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A STUDY OF THE NATRIURETIC PEPTIDE HORMONE SYSTEM
IN PLANTS

By

MADE PHARMAWATI, Ir. (Bogor Agricultural University)

Submitted in fulfilment of the requirements for the degree of
Master of Science

School of Biological and Chemical Sciences
DEAKIN UNIVERSITY
February, 1999
I certify that the thesis entitled

A STUDY OF THE NATRIURETIC PEPTIDE HORMONE SYSTEM IN PLANTS

submitted for the degree of

Master of Science

is the result of my own research, except where otherwise acknowledged, and that this thesis in whole or in part has not been submitted for an award, including a higher degree, to any other university or institution.

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ABSTRACT

In this study, both physiological and cellular effects are elicited by natriuretic peptides (NPs), a novel type of plant hormone. It was found that rat ANP (rANP) influenced stomatal movement in Tradescantia sp., where a significant increase in stomatal opening was observed in the presence of 1 μM rANP. Furthermore, this effect is mediated by cGMP, a (putative) second messenger of NPs. Two inhibitors of guanylyl cyclase, LY 83583 and methylene blue, inhibited rANP-induced stomatal opening. In contrast, stomatal opening is induced in a concentration dependent manner by the cell permeant cGMP analogue 8-Br-cGMP. In addition it was found, that like in animals, the secondary structure of rANP is essential for rANP responses. Linearised rANP is biologically inactive.

Since ANP elicit plant responses, an attempt was made to isolate NP analogues from plants. A protocol for partially purifying NP from plants was developed. It was found that two fractions eluted from an immunoaffinity chromatography column (0.5 M KCl eluted fraction and 0.75 M KCl eluted fraction) were biologically active.

The level of cGMP in response to NPs was also tested. It is suggested that the receptor of NP is specific since only 0.75 M KCl eluted fractions increased cGMP levels in Zea mays root stele tissue. rANP did not elicit an effect on cGMP levels in this tissue and LY 83583 did not affect this response. It is therefore argued that a plant specific biologically active NP system is present in the stele and it is predicted that NPs modulate solute movement in this tissue.

NPs also influence K⁺, Na⁺ and H⁺ fluxes in Zea mays root stele. Increase in both K⁺ and Na⁺ uptake were observed after 30 min., while H⁺ flux shifted immediately toward influx in the presence of both 0.5 and 0.75 KCl eluted fractions. Finally, a model is proposed for the effect of NPs on solute movement and its signalling system in plants.
ACKNOWLEDGEMENT

I acknowledge Australian Agency for International Development (AusAID) for providing an Australian Sponsored Training Scholarship (ASTAS).

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My high appreciation to Dr. Helen Irving from Department of Pharmaceutical Biology and Pharmacology, Victorian College of Pharmacy, Monash University for her guidance during cGMP experiment and for her support and helpful advice throughout my study. I also thank Dr. Evi Eitle from Department of Physiology, Melbourne University for her help in establishing cGMP extraction and radioimmunoassay.

I would like to thank Dr. Ian Newman from School of Mathematics and Physics and Dr. Sergey Shabala from School of Agricultural Science, University of Tasmania for allowing me to do experiment with their MIFE system. My grateful acknowledgement for their assistance and their patience during my study in their lab.

I also thank Gail Dyson for her assistance with the Mass Spectrometry, Dr. Anne Drake for her advice during my study, and all friends in SA 148 for sharing their respective skills in plant physiology and biochemistry.

I thank the academic staff, the technical staff and my fellow postgraduate students at the School of Biological and Chemical Sciences, Deakin University who provided their support and friendship throughout my study.

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Finally, I would like to thank my family for their love and support. My parents and my sister who always pray for me and encourage my study.
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<tr>
<td>ABA</td>
<td>abscisic acid</td>
</tr>
<tr>
<td>AdoMet</td>
<td>S-adenosyl-L-methionine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>rANP</td>
<td>rat atrial natriuretic peptide</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic-adenosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
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<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine 5'-triphosphatase</td>
</tr>
<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>BNP</td>
<td>brain natriuretic peptide</td>
</tr>
<tr>
<td>BR</td>
<td>brassinosteroid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin (albumin, bovine)</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celcius</td>
</tr>
<tr>
<td>[Ca^{2+}]_{cyt}</td>
<td>cytosolic free calcium</td>
</tr>
<tr>
<td>C_i</td>
<td>intracellular CO₂ concentration</td>
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<tr>
<td>cCMP</td>
<td>cyclic-cystidine 3',5'-monophosphate</td>
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<tr>
<td>CNP</td>
<td>C-type natriuretic peptide</td>
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<tr>
<td>D</td>
<td>dalton</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
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<td>DNA</td>
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<td>DTT</td>
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<td>ENOD 40</td>
<td>early nodulin</td>
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<td>ESMS</td>
<td>electrospray mass spectrometry</td>
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<td>g</td>
<td>gram(s)</td>
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<td>GA</td>
<td>gibberellin</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>GDPβS</td>
<td>guanosine-5'- (2-thiodiphosphate)</td>
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<td>cGMP</td>
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<td>GTP</td>
<td>guanosine 5'- triphosphate</td>
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<td>GTPγS</td>
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<td>GGPP</td>
<td>geranyl geranyl pyrophosphate</td>
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<td>8-br-cGMP</td>
<td>8'-bromo-cyclic-guanosine, 3'-5' monophosphate</td>
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<tr>
<td>h</td>
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</tr>
<tr>
<td>Hz</td>
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<td>horseradish peroxidase</td>
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<td>labelled iodine</td>
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<td>indole-3-acetic acid</td>
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<td>IAC</td>
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<td>IBMX</td>
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<tr>
<td>cIMP</td>
<td>cyclic-inosine 3',5'-monophosphate</td>
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<tr>
<td>IP</td>
<td>isopentenyl adenine</td>
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<td>jasmonate</td>
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<tr>
<td>μL</td>
<td>microliter(s)</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter(s)</td>
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<tr>
<td>LY 83583</td>
<td>6-amininoquinoline-5,8-quinone</td>
</tr>
<tr>
<td>μM</td>
<td>micromolar</td>
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<td>femtomole(s)</td>
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<tr>
<td>μmol</td>
<td>micromole(s)</td>
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<td>MB</td>
<td>methylene blue</td>
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<td>MeJa</td>
<td>methyl jasmonate</td>
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<td>MES</td>
<td>2-[N-morpholino]ethanesulfonic acid</td>
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<tr>
<td>MIFE</td>
<td>microelectrode ion flux estimation</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
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<tr>
<td>MOPS</td>
<td>3-[N-morpholino]propanesulfonic acid</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NAA</td>
<td>1-naphthalene acetic acid</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NP(s)</td>
<td>natriuretic peptide(s)</td>
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<tr>
<td>NPR</td>
<td>natriuretic peptide receptor</td>
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<tr>
<td>ΔpH</td>
<td>pH difference</td>
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<tr>
<td>pH_{cyt}</td>
<td>cytosolic pH</td>
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<td>P_{i}</td>
<td>inorganic phosphate</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<tr>
<td>irPNP</td>
<td>immunoreactive plant natriuretic peptide</td>
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<tr>
<td>PSK</td>
<td>phytosulphokine</td>
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<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
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<td>RIA</td>
<td>radioimmunoassay</td>
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<td>RNA</td>
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<td>mRNA</td>
<td>messenger RNA</td>
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<td>s</td>
<td>second(s)</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SE</td>
<td>standard error</td>
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<td>SNP</td>
<td>sodium nitroprusside</td>
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<td>TBS</td>
<td>tris buffered saline</td>
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<td>c-dTMP</td>
<td>cyclic-2'-deoxythymidine 3',5'-monophosphate</td>
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<td>TTBS</td>
<td>tween, tris buffered saline</td>
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<td>cUMP</td>
<td>cyclic-uridine 3',5'-monophosphate</td>
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<tr>
<td>v/v</td>
<td>volume/volume</td>
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<tr>
<td>V</td>
<td>volt(s)</td>
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<td>VNP</td>
<td>ventricular natriuretic peptide</td>
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<td>Description</td>
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<tr>
<td>w/v</td>
<td>weight/volume</td>
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<tr>
<td>Wm$^{-2}$</td>
<td>watt(s) per meter square</td>
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1. GENERAL INTRODUCTION

1.1. BACKGROUND

1.1.1. Definition of Plant Hormones

Plant hormones are defined as "a group of naturally occurring organic substances which influence physiological processes at low concentration" (Davies, 1995). Plant hormones coordinate growth, cell differentiation, and development. Both cell division and cell elongation are influenced by plant hormones. They also regulate responses to environmental stimuli such as stomatal movement (Davies, 1995).

1.1.2. Main Group of Plant Hormones

There are five groups of plant hormone, which are considered "classical" plant hormones. They are auxins, cytokinins, gibberellins, abscisic acid, and ethylene (Kende and Zeevaart, 1997).

Auxins

One example of an auxin is indole-3-acetic acid (IAA) which is the main chemical form of an auxin in plants. Other auxins include indole-3-butyric acid, phenyl acetic acid, and 4-chloro IAA, but their physiological action remains unclear (Kende and Zeevaart, 1997).

IAA is synthesised from tryptophan through several different routes such as indole-3-acetamide, tryptamine and indole-3-pyruvic acid (Bandurski, 1995). IAA biosynthesis can occur also through a non-tryptophan pathway. A recent study found that IAA could be synthesised from indole or indole-glycerol phosphate (Normanly et al., 1995) and a study using radioactive indole confirmed that IAA can be synthesised from indole (Rckoslavskaya and Bandurski, 1994).
IAA induces cell enlargement, cell division, root initiation and apical dominance, and also causes stomatal opening (Davies, 1995). The structure of IAA is shown in Fig. 1.

**Cytokinins**

Cytokinins are plant hormones, whose major function to promote cell division in combination with auxin, and also to induce morphogenesis and to inhibit apical dominance. Cytokinins also promote stomatal opening (Davies, 1995).

Naturally occurring cytokinins are synthesised from purine. They are N6-substituted adenine derivatives (McGaw and Burch, 1995). The first naturally occurring cytokinin was extracted from immature kernels of *Zea mays* and named zeatin or 6-(4-hydroxy-3-methylbut-trans-2-enylamino)purine. The structure of zeatin can be seen in Fig. 1. Other common cytokinins are benzyl adenine (BAP) and isopentenyl adenine (IP).

Incidentally, the most biologically active cytokinin is not found in plants, but was extracted from autoclaved herring sperm DNA and called kinetin or 6-(furfurylamino)purine (McGaw and Burch, 1995).

**Gibberellins (GAs)**

To date more than 100 GAs have been identified (Hisamatsu *et al.*, 1997). They are numbered from GA₁ to GA₁₄. GA₃ or gibberellic acid, is the most common form, and originally isolated from the fungus *Gibberella fujikuroi* and is known to have high activity in promoting growth of dwarf plants, dormant buds or dormant seed (Sponsel, 1995). A representative member of the GA family (GA₄) is shown in Fig. 1.

Gibberellins are synthesised from mevalonic acid to geranylgeranyl pyrophosphate (GGPP). GGPP is subsequently converted to an ent-kaurene through cyclization. Ent-kaurene then is converted to GA₁₂-aldehyde. The last stage is the conversion of GA₁₂-aldehyde to various GAs (Sponsel, 1995; Kende and Zeevaart, 1997).
The main functions of GAs are to promote cell elongation and cell division especially in stem tissues and to induce seed germination (Davies, 1995).

*Abscisic acid (ABA)*

ABA is plant hormone which promotes stomatal closure. When plants are water-stressed the cytosolic concentration of ABA increases and causes stomatal closure. ABA also inhibits shoot growth and induces storage protein synthesis in seed (for review see: Davies, 1995; Kende and Zeevaart, 1997).

In higher plants, ABA is derived from carotenoids, with xanotxin as an intermediate. Xanotxin is converted to ABA-aldehyde which then forms ABA. The formula of ABA is shown in Fig. 1.

*Ethylene*

Ethylene is a gaseous plant hormone, which affects shoot and root growth and differentiation. It influences adventitious root formation, leaf and fruit abscission, fruit ripening and flower and leaf senescence (Davies, 1995).

Ethylene is synthesised from methionine, which is converted to S-adenosyl-L-methionine (AdoMct). AdoMct then forms 1-aminocyclopropane-1-carboxylic acid from which ethylene is derived (McKeon et al., 1995). Fig. 1 shows the structure of ethylene.
Fig. 1. The structure of representative five "classical" plant hormone. Shown above are indole-3-acetic acid, ethylene, s-(+)‐abscisic acid, zeatin and gibberellin A1.

1.1.3. Non-traditional Plant Hormones

There are several other hormonal compounds which regulate plant growth and development. Creelman and Mullet (1997) have classified these compounds as "non traditional" regulators such as oligosaccharins, brassinolides, jasmonates, salicylic acid and polyamines.

Oligosaccharins consist of several groups such as pectic oligosaccharins and xyloglucan oligosaccharins. Pectic oligosaccharins inhibit auxin action and function as elicitors to initiate pathogen defense responses. The concentration of pectic oligosaccharins required for inhibition of auxin-induced functions is 10 to 100 fold lower than the concentration required to induce plant defense responses (Creelman and Mullet, 1997).

Xyloglucan oligosaccharins can inhibit auxin-, gibberellin-, proton-, and fussicoccin-induced growth and inhibit endogenously regulated growth at
concentration of 1 nM (McDougall and Fry, 1989). Some oligosaccharins can also promote stem elongation at higher concentration (McDougall and Fry, 1990).

The second group of non-traditional hormones are the brassinolides, which have a steroid-like structure. Other compounds, which have similar activity, are called brassinosteroids (BRs). They inhibit root growth, enhance gravitropism, inhibit leaf abscission, enhance resistance to stress, promote xylem differentiation and cause cell elongation (Sakurai and Fujioka, 1993).

The third group are the jasmonates. Jasmonate (JA) and methyl jasmonate (MeJA) are linolenic acid derived. The functions of JA in plants are varied and include inhibition of plant growth, seed germination, callus growth and photosynthesis. JA also promotes senescence, abscission, fruit ripening and pigment formation (Staswick, 1995). Another activity, which is influenced by JA, is plant insect and disease resistance. Creelman and Mullet (1997) reviewed several roles of JA action in plant defense. It is observed that there is an accumulation of JA in wounded plants as well as in plant cell culture treated with elicitors of pathogen defense. Genes encoding protease inhibitors are induced and their products help protect plants from insect damage. JA can also activate genes coding for antifungal proteins and modulate genes that express cell wall protein.

The fourth group are the polyamines. Polyamines are found in every plant cell in the form of diamine putrescine, triamine spermidine and tetramine spermine (reviewed by Galston and Sawhney, 1990). Exogenous application of polyamines affect growth and developmental processes such as vascular differentiation in tissue culture, root initiation, adventitious shoot formation and flower initiation. Polyamines prevent senescence-related processes such as chlorophyll degradation at millimolar concentrations (Galston and Sawhney, 1995).

Another group contains salicylic acid which is a plant phenolic compound. Salicylic acid inhibits ethylene biosynthesis and seed germination, blocks wound responses, induces rapid membrane depolarisation and collapse of transmembrane electrochemical potential, reduces transpiration and reverses ABA-induced stomatal closure (Raskin, 1995).
1.1.4. Plant Signal Peptides

There is now growing evidence that plants, like vertebrates, have peptide signalling systems. The three plant peptides established as signal molecules are systemin, ENOD 40 and phytosulphokines. (McGurl et al., 1992; Cooper and Long, 1994; Charon et al., 1997; Matsubayashi and Sakagami, 1996).

Systemin

Systemin is an 18 amino acid polypeptide known as a wound hormone. When systemin is applied in concentrations as low as fmols/plant, the defense genes in tomato leaves are activated (Schaller and Ryan, 1995).

Systemin is synthesised from a 200 amino acid precursor protein prosystemin (McGurl et al., 1992). The translocation pattern of systemin was found to be similar to the translocation of sucrose in plants. Moreover, a study using radiolabelled systemin showed that systemin was either stable in the phloem for long periods of time or was possibly continuously loaded into the phloem from the leaf source (Narváez-Vásquez et al., 1995).

ENOD 40

Early nodulins are peptide-signals involved in the early responses of (non-) legume hosts to nodulation factors and/or early nodulation steps. ENOD 40 is at least partially responsible for the induction of cortical cell divisions in the early phase of root nodule morphogenesis (Cooper and Long, 1994; Minami et al., 1996). During nodule formation there is a hormonal imbalance in the root cortex which is affected by ENOD 40 (Charon et al., 1997).

Recently, ENOD 40 was proposed to act as plant growth regulator by modifying phytohormone responses in legumes and in a non-legume resulting in reduced apical dominance (van de Sande et al., 1996). In tobacco protoplast the application of high concentration of 1-naphthalene acetic acid (NAA)
showed that the protoplast which express the ENOD 40 peptide can tolerate unusually high auxin concentrations.

Phytosulphokines

Phytosulphokines (PSKs) are disulphated pentapeptides that have mitogenic activities which can stimulate colony formation of rice protoplasts at concentrations > 10^{-8} M (Matsubayashi and Sakagami, 1996). It was also demonstrated with [^{35}S]PSK-\alpha binding assays that rice suspension cells contain both specific high- (K_d 10^{-8}) and low-affinity (K_d 10^{-7}) saturable PSK binding sites on the surface (Matsubayashi et al., 1997). PSK shows high ligand specificity and it was estimated that each cell contains \geq 10^5 binding sites for each type (Matsubayashi et al., 1997). Since PSK binding is not affected by either auxin or cytokinin it was suggested that novel signal transduction pathways may be involved in PSK-dependent activation of gene induction (Matsubayashi et al., 1997).

