CMLS, Cell. Mol. Life Sci. 54 (1998) 1161–1167
1420-682X/98/101161-07 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Article

Natriuretic peptide hormones promote radial water movements from the xylem of *Tradescantia* shoots

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Received 15 June 1998; received after revision 7 August 1998; accepted 26 August 1998

Abstract. Immunological evidence suggests that plants, like vertebrates, contain natriuretic peptides (NPs) and that rat atrial NP (rANP) binds specifically to plant membranes and promotes concentration and conformation-dependent stomatal opening. Stomatal opening and specific increases in cGMP levels were also observed in response to immunoreactive plant NP (irPNP). Here we report that both 1 μM rANP and irPNP (100 ng total protein/100 μL) significantly increase radial water movements out of the xylem of shoots of *Tradescantia multiflora*. Enhanced radial water movements are also observed in response to the cell permeant cGMP analogue 8-Br-cGMP (100 nM). The water channel inhibitor mercuric chloride ($HgCl_2$) sig-nificantly inhibits radial water movements at concentrations of 50 μM, while the presence of 10 μM 2-hydroxyethylmercaptoethanol (ME) prevents the inhibitory effect of the mercurial. The guanylate cyclase inhibitor LY 83583 at a concentration of 20 μM and sodium azide (NaN_3) at concentrations of ≥ 1 μM both also reduce radial water movements. We therefore conclude that the regulation of radial water movement out of the xylem involves modulation of cGMP levels, water channels and respiration-dependent processes. In addition, we propose that NPs have a critical role to play in radial water movements out of the xylem and speculate that as in vertebrates, NP effects might, at least in part, be mediated via the regulation of guanylate cyclases and water channels.

Key words. Natriuretic peptides in plants; water channels; cGMP; $HgCl_2$; ^2H NMR.

Maintenance of water and solute homoeostasis is a key requirement for living systems. In vascular plants, water and solutes are taken up mainly via the root system and are actively or passively transported to different plant organs and cells. The bulk of water is lost via the stomatal pores, whereas ions generally remain in the system and fulfil specific structural, metabolic or signalling roles and/or are sequestered in specific organelles. Water and solute homoeostasis are coregulated by different plant hormones that, among other tasks, regulate stomatal guard cell movements, ion channel activities, compatible solute synthesis and other drought and salt stress responses (for review, see ref. 1). In this paper we demonstrate that rat atrial natriuretic peptide (rANP) and a novel immunoreactant plant natriuretic peptide hormone (irPNP) have a role in radial water movements from the xylem of *Tradescantia multiflora* stems and hence in water and solute homoeostasis.

In vertebrates natriuretic peptide hormones are strongly implicated in the regulation of salt and water balance. Na^+ reabsorption occurs predominantly via apical

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amiloride-sensitive Na^+ channels and basolateral Na^+,K^+-ATPases in renal tubular cells [2]. The effects of NPs are mediated by two types of receptors (NPR-A and NPR-B) which operate via intracellular guanylate cyclase domains. Binding of ligand results in augmented intracellular cyclic guanosine-3′,5′-mono-phosphate (cGMP) levels. A third receptor, NPR-C, does not contain a guanylate cyclase domain but is probably linked to a cAMP-dependent pathway. NPR-C also functions as a 'clearance' receptor by internalizing and metabolizing NPs (for review, see ref. 3).

Several peptides of the NP family mediate inhibition of the apical Na^+ channels [2], and deactivation of Na^+,K^+-ATPases [4] and rANPs stimulates [5] or inhibits [6] Na^+/H^+ antiporters in different cell types. NPs have also been shown to increase conductance of K^+ channels in rat mesangial cells [6] and to inhibit slow inward Ca^{2+} channel activity as well as to facilitate K^+ channel activity in atrial ventricular papillary muscle [8].

In plants it has been shown that antibodies to rANPs recognize a putative plant rANP analogue [9, 10], and radioimmunoassay data predict that NPs isolated from plants show a high degree of amino acid similarity to vertebrate ANPs [10]. Furthermore, it was reported that rANP binds specifically to isolated leaf membranes from *Tradescantia*, suggesting the presence of NP-binding sites [11]. It was also demonstrated that rANP causes stomatal opening in a concentration-dependent manner in *Tradescantia* [11] and that this effect of rANP is critically dependent on the secondary structure of the peptide hormone. The native circular molecule is active, whereas the linearized molecule shows no biological activity [12]. Recent reports also suggest that cGMP is a second messenger in plant signal transduction and is involved in light sensing and phytochrome signalling (for review, see ref. 13), stomatal guard cell movements [12] as well as in gibberellic acid (GA)-induced gene expression in barley aleurone [14]. In addition, it has been demonstrated that the voltage dependence of a K^+ channel in *Arabidopsis thaliana* (KAT1) is modulated not just by pH and ATP but also by cGMP [15]. Since some NPs appear to signal via cGMP and affect cation and water transport in animal and plant cells [12], we are interested in further elucidating NP responses in plants with a view to characterizing NP effects on water and solute movements.

