGTA (1 mM) was added to the Ca^{2+} free saline to ensure that any trace amounts of Ca^{2+} were chelated, and this saline gave similar results as Ca^{2+} free saline without EGTA (data not shown). When rectal sacs were incubated in saline with 10 mM EGTA, J_V was much lower during the first hour (5.1 ± 0.9 $\mu L.h^{-1}$, n=6; compared to the control value of 18.7 ± 0.8 $\mu L.h^{-1}$, n=8) and on the third hour Jv was completely abolished (0.1 ± 0.4 $\mu L.h^{-1}$, n=6; compared to the control value of 9.8 ± 0.6 $\mu L.h^{-1}$) . By the third hour a white foamy liquid was being collected from the hemocoel side of these sacs and the lumen side appeared whitish instead of the usual yellow/brown. It seems that 10 mM EGTA is toxic to rectal cells and causes cell dissociation on the hemocoel side. Cell dissociation was not seen on the lumen side probably because the cuticle helped to hold the rectal tissue together.

Both NCC and DBcAMP increased J_V to a greater extent in Ca^{2+} free saline than in complex saline with Ca^{2+} (Figs. 3.2 & 3.4). Stimulation of recta with 2 NCC in Ca^{2+} free saline also caused a slight increase in rectal tissue swelling ($\approx$ 1 μL.) compared to NCC controls (Fig. 3.3). Exposure of unstimulated recta to Ca^{2+} free saline had no effect upon I_{SC} and subsequent stimulation of recta with NCC gave similar changes in electrical variables as seen with NCC controls in normal saline (Table 3.1).

Table 3.1. Effect of Calcium free saline upon electrical variables

	I_{sc} ($\mu eq.cm^{-2}.h^{-1}$)	V_t (mV)	R_t ($\Omega.cm^2$)	n
Control	1.44 ± 0.22a	8.48 ± 0.80a	265.41 ± 48.82a	8
+ 2NCC	7.27 ± 0.92b	20.89 ± 1.77b	120.88 ± 18.85b	
Ca^{2+} free	2.54 ± 0.40a	10.94 ± 2.30a	166.83 ± 22.83ab	7
+ 2NCC	10.07 ± 2.10 b	20.36 ± 2.51b	88.55 ± 13.91b	

Values sharing the same letter are not significantly different (Tukey test, $p > 0.05$)

V_t values are lumen side positive

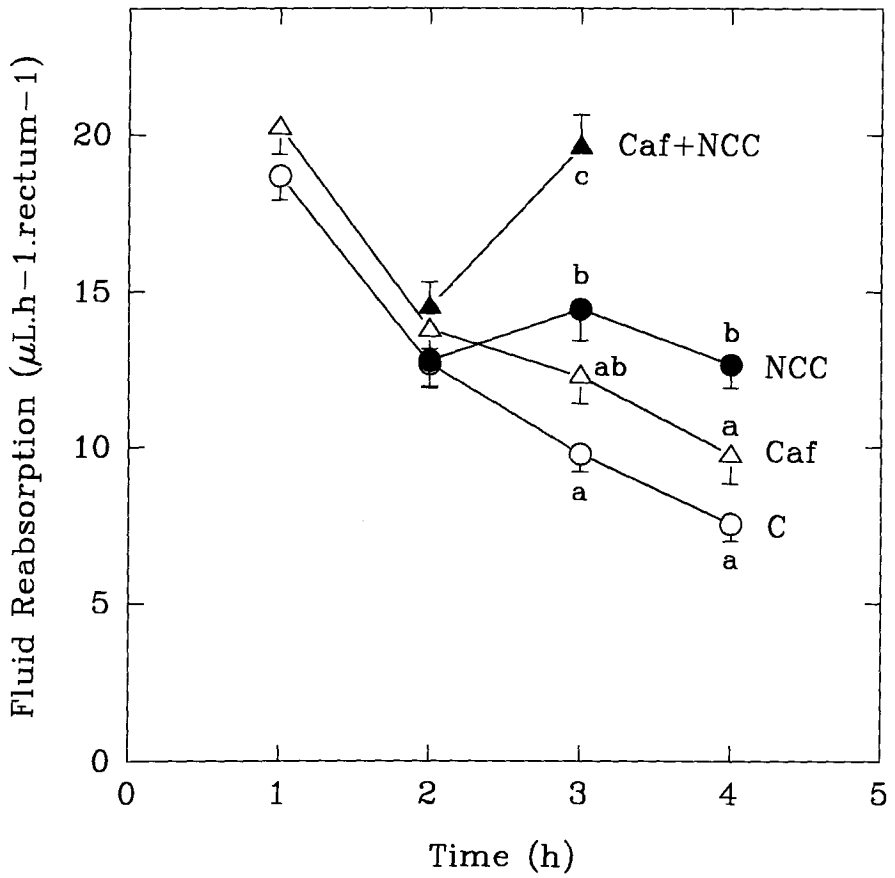


Fig. 3.2. Fluid reabsorption by rectal sacs under Ca^{2+} free conditions. Control complex saline hours 1-4 (C), complex saline with 2 NCC added to hemocoel side on hours 3+4 (NCC), Ca^{2+} free saline hours 1-4 (Caf), Ca^{2+} free saline for hours 1-3 with 2 NCC added to hemocoel side on third hour (Caf+NCC). Mean ± S.E. (n=8-13). Values from similar times that share the same letter are not significantly different (Tukey test, $p > 0.05$).

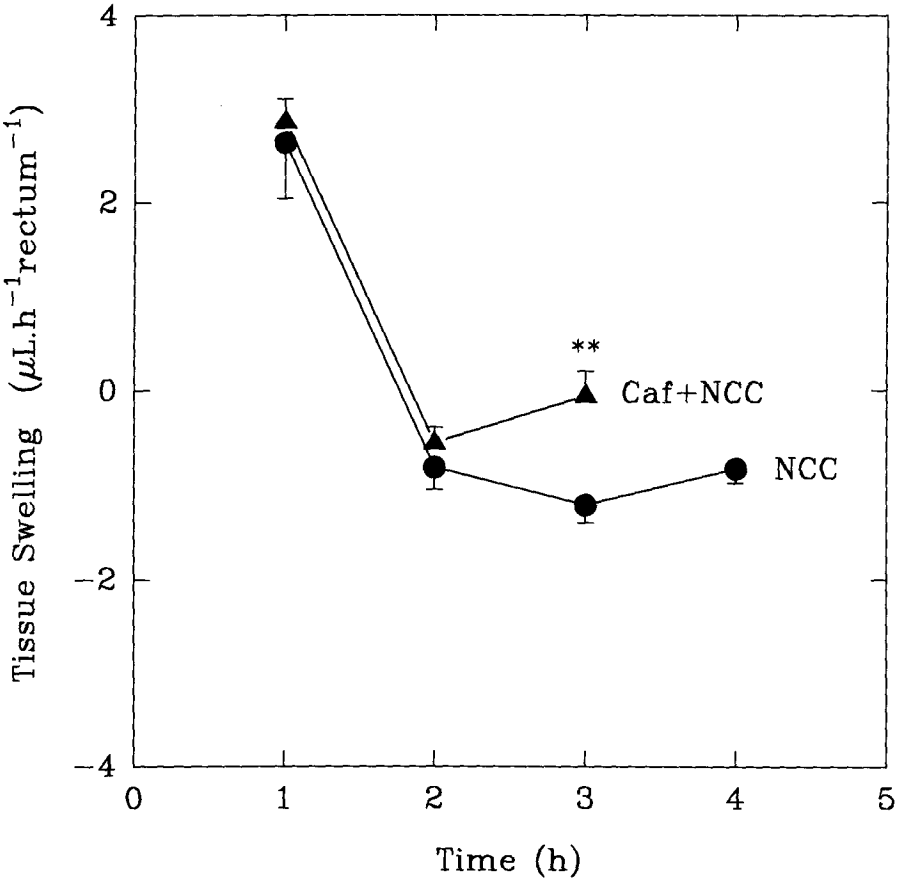


Fig. 3.3. Tissue swelling of rectal sacs under Ca^{2+} free conditions. 2 NCC added on hours 3+4 in presence of Ca^{2+} (NCC), Ca^{2+} free saline for hours 1-3 with 2 NCC added on third hour (Caf+NCC). Mean ± S.E. (n=11-13). ** Caf+NCC is significantly different from NCC (independent t-test, $p< 0.01$).

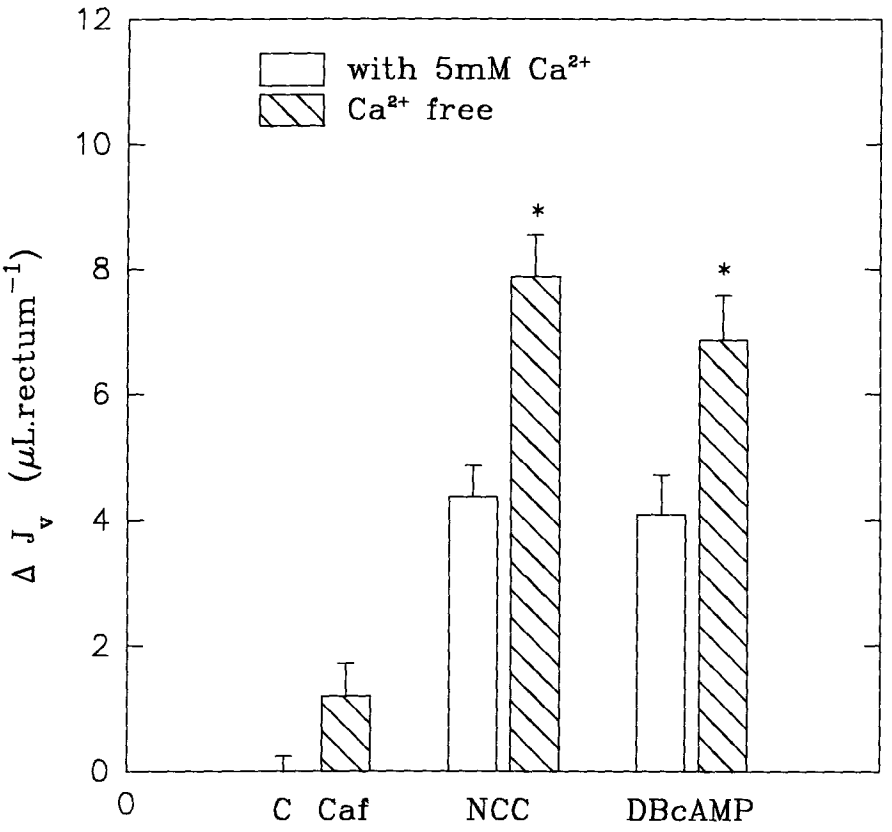


Fig. 3.4. Effect of Ca^{2+} free saline (Caf) upon J_V stimulated by 2 NCC and 1 mM DBcAMP. Mean ± S.E. (n=10-13). * values are significantly different from similar treatments with Ca^{2+} in bathing saline (Tukey test, $p< 0.05$).

Effect of agents that modulate cytosolic Calcium

The calcium ionphore ionomycin, an agonist of intracellular Ca^{2+}, had a partial stimulatory effect upon J_V (40% of maximum) when used at 4 μM, but no effect at 1μM (Fig. 3.5). Fournier (1990b) claims that elevating the bathing saline Ca^{2+} concentration by 10% increases the effectiveness of ionomycin; however when this procedure was tried there was no additional stimulation of J_V by 4 μM ionomycin (Fig. 3.5). No increase in J_V was seen when sacs were incubated in Ca^{2+} free saline and then exposed to 4 μM ionomycin.

Addition of 1 μM ionomycin to unstimulated short-circuited recta, with calcium present, had no effect on I_{SC} but reduced the stimulatory action of NCC by over 40% (4.1 ± 0.24 $\mu eq.cm^{-2}.h^{-1}$ compared to 7.3 ± 0.9 $\mu eq.cm^{-2}.h^{-1}$ for the NCC control, see Fig. 3.6). Thapsigargin, another agonist of intracellular Ca^{2+}, had no effect upon resting I_{SC} or NCC stimulated I_{SC} when used at 10-50 μM (Fig. 3.6). TMB-8, an antagonist of intracellular Ca^{2+}, had no effect upon rectal I_{SC} or subsequent stimulation with cAMP when used at 200 and 1000 μM concentrations. Although 1 mM TMB-8 reduced cAMP stimulated I_{SC} by almost 50%, this value was not statistically different from the control (Tukey test, $p > 0.05$). However, 1 mM TMB-8 significantly reduced NCC stimulated I_{SC} by 40% (4.3 ± 0.6 $\mu eq.cm^{-2}.h^{-1}$ compared to 7.27 ± 0.92 $\mu eq.cm^{-2}.h^{-1}$ for the NCC control, see Fig. 3.8).