1.1.5. Natriuretic Peptides Hormones (NPs)

The NPs system was first discovered in animal (deBold et al., 1981). However, it has been reported that a NPs system also operate in plants and that plants may also contain biologically active NPs (Vesely and Giodano, 1991; Vesely et al., 1993; Gehring et al., 1996). However, the biological role(s) of such a system in plants remains to be elucidated.

NPs in vertebrates

NPs are known to play important roles in salt and water homeostasis (Brenner et al., 1990; Rosenzweig and Seidman, 1991). These peptides have natriuretic, kaliuretic and diuretic effects thus they increase urine volume, inhibit sodium reabsorption and increase glomerular filtration rate (Martin et al., 1990). NPs also inhibit synthesis of aldosterone in the adrenal gland and
inhibit the release of vasopressin and the secretion of renin (Rosenzweig and Seidman, 1991). Furthermore, NPs cause vasodilation (Vesely et al., 1987).

There are three major types of NPs. These are the A-type (Atrial Natriuretic peptide; ANP), the B-type (Brain Natriuretic Peptide; BNP) and the C-type (CNP). The classification is based on the genetic and structural homology of their amino acid composition (for review see: Rosenzweig and Seidman, 1991). To date, several novel members of the natriuretic peptide family have been isolated such as urodilatin (Schulz-Knappe et al., 1988) and ventricular natriuretic peptide (VNP) which was identified e.g. in eel cardiac ventricles (Takei et al., 1991) and trout ventricles (Takei et al., 1994).

*Synthesis and structure of NPs*

ANP was first isolated from cardiac atria (deBold et al., 1981) but was later also found in several other organs such as the lung, anterior pituitary, hypothalamus, brain, adrenal, kidney, spleen, stomach, intestine, liver and pancreas (Brenner, 1990; Vesely et al., 1991).

ANP is synthesised from a single precursor called preproANP which consists of 149 to 153 amino acids depending on the species. The removal of the amino acid signal sequence leads to the formation of a 126 amino acid peptide called proANP. Proteolytic cleavage of proANP results in the active form of ANP which consists of a 28 amino acid C-terminus called ANP99-128 (or ANP1-28) while the N-terminal, (amino acids 1-99) is believed to be inactive (Koller and Goeddel, 1992; Ruskoaho, 1992). The active form of ANP has a disulfide bridge between cysteines 105 and 120. All analogues of ANP have this common ring structure.

The second type of NPs is BNP. BNP was originally isolated from porcine brain (Sudoh et al., 1988), however, it was subsequently also found in heart, where the concentration of BNP is higher than in brain (Ogawa et al., 1995).

BNP is formed from a common precursor termed preproBNP which contain between 121 and 134 amino acids. Similar to ANP, the removal of a 25 residue signal peptide from a preproBNP precursor yields a proBNP from
which BNP is formed (Ruskoaho, 1992). BNP also has a 17 member ring, which is formed by a disulphide bond.

CNP, the third member in the NP family was first extracted from porcine brain (Sudoh et al., 1990). The major difference between CNP and the two other NPs (ANP and BNP) is that CNP does not have COOH tail. It is known that CNP is also found in rat pituitary glands (Ohta et al., 1994) and in dogfish heart (Bjenning et al., 1992). However, CNP transcripts in the rat and human heart cannot be detected using northern blot and/or polymerase chain reaction (Nakao et al., 1992). It seems likely that CNP functions as a neuropeptide and the major natriuretic peptide in the central nervous system.

The precursor of CNP is preproCNP, which consists of 126 amino acids and cleavage results in proCNP with 103 amino acids. ProCNP is then further processed to CNP (Nakao et al., 1992).

The structures of human ANP, BNP and CNP are shown in Fig. 2, and a summary of the synthesis of ANP is shown in Fig. 3.
Fig. 2. Structure of human natriuretic peptides, modified from Jamison et al. (1992). The dark circles indicate identical amino acids in the ring of the three peptides. The lines between two cysteines indicate the cysteine-cysteine disulphide bond.
Fig. 3. Summary of synthesis of ANP, adapted from Koller and Goeddel (1992).
Function and modes of action of NPs

ANP has several roles in salt-water homeostasis. These include increased glomerular filtration rate and inhibition of net Na\(^+\) reabsorption and vasopressin-mediated water reabsorption in the cortical and inner medullary portion of the collecting duct (for review see: Brenner et al., 1990).

The specific effects of ANP on Na\(^+\)/H\(^+\) exchange depend on cell type. ANP inhibits the amiloride-sensitive cation (Na\(^+\)) channel in the inner medullary collecting duct (IMCD) by reducing the open time of this channel (Light et al., 1989). In rat synaptosomes, ANP decreases Na\(^+\) uptake by inhibiting Na\(^+\)/H\(^+\) exchange (Kanda et al., 1992) and in vascular smooth muscle cells, Na uptake was decreased by inhibiting Na\(^+\)/H\(^+\) antiporter activity (Caramelo et al., 1994). ANP also inhibits Na\(^+\)/H\(^+\) exchange in human neuroblastoma NB-OK-1 cell through an amiloride-sensitive mechanism (Delporte, 1993). However, the opposite result was observed in human blood cells, i.e. erythrocyte, where ANP stimulates Na\(^+\)/H\(^+\) exchange (Petrov et al., 1994).

ANP was also found to reduce the activity of angiotensin II to stimulate Na\(^+\)/K\(^+\)-ATPase activity in rabbit IMCD (Scavone et al., 1995) while Na\(^+\)/K\(^+\)-ATPase is also deactivated in the single proximal tubule segment of the rat kidney (Aperia et al., 1994). This suggests that the effect of ANP on natriuresis is not only due to regulation of Na\(^+\) transport through amiloride sensitive cation channels, but is also due to active Na\(^+\) transport by tubular epithelial Na\(^+\)/K\(^+\)-ATPase (Scavone et al., 1995).

ANP also has kaliuretic effects. It was shown that proANP(1-30), proANP(70-98) and ANP significantly increased urinary potassium excretion in rat (Martin et al., 1990). In humans, all atrial natriuretic peptides (proANP1-30, 31-67, 79-98 and ANP) can promote potassium excretion and proANP79-98 is the strongest stimulator of potassium excretion (Vesely et al., 1994). In addition, ANP in adrenal glomerulosa cells lowered intracellular K\(^+\) through a Ca\(^{2+}\)-activatable K\(^+\)-conductive pathway (Ganz et al., 1994). ANP increased K\(^+\) conductance and hyperpolarized membrane voltages of rat mesangial cells (Cermak et al., 1996). However, opposite results were also reported on
potassium homeostasis. ANP increased K⁺ current in isolated right ventricular papillary muscle of guinea-pig (Kecskemeti et al., 1996) and was found to delay outward K⁺ current in chick embryo (Bkaily et al., 1993).

BNP, like ANP can cause vaso-relaxing, natriuretic and diuretic responses (Pidgeon et al., 1996; Rademaker et al., 1997), while CNP contributes to the regulation of vascular tone, growth and hormone release (Shimekake et al., 1994; Amin et al., 1996).

The effects of NPs are mediated through interaction with receptors (NPRs). NPR-A is a receptor for ANP but also responds to BNP. NPR-B is a CNP receptor. Both NPR-A and NPR-B are guanylyl cyclase linked receptors i.e. the interaction with the ligand leads to the increase of cyclic guanosine 3', 5'-monophosphate (cGMP) (Anand-Srivastava and Trachte, 1993).

NPR-A and NPR-B are single trans-membrane proteins that have extracellular domains with 44% homology. The intracellular part of these two receptors consists of 2 domains. The first is a guanylyl cyclase catalytic domain that consists of approximately 250 amino acids and the second has homology to kinases and comprises 280 amino acids (Koller and Goeddel, 1992).

NPR-C is the third receptor and contains a very short amino acid tail in its intracellular region. NPR-C does not signal via cGMP. The primary function of this receptor is to clear NPs from circulation. It is now understood that the interaction between ANP and NPR-C can inhibit adenylyl cyclase thus reducing cellular cyclic adenosine monophosphate (cAMP) (Koller and Goeddel, 1992). The structures and properties of NPR-A, NPR-B and NPR-C are shown in Fig. 4.

Recently another natriuretic peptide receptor, NPR-D, has been isolated and characterised from eel. The sequence of NPR-D shares 70% amino acid homology with the eel NPR-C. Like NPR-C, NPR-D has a short cytoplasmic tail and does not signal via guanylyl cyclase. However, their structures differ in that NPR-D has a disulphide-linked tetramer structure while NPR-C has a homodimeric configuration (Kashiwagi et al., 1995).

In ANP action cGMP acts as second messenger. In animal cells it was observed that the level of cGMP increased in response to ANP, e.g. in smooth
muscle cells (Anand-Srivastava and Trachte, 1993), in colon mucosa (Argenzio and Armstrong, 1993) and in rat brain synaptosomes (Kanda et al., 1992). Furthermore, a cell permeant analogue of cGMP, 8-Br-cGMP, can mimic the effect of ANP in these tissues.

Fig. 4. Structure and properties of NPR-A, NPR-B and NPR-C, modified from Köller and Goeddel (1992)
1.1.6 Effects of NPs in Plants

Many functions and modes of action of ANP in animals are well understood. In contrast, there is only scarce information on NPs system in plants. Since the primary function of NPs in animal appear to be the regulation of water and salt balance, and plants also carry out homeostatic processes, it can be speculated that similar systems operate in plants.

In plants, it has been demonstrated by Vesely et al. (1993) that exogenously added proANP (1-30), proANP (31-67) and proANP (78-98) at concentration < 5.9 pg/mL increased longitudinal movement of solute. The effects of these three peptides occur via three different ways. Firstly, the peptides caused an increase in solute uptake. Secondly, they increased solute flow up the stem of plants and thirdly increased transpiration by increasing the loss of water from the leaves.

Vesely et al. (1993) also reported that the addition of ANP at the same concentration did not increase either the rate of flow of solute, solute absorption or the rate of transpiration. However, it has recently been shown that rANP at concentration of ≥ 1 μM opened stomatal guard cells of Kalanchoe daigremontiana, Vicia faba and Tradescantia sp. (Gehring et al., 1996). In addition, rANP was found to bind specifically to plant membranes isolated from Tradescantia sp. leaves (Gehring et al., 1996).

In plants, ANP may also influence Na⁺ homeostasis. Gehring et al. (1996) observed that in the presence of high salt (100 mM NaCl) auxin-induced stomatal opening is inhibited. However, this inhibition can be partly overcome by the addition of the Na⁺ channel blocker amiloride as well as by rANP. This finding provides indirect evidence that rANP may inhibit Na⁺ uptake into guard cells thus preventing inhibition of stomatal opening by Na⁺.

Gehring et al. (1996) proposed that NPs are part of a multifactorial control system that regulates transpiration and solute movements in plants.
1.2. **RESEARCH SUMMARY**

1.2.1. Aims and Significance

The following research was conducted to investigate the physiological roles of NPs in plants. Furthermore, the research aimed to find evidence for the presence of endogenous NPs in plants. Specifically, the aims were:

1. To elucidate if the second messenger cGMP was, as in vertebrate system, involved in NP signalling in plants.

2. To isolate NP analogues from ivy (*Hedera helix*) leaves.

3. To characterise the physiological and biochemical effect of isolated NP analogues from plants.

This study which proposed to isolate and characterise a novel plant peptide hormone will thus challenge traditional concepts of plant hormone action and their physiological and biochemical mechanisms.

1.2.2. Summary

A protocol was developed in this study for the partial purification of NP analogues from *Hedera helix* leaves. This included homogenisation of leaf material in phosphate buffered saline, extraction with ethanol and diethylether, then size-separation in Sephadex G25-fine column and further purification by immunoaffinity column chromatography.

It was also investigated what effect ANP and NP analogue from *Hedera helix* (termed irPNP for immunoreactive plant natriuretic peptide) had on plant signalling and in particular the levels of endogenous cyclic guanosine 3’-5’ monophosphate (cGMP) in the presence and absence of guanylyl cyclase inhibitors methylene blue and LY 83583.

Since in vertebrates ANP signals via cGMP, experiments were designed to determine the effect of ANP and irPNP on cGMP levels in plants. Furthermore, in vertebrates, ANP regulates ion transport. It is predicted that in plants also ANP and irPNP will influence ion transport. This hypothesis was
tested by measuring cation (e.g. potassium, sodium) fluxes, net-uptake and ATPase activity in different biological preparations. A model is suggested to explain NPs effects on ion transport in *Zea mays* root stele tissue.
II. STOMATAL OPENING BY ANP AND KINETIN IS MEDIATED BY cGMP

2.1. INTRODUCTION

2.1.1. Aspects of Stomatal Physiology

Stomata or stomatal pores consist of two specialised cells - the guard cells. Each pore is surrounded by several neighbouring cells. Stomata are located on the (lower) surface of plants. The pore between those two guard cells is the place where gases and water diffuse. There are many signalling systems in stomatal guard cells that regulate the movement of those cells. The signals include light, humidity, carbon dioxide concentration and plant hormones (Kearns and Assmann, 1993).

Stomata open when protons (H\textsuperscript{+}) are pumped out of guard cells and thus hyperpolarise the plasma membrane. This causes K\textsuperscript{+} as well as Cl\textsuperscript{-} to be taken up and accumulate in vacuoles. Guard cells then take up water osmotically and start swelling (Kearns and Assmann, 1993).

The plasma membrane H\textsuperscript{+}-ATPase is critically involved in H\textsuperscript{+} extrusion. The addition of the H\textsuperscript{+}-ATPase inhibitor vanadate significantly reduces proton pumping e.g. in guard cells of fava beans (Becker et al., 1993; Kinoshita et al., 1995). The H\textsuperscript{+}-ATPase in guard cell function is particularly important indicated by H\textsuperscript{+}-ATPase dependent currents being higher than those in the mesophyll protoplasts (Lohse and Hedrich, 1992). Moreover the H\textsuperscript{+}-pumping activity (at equal proton concentrations) in isolated membranes from guard cells is higher than in those from mesophyll cells (Becker et al., 1993).

K\textsuperscript{+} enters the guard cells through specific (inward) K\textsuperscript{+} channels. These are K\textsuperscript{+}-selective ion channels gated by voltage and hence open at specific voltage to drive K\textsuperscript{+} influx. The stretch-activated (SA) K\textsuperscript{+} channels is an additional K\textsuperscript{+} channel which could involve in K\textsuperscript{+} uptake (Assmann, 1993).
In addition to ion channels, the production of malate from starch during stomatal opening increases the osmotic potential and also drives \( \text{H}_2\text{O} \) uptake (Assmann, 1993).

Stomata close if cations and anions are released and/or \( \text{Ca}^{2+} \) is taken up. The latter presumably depolarises the plasma membrane causing \( \text{K}^+ \) efflux through outward \( \text{K}^+ \) channels. The movement of \( \text{K}^+ \) out of the guard cells causes the release of \( \text{H}_2\text{O} \) and consequent shrinkage (Kearns and Assmann, 1993).

Anion channels also contribute to stomatal closing. Opening of plasma membrane anion channels will depolarize membranes and promote \( \text{K}^+ \) efflux (Assmann, 1993). There are several types of anion channels such as the R-type (rapid) which activate rapidly in response to depolarization. In response to hyperpolarization this channel will deactivate quickly (Schroeder and Keller, 1992). In contrast to the R-type, the S-type (slow) anion channels activate and deactivate slowly in response to voltage changes. Another anion channel is the SA anion channel which exhibits decreased closing times as stretch is increased (Cosgrove and Hedrich, 1991).

*Environmental signals*

Stomatal movement is influenced by environmental stimuli such as \( \text{CO}_2 \), light, temperature and pollutants. Blue and red light stimulate stomatal opening, and those stomatal responses are mediated by photosynthetic photoreceptors such as chlorophyll (Gorton *et al.*, 1993). However, there is much evidence for a blue light specific receptors in the guard cell which distinct from chlorophyll (Gorton *et al.*, 1993; Kearns and Assmann, 1993). Blue light induces \( \text{H}^+ \) efflux from guard cells through activation of \( \text{H}^+\text{-ATPase} \) at the plasma membrane, which in turn results in hyperpolarizing the plasma membrane. This condition is a prelude for opening.

The response of stomata to \( \text{CO}_2 \) is determined by intracellular concentration of \( \text{CO}_2 \) (\( \text{C}_i \)) and not by the concentration at the surface of the leaf or \( \text{CO}_2 \) concentration at the stomatal pore (Mott, 1988). Stomata open in
response to decreasing intracellular CO₂ and close if CO₂ the concentration increases.

Cᵢ is influenced by both assimilation rate and concentration of CO₂ at the leaf surface which means that Cᵢ may be the signal of stomatal opening caused by demand for CO₂ of the mesophyll (Morrison, 1998). The cellular mechanism of guard cells responses to CO₂ is still unclear.

*The role of plant hormones in stomatal pore size regulation*

Many plant hormones in particular abscisic acid (ABA) and auxin are known to influence stomatal movements. ABA stimulates K⁺ efflux from the guard cells which in turn leads to stomatal closure (MacRobbie, 1990). Increases in ^86⁰Rb⁺ (as a tracer for K⁺) efflux in response to ABA are due to the activation of the ^86⁰Rb⁺ efflux channel caused by the attachment of the ligand to the channels or a chemical change in the channel. The activation of one or more other channels depolarizes the plasmalemma in the areas where the outward K⁺ channel is activated. Ions are released from the vacuole which cause an increase in cytoplasmic K⁺ (MacRobbie, 1990). ABA also prevents stomatal opening by inhibiting inward K⁺ currents (Blatt, 1990).

The sensitivity of stomata to exogenously added ABA is variable (Trejo *et al.*, 1993; Tardieu and Davies, 1992). Trejo *et al.* (1993) showed that in isolated epidermis of *Commelina communis* 1 µM ABA caused complete stomatal closure. However, if pieces of whole leaves were floated in the same concentration of ABA, stomata open, and when ABA (1 µM) was applied to the detached leaves through the midrib, the stomatal response was intermediate. This is caused by the limitation of ABA accumulation in the epidermis by mesophyll. Therefore, stomatal responses to ABA depend on accumulation of ABA in the epidermis (Trejo *et al.*, 1993).

Another condition, which affects stomatal sensitivity to ABA is the epidermal water status (Tardieu and Davies, 1992). It was observed that in epidermal pieces of *Commelina communis* the stomatal mean aperture decreased if water potential was lowered and ABA concentration increased,
which suggest that epidermal water relations could modulate stomatal responses to ABA.

Auxin can cause both stomatal opening and stomatal closure, depending on the type and concentration of auxin. The response of stomatal guard cells to different concentration of NAA showed that the greatest opening occurs at concentration of 5 μM to 10 μM, and higher concentration (0.5 mM) caused inhibition (Marten et al., 1991). While another auxin, IAA, still induced stomatal opening at high concentration (10 μM to 1 mM) in epidermal strip of *Vicia faba* (Levitt et al., 1987).

The mechanism of auxin induced stomatal movement is not achieved by modulating activity of inward and outward K⁺ channels in the guard cells (Marten et al., 1991). Auxin stimulates the plasma membrane H⁺-ATPase which in turn leads to membrane hyperpolarization. Therefore, auxin concentrations that stimulate stomatal opening may be those which are more effective in stimulating the guard cell proton pump (Kearn and Assmann, 1993).

Jasmonates (jasmonic acid and methyl jasmonate) have similar effects as ABA on stomatal movement. Methyl jasmonate inhibited stomatal opening in abaxial epidermis of *Commelina benghalensis* at concentration as low as 10⁻⁶ M (Raghavendra and Reddy, 1987) and in olive leaves treated by 45 μM MeJA (Sanz et al., 1993). Jasmonic acid (JA) and MeJA were also found to promote stomatal closure in *Paphiopedilum supersuk* and *P. tonsur* through an intracellular alkalinization mechanism (Gehring et al., 1997). However, in barley, MeJA does not function as primary controller of stomatal movement as it had no significant effect on transpiration rate when used at concentration up to 100 μM (Horton, 1991).

Cytokinins also function as modulators of stomatal movements. In isolated epidermis of *Antheaphora pubescens*, it was found that kinetin caused stomatal opening (Incoll and Whitlam, 1977). Kinetin at a concentration of 100 μM also caused an increase in transpiration in barley (Horton, 1991).

The response of stomata to kinetin is dependent on the age of the leaves. Stomata of *Zea mays* from the young leaves do not respond to kinetin,
while the opening was widen in more mature leaves when kinetin was applied (Blackman and Davies, 1984).

There is an interaction between cytokinins and other plant hormones to affect stomatal movement. When kinetin and zeatin were applied to young leaves of Zea mays, there was no significant effect, but if these plant hormones were added in combination with ABA, they overcame ABA-dependent inhibition (Blackman and Davies, 1983). However, there is a competitive action of kinetin and ABA as in the presence of 1 μM ABA, kinetin up to 10 μM decreased stomatal closure, but kinetin could not overcome closure caused by 3 μM ABA (Radin et al., 1982).

Second messengers in stomatal movement

Ca\(^{2+}\) is known to be critical in stomatal guard cell signalling system. It acts as second messenger in stomatal signal transduction. It was observed that the movement of stomatal guard cells in response to IAA, kinetin and ABA in epidermal strips of orchid Paphiopedilum tonsurn is preceded by changes in guard cell cytosolic free Ca\(^{2+}\) ([Ca\(^{2+}\)\(_{cyt}\)]\) (Irving et al., 1992). Guard cell [Ca\(^{2+}\)\(_{cyt}\)]\) increases between 1.5 to 3-fold in response to 10-100 μM ABA which induces closure. IAA and kinetin also increase [Ca\(^{2+}\)\(_{cyt}\)]\) i.e. 1.5 to 2-fold and 1.3 to 2.5-fold respectively.

Besides [Ca\(^{2+}\)\(_{cyt}\)], cytosolic pH (pH\(_{cyt}\)) also acts as a signal in guard cell movement. During stomatal closure induced by ABA, pH\(_{cyt}\) increases by 0.04 to 0.3 pH unit, while during stomatal opening induced by IAA and kinetin, pH\(_{cyt}\) decreases by 0.2 to 0.4 unit and 0.1 to 0.4 unit respectively. Cytosolic pH was also increased in response to jasmonates up to 0.5 unit within 5 to 15 min. in Paphiopedilum, which lead to stomatal closure (Gehring et al., 1997).

Irving et al. (1992) have proposed that this antagonism in response to plant hormones in stomatal guard cells is reflected in their effect on pH\(_{cyt}\) and postulated that opening agents will lower and closing agents will elevate pH\(_{cyt}\).

Further evidence for a contribution of pH\(_{cyt}\) in guard cell signal transduction is that if pH\(_{cyt}\) in the guard cell is increased, it will activate the
outward rectifying K⁺ channel (Blatt, 1992; Blatt and Armstrong, 1993), while lower pHcyt will inhibit the outward current. The inward rectifying K⁺ channel is activated when pHcyt is decreased (Blatt, 1992) and blocked when pHcyt is elevated (Blatt and Armstrong, 1993).

Other signalling molecules involved in guard cell movement are the G proteins (GTP-binding proteins). Using the patch clamp technique, Fairley-Grenot and Assmann (1991) showed that G-proteins modulate inward K⁺ current through K⁺ selective ion channels in the plasma membrane. GDPβS increases inward K⁺ currents, while GTPγS decreases inward current in Vicia faba guard cell protoplasts. The G-protein regulators, cholera toxin and pertussis toxin decrease inward K⁺ current (Fairley-Grenot and Assmann, 1991), but Lee et al. (1993) found that the G-protein regulator pertussis toxin promotes stomatal opening in intact guard cell of Commelina communis. This means G-proteins are involved both the stimulation and inhibition of inward K⁺ currents.

There is little information about cyclic-adenosine, 3'-5' monophosphate (cAMP) in plants. However, several lines of evidence indicate that cAMP may play a role in ion channel modulation in plants. In mesophyll cells of Vicia faba, cAMP can modulate outward K⁺ channels (Li et al., 1994). Furthermore, exogenously added cAMP and several agents capable of stimulating adenyl cyclase activity or increasing levels of endogenous cAMP can enhance stomatal opening in the light and promote opening in the dark (Curvetto and Delmastro, 1990).

2.1.2. Aim

The aim of this study was to confirm the effect of ANP and kinetin on stomatal opening and to study their signalling system. Specifically, it was examined if ANP- and kinetin-dependent stomatal opening is mediated by cGMP. The study also aimed at testing if the secondary ring structure of ANP is essential for ANP activity in plants.
2.2. MATERIALS AND METHODS

2.2.1. Materials

Kinetin (6-furfurylaminopurine), methylene blue (MB : 3,7-bis[Dimethylamino]phenazothionium chloride) and 8-bromo-cyclic-guanosine-3',5'-monophosphate (8-Br-cGMP) were purchased from Sigma Co. (St Louis, MO), LY 83583 (LY; 6-anilinoquinolinc-5,8-quinone) was obtained from Calbiochem (La Jolla, USA), rat-28 ANP (H-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH) was synthesised by Auspep (Parkville, Victoria, Australia). All other chemicals used were commercially available and Analytical Grade reagents unless otherwise stated. They were mainly purchased from Sigma Co. (St Louis, MO) and from Merck Pty Ltd. (Kilsyth, Vic).

2.2.2. Plant Material, Stomatal Measurement and Data Analyses

*Tradescantia* sp. was grown in garden soil in a growth chamber in incandescent light (12 h/day) at constant temperature of 22°C. Young and fully expanded leaves were cut (2 mm x 5 mm). Each leaf segment was rinsed and submerged at 20-25°C in "opening buffer" {10 mM MES (pH 6.3), 50 mM KCl, 1 mM MgCl2 and 100 μM CaCl2}. Leaf segments were treated in microtiter plate wells at 20-25°C under incandescent light (λ=430 nm at 35 W/m²) for ≥ 30 min. The aperture of ≥ 30 stomata from 3 separate segments and 3 different location in each segment for each treatment were measured under the microscope with a calibrated ocular micrometer. The results then were analysed by Student's t-test to measure the statistical significance between data sets.
2.2.3. Reduction and S-carboxymethylation of rat ANP (rANP)

The reaction was performed essentially as described previously by *Misono et al.*, (1984). One hundred μg of rANP were taken up in 200 μL of 0.1 M Tris-HCl (pH 8.5) containing 5 M guanidine hydrochloride prior to adding 20 μL of 0.5 mM dithiothreitol. The mixture was flushed with nitrogen and incubated in a sealed vessel at 30°C for 30 min. Then 20 μL of 0.5 M iodoacetic acid was added and the mixture flushed with nitrogen prior to incubation in the dark at 30°C for 30 min. Then 10 μL of β-mercaptoethanol was added to react with the excess iodoacetic acid. The reaction mixture was dialysed overnight against 1% acetic acid or H₂O.

2.2.4. Molecular Masses Determination

Molecular masses of rANP and the reduced rANP were established by electrospray mass spectrometry (ESMS) using a Micromass Platform (MassTech, Melbourne 3186, Australia) single quadrupole mass spectrometer. The carrier reagent was acetonitrile:water (60:40) flowing at 10 μL per min. Spectra were acquired using a cone voltage of 70 V.

2.3. RESULTS

2.3.1. Effect of rANP, Kinetin and eGMP on Stomatal Opening

Rat ANP and kinetin both at a concentration of 1 μM significantly increase stomatal aperture of *Tradescantia* sp. (p < 0.02). Fig. 5, column 5 and 9 show the opening effect of ANP, while column 3 and 7 show the effect of kinetin. As a control for the light effect, the measurement of stomatal aperture was done to *Tradescantia* sp. leaf segments before and after the 30 min. illumination regime. As can be seen in Fig. 5, column 1 and 2, the illumination increases mean stomatal aperture significantly (p < 0.05). However the
treatment with rANP as well as kinetin increase stomatal aperture further than the illuminated control level. An open stomatal pore of \textit{Tradescantia} sp. in the presence of 1 \mu M ANP and the untreated stoma are presented in Fig. 6.

![Stomatal aperture graph](image)

Fig. 5. Mean stomatal aperture in \mu m in response to \geq 30 min. exposure to kinetin and rANP in the presence and absence of the guanylyl cyclase inhibitors MB and LY.

Column 1 represents the mean pre-illumination stomatal aperture and column 2 shows the effect of light on the aperture. Responses to 1 \mu M kinetin (columns 3 and 7), 1 \mu M kinetin and 10 \mu M MB (column 4), 1 \mu M kinetin and 20 \mu M LY (column 8), 1 \mu M rANP (columns 5 and 9), 1 \mu M rANP and 10 \mu M MB (column 6), and 1 \mu M rANP and 20 \mu M LY (column 7) are compared with control pre-illumination (column 1) and post-illumination (column 2) apertures. Each bar represents the mean of \geq 60 stomatal measurements from three leaf segments and the error bars show standard errors (SE).
Fig. 6. The open and close stomatal pore of *Tradescantia* sp. A is the open stoma in the presence of 1 μM ANP and B is untreated stoma (400 x).

The cell permeant cGMP analogue 8-Br-cGMP increases stomatal opening, and the effect of cGMP to open stomata is concentration dependent. Fig. 7 demonstrates the opening of stomata in the presence of different concentration of cGMP. Results are expressed as percentage increase over untreated control.

The maximal opening was reached at the highest tested concentration of 8-Br-cGMP i.e. 100 μM. As the concentration of 8-Br-cGMP was decreased, the stomatal aperture also decrease, and concentration ≤ 100 nM did not lead to significant aperture changes (p > 0.05).

Fig. 7 also shows the effect of 8-Br-cGMP in the presence of ABA. The effect of 8-Br-cGMP to induce stomatal opening is completely prevented by 1 μM ABA.
Fig. 7. Mean stomatal aperture increase (%) in response to 30 min. exposure to decreasing (100 μM, 10 μM, 1 μM, 0.10 μM and 0.01 μM) concentrations of 8-Br-cGMP in the presence (□) or absence (●) of 1 μM ABA (n > 60). Error bars indicate SE.

2.3.2. Effect of Guanylyl Cyclase Inhibitors on rANP and Kinetin Induced Stomatal Opening.

The effect of rANP and kinetin to induce increases in stomatal aperture are prevented by the guanylyl cyclase inhibitors methylene blue (MB) and LY 83583. This inhibitory effect is shown in Fig. 5. MB at concentration of 10 μM or LY 83583 at concentration of 20 μM significantly (p < 0.02) reduce the mean aperture of stomata mediated by rANP and/or kinetin. The mean stomatal aperture in this particular treatment is lower than in the illuminated control. An additional experiment was done by adding MB and LY 83583 alone and under similar light conditions. Fig. 8 shows that MB and LY 83583 alone reduce light induced opening.
Fig. 8. Effect of MB and LY 83583 on illuminated stomata. Column 1 shows stomatal aperture after illumination, column 2 and 3 represent the effect of MB and LY 83583. Bars represent the mean of > 60 stomatal measurements from three leaf segments and the error bars show standard errors (SE).

The inhibitory effect of MB and LY 83583 on kinetin induced stomatal opening is completely reversible. When the leaf segments are treated with MB or LY 83583 in the presence of kinetin, then rinsed three times for five min. each with three volumes of inhibitor free buffer followed by exposure to 1 μM kinetin, opening could be restored (Fig. 9). The same experiment for ANP gave similar results (Fig. 10). This would suggest that the effect of the inhibitors is reversible under the described experimental conditions.
Fig. 9. Reversibility of MB and LY effects on kinetin induced stomatal opening. Column 1 represents the illuminated control aperture, columns 2 and 5 are the mean aperture induced by 1 µM kinetin and column 3 and 6 show the aperture in response to 1 µM kinetin in the presence of 10 µM MB (3) and 20 µM LY respectively (6). Column 4 shows a 1 µM kinetin induced aperture after 90 min. MB treatment followed by three 5 min. rinses in 3 volumes of MB free buffer. Column 7 represents mean apertures induced by 1 µM kinetin after 90 min. LY treatment followed by three 5 min. rinses in 3 volumes of LY free buffer. Bars represent the mean of > 60 stomatal measurements from three leaf segments and the error bars show standard errors (SE).
Fig. 10. Reversibility of MB and LY effects on ANP induced stomatal opening. Column 1 represents the illuminated control aperture, columns 2 is the mean aperture induced by 1 μM ANP and column 3 and 5 show the aperture in response to 1 μM ANP in the presence of 10 μM MB (3) and 20 μM LY respectively (5). Column 4 shows a 1 μM ANP induced aperture after 30 min. MB treatment followed by three 5 min. rinses in 3 volumes of MB free buffer. Column 6 represents mean apertures induced by 1 μM ANP after 90 min. LY treatment followed by three 5 min. rinses in 3 volumes of LY free buffer. Bars represent the mean of > 60 stomatal measurements from three leaf segments and the error bars show standard errors (SE).

2.3.3. Effect of Linearised rANP on Stomatal Opening

Fig. 11 provides evidence that the rANP effect is crucially dependent on the secondary structure of the molecule. In its native form (N) the 28-mer with a molecular weight of 3060 (Fig. 11b.) is circularised due to a disulfide bond formed between the cysteines in position 7 and 23 (Fig. 11a.). Reduction and S-carboxymethylation has linearised (L) the molecule (Fig. 11a.) and increased the molecular mass to 3181 (Fig. 11b.).

As can be seen in Fig. 11c that the native form (N-form) of rANP promotes significant stomatal opening. The L-form when applied to Tradescantia sp. leaf segments shows no biological activity at the same concentration.
Fig. 11. Dependence of the biological activity on the secondary structure of the rANP molecule. Schematic representation of the molecule in its native (N) form and in its linearised form (L) after S-carboxymethylation (a). Electrospray mass spectra determining the molecular masses of the N- and L-form of rANP. The x-axis signifies the ion count in % (b). Stomatal opening in the control (C), after treatment with 1 μM rANP in the N-form (N) and 1 μM rANP in the L-form (L) (c).
2.4. DISCUSSION

The data from Figs. 5 shows that the synthetic plant hormone kinetin opens stomata in *Tradescantia* sp. at concentration of 1 μM. Furthermore, it confirms induction of opening by the animal peptide hormone rANP. Gehring *et al.* (1996) have demonstrated that rANP opens stomatal guard cell in a concentration dependent manner.

The classic plant hormones auxin and cytokinins, that have been shown to promote stomatal opening are relatively small molecules (MW < 400), auxin being synthesized from tryptophan or indol and kinetin being an adenine derivative. While ANP, which used in this study is an animal peptide hormone. ANP is the first peptide hormone that has been reported to affect stomatal guard cells and conceivably the first animal peptide hormone with biological activity in plants.

To date, there are several lines evidence to suggest that plants have peptide signaling systems e.g. the presence of *ENOD* 40 and sytemin. The fact that rANP contributes to regulating plant and in particular stomatal responses at low concentrations also suggest the presence of specific NP receptors in plants.