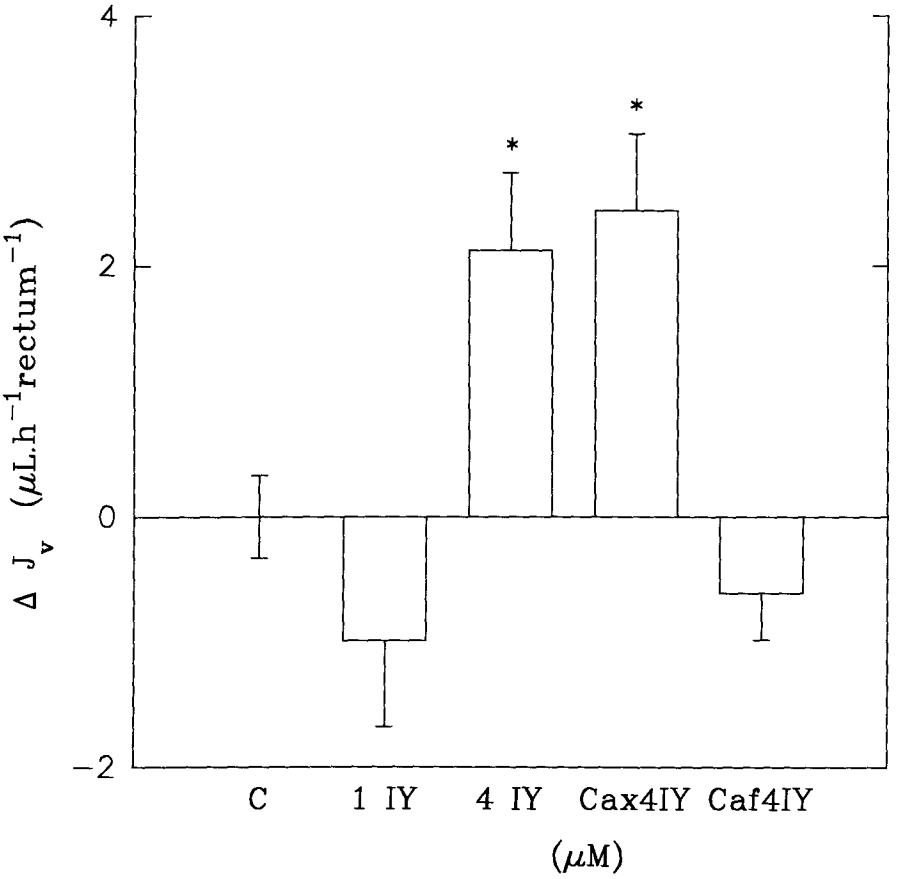


Fig. 3.5. Effect of Ionomycin (IY), IY with 5.5 mM Ca^{2+} (+10%) bathing saline (Cax4IY) and IY with Ca^{2+} free saline (Caf4IY) upon rectal J_V. Mean ± S.E. (n=7-11). * values are significantly different from 1% DMSO controls (C), (Tukey test, $p< 0.05$).

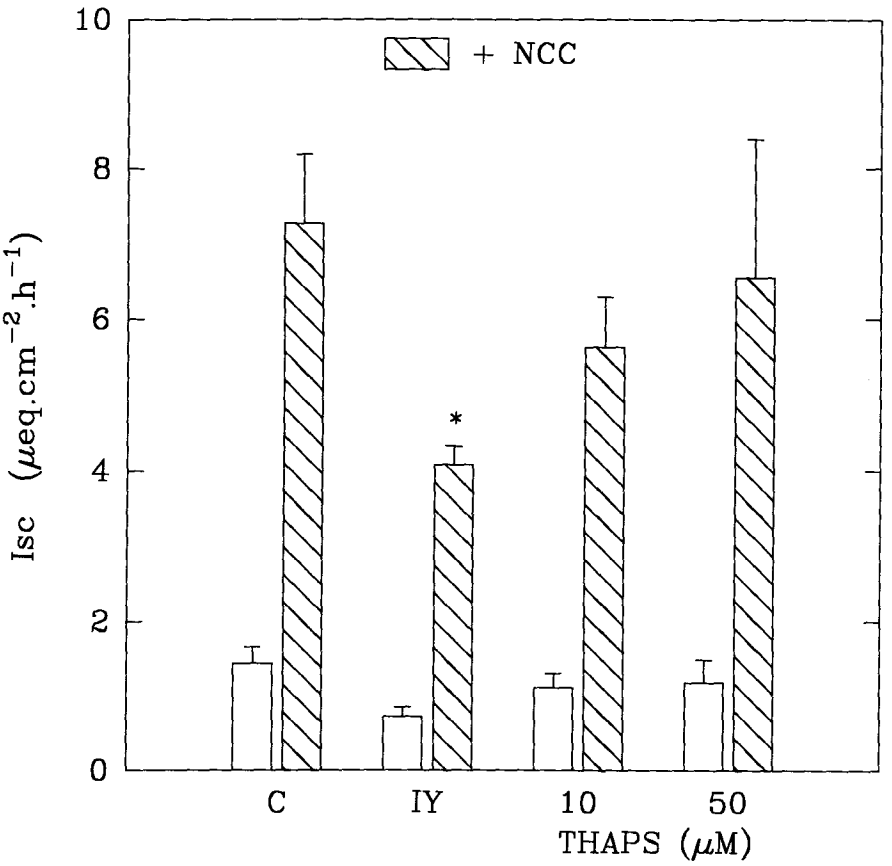


Fig. 3.6. Effect of 1 mM IY and Thapsigargin (THAPS) upon rectal Isc. At steady-state agents were added bilaterally (*open bars*), and then 30 minutes later 2 NCC were added (*hatched bars*). Mean ± S.E. (n=5-8). * value is significantly different from corresponding NCC control value (Tukey test, $p < 0.05$).

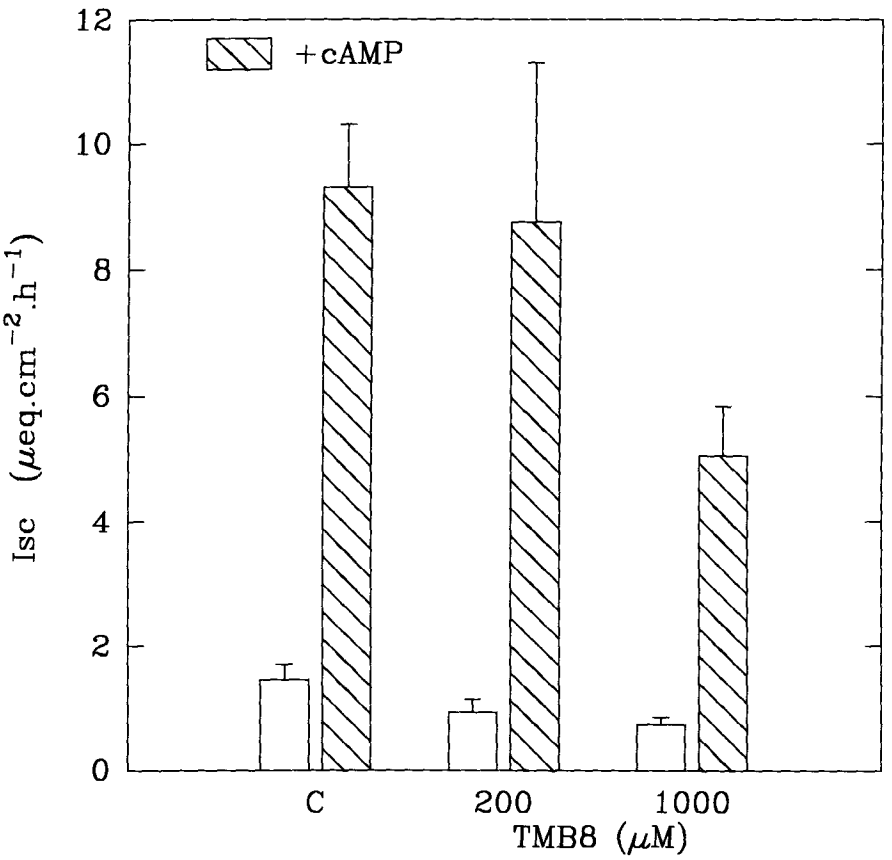


Fig. 3.7. Effect of TMB-8 upon 5 mM cAMP stimulated I_{SC}. At steady-state TMB-8 was added bilaterally, 30 min. later cAMP was added. Mean ± S.E. (n=8-10).

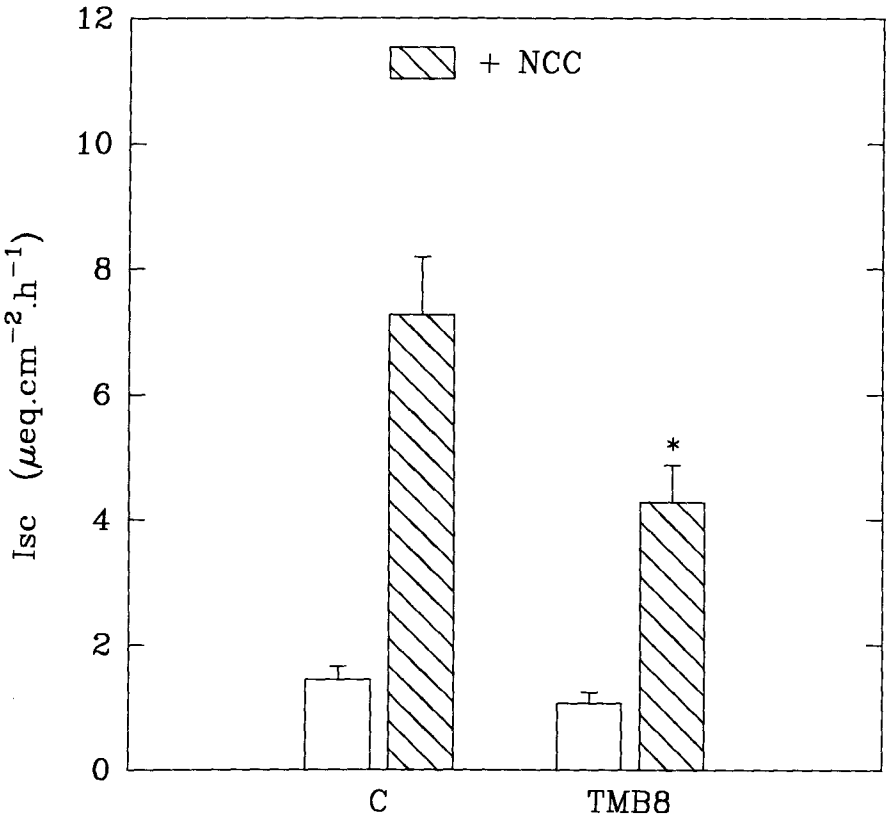


Fig. 3.8. Effect of TMB-8 upon 2 NCC stimulated I_{SC}. At steady-state 1 mM TMB-8 was added bilaterally, 30 min. later 2 NCC were added. Mean ± S.E. (n=8-10). * value is significantly different from NCC control (Tukey test, $p< 0.05$).

DISCUSSION

The removal of external Ca^{2+} from the bathing medium did not reduce either the fluid or ion reabsorbing ability of the recta. When recta were stimulated with either NCC or cAMP under Ca^{2+} free conditions, there were larger increases in rectal J_V compared to increases seen when Ca^{2+} was present. Calcium-free conditions did not have any effect upon the stimulation of I_{SC} or V_t with either cAMP or NCC. Since Ca^{2+} free conditions increased stimulated J_V without changing values for stimulated electrical variables, it would appear that the absence of Ca^{2+} may have an extracellular rather than an intracellular effect upon rectal cells. A possible effect of removing Ca^{2+} from the bathing saline could be the loosening of the tight junctions at the apical and basal complexes that hold adjacent rectal pad cells closely together (refer to Fig. 1.2). The opening of these complexes may facilitate the movement of fluid through the intercellular spaces toward the basal membrane.

Fournier (1990b,1991) has reported that bathing Ca^{2+} is essential for the maintenance of resting rectal J_V in *L. migratoria*, since the incubation of tissues with the Ca^{2+} chelator EGTA dramatically reduced J_V by 90% within the first hour. Fournier (1990b) proposes that this apparent sensitivity of the rectum to external Ca^{2+} is a result of "an insufficient mobilization or lack of calcium from intracellular stores". It seems unlikely that these two closely related insects can possess recta that have differing dependencies upon external Ca^{2+}, so why are Fournier's findings so different from the results of this study? A possible reason for Fournier's different results, is that he may have used a high concentration of EGTA in his Ca^{2+} free bathing medium that proved toxic to the rectal cells. It is unclear what actual concentration of EGTA was used, but a Ca^{2+} free bathing saline containing 0.8 M Ca^{2+} with 1.2 M EGTA has been reported in numerous papers (see Fournier 1990b,1991; Fournier et al., 1992). In my study, 10 mM EGTA in Ca^{2+} free saline proved toxic to the rectal cells, almost abolishing all J_V by the third hour of incubation. It also

seems unlikely that intracellular rectal Ca^{2+} levels, which are regulated very closely by the cell and are essential for the maintenance of cellular homeostasis, are so dependant on external Ca^{2+}. In support of the findings of this study, Berridge (1977) has also shown that fluid secretion by the salivary glands of the blow fly (*Calliphora*) was independent of external bathing Ca^{2+}.