In animals, rANP signals via cGMP. rANP increases intracellular cGMP levels and 8-Br-cGMP mimics the effect of rANP in animals. Therefore, experiments were designed to find evidence for cGMP effects and cGMP dependent signalling by kinetin and rANP, in stomatal guard cells. The cell permeant cyclic GMP analogue 8-Br-cGMP, by itself opens stomata, and the opening depends on the concentration of 8-Br-cGMP. This finding suggest that in stomatal movement, cGMP acts as second messenger. This evidence is further supported by data from treatments with the guanylyl cyclase inhibitors, MB and LY 83583.

MB is a guanylyl cyclase inhibitor which has been reported to block the nitric oxide (NO)-induced enhancement of EPSCs (excitatory post-synaptic currents) which is mediated by cGMP (Mothet *et al.*, 1996). MB also inhibited both the relaxation and the increase on cGMP levels induced by acetylcholine
in rabbit aorta ring (Martin et al., 1985) and can abolish CNP-induced vasodilation on rat cerebral arterioles (Mori et al., 1997).

LY 83583 is a specific inhibitor of guanylyl cyclase. It has been reported that LY 83583 inhibits the relaxation of histamine-supported tone by ANP and can cause reduction in basal cGMP accumulation in tracheal smooth muscle as well as inhibit the effect of sodium nitroprusside on cGMP accumulation (Ijioma et al., 1995).

As can be seen in Figs. 5 and 9 both MB and LY 83583 completely prevent the effects of kinetin and rANP. The stomatal opening induced by kinetin and rANP is reduced in the presence of MB or LY 83583. This MB and LY 83583 dependent reduction of aperture suggests that kinetin and ANP induced opening are cGMP dependent. Furthermore, the fact that MB and LY 83583 reduce opening significantly below the illuminated control opening level indicates that the promotion of opening by both hormones and light requires a functional guanylyl cyclase.

If hormones and light induced stomatal opening are truly cGMP dependent, there must be guard cell K⁺ channels that are directly or indirectly dependent on cGMP. Two plant (Arabidopsis thaliana) selective inwardly rectifying voltage dependent K⁺ transporters, KAT1 and AKT1 have indeed been shown to be regulated by cGMP (Hoshi, 1995; Gaymard et al., 1996).

Since kinetin and rANP both promote stomatal opening and are both dependent on cGMP, the results from experiment with ABA in combination with cGMP lead to the speculation that closure inducing hormones such as ABA might use cGMP independent signal pathways or possibly operate via guanylyl cyclase down-regulation. The finding shown in Fig. 7 would support such a hypothesis.

There is another system where two antagonistic hormones regulate and integrate different processes such as α-amylase expression in the barley aleurone layer. In this tissue, GA but not ABA increases cGMP levels. In addition, LY 83583 inhibits GA but not ABA stimulated accumulation of specific and inducible mRNAs (Penson et al., 1996). It was proposed that ABA acts in a way which is either cGMP independent or at least does not require guanylyl cyclase up regulation.
The effect of both inhibitors of guanylyl cyclase, MB and LY 83583 on ANP and kinetin induced stomatal opening, were completely reversed upon wash out (Figs. 9 and 10) proving that these inhibitors do not exert irreversible adverse effects on stomatal functioning in the experimental system.

The peptide hormone rANP is a 28 amino acid molecule forming a loop due to a disulfide bridge between cysteines in position 7 and 23 and contains six charged amino acids (5 arginines and one aspartic acid). In order to show that the observed effects of rANP in plants are not solely due to its charges but reside in the native conformation, the native rANP molecule was linearised. Experiments with the linearised molecule showed that biological activity was lost, thereby excluding a non-specific charge effect residing in the primary structure only. The loss of biological activity after irreversible reduction of rANP molecule has previously been demonstrated in an animal system, Misono et al. (1984) demonstrated that the reduced and S-carboxymethylated peptide did not cause natriuretic and diuretic responses and vascular smooth muscle relaxation in rat. The evidence that reduced ANP fail to increase stomatal aperture in Tradescantia sp. indicates that the ring structure of ANP is crucial for stomatal opening activity.

2.5. SUMMARY

Results from this study provide indirect evidence for a cGMP dependent signal pathway in plants. The addition of guanylyl cyclase inhibitor MB and LY 83583 inhibit stomatal opening induce by kinetin and ANP. Furthermore cGMP analogue 8-bromo cGMP itself is able to mimic biological processes such as stomatal opening. Therefore it can be concluded that opening of stomata by kinetin and ANP is cGMP dependent.

It was demonstrated in this study that the activity of ANP to open stomata is essentially dependent on the ring structure of ANP as the reduced ANP is ineffective in stomatal opening. This may also suggest that ANP have similar receptor and signalling requirements in plants as in vertebrates.
III. ISOLATION OF BIOLOGICALLY ACTIVE NATRIURETIC PEPTIDE FROM HEDERA HELIX LEAVES

3.1. INTRODUCTION

3.1.1. Evidence of Endogenous NPs in Plants

As previously described in Chapter I, exogenously added animal NPs, affect some physiological mechanism in plant including increased solute movement and transpiration (Vesely et al., 1993) and increased stomatal opening (Gehring et al., 1996). This finding strongly suggest that plants have an endogenous NP signalling system.

Evidence for the presence of NPs in plants came from the finding of Vesely's group (Vesely and Giordano, 1991; Vesely et al., 1993). Radioimmunoassays (RIA) have revealed that plant homologues of ANP and its prohormones are present throughout the plant kingdom (Thallophyta, Bryophyta and Tracheophyta). The peptides are also present in Euglena, chlorophyll-containing flagellate (Vesely and Giordano, 1991).

Among the plants species tested by Vesely et al., (1993), Florida Beauty (Dracena godseffiana) has been found to contain the highest concentration of NPs. The concentration of NPs in plants is lower than the concentration of ANP and proANP in rat atria, but significantly higher than in the rat ventricle. NP immunoanalogues were found to be present in the leaf and stem, root, flower petal of higher plants.

The peptides isolated from plants have high similarity as revealed by comparison to RIA standard curves devised to the human sequence of NPs. This suggests that a putative NPs in plants may have significant amino acid similarity to human ANP (Vesely et al., 1993). Moreover, using high performance gel permeation chromatography, it was proposed that the plant peptides have similar elution profiles and molecular weights compared with the human peptide (Vesely et al., 1993).
3.1.2. Immunoaffinity Chromatography

Immunoaffinity chromatography (IAC) is a technique to separate or isolate immunoreactant substances (Mohr and Pommerening, 1985) which can be purified with polyclonal or monoclonal antibodies (Pepper, 1992). There are two ways of using IAC as described by Hill et al. (1989). The first way is positive immuno-purification using specific immobilized antibodies, thus purifying the antigen from a crude extract. The second way is negative purification which aims to remove a specific component from a solution. In this case, the immobilized antibody should be specific for a particular component.

Immunoaffinity chromatography is widely and successfully used for purification of human hormones such as thyrotrophin (Jack and Blazek, 1987), pituitary glycoprotein hormones, thyrotropin, follitropin and lutropin (Jack et al., 1987) and growth hormone from African catfish (Berghman et al., 1996).

In plants, IAC has been used to purify cytokinins from young plants of *Phaseolus vulgaris* (Hammerton et al., 1996). It is also used to extract other plant hormones such as auxin and ABA (Fernandez et al., 1995) and to analyse gibberellins in plants (Pearce et al., 1994).

The technique of IAC includes the coupling of the antibody to the insoluble support matrix. There are several matrices available, including cellulose, polyacrylamide, polyhydroxyethylmethacrylate, and the most frequently and commonly used are agarose gels activated by CNBr (Mohr and Pommerening, 1985). The next step is packing the coupled gel into a column. After the antigen is applied, then antigen-antibody complexes are separated by breaking the bond which form the complex by either eluting with solutions with different ionic strength, pH, dielectric constants, surface tension or temperature.
3.1.3. Aim

This following experiment aimed at isolating NPs from *Hedera helix* leaves. The experiment also aimed at establishing a protocol for isolating and partially purifying NPs from plants and test bio-activity and mediating signals of NPs isolated from *Hedera helix*.

3.2. MATERIALS AND METHODS

3.2.1 Materials

Sephadex G25-fine was purchased from Sigma (St. Louis, MO), CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden), rabbit anti-alpha-ANP 1-28 (human, canine) was obtained from Peninsular Laboratories, Inc. (Belmont, CA). LY 83583 (LY; 6-anilinoquinoline-5,8-quinone) was obtained from Calbiochem (La Jolla, USA). All other chemicals used were commercially available and Analytical Grade reagents unless otherwise stated. They were mainly purchased from Sigma Co. (St Louis, MO) and from Merck Pty Ltd. (Kilsyth, Vic). The plant material, ivy (*Hedera helix*) leaves were harvested from the outside Biology Laboratory wall, Deakin University, Geelong.

3.2.2 Preparation of Crude Extract

Crude extraction of putative NPs from ivy leaves was essentially done as previously describe by Vesely et al., (1993). However, several modifications were introduced.

One hundred grams of ivy leaves were placed in a Yastral Blender (Ballrechten Dottingen, Germany) and blended (3 X 30 s) to a fine powder in liquid nitrogen. Two hundred mL of precooled phosphate buffered saline pH 7.4 (in g: 8 NaCl, 0.2 KCl, 1.15 Na2HPO4, 0.2 KH2PO4, containing 150 tetrasodium EDTA perL) were added prior to re-blending for 1 min. This leaf
extract was centrifuge at 6000 g at 4°C for 15 min. The supernatant was filtered through a 100 μM nylon mesh. The filtrate was then mixed with equal volume of ice-cold absolute ethanol and allowed to stand for 30 min. at 4°C. Centrifugation and filtration steps were then repeated. To remove plant lipids the supernatant was extracted with diethyl ether (1:1) in a separating funnel. The aqueous phase was drawn off and any organic solute remaining in it was evaporated at 50°C in a water bath under controlled nitrogen flow. This aqueous extract was then freeze dried.

3.2.3 Purification

To purify the crude extract, the protein was separated chromatographically using a Sephadex G25-fine column (1.6 cm ID x 50 cm length). The freeze dried pellet was reconstituted in 20 mL dH₂O. Five mL of this sample was applied to the column. The column was eluted with opening buffer [10 mM MES (pH 6.3), 50 mM KCl, 1 mM MgCl₂ and 100 μM CaCl₂] and 1 mL fractions were collected. Absorbance of each fraction was measured at λ=280 nm. Biological activity was assayed by assessing the activity to open stomatal guard cells. The stomatal opening bioassay was performed on selected individual fractions. Active fractions were then pooled, freeze dried and taken up in 1 mL dH₂O.

3.2.4 Further Purification using Immunoaffinity Chromatography

One gram of CNBr-activated Sepharose 4B was added to 0.1 M sodium hydrogen carbonate pH 8.3 in 0.5 M NaCl (coupling buffer) and washed several times on a sintered glass filter. It was then transferred to a polypropylene tube to which was added ANP antiserum reconstituted in the same buffer. Two gel volumes of coupling buffer were added and the tube was capped and incubated for 16 h at 4°C on a gently rocking platform. Excess antibody was washed out of the adsorbent complex with 5 gel volumes of coupling buffer. Unreacted groups were blocked by the addition of 0.1 M Tris-
HCl buffer pH 8 and the gel was allowed to remain in this buffer for 2 h. The adsorbent gel was poured into an appropriate column and washed with three repeated cycles of acetic acid pH 3.5 in 0.5 M NaCl alternating with 0.1 M sodium carbonate in 0.5 M NaCl pH 10 (8 gel volumes each). Finally, the column was equilibrated in opening buffer for 5 passages of 8 gel volumes.

An aliquot of the resuspended pooled active fractions from the Sephadex G25-fine column was loaded onto the adsorbent and then washed into the gel bed with 8 gel volumes of opening buffer. The column was then eluted with a stepwise series (8 mL each) of KCl solutions (0.25, 0.5, 0.75 and 1 M KCl in opening buffer). One mL fractions were collected and the absorbance was measured at \( \lambda = 280 \) nm. The protein concentration of each fraction was determined and then each fraction was bio-assayed for stomatal opening ability.

3.2.5 Protein Determination

The protein concentration of each fraction from the immunoaffinity column was determined according to Lowry (Lowry et al., 1951).

BSA standards (1, 2.5, 5, 10, 15, 20, 25 \( \mu \)g) were prepared, as well as samples and blanks. They were then brought to a final volume of 0.2 mL. One mL of reagent 1 [prepared by adding 1.0 mL of 2% (w/v) sodium tartrate solution and 1.0 mL of 1% (w/v) CuSO\(_4\) solution to 100 mL of 2% (w/v) Na\(_2\)CO\(_3\) in 0.1 M NaOH] was added and vortexed and let stand for 10 min. Subsequently 0.1 mL of reagent 2 (Folin-Ciocalteau reagent and H\(_2\)O in a 1:1 ratio) was added and vortexed immediately. This reaction was allowed to stand for 1 hour, and absorbance was read at \( \lambda = 740 \) nm.

A standard curve was plotted between absorbance and BSA concentration. The protein content of the samples was determined from the BSA standard curve.
3.2.6 Stomatal Bioassay and Data Analyses

Stomatal bio-assay for the fractions from the G25 column and fractions from the immunoaffinity column was performed in *Tradescantia* sp. leaf segments. The preparation, stomatal aperture measurement and data analyses were done as described in Chapter II.

3.2.7 Summary of the Protocol

A summary of the protocol for isolating and partially purifying natriuretic peptide-like hormone from plants which is established in this experiment is shown in Fig. 12.
**Hedera helix** leaves were extracted in phosphate buffered saline pH 7.4

↓

Centrifugation

↓

Supernatant extraction in ethanol (1:1) and keep for 30 min. at 4°C

↓

Centrifugation

↓

Extraction of supernatant with diethyl ether (1:1)

↓

Freeze drying

↓

Size separation with G25 column chromatography

↓

Measurement absorbance of fractions (λ=280nm)

↓

Test for stomatal opening activity

↓

Immunoaffinity column purification

(elution with 0.25, 0.5, 0.75 and 1 M KCl solution)

↓

Test bio-activity

↓

Determination of protein concentration

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Fig. 12. Summary of protocol for partial purification of natriuretic peptide-like hormones from *Hedera helix* leaves.
3.3 RESULTS

3.3.1. Gel Permeation Chromatography of *Hedera helix* Leave Extract

A crude preparation of NP from ivy (*Hedera helix*) leaves was obtained according to the procedure outlined above. Size separation proceeded with a Sephadex G25-fine column and fractions were eluted with opening buffer. Fig. 13 shows the optical density of 1 mL fractions collected from G25-fine column.

![Graph showing absorbance over fraction number]

Fig. 13. Post void volume Sephadex G25-fine elution profile of a *Hedera helix* leaf extract. The absorbance values at 280 nm refer to 1 in 10 dilutions of each fraction. Fractions under the bar (20-38) were pooled for subsequent immunoaffinity purification.

Selected individual fractions over the whole elution range were tested for their ability to open stomatal guard cells. Fractions 20 to 38 were found to contain the main activity and were retested individually. The activity of those
fractions is showed in Fig. 14. The active fractions were then pooled for further purification with an immunoaffinity column.

Fig. 14. Mean stomatal aperture in µm in response to a 90 min exposure to opening buffer for (control; c) and fractions 20 to 38 respectively. The hatched column represents control aperture. The error bars represent the SE of >30 stomatal aperture measurements from three leaf segments from *Tradescantia* sp.

3.3.2. Immunoaffinity Purification

Figs. 15 and 16 represent the result of the immunoaffinity purification. Elution was achieved by a change in ionic strength. A four step gradient (0.25, 0.5, 0.75 and 1 M KCl in opening buffer) released the immunoaffinity bound NP. The optical density and protein concentration of each eluted 1 mL fraction was measured (Fig. 15). Fractions that eluted at a given ionic strength were subsequently assayed for induction of stomatal opening. The ability of each fraction to open stomatal guard cells is plotted in Fig. 16. The leaf segments for each control were incubated in the respective KCl concentration.

As can be seen in Fig. 16, no opening activity was induced with the fractions that contained the bulk of the protein eluted with opening buffer (contain 0.05 M KCl). Similarly, no opening activity was observed at the 0.25 M KCl elution step. In contrast, significant induction of stomatal opening
(p < 0.05) was caused by the first and second fractions (fraction 32 and 33) in the 0.5 M KCl elution step, while subsequent fractions did not contain any activity. The third step (0.75 M KCl) also released immunoaffinity bound NP in the first three eluted fractions (fraction 40 – 42). None of the fractions eluting at the fourth step (1.0 M KCl) registered protein or showed any opening induction. (Fig. 17).

Fig. 15. Post void volume elution profile of the pooled fractions (20-38) from the CNBr-activated Sepharose 4B column, expressed as absorbance at 280 nm (●) and as protein in μg/mL (□). Open horizontal bars indicate fractions eluting with increasing KCl concentrations (0.25, 0.5, 0.75 and 1.0 M).
Fig. 16. Mean stomatal aperture of *Tradescantia* sp. in μm in response to a 90 min exposure to the indicated fractions from the immunoaffinity column. Controls (c) represent stomatal opening at the respective KCl concentrations. Means and percentages were calculated from > 60 aperture measurements. Error bars indicate SE.
Fig. 17. Stomatal aperture from fractions eluted by 1 M KCl solution. Control (c) represents stomatal opening at the 1 M KCl concentrations. Means was calculated from > 60 aperture measurements. Error bars indicate SE.

Fig. 18 shows the stomatal response to increasing dilution of the most active of the recovered NP fractions (fraction 33) eluted with 0.5 M KCl. The result is expressed in percent increase of mean stomatal aperture.
Fig. 18. Mean stomatal aperture increase (%) of *Tradescantia* sp. in response to a 90 min exposure to increasing dilutions of fraction 33. Means and percentages were calculated from > 60 aperture measurements.