The use of agents that modulate intracellular Ca^{2+} levels gave mixed results. Firstly, the addition of ionomycin to recta submaximally increased rectal J_V (~40%) compared to the increases seen with cAMP and NCC. Ionomycin had a small inhibitory effect upon resting I_{SC} and reduced the stimulatory effect of NCC by 50%. This suggests that high intracellular Ca^{2+} partially inhibits the stimulation of Cl^- transport by cAMP. In addition, TMB-8 slightly reduced resting I_{SC} and reduced the stimulation of I_{SC} by both NCC and cAMP by 40-50%. These TMB-8 results suggest that the stimulation of Cl^- transport by cAMP is dependant upon the elevation of cytosolic Ca^{2+} levels by release from intracellular stores. Since thapsigargin had no effect upon resting or stimulated I_{SC}, it must be concluded that either the agent did not enter the cell or that it could not elevate cytosolic Ca^{2+} levels to an extent where changes in I_{SC} could be observed.

In summary, it appears as though Ca^{2+} does play a role in the control of rectal transport processes. However it seems that the role of Ca^{2+} is quite complex, and that Ca^{2+} may have differing cellular effects depending upon its cytosolic concentration. Cytosolic Ca^{2+} alone was not able to cause a full stimulation of either J_V or I_{SC} as suggested by Fournier (1990b,1991). Earlier evidence suggests that rectal J_V results from Cl^- transport (I_{SC}, Goh and Phillips, 1978). The seemingly different effects of Ca^{2+} on rectal J_V and I_{SC} suggest that Ca^{2+} may control processes other than Cl^- transport which influence J_V (e.g. movement of counter ions such as K^+ and Na^+, or ion recycling in the lateral spaces of the rectal epithelium).

CHAPTER 4: THE ROLE OF PKC AND THE PI CYCLE IN CONTROLLING RECTAL TRANSPORT

INTRODUCTION

In the previous chapter there were some indications that intracellular Ca^{2+} was involved with the control of rectal transport. Although the adenylate cyclase signal transduction pathway can alter cellular Ca^{2+} levels indirectly by opening Ca^{2+} channels, the phospholipase C (PLC) pathway, via inositol 1,4,5-trisphosphate ($InsP_3$), is thought to be the primary method of elevating intracellular Ca^{2+} (Berridge and Irvine, 1984). PLC is activated by a G-protein subunit that is released from an activated receptor complex. When PLC is activated, it catalyzes the breakdown of phosphatidylinositol 4,5-bisphoshate (PIP_2) into $InsP_3$ and sn-1,2-diacylglycerol (DAG). While $InsP_3$ activates the release of Ca^{2+} from intracellular stores and the entry of exogenous Ca^{2+}, DAG stimulates the Ca^{2+} dependant protein kinase C (PKC) which then phosphorylates various cellular proteins (see Fig. 4.1; Berridge and Irvine, 1989; Nishizuka, 1986).

There is some evidence that PLC-mediated events are involved in the control secretion in insects. Berridge (1986) reported that $InsP_3$ levels rose in *Calliphora* salivary glands when serotonin (5-HT) stimulated secretion *via* the 5 HT_1 receptor. Fogg et al. (1990) showed that there was an increase in $InsP_3$ and cAMP levels following stimulation of *L. migratoria* Malpighian tubule secretion with CC homogenate. The elevation of cAMP and $InsP_3$ levels occurred between 30-300 s for cAMP and 15-300 s for $InsP_3$. Fournier (1990b), using pharmacological methods, provided evidence that *L. migratoria* neuroparsins (Nps) stimulated rectal J_V through PLC-mediated events. When Li^+ was used to inhibit the phosphotidylinositol (PI)

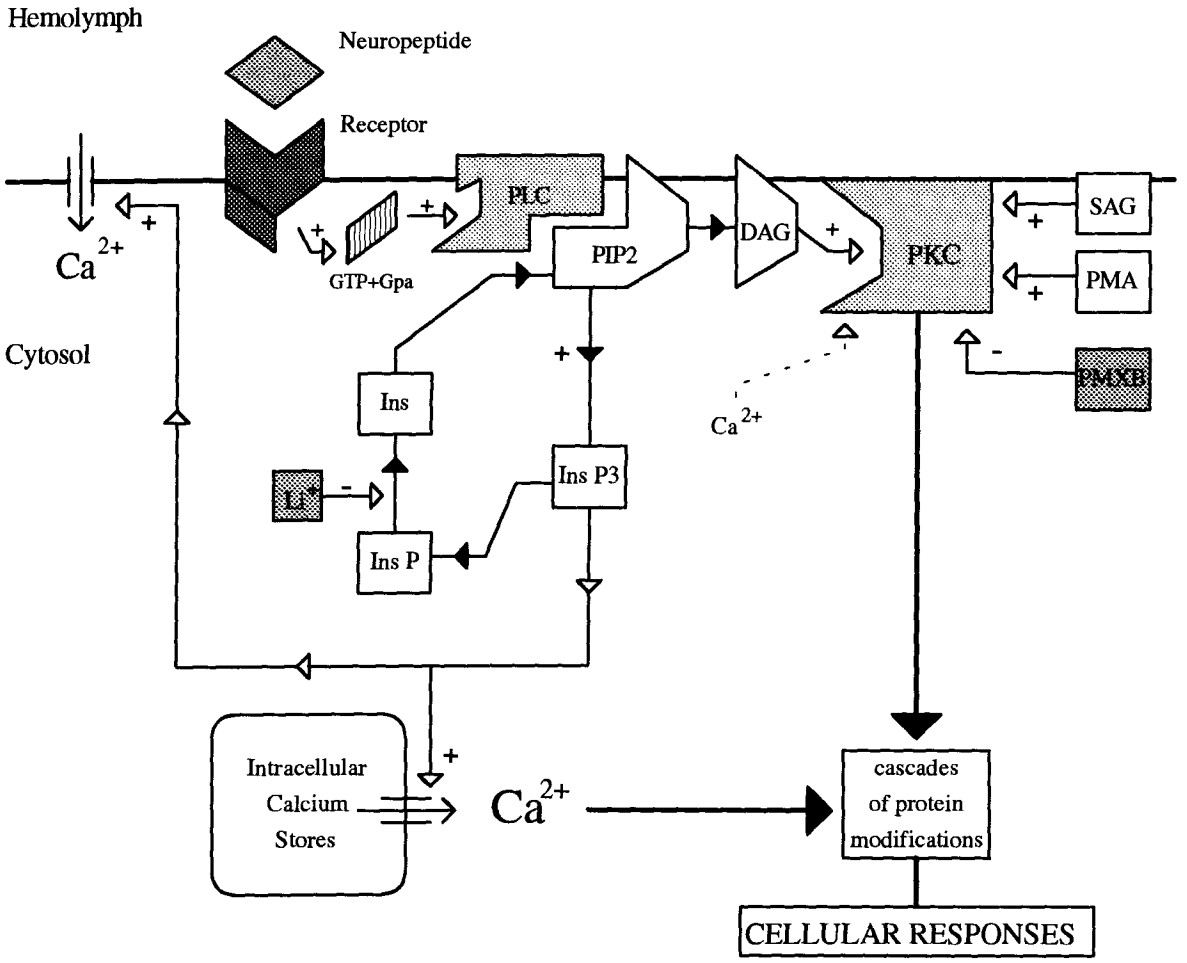


Fig. 4.1. Diagram of the phosholipase C (PLC) mediated signal transduction pathway. The G protein activated PLC breaks down phosphatidylinositol 4,5-bisphoshate (PIP_2) into diacylglycerol (DAG) and inositol trisphophate ($InsP_3$). $InsP_3$ then opens Ca^{2+} channels that are represented by *an arrow passing between two parallel lines*. The cellular action of agents added to the rectal preparations are shown by *arrows with associated +/- signs*.

cycle, the stimulatory effect of Nps was abolished. The addition of the PI cycle component *myo*-inositol was sufficient to maximally stimulate rectal J_V. Fournier (1990b) also suggested that PKC was involved in the stimulation of *L. migratoria* J_V since addition of specific PKC stimulators maximally stimulated J_V, while addition of the PKC inhibitor Polymyxin B (PMXB) abolished the stimulatory effect of Nps.

Fournier (1991) has recently demonstrated that Nps stimulate $InsP_3$ and inositol 1,3,4,5 tetrakisphosphate ($InsP_4$) production in *L. migratoria* rectal cells. In addition it was suggested that PKC activity is necessary for the phosphorylation of enzymes involved with inositol phosphate metabolism, since the inositol phosphate cascade was mimicked using PKC stimulators and inhibited with PMXB. It was also reported that neither the adenylate nor guanylate cyclase systems modified inositol phosphate metabolism.

In this chapter the role of the PLC-mediated signal transduction pathway in controlling rectal transport was studied. Specific stimulators and inhibitors of protein kinase C were used to explore its role in controlling rectal transport. The PKC stimulators used were 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) and phorbol 12-myristate-13-acetate (PMA), while polymyxin B sulfate was used to inhibit PKC. The involvement of the phosphatidylinositol (PI) cycle in the control of rectal transport was examined by attempting to stimulate it with *myo*-inositol, a component of the PI cycle. In addition, the effects of inhibiting the PI cycle were investigated using Li^+. Lithium is known to block the conversion of inositol phosphate to inositol and hence synthesis of inositol trisphosphate ($InsP_3$; see Fig. 4.1; Berridge and Irvine, 1989). *L. migratoria* rectal preparations were used to investigate the role of the PI cycle in controlling rectal J_V and I_{SC}, since Fournier (1990b, 1991) had previously indicated that *L. migratoria* neuroparsins increased rectal J_V by increasing $InsP_3$ levels. Neuroparsins has also been shown to have anti-juvenile hormone activity and increase hemolymph trehalose and lipid levels (Girardie et al., 1987; Moreau et al., 1988) Finally, neuroparsins was tested on *S. gregaria* rectal preparations to test for any cross-reactivity between these species.

MATERIALS AND METHODS

Most of the methods used in these experiments were similar to those described in Chapter 2, unless otherwise stated. The *Locusta migratoria* colony was maintained as previously described for the *Schistocerca gregaria* colony. Adult 6 week old male *Locusta migratoria* were used for the *Locusta* fluid transport experiments, while 6 week old female *Locusta migratoria* were used for the *Locusta* ion transport experiments.

All chemicals were supplied by the Sigma Chemical Company (St. Louis MO, USA). Most of the tested substances were dissolved in complex saline. However, 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG) and phorbol 12-myristate-13-acetate (PMA) were initially dissolved in 100% dimethyl sulfoxide (DMSO) and these stock solutions were then diluted with complex saline (final DMSO concentration $\leq$ 1%). Neuroparsins (Nps), purified from NCC of *Locusta migratoria* (Girardie et al. 1987b), was a gift from J. Proux (Laboratoire de Neuroendocrinologie, Université de Bordeaux, Talence, France).

RESULTS

Effect of agents acting upon protein kinase C

Stimulators of PKC, SAG and PMA had no stimulatory activity upon *S. gregaria* rectal J_V and I_{SC} (Figs. 4.2 & 4.3). Polymyxin B sulfate (PMXB), a PKC inhibitor, slightly increased NCC stimulated J_V (6.1 $\pm$ 0.6 $\mu L.h^{-1}$ compared to 4.4 $\pm$ 0.5 $\mu L.h^{-1}$ with NCC alone), but had no effect upon unstimulated or NCC stimulated I_{SC} (Figs. 4.4 & 4.5).

Effects of agents acting upon the Phosphotidyl-Inositol cycle

Myo-inositol (MI) is a component of the PI cycle and was shown to have no effect upon *S. gregaria* ΔJ_V (-1.7 ± 0.6 $\mu L.h^{-1}$) when used at 0.1-50 mM (Fig. 4.6). In addition, MI had no effect upon rectal I_{SC} at either low or high concentrations (Fig. 4.7). LiCl is an inhibitor of the PI cycle, and its effects upon J_V and I_{SC} were studied using a 10 minute pulse of 10 mM LiCl applied to recta immediately before adding NCC (as described by Fournier 1991). LiCl had no effect upon NCC stimulated J_V (Fig. 4.6), did not change unstimulated I_{SC} nor alter NCC stimulated I_{SC} (Fig. 4.7). In addition when LiCl (1mM) was present in the incubating saline throughout the experiment there was no effect upon NCC stimulated ΔJ_V (3.9 ± 1.0 $\mu L.h^{-1}$ compared to 4.4 ± 0.5 $\mu L.h^{-1}$ for NCC alone; both n=11). However, the same LiCl treatment did increase NCC stimulated I_{SC} and V_t compared to control values (see Table 4.1.).

Table 4.1. Effect of 1mM LiCl upon electrical variables

	I_{sc} ($\mu eq.cm^{-2}.h^{-1}$)	V_t (mV)	R_t ($\Omega.cm^2$)	n
Control	1.4 ± 0.2	8.5 ± 0.8	265.4 ± 48.8	8
+ 2NCC	7.3 ± 0.9	20.9 ± 1.8	120.9 ± 18.9	
LiCl	2.7 ± 0.6	20.7 ± 5.1	272.2 ± 25.9	8
LiCl + 2NCC	12.8 ± 1.5*	52.9 ± 8.4*	150.0 ± 8.6	

* values are significantly different from the respective control values (Tukey test, $p < 0.05$).

V_t values are lumen side positive

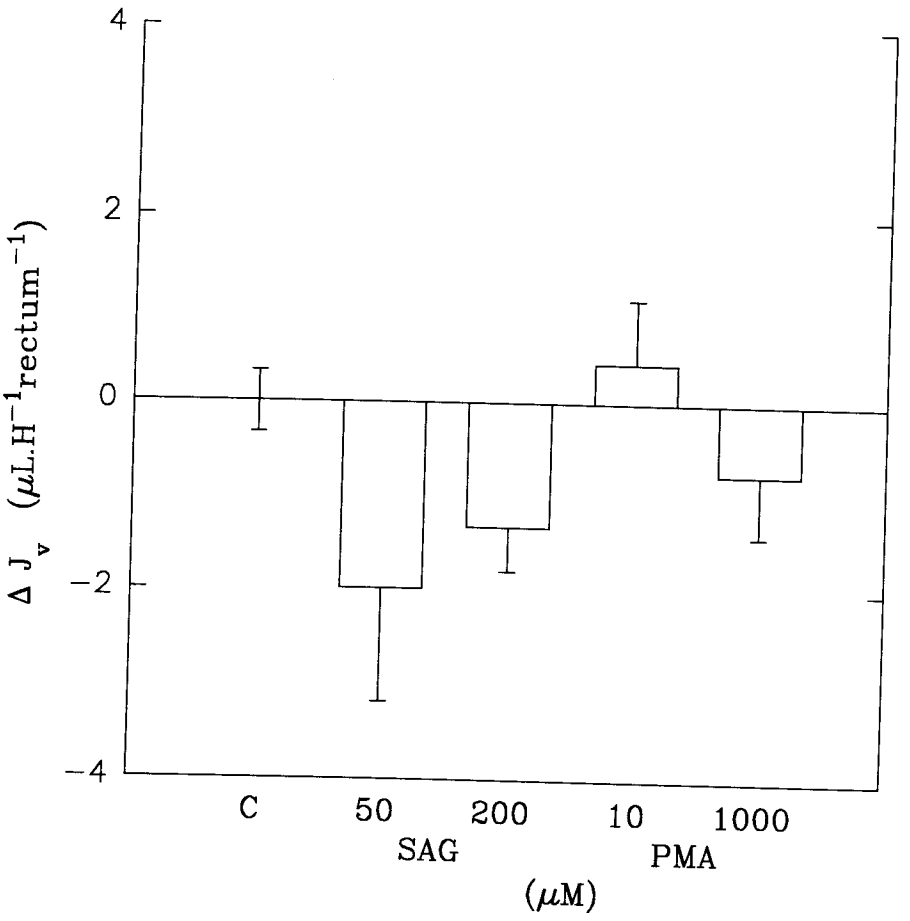


Fig. 4.2. Effect of SAG and PMA upon rectal J_V. Mean ± S.E. (n=5-11).

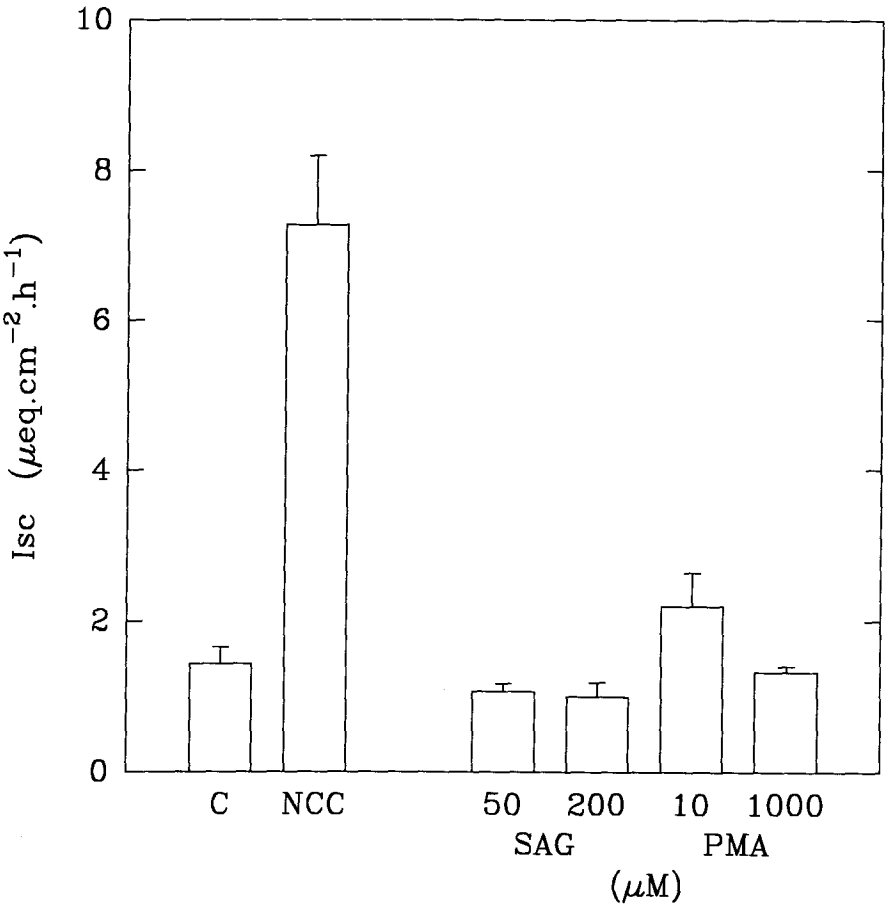


Fig. 4.3. Effect of SAG and PMA upon rectal I_{SC}. Mean ± S.E. (n=4-8).

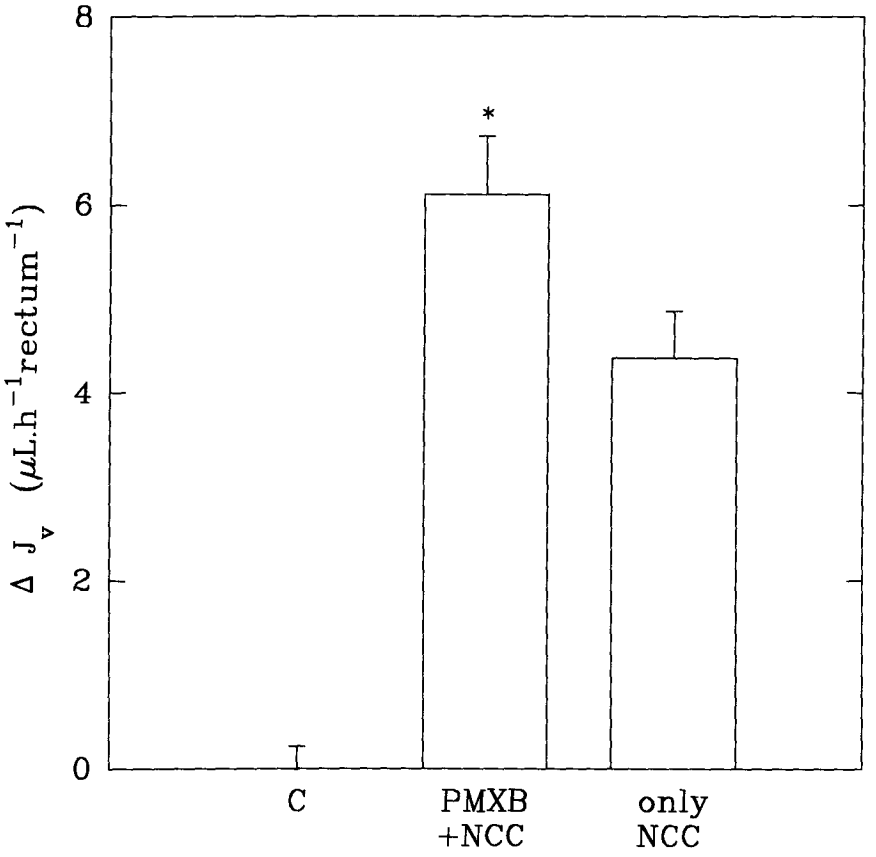


Fig. 4.4. Effect of a 100μM PMXB (1000 iu/mL.) upon 2 NCC stimulated J_V. Recta were pre-incubated with PMXB for 1 hour before stimulation with NCC. Mean ± S.E. (n=11-16). * PMXB+NCC value is significantly different from both control (C) and NCC values (Tukey test, $p< 0.05$).

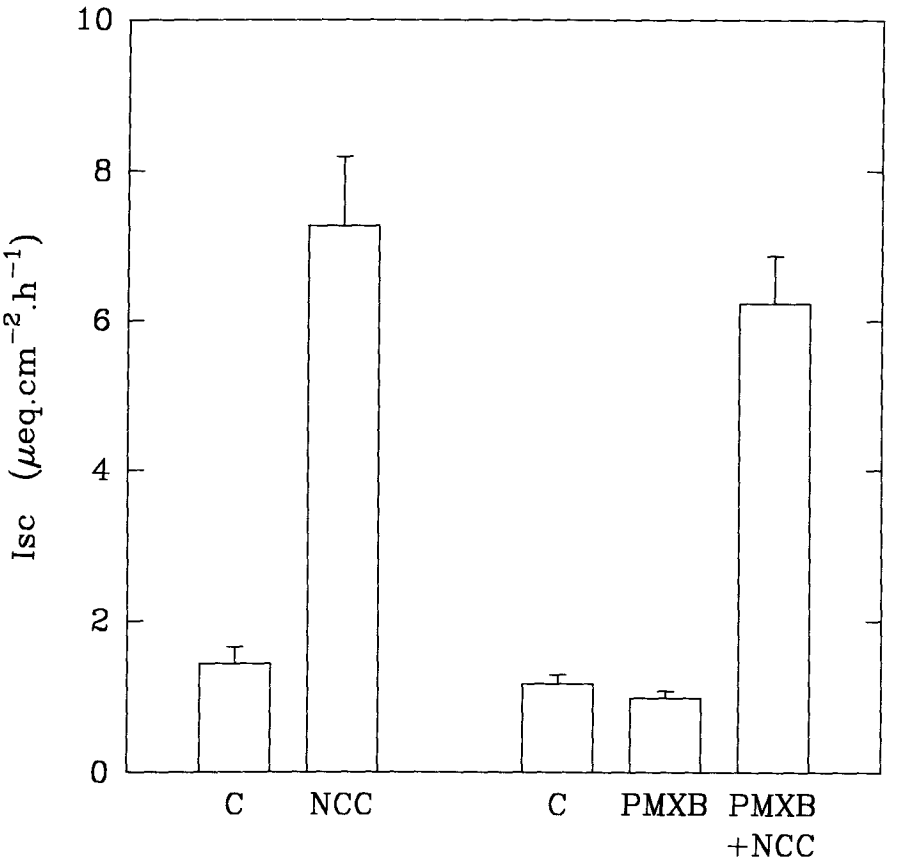


Fig. 4.5. Effect of 100 μM (1000 iu/mL.) PMXB upon rectal I_{SC}. PMXB was added bilaterally at steady-state, and then 30 minutes later 2 NCC were added. Mean ± S.E. (n=7-8).

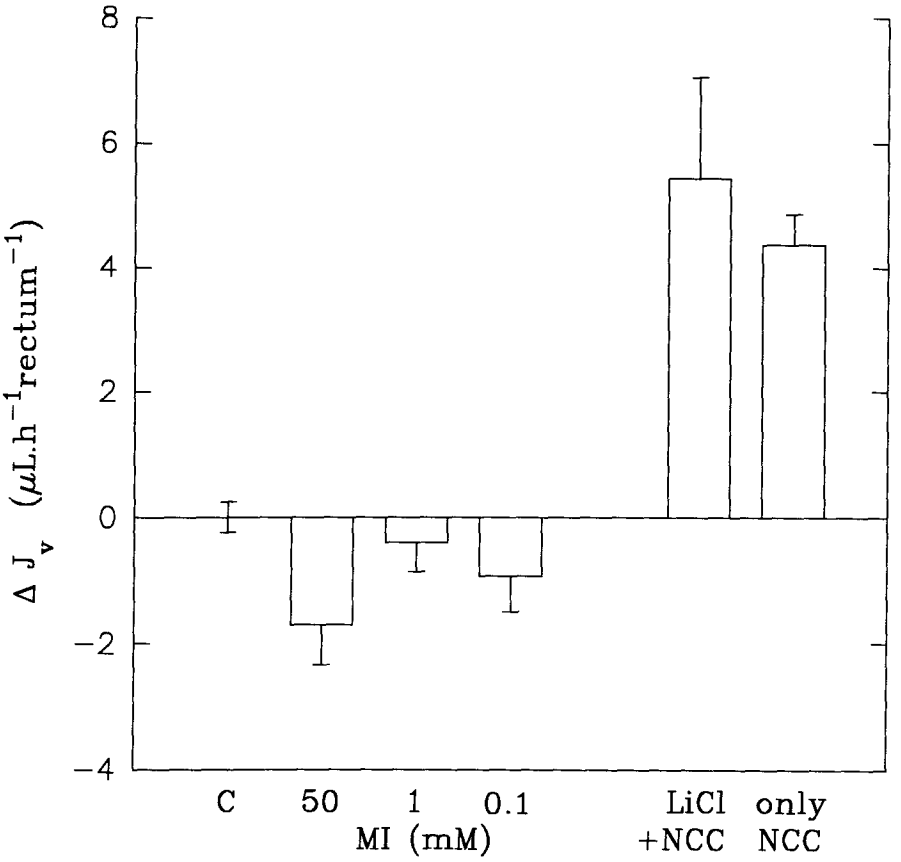


Fig. 4.6. Effects of *myo*-inositol (MI) and LiCl upon rectal J_V. In the LiCl experiment recta were exposed to a 10 minute pulse of 10 mM LiCl and then immediately stimulated with 2 NCC. Mean ± S.E. (n=6-11).