3.3.3. Effect of LY 83583

A set of experiment was performed to test if immunogaeant plant NP (ir PNP) induced stomatal opening also required cGMP (Fig. 19). Since the biological activity from immunoaffinity column was in the 0.5 M KCl and the 0.75 M KCl eluted fractions, both were pooled separately. Pooled fractions were dialysed against H$_2$O at 4°C for 24 hours with several changes of H$_2$O during the dialysis period then freeze dried overnight, resuspended in 1 mL H$_2$O and divided into several Eppendorf tubes after protein determination. The pooled fractions showed biological activity which could be inhibited by guanylyl cyclase inhibitor (Fig. 19).
Fig. 19. Mean stomatal aperture of *Tradescantia* sp. in μm in response to a 90 min exposure of 0.5 KCl eluted fraction and 0.75 KCl eluted fraction (column 2 and 4). Column 3 and 5 demonstrate that the addition of LY 83583 at 20 μM inhibits stomatal opening induced by both rPNT fractions. Means were calculated from > 60 aperture measurements. Error bars indicate SE.

3.4 DISCUSSION

Gel permeation chromatography was used to separate molecules of different sizes. Since it was assumed that a putative NP in plants would have similar molecular weight as animal NPs, a suitable media for separating relatively low molecular weight was used. Sephadex G25-fine has a small pore size and separates proteins in the molecular weight range from 1000 to 5000 D. Therefore, Sephadex G25-fine was used to size-separate the ivy crude extract. The choice of opening buffer as eluant for the Sephadex G25-fine column permitted the eluate to be used directly in a stomatal aperture bioassay.

Fractions between 20 and 38 which were collected from Sephadex G25-fine contained one or several compounds that induced significant (p < 0.05) stomatal opening when compared with the control. The location of biological activity obtained from G25-fine column, which is in the deep of the elution profile, is specific. The activity was found in this range in all experiments performed.
Further purification of pooled active fractions was done by immunoaffinity column using rabbit anti-alpha-ANP (1-28) (human, canine) coupled to CNBr-activated Sepharose.

The early fractions collected from the immunoaffinity column eluted with opening buffer contain the bulk of protein. However, these fractions have no activity. This step removed non-bound protein.

Stomatal opening was not induced by fractions eluted with 0.25 M KCl in opening buffer. This means that the ionic strength of 0.25 M KCl in opening buffer did not release the bound NPs.

The immunoaffinity bound was released by elution with 0.5 M KCl in opening buffer and the first two fractions showed opening stomata activity (Fig. 16.). Activity was also observed in the first three fractions eluted with 0.75 KCl in opening buffer. As can be seen in Fig. 15, while at 0.75 KCl in opening buffer control opening was significantly reduced (p < 0.05) when compared with lower KCl concentration, the stomatal aperture increases induced by fraction 40 to 42 were significant (p < 0.05). In fact fractions 41 and 42 induced a 2-fold aperture increase.

If it is assumed, that the molecular weight of immunoreactive plant natriuretic peptide (irPNP) is about the same as rANP (MW 3063), then fraction 33 and fraction 41 would each contain \( \approx 3.2 \mu M \) of irPNP. Since these two fractions could contain several compounds, it is suggested that the actual concentration of irPNP required to open stomata could be lower.

Also it is conceivable, that the immunoaffinity purified fractions contain two or more different molecules one of which being the natriuretic peptide, the others uncleaved precursors. Further structural and functional characterisation of irPNP is presented in Chapter IV.

3.5 SUMMARY

Biologically active immunoreactive PNP (irPNP; 0.5 M KCl eluted fraction and 0.75 KCl eluted fraction) was obtained in this study. The two fractions of irPNP show rANP-like characteristics such as promotion of stomatal opening and cGMP dependent signalling. This study provides
evidence that NPs contribute to plant physiological processes and suggests that plants itself contain NP analogues. The data indicates that irPNP is part of a highly conserved family of natriuretic peptide hormones common to both kingdoms. Conserved NP hormones seem to regulate solute transport since early in evolution, a speculation strengthened by the presence of ANP-like hormone in Paramecium (Vesely and Giordano, 1982) and in the unicellular green algae Euglena sp. (Vesely et al., 1993).
IV. DETERMINATION OF cGMP LEVELS IN ZEA MAYS ROOT STELE AND THE EFFECT OF NPs AND KINETIN

4.1. INTRODUCTION

4.1.1. Cyclic Nucleotides as Second Messengers

The binding of a hormone or ligand to a receptor at the outer surface of the cell may trigger release of substances called second messengers. These substances can stimulate many cellular activities. Cyclic AMP and cyclic GMP are two of several second messengers known.

In signalling systems in animals, cyclic nucleotides are known to be involved in the mediation of hormone responses. It has been reported that binding of hormones such as glucagon, catecholamines, angiotensin, vasopressin (Sutherland et al., 1968) to their receptors leads to an increase in intracellular cAMP. The action of hormones occurs by stimulating adenyl cyclase and the binding of cAMP to cAMP dependent protein kinases which then phosphorylate specific substrates.

The formation of cAMP and components involved in controlling cAMP levels are illustrated in Fig. 20.

```
\[ \text{ATP} \xrightarrow{\text{adenyl cyclase, Mg}^{2+}} \text{cyclic 3',5'-AMP} + \text{PPi} \]

\[ \text{cyclic 3',5'-AMP} \xrightarrow{\text{phosphodiesterase, Mg}^{2+}} \text{5'-AMP} \]
```

Fig. 20. The formation of cAMP

Cyclic GMP (cGMP) is another cyclic nucleotide which mediates many cellular and physiological processes. It has been confirmed that cGMP serves as a second messenger system for the actions of ANP. The addition of atrial extract can elevate cGMP levels in urine, plasma, kidney slices and primary
cultures of renal tubular cells (Hamet et al., 1984), however it has no effect on urinary cAMP excretion. When synthetic ANP is administered (ANP 8-33) it similarly increases urinary plasma and kidney tubular cGMP levels (Hamet et al., 1984).

ANP activates particulate guanylyl cyclase in crude membranes obtained from homogenates of many tissues such as rat kidney, aorta, intestine, while soluble guanylyl cyclase obtained from these tissues is not activated by ANP (Waldman et al., 1984). It was also reported that the increase of cGMP levels correlates with the distribution of particulate guanylyl cyclase. (Tremblay et al. 1985). In glomeruli the presence of soluble guanylyl cyclase is much lower compared with the particulate guanylyl cyclase. Consequently, the increase of cGMP levels is higher following addition of ANP as compared to SNP (sodium nitroprusside), an agonist of soluble guanylyl cyclase (Ding et al., 1999).

4.1.2. Cyclic Nucleotides in Plants

Compared with animal systems, where the presence and the roles of cyclic nucleotides are well established, the precise roles of these nucleotides in plants are not well understood. In plants (e.g. Pisum sativum) endogenous cyclic nucleotides have been identified and include cAMP, cGMP, cUMP, cCMP, cIMP and c-dTMP (Newton et al., 1989). In addition, cGMP was found both in meristematic and non-meristematic tissue (Newton et al., 1989).

In addition to the finding that plants contain cyclic nucleotides, it was also reported that plants contain enzymes related to cGMP signalling systems such as cGMP phosphodiesterase (Newton et al., 1984; Chiatante et al., 1988) and guanylyl cyclase (Newton et al., 1984). Similar to cGMP, the enzyme of cAMP synthesis, adenylyl cyclase has been found in roots of Pisum (Pacini et al., 1993). Although the function and mode of action of cyclic nucleotides in plants are not well established, several reports suggest that cyclic nucleotides regulate a diverse range of cellular and physiological mechanisms in plants.

Cyclic AMP (but not cGMP) has been found to stimulate $K^+$ channel activity in Vicia faba mesophyll cells by increasing an outward $K^+$ current,
which occurs through a cAMP-regulated protein kinase (Li et al., 1994). cAMP was also found to stimulate flowering and development in *Lemna paucicostata* (Khurana et al., 1988). It was also reported that cAMP stimulates protein kinase activity in rice leaves (Komatsu and Hirano, 1993) and activates Ca$^{2+}$ uptake in cultured carrot cells (Kurosaki and Nishi, 1993; Kurosaki et al., 1994). cGMP was found to mediate light regulated metabolic events (Brown et al., 1989) and to be involved in phytochrome signal transduction pathways which induce anthocyanin production. In combination with Ca$^{2+}$, cGMP induces complete chloroplast development (Bowler et al., 1994).

It has been demonstrated that cGMP modulates the KAT1 channel which is a hypolarization-activated K$^+$ channel from *Arabidopsis thaliana* (Hoshi, 1995) and modulates the AKT1 K$^+$ channel (Gaymard et al., 1996). Both of the above K$^+$ channels contain a putative cyclic nucleotide binding domain in the carboxy terminus (Anderson et al., 1992; Sentanac et al., 1992). Recently, it has been reported that cGMP was found to play an important role in gibberellic acid (GA) but not abscisic acid (ABA) signalling in the barley aleurone layer (Penson et al., 1996).

4.1.3. Aim

The aim of the following experiment was to measure cGMP levels in *Zea mays* root stele and to examine possible modulation of cGMP levels by ANP, irPNP and kinetin.

4.2. MATERIALS AND METHODS

4.2.1. Materials

3-isobutyl-1-methylxanthine (IBMX), 6-furfuryliminopurine (kinetin) and molecular weight standard (ultra low colour marker) were purchased from Sigma Chemical Co. (St. Louis, MO). LY 83583 (6-anilinoquinoline-5,8-quinone) was obtained from Calbiochem (La Jolla, CA) and rat 1-28 ANP (H-
Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-
Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH) was synthesised by
Auspep (Parkville, Victoria, Australia). The rabbit anti-\(α\)-ANP 1-28 (human,
canine) antibody was obtained from Peninsula Laboratories Inc. (Belmont CA)
and the immuno-blot assay kit was purchased from Bio-Rad (Regents Park,
Australia). Calmodulin and phosphodiesterase were kindly donated by Dr G.
Polya (La Trobe University, Bundoora, Australia). All other chemicals used
were commercially available and Analytical Grade reagents unless otherwise
stated. They were mainly purchased from Sigma Co. (St Louis, MO) and from
Merck Pty Ltd. (Kilsyth, Vic). Leaves of Hedera helix (ivy) were harvested
from plants growing on the outer Plant Biology Laboratory wall at Deakin
University, Geelong. Seeds of Zea mays L. (Punchline hybrid) were purchased
from Arthur Yates and Co (Milperra, Australia).

4.2.2. Isolation and Measurement of Molecular Mass of irPNP

Immunoreactive PNP (irPNP) was extracted from ivy leaves as
described in Chapter III. Molecular masses of the irPNP fractions were
established by electrospray mass spectrometry (ESMS) using a Micromass
Platform (MassTech, Melbourne 3186, Australia) single quadrupole mass
spectrometer. The carrier reagent was acetonitrile:water (60:40) flowing at 10
\(\mu\)L per min. Spectra were acquired using a cone voltage of 75 V.

4.2.3. Western Dot Blot

Western dot blots were performed on various isolated fractions using
anti-ANP antibody and a Bio-Rad immuno-blot assay detection system.

Nitrocellulose sheet was cut 1 x 5 cm and grids of 1 cm\(^2\) were drawn
with a pencil. Nitrocellulose was then wetted into Tris buffered saline (TBS)
and dried on filter paper for 5 min. Four to five \(\mu\)g of irPNP (0.5 fraction and
0.75 fraction) were applied to each grid square. ANP was applied as a positive
control and insulin was used as negative control. After the nitrocellulose was
dry, the membrane was immersed in blocking solution (3% gelatin in TBS) and agitated at room temperature for 1 hour. The membrane was washed in TTBS for 10 min. TTBS was prepared by adding 350 µL Tween-20 to 700 mL TBS.

The first antibody solution (anti-ANP antibody in 1:1000 dilution) in 1% gelatin in TTBS was added to the membrane and incubated overnight at room temperature with gentle agitation. The membrane then was washed twice in TTBS for 5 min. each at room temperature to remove excess unbound first antibody.

The second antibody conjugate solution was added. After 2 hours incubation at room temperature with gentle agitation, the membrane was then washed in TTBS for 5 min., a process repeated and a final wash to remove residual Tween-20 was done by immersing the membrane in TBS for 5 min.

To detect protein-antibody complexes, the colour development was obtained with a horseradish peroxidase colour reagent. The membrane was immersed in colour development solution until purple dots became visible. Purple dots indicate the presence of ANP/irPNP complexed with antibody. The development process was terminated by immersing the membrane in distilled water for 5 min. with gentle agitation and repeated for another 5 min. to remove residual colour development solution.

4.2.4. Gel Electrophoresis

The peptides of the eluted irPNP fractions were separated by tricine-SDS-polyacrylamide gel electrophoresis using a stacking, spacer and 15% separating gel as described by Schagger and von Jagow (1987) in a Penguin™ water-cooled dual-gel electrophoresis system model P8DS (OWL Scientific Inc, USA).

Gel preparation

The percentage concentration of the separating gel used in this experiment was 15%. The mini gel was prepared by mixing 2 mL of stock solution separating acrylamide (46.5% acrylamide and 1.5% bis-acrylamide),
2 mL gel buffer (3.0 M Tris pH 8.45 and 0.3% SDS) and 2 mL 50% glycerol and polymerized with 75 μL of 10% ammonium persulfate (APS) and 7.5 μL TEMED (N,N,N',N'-tetramethylethylenediamine).

The separating gel was overlaid by a spacer gel (2 cm) which was prepared by adding 6.1 mL of stock solution separating acrylamide, 10 mL gel buffer and water to a final volume of 30 mL, 100 μL of 10% APS and 10 μL TEMED. Finally, the spacer gel was overlaid by the stacking gel with a composition of 0.25 mL of stock solution stacking acrylamide (48% acrylamide and 1.5% bis-acrylamide), 0.75 gel buffer, 2.0 mL water, 20 μL of 10% APS and 2 μL TEMED.

Sample application

The protein samples (ANP, irPNI fraction 0.5 and irPNP fraction 0.75) were incubated for 30 min. at 40°C in the sample buffer (5 mL 0.5 M Tris pH 6.8, 4 mL 20% SDS, 1 mL β-mercaptoethanol, 4 mL 50% glycerol, 0.004 g bromophenol blue, 6 mL water) with equal volume of samples.

The samples and the molecular weight standard (ultra low colour marker, native molecular weights: 1,060-26,600 D) were then loaded into the wells of the gel under the cathode buffer with a micropipette.

Electrophoresis conditions

The electrophoresis was performed at room temperature with 200 mM Tris pH 8.9 as anode buffer and 100 mM Tris, 100 mM Tricine, 0.1% SDS as cathode buffer and cooled by running tap water through the cooling jacket of the electrophoresis apparatus. The electrophoresis was run at 100 V for 4-5 hours.
Fixing and staining

Silver staining was used to visualize the protein bands. The separating gel was fixed in fixative solution 1 (80 mL methanol, 20 mL acetic acid, 100 mL distilled water) for 30 min. on a rocking platform. Then the gel was washed in fixative solution 2 (40 mL ethanol, 20 mL acetic acid, 340 mL distilled water) twice for 15 min. each. The gel was oxidised in 1% nitric acid for exactly 5 min. and rinsed with excess distilled water for 5 min., then soaked in impregnation solution (200 mg AgNO₃, 150 μL formaldehyde, 100 mL distilled water) for 30 min. and washed with distilled water for exactly 20 s.

The gel was then placed in precooled development solution [6 g Na₂CO₃, 200 mL distilled water, 150 μL formaldehyde, 20 μL Na₂S₂O₃·5H₂O (20g/L)]. Initially, the gel was soaked for exactly 30 s., followed by exactly 3 min. immersion in fresh development solution and finally until satisfactory bands and contrast was achieved. This development stage was performed at 4°C.

After rinsing the gel with distilled water, developing reaction was stopped with 10% glacial acetic acid for 5 min., then the gel rinsed again with an excess of distilled water for 5 min.

4.2.5. Preparation and Incubation of Maize Stele

Maize seeds were imbibed overnight in deionised water and germinated in moist vermiculite at 27°C for three days in the dark. Roots were collected individually and briefly rolled on a Petri dish to separate the vascular tissue and the stele dissected from the cortex. The dissected stele tissue was incubated in "incubation buffer" (10 mM MES (2-[N-morpholino]ethanesulfonic acid) pH 6; 1 mM KCl; 0.5 mM CaSO₄). Stele tissue was blotted dry, pooled into 50 mg lots and cut into 5 mm lengths (about 100 segments). Segments were then placed in 200 μL incubation buffer.
4.2.6. Effect of NPs and Kinetin on cGMP Levels

For NPs treatments, the stele tissue was preincubated in 0.5 mM IBMX for 30 min. at room temperature (22-25°C). ANP at concentration of 1 µM and 10 µM were then added and the incubation continued for another 10 min. The same procedure was done for treatments with irPNP and kinetin. The two concentrations of kinetin tested were 1 µM and 10 µM, while treatments with either the 0.5 M KCl eluted fraction or the 0.75 M KCl eluted fraction of irPNP were done with 2 µg protein per 200 µL incubation buffer.

A time course experiment on the effect of ir PNP on cGMP levels was also done by incubating the tissue in the presence of irPNP for zero min. up to 180 min. in the absence of IBMX.

4.2.7. Effect of Guanylyl Cyclase Inhibitor (LY 83583)

The stele was preincubated in 0.5 mM IBMX for 30 min at room temperature. ANP, irPNP and kinetin were then added as well as the guanylyl cyclase inhibitor, LY 83583, at concentration of 20 µM and 200 µM. The incubation then was continued for another 10 min.