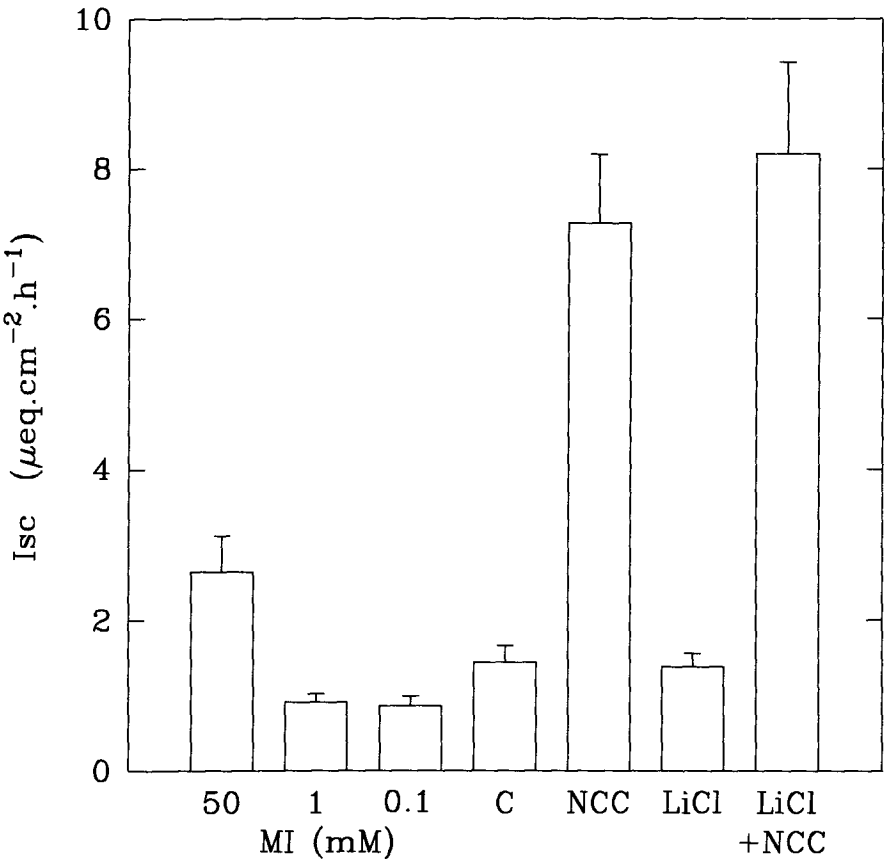


Fig. 4.7. Effects of MI and LiCl upon rectal I_{sc}. In the LiCl experiment recta were exposed to a 10 minute pulse of 10 mM LiCl and then immediately stimulated with 2 NCC. Mean ± S.E. (n=5-17).

***Locusta* experiments**

Locusta rectal sacs transported fluid at a slower rate overall than those of *Schistocerca*. *Locusta* rectal sacs transported 8.1 ± 0.9 $\mu L.h^{-1}$ and 6.4 ± 0.9 $\mu L.h^{-1}$ during hours one and two respectively (Fig. 4.8), while *Schistocerca* sacs transported 18.7 ± 0.8 $\mu L.h^{-1}$ and 12.7 ± 0.7 $\mu L.h^{-1}$ at the same time intervals. *Locusta* rectal sacs exhibited a small amount of tissue swelling (1.0 ± 0.5 μLh^{-1}.) during the first hour and a negligible tissue tissue volume change over the following two hours (Fig.4.8), similar to the response observed for *Schistocerca* sacs (Fig. 2.5). Addition of 50 mM MI to *Locusta* rectal sacs on the third hour had no effect upon J_V, while the addition of 1 mM DBcAMP during the fourth hour significantly increased J_V (Fig. 4.8).

Unstimulated electrical variables for *Locusta* recta (Table 4.2) closely resembled equivalent *Schistocerca* values (Table 2.1). The addition of 50 mM MI had no effect upon *Locusta* electrical variables, while subsequent additon of 1 mM DBcAMP stimulated I_{SC}, V_t and reduced R_t. *Locusta* electrical variables after addition of DBcAMP closely resemble *Schistocerca* electrical variables after stimulation with cAMP or NCC. Unfortunately further experiments using *Locusta migratoria* recta could not be performed because the colony perished.

Effect of Neuroparsins on *S.gregaria* recta

Neuroparsins applied at a very high concentration of 5CC gland equivalents had no effect upon *S. gregaria* rectal J_V or I_{SC} (Figs. 4.9 & 4.10). All preparations subsequently responded to *S. gregaria* NCC. Unfortunately we were unable to test the effect of Nps on *L. migratoria* recta since there were no animals available.

Table 4.2. Effect of MI and DBcAMP upon electrical variables of *L. migratoria* recta

(n=4)	I_{sc} ($\mu eq.cm^{-2}.h^{-1}$)	V_t (mV)	R_t ($\Omega.cm^2$)
Control	1.55 ± 0.42	11.68 ± 2.73	288.56 ± 17.10
50mM MI	1.44 ± 0.42	11.50 ± 3.50	289.01 ± 14.19
1mM DBcAMP	10.27 ± 2.17*	33.75 ± 5.25*	30.25 ± 13.44*

* value is significantly different from preceding value (paired t-test, $p< 0.05$).

V_t values are lumen side positive

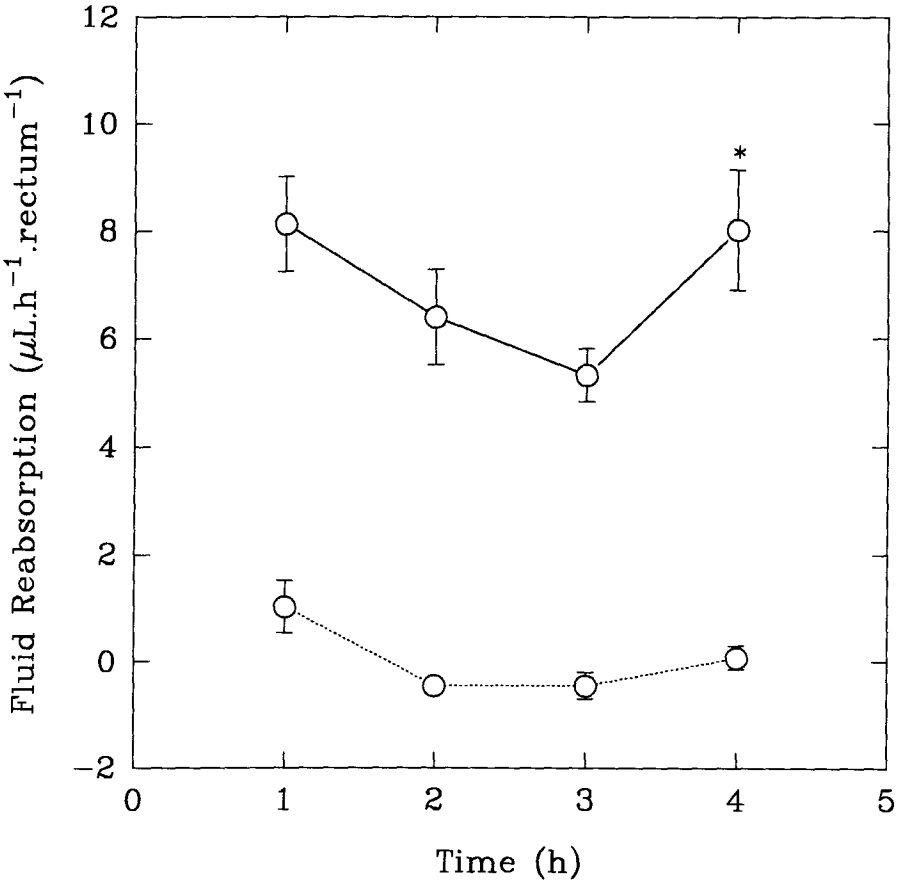


Fig. 4.8. Fluid reabsorption by *Locusta migratoria* everted rectal sacs. During the third hour 50 mM MI was added to the hemocoel side, then 1 mM DBcAMP was added to the hemocoel side during the fourth hour. Tissue swelling is represented by the dotted line. Mean ± S.E. (n=6). * fourth hour value is significantly different from third hour value ($p < 0.05$, paired t-test).

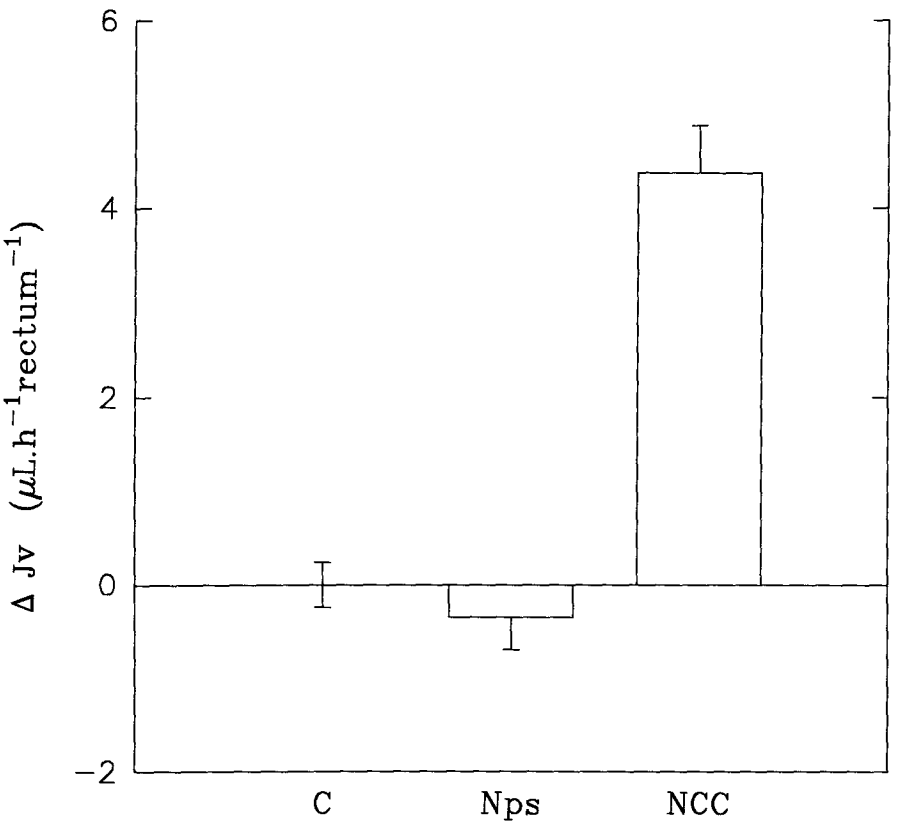


Fig. 4.9. Effect of Neuroparsins upon rectal *S. gregaria* J_V. Neuroparsins (Nps; 5CC gland equivalents) or 2NCC were added to the hemocoel side during the third hour. Mean ± S.E. (n=8-11).

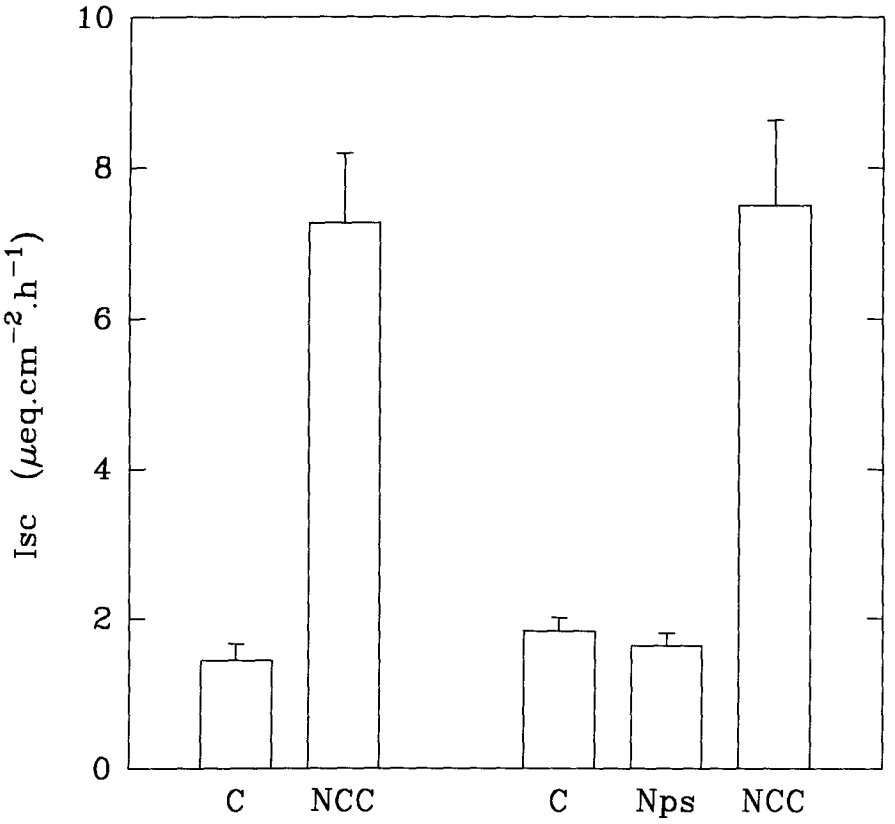


Fig. 4.10. Effects of Neuroparsins upon *S. gregaria* rectal I_{SC}. Neuroparsins (Nps; 5CC gland equivalents) was added to the hemocoel side of recta at steady-state, 30 minutes later 2NCC were then added to the same preparations. These results are compared to control preparations to which only NCC was added. Mean ± S.E. (n=8).

DISCUSSION

In this study, neither PKC nor the PI cycle were shown to play a stimulatory role in the control of *S. gregaria* rectal transport. However PKC may have a slight inhibitory effect upon rectal J_V, since the PKC inhibitor PMXB slightly increased NCC-stimulated J_V. It also seems that the PI cycle plays a slight inhibitory role in controlling rectal transport since long-term Li^+ exposure elevated NCC-stimulated I_{SC} and V_t. These results are markedly different from those with *L. migratoria* recta, where stimulants of PKC and the PI cycle were reported to maximally increase J_V, while specific inhibitors prevented stimulation of J_V with Nps (Fournier, 1990b).

Locusta migratoria rectal preparations were shown to possess similar electrical variables as *S. gregaria* recta, although *L. migratoria* recta transported fluid at slightly lower rates than *S. gregaria* recta, probably because *L. migratoria* recta are smaller. The patterns of *L. migratoria* J_V and tissue swelling with time were similar to patterns previously seen for *S. gregaria* recta. Although both *L. migratoria* J_V and I_{SC} responded to stimulation with Dibutyryl cAMP, *myo*-inositol had no effect. Since *myo*-inositol had no effect, it would seem that the PI cycle is not involved in controlling *L. migratoria* J_V or I_{SC}. This conclusion contradicts the findings of Fournier (1990b) who has reported that 50 mM *myo*-inositol can maximally increase *L. migratoria* rectal J_V. Finally, there was no cross-reactivity between *L. migratoria* neuroparsins and the *S. gregaria* rectal preparations, even though the same Nps sample was later shown to have significant fluid transporting activity on *L. migratoria* recta (J. Proux, personal communication). Since our lab was unable to demonstrate any transport activity with Nps on *S. gregaria* recta or ilea (J. Martin, unpublished results), we were unable to investigate the activation of signal transduction pathways by Nps using our bioassays.

It would initially appear that *L. migratoria* and *S. gregaria* recta are controlled by different antidiuretic peptides that employ separate signal transduction mechanisms.

However, Hérault and Proux (1987) showed that there is a factor in *L. migratoria* GCC that increases rectal J_V by elevating intracellular cAMP. In addition, *L. migratoria* CC homogenates have been shown to maximally stimulate *S. gregaria* rectal I_{SC}, suggesting that *L. migratoria* does possess factors with *S. gregaria* cross-reactivity (J. Marshall, unpublished results). This present study raises an important question: Is *L. migratoria* neuroparsins a true antidiuretic factor, or is its reported activity an artifact of the unusual bioassay procedure used to study rectal J_V? This unusual procedure was discussed in chapter 2, and involves the omission of Cl^- from the bathing saline during the first hour of rectal incubation. On the second hour, Cl^- was added back to the saline at the same time as the tissue is exposed to various treatments (e.g. neuroparsins, pharmacological agents). The re-introduction of Cl^- to the bathing medium combined with the action of the various treatments, including Nps, may trigger second messenger mechanisms that are involved with cell volume regulation, leading to apparent increases in rectal J_V. These experiments by Fournier's group need to be repeated using steady-state conditions, where Cl^- is present throughout the experiment, before neuroparsins can be considered a potential antidiuretic factor.

CHAPTER 5: GENERAL DISCUSSION

The findings of this study suggest that cAMP is the primary second messenger responsible for controlling rectal Cl^- and fluid transport in *Schistocerca gregaria*, and that other second messengers are also involved. The second messenger cGMP was also shown to have a partial stimulatory action in controlling rectal transport. The evidence for the role of Ca^{2+} in controlling rectal transport is interesting, suggesting that different cellular Ca^{2+} levels may have varying effects (e.g. stimulatory, inhibitory/negative feedback). Another interesting finding was that neither PKC nor the PI cycle, which are stimulated by PLC activation, were shown to have a role in stimulating rectal J_V or I_{SC}. Many of the results from this study are strikingly different from those previously reported, using the closely related species *L. migratoria* (Fournier, 1990b,1991). However, the unusual assay procedures used in these previous studies may be responsible for the observed differences.

There were some limitations of this study that may be overcome in future work. The first limitation was that a crude NCC aqueous extract was used to study the signal transduction mechanisms involved in controlling rectal J_V and I_{SC}. This crude extract may have contained multiple factors that control rectal transport. The use of individual purified peptides could simplify the interpretation of the results and allow the construction of more representative models. My justification for using crude NCC was the claim by Fournier and Girardie (1988) that neuroparsins is the only stimulant of rectal J_V in *L. migratoria* NCC and acts *via* the PI cycle to raise cytosolic Ca^{2+}. This is clearly not the case for *S. gregaria.* Unfortunately, no factor that stimulates *S. gregaria* rectal J_V and I_{SC} has yet been purified. Phillips et al. (1980) managed to partially purify CTSH from *S. gregaria* CC that was very unstable during HPLC, while ScgITP had no effect upon rectal J_V and only stimulated rectal I_{SC} to 40% of maximum at very high doses (Audsley, 1991). In addition, this study demonstrated that neuroparsins has no effect upon *S. gregaria* rectal I_{SC} or J_V.

Although another attempt to purify CTSH could be undertaken, a more promising approach may be to concentrate on signal transduction mechanisms involved with the stimulation of ileal transport by ScgITP. The advantages of using the ileum are: 1) it is a simple epithelium comprised of fewer cell types than the rectum; 2) there is no recycling of ions in the lateral intracellular spaces between the ileal epithelial cells 3) the major CC peptide that stimulates transport (ScgITP) has already been purified and partially sequenced.

Another limitation of this study was that the effects of NCC homogenate on second messenger levels were not monitored. The measurement of second messengers would provide direct evidence for the involvement of specific signal transduction pathways and confirm the proposed actions of pharmacological agents. Changes in tissue cAMP and cGMP levels could be measured using the cAMP binding protein technique and the cGMP radioimmunoassay to show that purified factors affect these pathways. These assays are both commercially available. In fact, Chamberlin and Phillips (1988) have already shown that crude CC homogenate elevated rectal cAMP and cGMP levels. The involvement of PLC-mediated events, namely the activation of the PI cycle and PKC, could also be monitored. The role of the PI cycle in the control of rectal transport could be investigated by loading cells with tritiated *myo*-inositol (a precursor of the PI cycle) and examining the amounts of labeled intermediates formed (e.g. $InsP_3$ and $InsP_4$) using standard HPLC methods (Kirk et al., 1990). Protrein kinase C enzyme activity could be measured using a standard assay technique described by Farese and Cooper (1990). The effect of antidiuretic factors on cytoplasmic Ca^{2+} could possibly be directly measured by using fura-2 spectrometry (see Hallett et al., 1990).

Lastly, most of the experiments conducted on *S. gregaria* recta could not be repeated using *L. migratoria*, because the animals were not available. A few experiments with *L. migratoria* recta were performed in this study but the results were completely different from Fournier's findings. For example Fournier (1990b) stated that myo-inositol maximally

stimulated rectal J_V in *L. migratoria*, whereas a similar experiment from this study showed no stimulatory effect of *myo*-inositol on *L. migratoria* rectal J_V. The only difference between these two experiments was the assay conditions. As previously stated, Fournier (1990b, 1991) used an unusual procedure that involved the incubation of the rectal tissue in a Cl^- free saline for the first hour of the experiment. Chloride was re-introduced on the second hour simultaneously with the exposure of the tissue to various treatments, either peptide factors or pharmacological agents. It would be interesting to see if the same results are obtained using a conventional incubation procedure.

The importance of the insect excretory system in maintaining hemolymph homeostasis is comparable to the role of the vertebrate nephron. Both of these systems eliminate toxic compounds and metabolic waste products, control blood volume and osmolarity, and are involved with acid-base regulation. The Malpighian tubules perform a function similar to that of the glomerular capsule and are responsible for the formation of the isosmotic primary urine. In vertebrates, the glomerular capsule and the endothelium form the endothelial capsular membrane that filters blood under pressure (60-100 mmHg) allowing the passage of fluid and ions across the membrane but blocking the entry of larger molecules such as proteins (see Tortora and Anagnostakos, 1984). Malpighian tubules actively secrete a primary urine that is rich in KCl and that contains most blood solutes. In contrast to the glomerulus, Malpighian tubule secretion is under direct endocrine control, with cAMP, $InsP_3$ and Ca^{2+} being implicated as second messengers. It has been shown that the ileum acts as the 'proximal tubule' of the locust excretory system, since it actively transports an isosmotic absorbate, is the major site of ammonia secretion, and is involved in acid-base regulation (Lechleitner, 1988; Peach, 1991; Thomson, 1990). In vertebrates, proximal tubule transport is stimulated by the steroidal hormone aldosterone. Aldosterone is released from the outer cortex of the adrenal glands, it then activates the synthesis of more Na^+ carrier proteins (Na^+/K^+ ATPases) and also increases the availability of ATP in the tubular epithelial cells (reviewed by Lote, 1987; Guyton, 1991). Locust ileal

transport is stimulated by a peptide factor from the insect's corpus cardiacum, ScgITP, which is thought to activate electrogenic Cl^- transport by elevating cellular cAMP levels. ScgITP also inhibits ileal acid secretion, but the adenylate cyclase signal transduction pathway is not involved (Audsley, 1991). Lastly the locust rectum is responsible for determining the final ion and water composition of excreta, and can selectively create strongly hypo- or hyper-osmotic urine. In the nephron, the loop of Henle, distal tubule and the collecting ducts are all required for the production of hypo- or hyper-osmotic urine. Both systems employ ion/solute recycling mechanisms to form hyper-osmotic urine, but this recycling is performed at different structural levels. The locust rectum can recycle solutes in the lateral channels between the principle epithelial cells. The late distal tubule and the collecting duct becomes permeable to water when antidiuretic hormone (ADH) is released by the posterior pituitary. ADH binds to the baso-lateral membrane of the target cells, elevating cAMP levels, leading to the fusing of vesicular structures with the apical membrane (see Lote, 1987; Guyton, 1991). These vesicles possess proteins with large water conducting channels. The locust rectum is also under hormonal control (e.g. CTSH) and from this study it appears that cAMP, cGMP and Ca^{2+} are all involved with the control of rectal ion and fluid transport.

Future studies should attempt to integrate the hormonal control mechanisms of the entire insect excretory system and address the following questions. Are these controlling factors actually liberated into the hemolymph, if so, where, when, and what are the sensory pathways controlling their release? Do multiple factors control transport in the same tissue/cell and if so, what are the combined effects of these factors? What conditions stimulate the release/activation of these factors; feeding, dehydration, satiation?. The answers to these questions and the ongoing purification and characterization of peptide factors, may eventually lead to the development of novel strategies to control insects that are deleterious to civilization.

REFERENCES

ALBARWANI, S.A. (1988). Effect of corpus cardiacum homogenates on absorption by the rectum of the house cricket (*Acheta domesticus*) *in vitro*. M.Sc. thesis, University of Southwestern Louisiana.

ALBERTS, B., BRAY, D., LEWIS, J., RAFF, M., ROBERTS, K. & WATSON, J.D. (1989). Molecular biology of the cell. Garland Publishing, New York. pp. 681-726.

ASTON, R.J. (1975). The role of adenosine 3',5'-cyclic monophosphate in relation to the DH of *Rhodnius prolixus*. J. Insect Physiol. 21, 1873-1877.

AUDSLEY, N. (1991). Purification of a neuropeptide from the corpus cardiacum of the desert locust which influences ileal transport. Ph.D. thesis, University of British Columbia, Vancouver.

AUDSLEY, N. & PHILLIPS, J.E. (1990). Stimulants of ileal salt transport in the desert locust. Gen. comp. Endocr. 80, 127-137.

AUDSLEY, N., McINTOSH, C. & PHILLIPS, J.E. (1992). Purification of a new neuropeptide from locust corpus cardiacum which influences ileal transport. Prog. Brain Res. 92, 157-162.

BALSHIN, M. & PHILLIPS, J.E. (1971). Active absorption of amino acids in the rectum of the desert locust (*Schistocerca gregaria*). Nature 233, 53-55.

BALSHIN, M. (1973). Absorption of amino acids *in vitro* by the rectum of the desert locust (*Schistocerca gregaria*). Ph.D. thesis. University of British Columbia, Vancouver.

BERRIDGE, M. J. (1986). Inositol phosphates as second messengers. Biochem. J. 220, 345-360.