4.2.8. Isolation and Measurement of cGMP

After the incubation period was completed, the stele tissue was then frozen with liquid N₂ and ground to a powder. The frozen powder was mixed with ice-cold 10% perchloric acid (200 µL) and incubated on ice for a further 15 min. The slurry was clarified by centrifugation at 14,000 g for 2 min. Duplicate 200 µL aliquots were taken from each sample for further processing to neutralise the perchloric acid (Sharps and McCarl, 1982). Briefly, the aliquots were diluted with 400 µL water and then 700 µL of tri-n-octylamine:trichlorotrifluoroethane (1:1 v/v) was added. The phases were vigorously mixed before separation by centrifugation at 14,000 g for 1 min.
A 300 µL aliquot of the aqueous phase was dried using a vacuum concentrator (Heto Lab Equipment, Denmark). Residues were resuspended in 300 µL of 50 mM acetate buffer pH 5.8 with 0.01% (w/v) sodium azide. cGMP concentration was determined using a [125I] radioimmunoassay commercially available as a kit (Amersham UK). Endogenous cyclic nucleotide was acetylated to increase the sensitivity of the assay.

The acetylation was done by adding 12 µL acetylation reagent that was prepared by mixing 1 volume of acetic anhydride with 2 volumes of triethylamine and vortexed immediately. Aliquots of 100 µL were pipetted into polypropylene assay tubes.

One hundred µL of antiserum solution (rabbit anti-cGMP serum in 50 mM acetate buffer containing 1% bovine serum albumine and 0.01% (w/v) sodium azide) was added and vortexed. The tubes were covered and incubated for 1 hour at room temperature.

Tracer solution (guanosine 3',5'-cyclic phosphoric acid 2'-0-succinyl-3-[125I] iodotyrosine methyl ester in 50 mM acetate buffer containing 0.01% (w/v) sodium azide) for 100 µL was pipetted into all assay tubes, mixed thoroughly, covered and incubated for 48 h. at 4°C.

On day 3, 200 µL of second antibody reagent was added to each tube. All tubes were vortexed and incubated for 10 min. at room temperature. The antibody bound fraction was separated using centrifugation at 10,000g for 10 min. The supernatant was discarded and the tubes were kept inverted on a pad of absorbent tissue and allowed to drain for 5 min.

Radioactivity was counted in a gamma counter (1261 Multigamma, Pharmacia-Wallac, Finland).

4.2.9. Hydrolysis of Endogenous Cyclic Nucleotides

Tissue was incubated as described above in the absence of IBMX for 1 min. and then frozen with liquid N₂ and ground to a powder. At this stage, 200 µL of phosphodiesterase buffer (50 mM MES pH 6.9, 2 mM MgSO₄, 0.2 mM dithiothreitol, 0.1 mM CaCl₂ containing 1.25 µM calmodulin and 120 µg
phosphodiesterase) was added and the mixture incubated at 30°C for 1 h. This incubation was stopped with 10% perchloric acid and processed as above.

4.3. RESULTS

4.3.1. Western Dot Blot

Two bioactive fractions irPNP were eluted from the immunoaffinity column by 0.5 M and 0.75 M KCl as described in Chapter III. These two fractions of irPNP are indeed immunoreactive with anti-ANP antibodies. The results are shown in Fig. 21. The positive signals were also observed with rANP, while insulin gave no signal.

Fig. 21. An immunoblot using the anti-ANP antibody showing the immunoreactivity of ANP and irPNP. Position 1 is ANP (1.5 μg; positive control), the 0.5 M KCl eluted fraction (5 μg) is in position 2, the 0.75 M KCl eluted fraction of irPNP (4 μg) is in position 3 and insulin (5 μg; negative control) is in position 4.

4.3.2. Mass-Spectrometry

Mass-spectrometry of the 0.5 M KCl eluted fraction shows that this fraction contains several species and the data is compatible with molecular weights of 2997 and 3025 (Fig. 22).
Fig. 22. The ESMS spectra of the low molecular weight peaks found in the 0.5 M KCl eluted fraction of irPNP.

The 0.75 M KCl eluted fraction also consists of several species and the data is compatible with two species of molecular weight of 3252 and 3278 respectively (Fig. 23). Fractions in this size range are expected if irPNP is a true structural analogue of vertebrate ANP.
Fig. 23. The ESMS spectra of the low molecular weight peaks found in the 0.75 M KCl eluted fraction of irPNP.

4.3.2. Tricine-acrylamide Gel Electrophoresis

Both irPNP fractions were separated by gel electrophoresis to determine their relative purity. Fig. 24 shows that both fractions contained several high molecular weight species in the order of 9 to 16 kD but were differentiated by the 0.75 M KCl fraction containing a just visible band of approximately 3-4 kD. Both the irPNP fractions and the ANP sample also contained bands at approximately 6-8 kD which could represent a dimer or trimer of the 3-4 kD band formed due to the particular running conditions used in this gel to resolve small peptides (Schagger and Jagow, 1987).
4.3.3. cGMP Levels in Zea mays Root Stele and the Effect of NPs

A radioimmunoassay was used to determine if cGMP levels could be detected in maize root stele tissue. Levels of cGMP in untreated root stele ranged from 0.27 to 0.45 pmol/g fresh weight. These levels are comparable to those reported in barley aleurone layers using a radioimmunoassay (Penson et al., 1996) but considerably lower than those reported previously for total maize seedlings using gas chromatography (Janistyn, 1983). Since a specific tissue was used, the root stele, this discrepancy possibly reflects tissue-related differences.

In order to detect if rapid and transient changes in cGMP occur in response to ANP or irPNP, the stele tissue was preincubated in the phosphodiesterase inhibitor IBMX. Then the stele tissue was exposed to treatments with buffer, ANP and two fractions of irPNP from the immunoaffinity column for 10 min. (Fig. 25). The treatment with the 0.75 M KCl eluted irPNP fraction resulted in a three-fold increase in cGMP levels. The other treatments, 0.5 M KCl eluted irPNP fraction and 1 µM ANP, did not increase cGMP levels. Even when the concentration of ANP was raised to 10 µM, there was no effect on cGMP levels (Table 2).
Fig. 25. Effect of natriuretic peptide treatments on cGMP levels in maize root stele tissue preincubated with IBMX. Root stele tissue was treated for 10 min. with buffer, 1 μM ANP or 0.5 M KCl eluted irPnP fraction or 0.75 M KCl eluted irPnP fraction. Results are expressed as mean ± SE of triplicate samples.

4.3.4. Time Dependence of the Effect of irPnP on cGMP Levels and Effect of Phosphodiesterase

To determine the temporal pattern of the effect of 0.75 M KCl eluted fraction of irPnP, cGMP levels were measured from zero to 180 min. in the absence of IBMX (Fig. 26). The response was rapid and maximal cGMP concentration was reached within one to two min. The cGMP levels remain elevated for approximately 10 min. before beginning to decline although they remained higher than control levels for up to 30 min. After this period there is
no detectable difference in cGMP levels between the control and treated tissues.

Since the response to the 0.75 KCl M eluted fraction of irPNP was rapid, it was important to ascertain that changes in cyclic nucleotides were truly measured and not an artefact from the irPNP fraction (the antiserum is specific for cGMP and detects negligible levels of cAMP; Amersham). Firstly the reaction was stopped at zero time by freezing the root stele tissue in liquid N$_2$ prior to adding the appropriate volume of the 0.75 M KCl eluted irPNP fraction. This procedure resulted in the zero time point for irPNP in Fig. 26 that is not significantly different from the control treatments. As a further test, root stele tissue was incubated for one min. and then the tissue was ground in liquid N$_2$ before incubating the ground material in the presence of a phosphodiesterase. The phosphodiesterase treatment resulted in a marked reduction of cGMP to a similar basal level in both the control and 0.75 M KCl eluted irPNP treatment (Table 1).

Fig. 26. A time course of the effect of the 0.75 M KCl eluted irPNP fraction (10 µg/mL) on cGMP levels in maize root steles. Results are expressed as mean ± SE of at least three samples except points without an error bar which only contained duplicate samples.
Table 1. Effect of hydrolysis of endogenous cyclic nucleotides on cGMP levels in maize root stele.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP (pmol/g fresh weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No further treatment&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.48 ± 0.23 (3)</td>
</tr>
<tr>
<td>0.75 M KCl eluted irPNP</td>
<td>1.02 ± 0.25 (3)</td>
</tr>
</tbody>
</table>

<sup>1</sup> Maize root stele tissue was incubated in the presence of buffer ± irPNP for 1 min. before processing as described in Materials and Methods.

<sup>2</sup> In this treatment, maize root stele tissue was incubated in the presence of buffer ± irPNP for 1 min. then ground and incubated in the presence of phosphodiesterase for 1 h before processing as described in Materials and Methods.

4.3.5. Effect of LY 83583

In the previous chapter it was shown that the guanylyl cyclase inhibitor LY 83583 inhibits stomatal responses to ANP. Therefore the effect of LY 83583 on cGMP levels in root stele tissue was tested to see if the inhibitor would effect the cGMP levels. These experiments were undertaken with root stele tissue preincubated with IBMX to ensure that transient changes in cGMP were detected (Table 2). In the presence of the LY 83583 the treatments with ANP and the two irPNP fractions mirror the result in Fig. 25 where the inhibitor is not present. Again the 0.75 M KCl eluted irPNP fraction showed a several fold increase over the other hormone treatments. Since it was also reported in the previous chapter that LY 83583 also affects kinetin induced stomatal movement, the effect of kinetin (1 and 10 μM) on cGMP levels was tested. In the presence or absence of LY 83583, kinetin did not significantly alter cGMP levels from control treatments in maize root stele tissue (Table 2).
Table 2. Effect of LY 83583 on cGMP levels in maize root stele tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cGMP (pmol/g fresh weight)¹</th>
<th>20 µM</th>
<th>200 µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>20 µM</td>
<td>200 µM</td>
</tr>
<tr>
<td>Control</td>
<td>0.29 ± 0.05</td>
<td>nd²</td>
<td>nd</td>
</tr>
<tr>
<td>1 µM ANP</td>
<td>0.27 ± 0.02</td>
<td>0.43</td>
<td>nd</td>
</tr>
<tr>
<td>10 µM ANP</td>
<td>0.32</td>
<td>nd</td>
<td>0.32</td>
</tr>
<tr>
<td>0.5 M KCl eluted irPNP</td>
<td>0.33 ± 0.04</td>
<td>0.38</td>
<td>0.35</td>
</tr>
<tr>
<td>0.75 M KCl eluted irPNP</td>
<td>1.38 ± 0.08</td>
<td>1.10</td>
<td>1.58</td>
</tr>
<tr>
<td>1 µM kinetin</td>
<td>0.30 ± 0.03</td>
<td>0.30 ± 0.01</td>
<td>nd</td>
</tr>
<tr>
<td>10 µM kinetin</td>
<td>0.29</td>
<td>nd</td>
<td>0.31</td>
</tr>
</tbody>
</table>

¹Results are mean of duplicate or the mean ± SE of triplicate samples.
²nd - not determined

4.4. DISCUSSION

In this study it was shown that cGMP in maize root stele tissue is specifically modulated by an immunoaffinity isolated irPNP fraction. In response to the 0.75 M KCl eluted irPNP fraction, rapid increases in cGMP occurred that returned to control levels over a period of 30 min. Neither the 0.5 M KCl eluted irPNP fraction nor ANP nor kinetin treatment induced cGMP increases in maize root stele tissue. This result appeared at first a little surprising as it had been previously shown that kinetin and rANP effects on stomatal movement were modulated by cGMP. However, these responses have not been examined in maize. Furthermore Lundeen et al. (1973) reported that intracellular cGMP levels appear not to be affected by either auxin or kinetin or both during the cell enlargement or cell division phases of the cell cycle in the tobacco pith tissues. In addition, it is possible that maize and the particular tissues tested have different and more specific receptor requirements for NPs.
The fact that ANP at 10 μM does not alter cGMP levels would indicate, that no unspecific (e.g. charge) effect can be responsible for the response but that the receptor ligand interaction is highly specific and dependent upon a configuration requirement which is not met by ANP. While both the 0.5 M and the 0.75 M KCl eluted irPNP fractions are recognised by antibodies to rANP in a western dot blot and thus have similarities to ANP there is no reason to assume that the epitope recognised in irPNP is identical with the biologically active site in the molecule.

The ESMS data of the irPNP fractions indicates that species of molecules in the mass range of 3250 are present in the 0.75 M KCl eluted fractions. This result also suggests that the small molecular species in the 0.75 M KCl eluted irPNP fraction probably contains an additional amino acid residue. This molecular mass value corresponds well to the value in vertebrates where for example rANP has a molecular weight of 3063. This similarity in molecular mass between vertebrate NPs and plant analogues has already been predicted from radioimmunoassay data (Vesely et al., 1993). The data of Vesely et al. (1993) indicated precursor molecules of NP were also present in plants. The higher molecular weight species is speculated to form precursors of active irPNP. Furthermore, several bands of approximately 9,000 to 16,000 molecular weight are visualised on the silver stained polyacrylamide gel. Such bands are not unexpected as vertebrate NPs represents the carboxy terminus of a precursor peptide of 10 kD or more and products from the amino terminus may also have some hormonal activity (Zeidel, 1993; Anand-Srivastava and Trachte, 1993). Since the 3000 kD molecular weight fraction is only just faintly visible in the 0.75 M but not the 0.5 M KCl fraction of irPNP, it is speculated that this is the active part of the fraction.

All smaller peptides and non-peptide plant hormones would have been removed from the irPNP fractions by dialysis using a membrane with a 1 kD cut-off. Moreover, the ESMS data allows us to estimate the amount of protein present in the experiment. The protein concentration in the cGMP assay mix of the 0.75 M KCl eluted irPNP fraction was estimated to be at the very most 3 μM if we assume a molecular weight of 3250 and molecular homogeneity. Since this is evidently not the case, it is likely that the actual active irPNP
concentration is significantly lower indicating that specific ligand receptor interactions with irPNP precede increases in levels of intracellular cGMP.

The 0.75 M KCl eluted irPNP fraction is stimulating specific increases in cGMP as the zero, 60, 120 and 180 min. time points induce cGMP levels indistinguishable from the control time points. Moreover digestion of the cGMP induced with the 0.75 M KCl eluted irPNP fraction by phosphodiesterase results in levels of cGMP below the control level. Since the control treatment was also reduced to this final level, it indicates that some non-specific binding in the order of approximately 0.3 pmol/g fresh weight may be occurring in the radioimmunoassay. When this non-specific binding was subtracted from the data a four-fold increase of cGMP is observed in response to irPNP eluted with 0.75 M KCl.

The temporal pattern of the cGMP increase induced by the 0.75 M KCl eluted irPNP fraction is noteworthy. A very rapid induction occurs within 30 s which is maintained for 10 min. before declining to basal levels. Interestingly, the speed and amplitude of induction as well as the duration of the tissue cGMP elevation are nearly identical to the ANP induced cGMP response in erythrocytes (Petrov et al., 1994).

Since each sample contains about 100 segments of root stele, an averaged response was observed in this experiment which may reflect several independent transient increases as the hormone penetrates the tissue. Unfortunately the limits of detection of the assay prevent the examination of the responses in single cells or tissues. Although the cGMP response to irPNP is similar to that reported for erythrocytes (Petrov et al., 1994), plant tissues may respond in distinctly different ways from animal tissues. For example, in barley aleurone layers cGMP levels do not increase for over 1 h after GA treatment but remain elevated for over 2 h (Penson et al., 1996). On the other hand, highly elevated cGMP levels were recorded in spruce needles in response to 10 min. exposure to 60 ppm gaseous nitric oxide (Pfeiffer et al., 1994).

The guanylyl cyclase inhibitor LY 83583 has been used extensively as a tool in NP research in animals even though there is considerable contradiction about its precise mode of action. Some reports suggest that LY 83583 antagonises the soluble guanylyl cyclase (e.g. Ruecker et al., 1989)
whereas others claim that LY 83583 is specific for the particulate form of the enzyme (e.g. Malta et al., 1988). The discussion is further complicated by different responses in different species and tissues; a situation which could be similar in plants. LY 83583 inhibits different cGMP dependent processes such as GA dependent gene induction in barley aleurone layers (Penson et al., 1996) and stomatal opening in Tradescantia sp. as reported in chapter II. Since the results are obtained with intact tissues or protoplasts in both cases, guanylyl cyclase activity could be due to either the soluble or particulate form of the enzyme. Similarly the results from this experiment have been obtained with intact tissues and could also reflect activity of either the soluble or particulate form of guanylyl cyclase. However, LY 83583 had no effect on cGMP levels in the experiments reported here. It is therefore possible that the root stele irPNP receptor is similar to vertebrate plasma membrane receptors containing an intracellular guanylyl cyclase domain that is not always susceptible to LY 83583. Similar results in animal were reported by Ding et al. (1999), where LY 83583 had no effect on ANP and CNP-induced cGMP formation in SV-40 transformed cat iris sphincter smooth muscle (SV-CISM-2) cells.

A physiological role for irPNP is as yet unidentified. However, the hypothesis can be proposed that the plant NP hormone system play an important role in the regulation of water and solute movements in and out of the phloem and xylem. Such a regulation could conceivably be achieved by modulation of cation and anion channel activities in the conductive tissue itself or the mesophyll cells adjacent to it. The channel activities may be modulated by signal transduction pathways involving increases in cGMP in response to irPNP binding specific receptors in these cells. Another possible mechanism recently reported in animal systems (Patil et al., 1997) might involve natriuretic peptide dependent changes in aquaporin activities. Changes in aquaporin activity are in some instances modulated by cyclic nucleotides in plants (Maurel et al., 1995) so that increases in cGMP stimulated by PNP could then directly influence intracellular water content. In summary, a plant natriuretic peptide hormone system may be revealed to coordinately regulate ion and solute channels and thus significantly contribute to the cellular homeostasis.
4.5. SUMMARY

Two fractions of immunoaffinity purified biologically active plant natriuretic peptide analogues (irPNP 0.5 M KCl eluted fraction and 0.75 M KCl eluted fraction) are immunoreactive with anti-ANP antibody. However only irPNP 0.75 M KCl eluted fraction rapidly and specifically increases cGMP levels in stele tissue isolated from Zea mays root within 30 s.

ANP and kinetin do not affect cGMP levels in Zea mays root stele tissue, which means that the receptor for the ligand which can elevate cGMP levels in this tissue is highly specific. Moreover LY 83583, an inhibitor of guanylyl cyclase does not affect any of these responses. Since LY 83583 does not always inhibit particulate guanylyl cyclase, the results support the existence of a membrane bound irPNP receptor containing intrinsic guanylyl cyclase activity analogous to animal NP receptors.

It is concluded from these experiments that a biologically active NP system is present in the stele of Zea mays and speculated that it modulates solutes movements in and out of this tissue.
V. MEASUREMENT OF $K^+$, $Na^+$ AND $H^+$ FLUXES AND ATP-ASE ACTIVITY IN ZEA MAYS ROOT STELE

5.1. INTRODUCTION

5.1.1. NPs Regulate Water and Solute Balance in Vertebrates

Water and solute homeostasis is a key requirement for living systems and in vertebrates this homeostasis is in part achieved by a family of natriuretic peptide hormones (NPs) (for review see Anand-Srivastava and Trachte, 1993; Zeidel, 1993). Recently, structural and functional evidence for the presence of such a NP hormone system in plants has been forthcoming (e.g. Vesely et al., 1993). Results from previous chapters support the presence of NP in plants and the immunoaffinity column has successfully purified biologically active plant natriuretic peptide analogue (irPNP) from Hedera helix leaves.

In animal tissues several peptides of the NP family mediate inhibition of the apical Na$^+$ channels (Zeidel, 1993) and deactivation of Na$^+$, K$^+$ -ATPases (Aperia et al., 1994). ANPs can also stimulate (Petrov et al., 1994) or inhibit (Carmelo et al., 1994) Na$^+$/H$^+$ antiporters in different cell types. It has also been observed that NPs can promote kaliuresis (e.g. Martin et al., 1990) and in particular that NPs increase a K$^+$ conductance in rat mesangial cells (Cermak et al., 1996) as well as facilitate a K$^+$ current in atrial ventricular papillary muscle (Kecskemeti et al., 1996).

In plants NPs also appear to regulate water and solute movement, since the proANP increased transpiration and solute uptake (Vesely et al., 1993). Furthermore, both ANP and irPNP induced stomatal opening, which indicate their contribution to water and K$^+$ movement. It is therefore important to determine the effect of NPs on ion movement.

5.1.2. ATPase in Solute Transport

The plasma membrane ATPase plays important role in many physiological processes in plants. This enzyme generates electrochemical
gradient by pumping protons and thus provide energy for transport of various solutes such as cations, anions, amino acids, sugar and growth regulators (reviewed by Sze, 1985; Michelet and Boutry, 1995).

The protons pumped by H⁺-ATPase create a pH difference (ΔpH) and potential difference (E_m). These drive solute transport via secondary active transport where solute transport is coupled to H⁺ flux via an antiport or symport mechanism. Fig. 27 shows the scheme of energization for secondary transport provided by the H⁺-ATPase.

![Diagram of H⁺ coupled solute transport systems](image)

**Fig. 27.** Theoretical model of H⁺ coupled solute transport systems energized by the H⁺-ATPase. Adapted from Michelet and Boutry (1995).

ATPase activity is modulated by several factors. In guard cells, the proton pump is stimulated by blue light and red light (Assmann et al., 1985; Serrano et al., 1988; Shimazaki et al., 1986), the plant hormone auxin, the second messenger 1,2-diacylglycerol and the fungal toxin fusicoxin (reviewed by Assmann, 1993). brassinolide activated the proton pump in maize root
(Romani et al., 1983) and epicotyls of Azuki bean (Cerana et al., 1983). In contrast, abscisic acid inhibited the H⁺-ATPase and H⁺ transport in tonoplast vesicles from barley roots (Zhang et al., 1998) and inhibited blue light-dependent H⁺ pumping in guard cell protoplast from Vicia faba (Goh et al., 1996).

5.1.3 Aim

This study was aimed to measure the net fluxes of Na⁺, K⁺ and H⁺ in the Zea mays root conductive tissue and test if NPs and cGMP, a putative second messenger of NP modulate those ion fluxes. It was also aimed to measure ATPase activity in the same tissue to assess the mechanisms that drive ion and water transport.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Rat 1-28 ANP (H-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Scr-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH) was synthesised by Auspep (Parkville, Victoria, Australia), 8-bromo-cyclic-guanosine-3',5'-monophosphate (8-Br-cGMP), adenosine triphosphate (ATP) were purchased from Sigma Chemical Co. (St. Louis, MO). Tributylchlorosilane (90796), ionophore cocktails (hydrogen 95297, potassium 60031, sodium 71178) were purchased from Fluka Chemical, Buchs, Switzerland. All other chemicals used were commercially available and Analytical Grade reagents unless otherwise stated. They were mainly purchased from Sigma Co. (St Louis, MO) and from Merck Pty Ltd. (Kilsyth, Vic). irPNP was isolated from Hedera helix leaves as described in previous chapter. Seeds of Zea mays L. (punchline hybrid) were purchased from Arthur Yates and Co (Milperra, Australia)
5.2.2. Plant Preparation and Experimental Procedure

Corn seedlings were grown in 1 L plastic containers filled with vermiculite at 25°C in the darkness. "Basic" solution containing 0.5 mM CaCl₂, 1.0 mM KCl and 1 mM NaCl was used for both growing the seedlings and ion flux measurements. When roots were 8-10 cm long (at 4-5 days), seedlings were gently removed from vermiculite, carefully washed in distilled water, and root segments of about 5 cm long were cut starting 25-20 mm from the root tip. Then the root stele was isolated, segments about 10 mm were cut and floated in a Petri dish in aerated "basic" solution for 3-4 h. One hour prior to measurements, stele segments were fixed in a plastic holder and placed in the measuring chamber of approximately 1 mL volume. The chamber was mounted on the hydraulic micro-manipulator, the electrodes were positioned and left to condition.

Ion flux measurements started 5 h after the stele tissue was extracted, and 1 h after placement in the chamber. By that time all transients were finished. The steady fluxes were measured in control (no treatment) for 20 min., prior to the addition of treatments. Stock solutions of ANP, iRPNP and cGMP were added to the bath and then sucked and expelled 10-12 times to assure proper mixing. This procedure typically required 2-2.5 min. For flux calculations this interval was disregarded for analysis. In control plants, the small amount of bath solution was added and mixed in the same way to confirm that changes e.g. in aeration conditions did not affect ion fluxes.

5.2.3. Quantification of Net K⁺, Na⁺ and H⁺ Fluxes

Net ion fluxes were measured non-invasively using ion-selective vibrating microelectrodes (the MIFE™ technique, Unitas Consulting, Hobart, Australia) generally as described before (Shabala and Newman 1997; Shabala et al., 1997; Shabala and Newman 1998). Microelectrodes were pulled from borosilicate glass capillaries (GC150-10, Clark Electromedical Instruments, U.K.), oven dried at 200°C for 5 h, and silanised with two drops (≈ 15 μg) of
tributylchlorosilane for 10 min. in the same oven under a steel cover. Dried and cooled electrode blanks were back filled with solutions (0.15 mM NaCl and 0.4 mM KH₂PO₄ adjusted to pH 6.0 using NaOH for the proton electrode; 0.5 M KCl for the potassium electrode; 0.5 M NaCl for the sodium electrode). The electrode tips (3 to 5 μm external diameter) were then filled with commercially available ionophore cocktails (hydrogen 95297; potassium 60031; sodium 71178; all from Fluka). The electrodes were calibrated in sets of standards (pH from 4.5 to 8; K⁺ and Na⁺ 0.5 to 5 mM) before and after use. Electrodes with a response of less than 50 mV per decade were discarded. A flattened glass microelectrode (tip diameter = 50 μm) containing 1 M KCl in 2% agar was used as a reference electrode.

The ion selective electrodes were mounted on a manipulator allowing 3-dimensional positioning. Electrodes were positioned parallel to the root axis 50 μm above the root surface. During measurements, the electrodes were virtually moved between 50 to 90 μm at a frequency of 0.1 Hz. That was achieved by fixing the experimental chamber on a 3-way hydraulic micromanipulator (Narashige WR-88) driven by a computer-controlled stepper motor (Superior Electric, Bristol CT, MO61-CE08). The recorded potential differences were converted into electrochemical potential using the calibrated Nernst slope of the electrodes. The initial one or two seconds post movement were ignored to allow for both the movement (0.3 s) and the electrochemical settling of the electrodes (Shabala et al. 1997). Ion fluxes were calculated assuming cylindrical diffusion geometry.

5.2.4. ATPase Activity Determination

Plant material preparation

Zea mays seeds were soaked overnight in distilled water and germinated in moist vermiculite at 27°C for three days in the dark. Roots were 5-8 cm long at this stage and were collected individually. Stele and cortex were separated manually by twisting the basal region of the root and pulling the
cortex out. Stele tissue was then kept in ice-cold distilled water prior to homogenization.

*Isolation of microsomal membrane*

Microsomal membranes were isolated as previously describe by Palmgren *et al.* (1990) with some modification. Stele tissue was chopped and ground in the homogenization medium containing 250 mM sucrose, 50 mM MOPS (pH 7.5), 5 mM EDTA, 5 mM DTT, 0.5 mM PMSF, 0.2% (w/v) BSA; protease free, 0.2% (w/v) casein, 0.6% (w/v) PVP, 4 mL per g tissue. The homogenate was filtered through 8 layers of miracloth and centrifuged at 13,000 g for 10 min. in a Beckman J2-21M/E centrifuge (California, USA).

The pellet was discarded and the supernatant was centrifuged at 100,000 g for 45 min. in a Beckman ultracentrifuge [model L5-65 (California, USA)]. The resulting microsomal pellet was resuspended in 250 mM sucrose, 5 mM K-phosphate (pH 7.8), 5 mM KCl, 1 mM DTT, 0.1 mM EDTA and protein concentration was determined according to Bradford (Bradford, 1976).

*Bradford protein determination*

Duplicate aliquots of 1 mg/mL BSA (5, 10, 15, and 20 μL) were added into microcentrifuge tubes. The volume in each tube was brought to 100 μL with distilled water. Two blank tubes were prepared which contained only 100 μL distilled water. The unknown protein (microsomal membrane) was added to H₂O to give a total volume of 100 μL.

One mL of Coomassie brilliant blue solution (100 mg Coomassie brilliant blue G-250, 50 mL of 95% ethanol, 100 mL of 85% phosphoric acid, diluted to 1 liter and filtered through Whatman No. 1 filter paper) was added to each tube and vortexed and left 2 min. at room temperature. Absorbance was measured at λ=595 nm using 1 mL cuvettes.
A standard curve was drawn by plotting absorbance versus BSA concentration and the protein concentration of the microsomal membrane was determined from the BSA standard curve.

Assay for ATPase activity

The activity of ATPase was determined by measuring the inorganic phosphate released from the substrate, essentially according to standard procedures described elsewhere (Shimazaki and Kondo, 1987). The assay mixture (1 mL) contained 50 mM MES (pH 6.8), 2 mM ATP, 5 mM MgSO₄, 50 mM KCl, 1 mM EDTA and 0.02% Triton X-100. Treatments, such as cGMP at several concentrations (0.1, 1, 10 and 100 μM), and vanadate (50, 100, 200, 300, 400 μM) were added to the assay mixture. The reaction was started by the addition of microsomal membrane (20 μg) at 25°C and allowed to proceed for 30 min. As a control, an assay was done where the membrane was added after stopping the reaction.

Inorganic phosphate was determined according to Briskin et al. (1987). The reaction was stopped by the addition of 2.6 mL stop/color reagent [6 volumes of 0.42% (w/v) ammonium molybdate in 1 N H₂SO₄ : 1 volume of 10% (w/v) ascorbic acid, and 0.5% SDS] to 1 mL reaction and incubated for 20 min. at room temperature. Then absorbance was read at λ=700 nm.

A standard inorganic phosphate curve was obtained by plotting absorbance versus inorganic phosphate concentrations (0.1, 0.2, 0.3, 0.4, 0.5 μmoles PO₄³⁻).
5.3. RESULTS

5.3.1. Effect of NPs and cGMP on $H^+$, $K^+$ and $Na^+$ Fluxes

A summary of the effects of natriuretic peptide hormones on net fluxes of $H^+$, $K^+$, and $Na^+$ is given in Table 3, with transient curves shown in Figs. 28-30. The two fractions of irPNP (0.5 M KCl eluted fraction and 0.75 M KCl eluted fraction) influence $H^+$, $K^+$ and $Na^+$ fluxes, while cGMP mimics the effect of irPNP on $K^+$ flux only, although there is a positive trend observed on $Na^+$ flux.

Table 3. Summary of effects of natriuretic peptide hormones and cGMP on net $K^+$, $H^+$ and $Na^+$ fluxes from the root stele tissue. Plus means statistically ($P < 0.05$) significant effect; asterisk indicate a possible trend; minus means no effect exists.

<table>
<thead>
<tr>
<th>Ion Treatment</th>
<th>PNP 0.5</th>
<th>PNP 0.75</th>
<th>ANP</th>
<th>cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$H^+$ flux</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>$K^+$ flux</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>+</td>
</tr>
<tr>
<td>$Na^+$ flux</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

Both irPNP fractions caused an almost immediate reduction of net $H^+$ efflux (Fig. 28). In both instances, $H^+$ fluxes reached a new steady level, shifted 20-25 nmol m$^{-2}$ s$^{-1}$ towards net influx within the first 7-10 min. Such shifts were not observed in response to ANP or cGMP (Fig. 28) indicating absence of endogenous rhythmical activity as reported for the root epidermis (Shabala et al., 1997; Shabala and Newman, 1997).

The absence of ANP or cGMP induced $H^+$ flux changes also validate the experimental protocol by indicating that results observed in response to irPNP treatments are not addition artefacts.

In addition to the rapid response of $H^+$ fluxes, significant changes in net $K^+$ fluxes were observed 30-35 min. after irPNP treatment (Fig. 29; Table 4). Furthermore, 8-Br-cGMP treatment also caused significant shifts in net $K^+$ flux from efflux to influx (Fig. 29D; Table 4). Similar trends were also recorded in
response to ANP (Fig. 29C), but the data remained below significance levels (P > 0.05). There was also a clearly pronounced and statistically significant trend towards irPNP-induced Na⁺ influx (Fig. 30A,B; Table 2) with a time delay similar to that of K⁺ flux responses (Table 4). In both cases, the delay significantly (P < 0.001) exceeded that for H⁺ flux which was always < 3 min.

Table 4. Parameters of the net K⁺ and Na⁺ flux kinetics measured near the Zea mays root stele tissue before and after various treatment with NPs and cGMP. Data are means ± SE (n = sample size). Probabilities compared with fluxes before treatments by Student's test (aP < 0.001; bP < 0.01; cP < 0.05). Time delay is defined as the moment then significant changes in net ion flux became evident.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Flux before treatment, nmol m⁻² s⁻¹</th>
<th>Flux 60 min after treatment, nmol m⁻² s⁻¹</th>
<th>Time delay, min</th>
<th>ΔK⁺/ΔNa⁺ ratio</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K⁺ flux</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNP 0.5</td>
<td>-27.5 ± 7.7</td>
<td>62.3 ± 20.9b</td>
<td>34.8 ± 5.5a</td>
<td>2.08</td>
<td>7</td>
</tr>
<tr>
<td>PNP 0.75</td>
<td>-35.6 ± 9.2</td>
<td>86.6 ± 12.8a</td>
<td>30.1 ± 4.0a</td>
<td>1.76</td>
<td>7</td>
</tr>
<tr>
<td>ANP</td>
<td>-23.8 ± 28.2</td>
<td>-9.2 ± 21.3</td>
<td>N/A</td>
<td>N/A</td>
<td>6</td>
</tr>
<tr>
<td>cGMP</td>
<td>-17.9 ± 25</td>
<td>108 ± 30.9c</td>
<td>28.8 ± 4.3a</td>
<td>N/A</td>
<td>5</td>
</tr>
<tr>
<td><strong>Na⁺ flux</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNP 0.5</td>
<td>-6.0 ± 5.0</td>
<td>37.4 ± 11.8c</td>
<td>24.8 ± 2.4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>PNP 0.75</td>
<td>-0.4 ± 7.1</td>
<td>68.9 ± 9.0a</td>
<td>31.0 ± 3.1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>ANP</td>
<td>7.8 ± 15.9</td>
<td>40.2 ± 13.3</td>
<td>N/A</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>cGMP</td>
<td>-19.3 ± 13.1</td>
<td>-0.7 ± 8.0</td>
<td>31.3 ± 3.9</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 28. Transient changes in net H⁺ flux near the maize root stele tissue caused by different treatments. Measurements were made at 5 s intervals. Each point represents the average value of 48 measurements (4 plants averaged over 1 min interval each). Numbers on the left side are steady flux values before treatment. Treatments (ANP, irPNP and cGMP) were added at 10 min. Bars are SE (n = 4-5). First 3 min after peptide was added are discarded from analysis. Statistically significant effect of irPNPs occurs within time resolution of experiment.
Fig. 29. Effect of peptide and cGMP treatments on net $K^+$ uptake into the maize root stele. Average values over 10 min intervals are given. Number of plants measured is 6, 7, 5, 5 respectively for A, B, C and D. Bars are SE. Statistically significant effect of irPNPs and cGMP is evident only after about 30 min after treatment. Arrows indicate the time when the treatment additions were made.
Fig. 30. Effect of peptide and cGMP treatments on net Na⁺ uptake into the maize root stelae. Average values over 10 min intervals are given. Number of plants measured is 6, 7, 5, 5 respectively for A, B, C and D. Bars are SE. Statistically significant effect of iPNPs is evident only after about 30 min after treatment. Arrows indicate the time when the treatment additions were made.
5.3.2. Effect of Vanadate and cGMP on ATPase Activity

In order to elucidate the mechanism of cGMP in affecting ion fluxes and the mechanisms governing iRPNP-dependent changes on ion transport, the cGMP-dependent effects on microsomal membrane ATPase activity isolated from maize root stele was tested. The microsomal membrane of maize root stele prepared according to a method Palmgren et al. (1990) has vanadate sensitive activity. Vanadium sulphate oxide (VOSO₄) was used in this experiment as source of vanadate and the dose response of the ATPase activity to vanadate is shown in Fig. 31.