BERRIDGE, M.J. & IRVINE, R.F. (1989). Inositol phosphates and cell signalling. Nature 341, 197-205.

BERRIDGE, M.J. & GUPTA, B.L. (1967). Fine-structural chamges in relation to ion and water transport in rectal papillae of the blowfly, *Calliphora*. J. Cell Sci. 2, 89-112.

BERRIDGE, M.J. (1983). Calcium as a second messenger. In Endocrinology of insects. Eds. H. Laufer and R. Downer. Alan R. Liss, New York. pp. 615-624.

BERRIDGE, M.J. & IRVINE, R.F. (1984). Inositol trisphosphate, a novel second messenger in cellular signal transduction. Nature 312, 315-321.

BERRIDGE, M.J. (1977). Cyclic AMP, calcium and fluid secretion. In "Transport of Ions and Water in Animals". B.L. Gupta et al. Eds. Acedemic Press, San Diego. pp. 225-238.

BEYENBACH, K.W. & PETZEL, D.H. (1987). Diuresis in mosquitos: role of a natriuretic factor. News Physiol. Sci. 2, 171-175.

BLACK, K., MEREDITH, J., THOMSON, B., PHILLIPS, J. & DIETZ, T. (1987). Mechanisms and properties of sodium transport in locust rectum. Can. J. Zool. 65, 3084-3092.

BODNARYK, R.P. (1983). Cyclic neucleotides. In Endocrinology of insects. Eds. H. Laufer and R. Downer. Alan R. Liss, New York. pp. 567-614.

BOURÊME, D., FOURNIER, B. MATZ, G. & GIRARDIE, J. (1989). Immunological and functional cross-reactivities between locust neuroparsins and proteins from cockroach corpora cardiaca. J. Insect Physiol. 35, 265-271.

BOURÊME, D., TAMARELLE, M. & GIRARDIE, J. (1987). Production and characterization of antibodies to neuroparsins A and B isolated from the corpora cardiaca of the locust. Gen. comp. Endocr. 67, 169-177.

CAZAL, M. & GIRARDIE, A. (1968). Contrôle humoral de l'equilibre hydrique chez Locusta migratoria migratorioides. J. Insect Physiol. 14, 665-668.

CHAMBERLIN, M.E. & PHILLIPS, J.E. (1983). Oxidative metabolism in the locust rectum. J. Comp. Physiol. 151, 191-198.

CHAMBERLIN, M.E. & PHILLIPS, J.E. (1988). Effects of stimulants of electrogenic ion transport on cyclic AMP and cyclic GMP levels in locust rectum. J. exp. Zool. 245, 9-16.

CHEUNG, W.Y. (1982). Calmodulin. Sci. Amer. 246, 48-56.

COAST, G.M., HAYES, T.M., & CHUNG, J-S. (1992). Effect of *Manduca sexta* diuretic hormone and realted peptides on isolated Malpighian tubules of the house cricket *Acheta domesticus*. J. exp. Biol. 162, 331-338.

COAST, G.M., WHEELER, C.H., TOTTY, N.F., PHILP, R.J. & GOLDSWORTHY, G.J. (1988). Isolation, characterization and synthesis of a diuretic peptide, AP-1 from the house cricket *Acheta domesticus*. Proc. Int. Cong. Ent. XVIII, 105.

COAST, G.M., CUSINATO, O., KAY, I. & GOLDSWORTHY, G.J. (1991). An evaluation of cyclic AMP as an intracellular second messenger in Malpighian tubules of the house cricket, *Acheta domesticus*. J. Insect Physiol. 37, 563-573.

COHEN, P. (1989). The structure and regulation of protein phosphatases. Annu. Rev. Biochem. 58, 453-508.

DE BESSÉ, N. & CAZAL, M. (1968). Action des extraits d'organes perisympathetiques et corpora cardiaca sur la diurese de quelques insectes. C. r. hebd. Seanc. Acad. Sci., Paris 266, 615-618.

FARESE, R.V. and COOPER, D.R. (1990). Lipid-related second messengers. In Peptide hormone action, a practical approach. Eds. K. Siddle and J.C. Hutton. Oxford University Press, New York. pp. 185-222.

FOGG, K.E., ANSTEE, J.H. & HYDE, D. (1990). Effects of corpora cardiacaextract on intracellular second messenger levels in Malpighian tubules of *Locusta migratoria* L. J. Insect Physiol. 36, 383-389.

FOURNIER, B., HÉRAULT, J.P. & PROUX, J.P. (1987) Antidiuretic factor from the nervous corpora cardiaca of the migratory locust: improvement on a an existing *in vitro* bioassay. Gen. comp. Endocr. 68, 49-56.

FOURNIER, B. (1990a). Functional cross-reactivities and transduction systems at the rectal level by antidiuretic peptides from coakroch and locust corpora cardiaca. J. Insect Physiol. 36, 537-544.

FOURNIER, B. (1990b). Neuroparsins induce phosphoinositide breakdown in the migratory locust rectal cells. Comp. Biochem. Physiol. 95B, 57-64.

FOURNIER, B. and DUBAR, M. (1989). Relationship between neuroparsins induced rectal fluid reabsorption & cyclic nucleotides in the migratory locust. Comp. Biochem. Physiol. 94A, 249-255.

FOURNIER, B. and GIRARDIE, J. (1988). A new function for the locust neuroparsins: stimulation of water reabsorption. J. Insect Physiol. 34, 309-313.

FOURNIER, B., VIRATELLE, O., HAMOUDI, N-E. & PROUX, J. (1992). Neurotransmitters and stimulation of fluid reasbsorption in migratory locust rectal cells. Gen. comp. Endocrinol. 87, 95-104.

FOURNIER, B. (1991). Neuroparsins stimulate inositol phosphate formation in locust rectal cells. Comp. Biochem. Physiol. 99B, 57-64.

GIRARDIE, J., BOURÊME, D. & GIRARDIE, A. (1987a). Production sited of the three neurosecretory proteins characterized in the corpora cardiaca of the migratory locust. Insect Biochem. 17, 29-36.

GIRARDIE, J., BOURÊME, D., COUILLAUD, F., TAMARELLE, M. & GIRARDIE, A. (1987b). Antijuvenile effect of Neuroparsin A, a new protein isolated from locust corpora cardiaca. Insect Biochem. 17, 977-983.

GIRARDIE, J., HUET, J.C., & PERNOLLET, J.C. (1990). The locust Neuroparsin A: sequence and similarities with vertebrate and insect polypeptide hormones. Insect Biochem. 20, 659-666.

GIRARDIE, J., GIRARDIE, A., HUET, J.C. & PERNOLLET, J.C. (1989). Amino acid sequence of locust Neuroparsins. FEBS Lett. 245, 4-8.

GIRARDIE, J., BOURÊME, D., COUILLAUD, F., TAMARELLE, M. & GIRARDIE, A. (1987). Anti-juvenile effect of neuroparsin A, a new protein isolated fronm locust copora cardiaca. Insect Biochem. 17, 977-983.

GOH, S.L. & PHILLIPS, J.E. (1978). Dependance of prolonged water absorption by *in vitro* locust rectum on ion transport. J. exp. Biol. 72, 25-41.

GOLDBARD G.A., SAUER, J.R. & MILLS, R.R (1970). Hormonal control of exretion in the American cockroach, II. Preliminary purification of a diuretic and an antidiuretic hormone. Comp. gen. Pharm. 1, 82-86.

GOODHUE, D. (1963). Some differences in the passage of food through the intestines of the desert and migratory locusts. Nature 200, 288-289.

GUYTON, A.C. (1991). Textbook of medical physiology. 8th edition. W.B Saunders, Philadelphia, PA. pp 286-319.

HALLETT, M.B., DORMER, R.L. and CAMPBELL, K. (1990). Cytoplasmic free calcium: measurement and manipulation in living cells. In Peptide hormone action, a practical approach. Eds. K. Siddle and J.C. Hutton. Oxford University Press, New York. pp. 115-150.

HANRAHAN, J.W. & PHILLIPS, J.E. (1983). Mechanism and control of salt absorption in the locust rectum. Am. J. Physiol. 244 (Regulatory and Integrative Comp. Physiol. 13): R131-R142.

HANRAHAN, J.W. & PHILLIPS, J.E. (1984a). KCl transport across an insect epithelium: I. Tracer fluxes and the effects of ion substitutions. J. Membrane Biol. 80, 15-26.

HANRAHAN, J.W. (1982). Cellular mechanisms and regulation of KCl transport across an insect epithelium. Ph.D. thesis, University of British Columbia, Vancouver.

HANRAHAN, J.W. & PHILLIPS, J.E. (1984b). KCl transport across an insect epithelium: II. Electrochemical potentials and electrophysiology. J. Membrane Biol. 80, 27-47.

HANRAHAN, J.W. & PHILLIPS, J.E. (1985). Further observations of the regulation of KCl absorption across locust rectum. J. exp. Biol. 116, 153-167.

HANRAHAN, J.W., MEREDITH, J., PHILLIPS, J.E. & BRANDYS, D.H. (1984). Methods for the study of transport and control in insect hindgut. In measurements of ion transport and metabolic rate in insects. Edited by T. J. Bradley and T. A. Miller. Springer Verlag, New York. pp 19-67.

HAYES, T.K., PANNABECKER, T.L., HINCKLEY, D.J., HOLMAN, G.M., NACHMAN, R.J, PETZEL, D.H. & BEYENBACH, K.W. (1989). In Chromatography and Isolation of Insect hormones and Pheromones. McCaffery, A.R. and Wilson, I.D., Eds. Plenum Press, New York. pp. 213-222.

HEILBRUNN, L.V. & WIERCENSKI, F.J. (1947). The actions of various cations on muscle protoplasm. J. Cell. Comp. Physiol. 29, 15-32.

HÉRAULT, J.P. & PROUX, J.P. (1987). Cyclic AMP: The second messenger of an antidiuretic hormone from the glandular lobe of the migratory locust corpora cardiaca. J. Insect Physiol. 33, 487-491.

HÉRAULT, J.P., GIRARDIE, J. & PROUX, J.P. (1985). Separation and characteristics of antidiuretic factors from the corpora cardiaca of the migratory locust. Int. J. Invert. reprod. Dev. 8, 325-335.

HIETTER, H., VAN DORSSELAER, A. & LUU, B. (1991). Characterization of three structurally-related 8-9 KDa monomeric peptides present in the corpora cardiaca of *Locusta*: a revised structure for the Neuroparsins. Insect Biochem. 21, 259-264.

HOLMAN, G.M., NACHMAN, R.J. & WRIGHT, M.S. (1990). Comparative aspects of insect myotropic peptides. In Chromatography and Isolation of Insect hormones and Pheromones. McCaffery, A.R. and Wilson, I.D., Eds. Plenum Press, New York. pp. 195-204.

HOLMAN, G.M., NACHMAN, R.J., SCHOOFS, L., HAYES, T.K., WRIGHT, M.S. & DeLOOF, A. (1991). The *Leucophaea maderae* hindgut preparation: a rapid and sensitive bioassay tool fot the isolation of insect myotropins of other insect species. Insect Biochem. 21, 107-112.

IRVINE, B., AUDSLEY, N., LECHLEITNER, R.A., MEREDITH, J., THOMSON, R.B. & PHILLIPS, J.E. (1988). Transport properties of locust ileum *in vitro*: Effects of cAMP. J. exp. Biol. 137, 361-385.

JONES, D.M., HARRISON, P.J., CLIFFORD, P.J., YIN, K. & ST. JOHN, M. (1991). A computer-based system for the aquisition of and display of continuous vertical profiles of temperature, salinity, fluorescence and nutrients. Wat. Res. 25, 1545-1548.

KATAOKA, H., TROETSCHLER, R.G., LI, J., KRAMER S.J., CARNEY, R.L. & SCHOOLEY, D.A. (1989). Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta*. Proc. natl. Acad. Sci., U.S.A. 86, 2976-2980.

KAY, I., WHEELER, C.H., COAST, G.M., TOTTY, N.F., CUSINATO, O., PATEL, M. & GOLDSWORTHY, G.J. (1991b). Characterization of a diuretic peptide from *Locusta migratoria*. Biol. Chem. Hoppe Seyler, 372, 929-934.

KAY, I., COAST, G.M., CUSINATO, O., WHEELER, C.H., TOTTY, N.F. & GOLDSWORTHY, G.J. (1991a). Isolation and characterization of a diuretic peptide from *Acheta domesticus*; evidence for a family of diuretic peptides. Biol. Chem. Hoppe Seyler, 372, 505-512.

KIM, I.S. & SPRING, J.H. (1992). Excretion in the house cricket (Acheta domesticus): relative contributions of distal and mid-tubule to diuresis. J. Insect Physiol. 38, 373-381.

KIRK, C.J., MORRIS, A.J. and SHEARS, S.B. (1990). Inositol phoshate second messengers. In Peptide hormone action, a practical approach. Eds. K. Siddle and J.C. Hutton. Oxford University Press, New York. pp. 151-184.

LAGUEUX, M., KROMER, E. & GIRARDIE, J. (1992). Cloning of a *Locusta* cDNA encoding neuroparsin A. Insect Biochem. Molec. Biol. 22, 511-516.