Vanadate is known as an inhibitor of the plasma membrane H⁺-ATPase. It inhibited ATPase activities in various plant tissue such as corn root (Gallagher and Leonard, 1982), guard cell of Vicia faba (Kinoshita et al., 1995) and maize coleoptile (Irving, 1998). Fig. 31 demonstrates that ATP hydrolisis in microsomal membrane of maize root stele reduce in the present of vanadate.

In this study, it is also demonstrated that the cGMP analogue 8-Br-cGMP inhibits microsomal membrane ATPase activity. The inhibition is small but consistent and appears to occur at low concentration of 8-Br-cGMP (0.1 and 10 μM). Fig. 32 shows that 8-Br-cGMP inhibits the enzyme activity.

The average activity of ATPase as measured by P₁ release per mg protein per min. in maize root stele microsomal membrane is 0.232 ± 0.024 µmoles/mg protein/min. This value is lower then the ATPase activity in the plasma membrane of maize root stele as observed by Cowan et al. (1993).

Irving (1998) showed that ATPase activity was present in both microsomal and plasma membrane preparations but that the plasma membrane shows higher levels. The use of microsomal membrane in this study is due to the small amount of plasma membrane isolated from maize root stele. Triton X-100 was included in the ATPase assay buffer to ensure that both sides of the vesicles were exposed to the ligand (cGMP) and substrate.
Fig. 31. Effect of vanadate on ATP dependent P$_i$ release. Column 1 is the control P$_i$ level, columns 2 to 7 represent P$_i$ levels in the presence of increasing amounts of vanadate. The concentrations are 50 μM (2), 100 μM (3), 200 μM (4), 300 μM (5), 400 μM (6) and 500 μM (7) vanadate. Each point is mean value from three replicates and from different membrane preparations.

Fig. 32. Effect of the 8-Br-cGMP on ATP dependent P$_i$ release. Column 1 is the control P$_i$ level in the absence 8-Br-cGMP, columns 2 to 5 represent P$_i$ levels in the presence of increasing amounts of 8-Br-cGMP. The concentrations are 0.1 μM (2), 1 μM (3), 10 μM (4) and 100 μM (5) 8-Br-cGMP. Each point is mean value from three replicates and from different membrane preparations.
5.4. DISCUSSION

In this study it was demonstrated that NPs and cGMP specifically modulate ion exchange in maize root stele tissue. This is therefore the first direct evidence of an effect of these peptides on cation ion movements and on K⁺ fluxes in particular. It has been shown previously that irPNP can stimulate radial water movements from the xylem of Tradescantia sp. stems and it has been hypothesised that this may be caused by enhanced K⁺ influx into mesophyll cells adjacent to the conductive tissue (Suwastika and Gehring, 1998). Such an enhanced K⁺ influx then causes water to follow and could account for the irPNP-induced promotion of radial water movements and thereby link NPs to a function and a mechanism in plant homeostasis.

A key observation is the striking difference between NP-dependent changes in H⁺ transport and K⁺ and Na⁺ transport. Only irPNPs caused rapid and significant changes in net H⁺ flux while changes in both K⁺ and Na⁺ fluxes were activated only 30 min. after NP application. This is possibly an indication that indirect or downstream effects modulate K⁺ and Na⁺ transport. It is postulated that some responses to NPs are mediated by second messengers and in particular cGMP. The NP-like effect of cGMP on K⁺ fluxes in the stele is consistent with this idea and so is the timing for both irPNP- and cGMP-dependent responses (Table 4). The findings are also consistent with three previous experimental observations and theoretical predictions based on our knowledge of NP systems in vertebrates. First, it was shown in Chapter II, that in stomata ANP dependent opening was dependent on cGMP and can be induced by 8-Br-cGMP alone. Second, radial water movement from the xylem of Tradescantia stems is also enhanced by 8-Br-cGMP (Suwastika and Gehring, 1998) and third and most importantly, cGMP levels rise rapidly, specifically and transiently in isolated Zea mays stele tissue in response to irPNP as described in Chapter IV. These three lines of evidence have suggested that some NP dependent responses require up-regulation of guanylyl cyclases and consequent increases in cellular cGMP levels.

Structure and function of putative NP receptors in plants are still largely unknown but it is reasonable to expect a receptor and signalling system that
shares at least some homology with the vertebrate system. Three vertebrate NP receptors, NPR-A, NPR-B and NPR-C have been cloned and two of them (NPR-A and NPR-B) constitute a new family of receptor guanylyl cyclases (Chinkers et al., 1989; Koller and Goeddel, 1992). The receptors are single transmembrane proteins with extracellular domains of \( \approx 440 \) amino acids. While the intracellular domain of NPR-C is short (37 amino acids), NPR-A and NPR-B both have bipartite intracellular domains of \( \approx 280 \) and \( \approx 250 \) amino acids each. The domains proximal to the membrane (\( \approx 280 \) amino acids) show significant homology to protein kinases while the distal fragments are constituted by the guanylyl cyclase domains (\( \approx 250 \) amino acids) (Fig. 33). It is therefore speculated that in plants also, NPs signal via receptors that contain guanylyl cyclase domains. The particulate guanylyl cyclase would be activated after ligand receptor binding and a consequent transient elevation of cytosolic cGMP levels is observed. While mechanisms that underlie NP responses in plants remain speculative, the following model will be useful to design critical experiments to elucidate components of the NP signal and response pathways.

There are several possible ways for irPNP to affect \( H^+ \) transport in the stele. The most obvious one is a direct inhibition of \( H^+ \)-pumping ATPase at the plasma membrane. However, such inhibition is expected to cause significant and rapid membrane depolarisation which would immediately affect transport of all other ions (including \( K^+ \) and \( Na^+ \)). No such immediate effects on \( K^+ \) and \( Na^+ \) were observed. This indicates absence of immediate and significant membrane potential changes and hence no rapid inhibition of the electrogenic ATPase.

An immediate effect of irPNP on net \( H^+ \) flux could be mediated by a \( H^+ \)-cotransport mechanism (\( H^+/Cl^- \) symport in the model in Fig. 33). If such a symporter is directly activated by irPNP one would expect the observed (Fig. 28) fast shift towards net \( H^+ \) influx. The less pronounced effect of ANP as compared to irPNP suggests that the interaction has very specific sequence requirements which are not met by the vertebrate analogue. The presence of \( H^+/Cl^- \) symporters in the plasma membranes of the root cells is well documented (Felle 1994; Mistrik and Ulrich, 1996). Cotransport with \( Cl^- \) is
proposed since Na\(^+\) and K\(^+\) fluxes do not change within 30 min. (Figs. 29, 30) and the remaining ions in the solution are Ca\(^{2+}\) and Cl\(^-\). Rapid activity changes of the H\(^+/\)Cl\(^-\) symporter after auxin treatment in oat coleoptile parenchyma have been demonstrated (Babourina et al., 1998) and interpreted as evidence for the presence of receptors in the plasma membrane. In analogy to vertebrate systems, it is then suggested that NP receptors are present in the plant plasma membranes and proposed that downstream signals can also directly or indirectly mediate rapid modulations of H\(^+/\)Cl\(^-\) symporters.

Simultaneously, receptor ligand interactions can trigger a series of slower processes including the up-regulation of the NP receptor guanylyl cyclase domain. Subsequent increases in cGMP levels may then directly or indirectly modulate K\(^+\) and Na\(^+\) fluxes (Table 4). A number of K\(^+\) transporters have been identified in plant cells and in the root plasma membranes in particular. Both low- and high-affinity transport mechanisms are known to mediate K\(^+\) uptake by plants (e.g. Smart et al. 1995; Tyerman et al. 1997). High-affinity K\(^+\) uptake requires an active mechanism since uptake is against an electrochemical gradient for K\(^+\). It has been suggested that such mechanisms could involve either K\(^+\)/H\(^+\) (HKT1 transporter, Schachtman and Schroeder, 1994) or K\(^+\)/Na\(^+\) symporter (Smith and Walker 1989; Rubio et al. 1995). NPs do not seem likely to affect K\(^+\)/H\(^+\) cotransport since changes in fluxes of both these ions are strikingly different (Figs. 28, 29). In contrast, there is a remarkable similarity between K\(^+\) and Na\(^+\) flux kinetics observed. It is therefore reasonable to postulate that a K\(^+\)/Na\(^+\) cotransporter could be activated by irPNP.

High-affinity transport mechanisms are only operating at low (≤ 0.2 mM) external K\(^+\) concentrations (Epstein, 1963) hence their contribution is unlikely to be major under the above experimental conditions (K\(^+\) = 1 mM). At concentrations ≥ 1 mM low affinity mechanisms are prevailing (Smart et al. 1996). In root cells, hyperpolarisation-activated inward-rectifying K\(^+\) channels (AKT1 in particular; Cao et al., 1995) are the main contributors to cellular K\(^+\) uptake. Incidentally, Roberts and Tester (1995) have found that such inwardly rectifying channels are present mainly in the root cortex while the most
commonly observed plasma membrane conductance in protoplasts isolated from the stele was a K⁺-selective outwardly rectifying conductance (Roberts and Tester, 1995). It follows that a contribution of the inward-rectifying K⁺ channels may also not be very significant in our experiments.

A further cellular pathway of K⁺ uptake leads via non-selective cation channels common in plant roots (White and Lemtiri-Chlieh, 1995; Tyerman, et al. 1997). Their selectivity for K⁺ and Na⁺ is slightly different, with P_K : P_Na ratio varying from 1.3 (Tyerman et al., 1997) to 5 (Garrill et al., 1994). The ΔK⁺/ΔNa⁺ ratio in the experiments conducted in this NPs study was around 2 (Table 4). The model (Fig. 28) predicts that the major effect of irPnP and cGMP on K⁺ flux is mediated by activation of these non-selective cation channels. This is consistent with the near identical time courses for K⁺ and Na⁺ flux changes.

The second messenger cGMP is likely to be involved in a number of NP dependent reactions. Firstly, cGMP inhibits microsomal membrane H⁺-ATPase activity in microsomal membrane preparations from stele tissue (Fig. 32). Such inhibition by 8-Br-cGMP on H⁺-ATPase activity was also reported in animals, where 1 mM cGMP inhibited the bafilomycin-sensitive H⁺-ATPase activity in rat cortical collecting duct cells (Tojo et al., 1994). It is speculated that this inhibition may involve in the regulation of proton and bicarbonate transport (Tojo et al., 1994). The inhibition of ATPase activity by cGMP in microsomal membrane of maize root stele tissue is small (Fig. 32) but this evidence could account for reduced net H⁺ efflux, at least post the very rapid initial H⁺ flux change. However, no further reduction in net H⁺ efflux was noticeable in the effect of NP on H⁺ flux (Fig. 28). Moreover cGMP itself appear to have no effect on H⁺ flux (Fig.28). Changing cGMP levels may also influence other processes such as ATP-driven Ca²⁺ pumps coupled with H⁺ cotransport. A H⁺/Ca²⁺ antiport mechanism is well documented in the tonoplast membrane (Bush, 1993) and there is increasing evidence that it may also exist at the plasma membranes of plant cells (Kasai and Muto, 1990; Evans et al., 1991). Inhibition of this Ca²⁺ pump may lead to decreased H⁺ uptake via antiport mechanism, balancing reduction of the net H⁺ efflux due to decreased activity of pumping H⁺-ATPase. Finally, evidence for direct cGMP
modulated K⁺ fluxes in plant systems is also forthcoming (e.g. Hoshi, 1995; Gaymar et al., 1996).

Taken together, the data suggest an important role of plant NPs in the regulation of numerous ion transporters at the plasma membrane level and point to an involvement of the messenger cGMP.
Fig. 33. Suggested model of NP effects on ion transport in the Zea mays root stele tissue. Only transporters involved in hypothesised mechanisms are shown. Factors that stimulate transporter activity are indicated by a normal arrow; those that inhibit by a blunt arrow.
5.4.1. SUMMARY

In this study it was found that both fractions of irPNP (0.5 M KCl eluted fraction and 0.75 KCl eluted fraction) caused an immediate shift of net H⁺ flux toward influx in Zea mays root stele. However, uptake of both Na⁺ and K⁺ in maize root stele showed significant increases only 30 min. later. This result was also observed in response to 8-Br-cGMP, but it was not accompanied by changes in H⁺ fluxes.

Results from these experiments suggest that irPNPs act via activating Na⁺/K⁺ co-transport since changes in fluxes between K⁺ and H⁺ are different, while the K⁺ and Na⁺ fluxes appear very similar.

The effects of irPNPs seem to be partly mediated by the second messenger cGMP, since cGMP mimics the effect of irPNP on K⁺ flux. Furthermore, in microsomal membrane of maize root stele, cGMP appear to inhibit H⁺-ATPase activity thus contributing to the reduction of H⁺ efflux. It is speculated that irPNP as well as cGMP affect K⁺ channels directly rather than driving K⁺ uptake in response to an increased trans-membrane H⁺ gradient.
VI. GENERAL CONCLUSION AND OUTLOOK

6.1. The Role of NPs in Plants

The presence of NP analogues in plants has been demonstrated by Vesely et al. (1993). Furthermore, Gehring et al. (1996) have shown that ANP increases stomatal opening in Tradescantia sp. and $^{125}$I ANP binds specifically to plasma membranes isolated from Tradescantia sp. leaves.

In stomatal movement studies, it was found that as in animals, the effect of ANP in plants is mediated by cGMP, a second messenger of ANP in vertebrates. The addition of LY 83583, a guanylyl cyclase inhibitor, reduces the opening of stomata induced by ANP. Furthermore, the ring structure of ANP, which is formed by two cysteines forming a disulphide bridge, is essential in ANP action. Breakage of this bridge causes loss of ANP activity.

Kinetin is a plant hormone that is known to influence stomatal movement. It was confirmed in this study, that kinetin can promote opening of stomata and that this ability of kinetin is also cGMP dependent. LY 83583, when added together with kinetin, significantly inhibits stomatal opening.

Extraction and immunoaffinity chromatography with anti-ANP antibody was used to isolate natriuretic peptide from Hedera helix leaves. Two fractions eluted from immunoaffinity column are biologically active. The 0.5 M KCl eluted fraction and the 0.75 M KCl eluted fraction significantly increase stomatal opening in Tradescantia sp. and this opening is prevented by LY 83583, suggesting that the effect of NPs to open stomata is mediated by cGMP.

One of the two fractions of irPNP, which is the 0.75 M KCl eluted fraction significantly increase cGMP levels in Zea mays root stele. The increase is rapid (within 30 s.) and the addition of phosphodiesterase causes the cGMP level to return back to basal levels. Different tissues may have different responses to NPs and kinetin. In Zea mays root stele, ANP and kinetin did not have an effect on cGMP levels. This evidence may suggest that NPs require very specific receptors to modulate cGMP levels.

IrPNP is suggested to influence the movement of water and solute as in vertebrates where ANP has an important role in water and salt homeostasis.
Both 0.5 M KCl eluted fraction and 0.75 M KCl eluted fraction cause a net increase in K⁺ influx in Zea mays root stele after 30 min. Furthermore, this effect is mimicked by the cGMP analogue 8-Br-cGMP.

Both irPnP fractions also increase net Na⁺ influx, and this flux is similar to K⁺ flux and there is a shift toward H⁺ influx in response to both fraction of irPnP. However, cGMP has no effect on this flux. These results suggest that irPnP modulates ion movements in this particular tissue (Zea mays root stele) acting via activating K⁺/Na⁺ cotransport and H⁺/Cl⁻ symport. It is proposed that not all irPnP responses are mediated by the second messenger cGMP.

6.2. Future Research

This study has yielded interesting results concerning the present of NP in plants (in particular in Hedera helix leaves) and their physiological as well as cellular characterisation such as its involvement in stomatal movement, solute transport and cGMP levels. Furthermore a model is proposed to explain ion movements and signalling systems of NP in plant. There are however, several key considerations, which must be addressed to further test this model.

It is predicted that NP affect H⁺/Cl⁻ symport in maize conductive tissue. This hypothesis needs further testing by measuring the effect of NP on Cl⁻ uptake. Furthermore, in this model, Ca²⁺ is also involved, therefore the role of this ion needs to be elucidated directly.

Importantly, the sequence of this plant peptide hormone has not yet been obtained. Even though the immuno blot has revealed that the two fractions eluted from immunoaffinity column are immunoreactive with anti-ANP antibody, the structure of this hormone remains to be elucidated.

It is hoped that these results on NPs in plant can stimulate further research in this area.
BIBLIOGRAPHY