LECHLEITNER, R.A. (1988). Properties of fluid transport and control in hidgut of the desrt locust (*Schistocerca gregaria*). Ph.D. thesis, University of British Columbia, Vancouver.

LECHLEITNER, R.A. & PHILLIPS, J.E. (1989). Effect of corpus cardiacum, ventral ganglia and proline on absorbate composition and fluid transport by locust hindgut. Can. J. Zool. 67, 2669-2675.

LECHLEITNER, R.A. & PHILLIPS, J.E. (1988). Anion-stimulated ATPase in the locust rectal epithelium. Can. J. Zool. 66, 431-438.

LECHLEITNER, R.A., AUDSLEY, N. & PHILLIPS, J.E. (1989a). Antidiuretic action of cAMP, corpus cardiacum, and ventral ganglia on fluid absorption across locust ileum *in vitro*. Can. J. Zool. 67, 2655-2661.

LECHLEITNER, R.A., AUDSLEY, N. & PHILLIPS, J.E. (1989a). Composition of fluid transported by locust ileum: influence of natural stimulants on luminal ion ratios. Can. J. Zool. 67, 2662-2668.

LEWIS, S. (1971). Charge properties and ion selectivity of rectal intima of the desert locust. M.Sc. thesis. University of British Columbia, Vancouver.

LOTE, C.J. (1987). Principles of renal physiology. 2nd edition. Croom Helm, London. pp 109-126.

MADDRELL, S.H.P. (1963). Excretion in the blood-sucking bug. *Rhodnius prolixus* Stål. I. The control of diuresis. J. exp. Biol. 40, 247-256.

MADDRELL, S.H.P. (1964a). Excretion in the blood-sucking bug. *Rhodnius prolixus* Stål. II. the normal course of diuresis and the effect of temperature. J. exp. Biol. 41, 163-176.

MADDRELL, S.H.P. (1964b). Excretion in the blood-sucking bug. *Rhodnius prolixus* Stål. III. The control and release of diuretic hormone. J. exp. Biol., 41, 459-472.

MADDRELL, S.H.P. & GARDINER, B.O.C. (1980). The permeability of the cuticular lining of the of the insect alimentary canal. J. exp. Biol. 87, 227-237.

MADDRELL, S.H.P. & KLUNSUWAN, S. (1973). Fluid secretion *in vitro* preparations of the Malpighian tubules of the desert locust *Schistocera gregaria.* J. Insect Physiol. 19, 1369-1376.

MEREDITH, J. & PHILLIPS, J.E. (1988). Na-independent proline transport in the locust rectum. J. exp. Biol. 137, 341-360.

MORDUE, W. (1969). Hormonal control of Malpighian tubule and rectal function in the desert locust, *Schistocera gregaria.* J. Insect Physiol. 15, 273-285.

MORDUE, W. (1970). Evidence for the existance of diuretic and antidiuretic hormones in locusts. J. Endocrin. 46, 119-120.

MORDUE, W. (1972). Hormones and exretion in locusts. Gen. comp. Endocr. (suppl.) 3, 289-298.

MORDUE, W., MORGAN, P.J. & SIEGERT, K.J. (1985). Strategies for the isolation of insect peptides. Peptides, (Suppl.) 6, 407-410.

MOREAU, R., GORDOUX, L. & GIRARDIE, J. (1988). Neuroparsin: a new energetic neurohormone in the African locust. Archs. Insect Biochem. Physiol. 8, 135-145.

MORGAN, P.J. & MORDUE, W. (1985). Cyclic AMP and locust diuretic hormone action. Hormone induced changes in cAMP levels offers a novel method for detecting biological activity of uncharacterized peptides. Insect Biochem. 15, 221-231.

MORGAN, P.J., SIEGERT, K. & MORDUE, W. (1987). Preliminary characterization of locust diuretic peptide (DP-1) and another corpus cardiacum peptide (LCCP). Insect Biochem. 17, 383-388.

NACHMAN, R.J., ROBERTS, V.A., HOLMAN, G.M. & trainer, J.A. (1990). Progress in Comparative Endocrinology. Wiley, New York. pp. 60-66.

NISHIZUKA, Y. (1984). The role of protein kinase C in cell surface signal transduction and tumor production. Nature 308, 693-698.

NISHIZUKA, Y. (1986). Studies and perspectives of protein kinase C. Science 233, 305-312.

OLSSON, R.A. & PEARSON, J.D. (1990). Cardiovascular purinoreceptors. Physiol. Rev. 70, 761-845.

PEACH, J.L. (1991). Substrate utilization and ammonia secretion in the locust ileum. M.Sc. thesis, University of British Columbia, Vancouver.

PEACH, J.L. & PHILLIPS, J.E. (1991). Metabolic support of chloride-dependent short-circuit current across the locust (*Schistocerca gregaria*) ileum. J. Insect Physiol. 37, 255-260.

PHILLIPS, J.E. (1981). Comparative physiology of insect renal function. Am. J. Physiol. 241: R241-257.

PHILLIPS, J.E. (1983). Endocrine control of salt and water balance; excretion. In Endocrinology of Insects (Eds. H. Laufer and R. Downer). Alan R. Liss, New York. pp. 411-425.

PHILLIPS, J.E., HANRAHAN, J.W., CHAMBERLIN, M.E. & THOMSON, R.B. (1986). Mechanisms and control of reabsorption in insect hindgut. Adv. Insect Physiol. 19, 329-422.

PHILLIPS, J.E., AUDSLEY, N., LECHLEITNER, R.A., THOMSON, B., MEREDITH, J. & CHAMBERLIN, M.E. (1988). Some major transport mechanisms of insect absorptive epithelia. Comp. Biochem. Physiol. 90A, 643-650.

PHILLIPS, J.E., MORDUE, W., MEREDITH, J. & SPRING, J.H. (1980). Purification and characteristics of the chloride transport stimulation factor from locust corpora cadiaca: A new peptide. Can J. Zool. 58, 1851-1860.

PHILLIPS, J.E. (1964a). Rectal reabsorption in the desert locust, *Schistocerca gregaria* Forskål. I. Water. J. exp. Biol. 41, 15-38.

PHILLIPS, J.E. (1964b). Rectal reabsorption in the desert locust, *Schistocerca gregaria* Forskål. II. Sodium, potassium and chloride. J. exp. Biol. 41, 39-67.

PHILLIPS, J.E. (1964c). Rectal reabsorption in the desert locust, *Schistocerca gregaria* Forskål. III. The nature of the excretory system. J. exp. Biol. 41, 68-80.

PHILLIPS, J.E. & DOCKRILL, A.A. (1968). Molecular sieving of hydrophillic molecules by the rectal intima of the desert locust (*Schistocera gregaria*). J. exp. Biol. 48, 521-532.

PHILLIPS, J.E. (1982). Hormonal control of renal function in insects. Federation Proc. 41, 2348-2354.

PROUX, B., PROUX, J. & PHILLIPS, J.E. (1984). Antidiuretic action of corpus cardiacum factor (CTSH) on longterm fluid absorption across locust recta *in vitro*. J. exp. Biol. 133, 409-421.

PROUX, J.P, MILLER, C.A., LI, J.P., CARNEY, R.L., GIRARDIE, A., DELAAGE, M. & SCHOOLEY, D.A. (1987). Identification of an arginine vasopressin-like diuretic hormone from *Locusta migratoria*. Biochem. Biophys. Res. Commun. 149, 180-186.

RAABE, M. (1989). Recent developments in insect neurohormones. Plenum Press, New York.

RAMSEY, J.A. (1954). Active transport of water by the Malpighian tubules of the stick insect, Dixippus morosus (Orthoptera: Phasmidae). J. exp. Biol. 31, 104-113.

ROTHERMEL, J.D. & PARKER-BOTELHO, L.H. (1988). A Mechanistic and kinetic analysis of the interactions of the diastereoisomers of adenosine 3',5'-(cyclic) phosphorothioate with purified cyclic AMP-dependant protein kinase. Biochem. J. 251, 757-762.

SCHRAM, S.B. (1980). The basic book on liquid chromatography. Milton Roy. St. Petersburg, Florida. pp 1-9.

SMITH, R.J. & IDEN, S.S. (1979). Phorbol myristate acetate induced release of granule enzymes from human neutrophils: inhibition by the calcium antagonist, 8-(n,n-diethylamino)-octyl 3,4,5,-trimethoxybenzoate hydrochloride. Biochem. Biophys. Res. Comm. 91, 263-271.

SPRING, J.H. & CLARK, T.M. (1990). Diuretic and antidiuretic factors which act on the Malpighian tubules of the house cricket, *Acheta domesticus*. Prog. Comp. Endocrinol. 11, 559-564.

SPRING, J.H., HAZELTON, S.R. & ALHARTHY, S.A. (1988a). Diuresis in crickets: the differential control of secretion and reabsorption by hormones from the corpus cardiacum. Proc. Int. Congr. Ent. XVIII, 105.

SPRING, J.H., MORGAN, A.M. & HAZELTON, S.R. (1988b). A novel target for antiduretic hormone in insects. Science 241, 1096-1098.

SPRING, J.H. & PHILLIPS, J.E. (1980a). Studies on locust rectum. I. Stimulants of electrogenic ion transport. J. exp. Biol. 86, 211-223.

SPRING, J.H. & PHILLIPS, J.E. (1980b). Studies on locust rectum. II. Identification of specific ion transport processes regulated by corpora cardiaca and cAMP. J. exp. Biol. 86, 225-236.

SPRING, J.H. (1990). Endocrine regulation of diuresis in insects. J. Insect Physiol. 36, 13-22.

SPRING, J.H. & PHILLIPS, J.E. (1980c). Studies on locust rectum. III. Stimulation of electrogenic chloride transport by hemolymph. Can. J. Zool. 58, 1933-1939.

SPRING, J.H., HANRAHAN, J.W. & PHILLIPS, J.E. (1978). Hormonal control of chloride transport across locust rectum. Can. J. Zool. 56, 1879-1882.

STREB, H., IRVINE, A.F., BERRIDGE, M.J. & SCHULTZ, I. (1983). Release of Ca^{2+} from a nonmitochondrial intracellular store in pancreatic acinar cells by inositol 1,4,5 trisphosphate. Nature 306, 67-68.

STRYER, L. (1988). Biochemistry. W.H. Freeman and Company, New York.

TAKEMURA, H., HUGHES, A.R., THASTRUP, O. & PUTNEY, J.W. (1989). Activation of calcium entry by the tumor promoter thapsigargin in parotid acinar cells. J. Biol. Chem. 264, 122266-12271.

TAMARELLE, M. & GIRARDIE, J. (1989). Immunohistochemical investigation of locust neuroparsin-like substances in several insects, in some other invertebrates, and vertebrates. Histochemistry, 91, 431-435.

THASTRUP, O., CULLEN, P.J., DRØBAK, B.K., HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}/ATPase. Proc. Natl. Acad. Sci. USA 87, 2466-2470.

THOMSON, R.B. (1990) Acid-base transport in locust hindgut. Ph.D. thesis, University of British Columbia, Vancouver.

THOMSON, R.B. & PHILLIPS, J.E. (1985). Characterization of acid/base transport in an insect epithelium. Federation Proc. 44, 643.

THOMSON, R.B., THOMSON, J.M. & PHILLIPS, J.E. (1988). $NH4^{+}$ transport in acid -secreting insect epithelium. Am. J. Physiol. 254, R348-R356.

THOMSON, R.B. & PHILLIPS, J.E. (1992). Electrogenic proton secretion in the hindgut of the desert locust, *Schistocerca gregaria*. J. Membrane Biol. 125, 133-154.

TORTORA, G.J. & ANAGNOSTAKOS, N.P (1984). Principles of anatomy and physiology, 4th edition. Harper & Row, New York. pp.658-683.

VIETINGHOFF, U (1966). Einfluss der Neurohormone C und D auf die Absorptionsleitstung der Stabheuschrecke (*Carausius morosus*. Br.). Naturwissenschaften. 53, 162-163.

WALL, B.J. & OSCHMAN, J.L. (1970). Water and solute uptake by the rectal pads of *Periplaneta americana*. Am. J. Physiol. 218, 1208-1215.

WALL, B.J (1967). Effects of dehydration and rehydration on *Periplaneta americana*. Am. J. Physiol. 36, 23-34.

WHEELER, C.H. & COAST, G.M. (1990). Assay and characterization of diuretic factors in insects. J. Insect Physiol. 36, 23-34.

WIGGLESWORTH, V.B. (1931). The physiology of excretion in a blood sucking insect, *Rhodnius prolixus*. I. The composition of the urine. J. exp. Biol. 8, 411-427.

WILLIAMS, D., PHILLIPS, J.E., PRINCE, W.T. & MEREDITH, J. (1978). The source of short circuit current across the locust rectum. J. exp. Biol. 77, 107-122.

WILLIAMS, J.C. & BEYENBACH, K.W. (1983). Differential effects of secretagogues on Na and K secretion in the Malpighian tubules of Aedes aegypti (L.). J. comp. Physiol. 149, 511-517.

WILLIAMS, J.C. & BEYENBACH, K.W. (1984). Differential effects of secretagogues on the electrophysiology of the Malpighian tubules of the yellow fever mosquito. J. comp. Physiol. 154, 301-309.