Materials and methods

Materials. Mercuric chloride ($HgCl_2$), sodium azide (NaN_3), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (neutral red), 2-hydroxyethylmercaptoethanol (ME), 8-bromo-cyclic-guanosine-3′,5′-mono-phosphate (8-Br-cGMP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LY 83583 (6-anilinoquinoline-5,8-quinone) was obtained from Calbiochem (La Jolla, CA, USA), rat 1-28 rANP (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 synthesized by Auspep (Parkville 3052, Australia) and deuterium oxide was supplied by CIL (Andover, MA, USA). The rabbit anti-α-ANP 1-28 (human, canine) antibody was obtained from Peninsula Laboratories Inc. (Belmont, CA, USA) and brilliant blue (E 133) was purchase from Safeway (Highton 3216, Australia).

Isolation of irPNP. irPNP was extracted from ivy leaves ground in liquid N_2, followed by centrifugation and ethanol liquid phase extraction and lipid removal with diethyl ether (1:1) as detailed elsewhere [16]. Aqueous extracts were freeze-dried and resuspended in double-distilled (dd) water prior to size fractionation on a Sephadex G25-80 column. Absorbance at 280 nm was measured, and a bioassay was performed on selected fractions. Active fractions were pooled, freeze-dried and resuspended in 1 mL of ddH_2O. CNBr-activated Sepharose 4B was prepared, unreacted groups were blocked and the adsorbent slurry was poured into an appropriate column. Aliquots were loaded, washed, then eluted with a stepwise series of 0.25 M, 0.5 M, 0.75 M and 1 M KCl solutions. The bioactivity was assayed [16], and irPNP was dialysed and concentrated prior to determining protein amounts by the method of Lowry [17].

Quantification of radial water movement. Stems from *T. multiflora* grown at room temperature and under natural daylight conditions were cut into approximately 4.5-cm-long segments between two internodes and transferred to 1.5 mL (Eppendorf) reaction tubes containing ddH_2O for equilibration. After 20 min the shoots were transferred to $\leq$0.5 mL of the respective treatment solutions in ddH_2O. The tissue was placed in such a way as to ensure 5-mm submersion of the shoot. Treatment times were 10 min followed by 30 min in ddH_2O containing 0.02% (w/v) brilliant blue. Post-treatment the 5-mm submersed segment of the stem tissue was cut off and discarded. Above the treatment zone, two 5-mm tissue segments were excised (at 10 mm and 30 mm), and longitudinal sections of $<$1 mm thickness were prepared, rinsed in ddH_2O and observed under the microscope. The widths of the dye fronts extending from single xylem cells were measured under the microscope with a calibrated ocular micrometer. Measurements were only taken in sections where the conductive tissue was intact, and results were analysed using a *t* test and/or one-way analysis of variance (ANOVA). Changes from the standard treatment procedure are detailed in the text and figure legends, and viability of the tissue and cells was assessed with neutral red (an indicator of plant cell viability) stains (0.01% w/v).

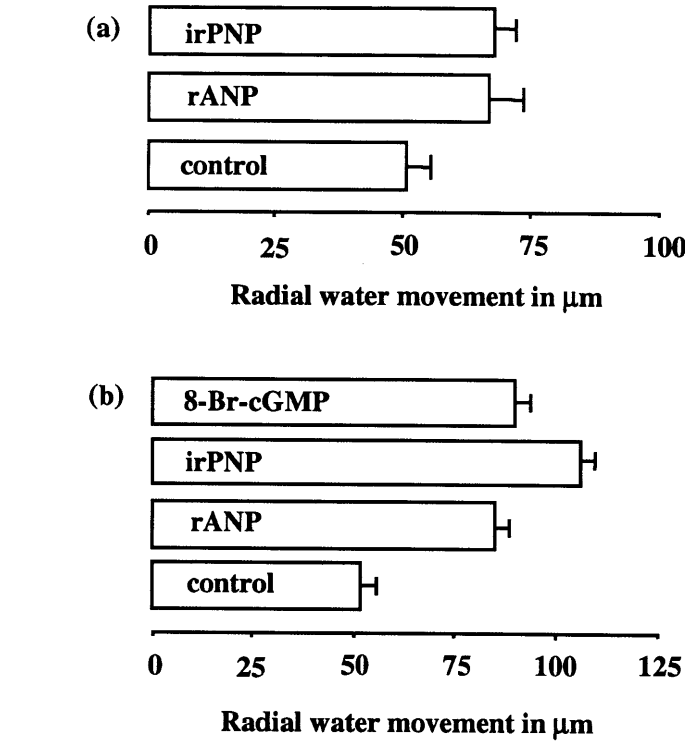

Figure 1. Mean lateral water movement in microns in response to 10-min exposure to 1 µM rANP and irPNP (100 ng total protein/100 µL) (*a*). In (*b*) the exposure time was increased from 10 min to 30 min, and a treatment with 100 nM 8-Br-cGMP was included. Each bar represents the mean of ≥ 12 measurements per shoot segment, and the error bars show standard errors (SE). An example of a (0.02% w/v) brilliant blue-carrying cell (*c*) is optically sectioned with confocal laser scanning microscope (excitation: 488 nm) to reveal typical helical wall thickenings of a xylem cell (*d*). (*e*) shows a ^{2}H NMR trace; the left peak represents the signal from the internal standard, and the right peak represents the sample. The inset compares mean ratio between internal reference and controls (■) with mean ratios between internal reference and 1 µM rANP treatments (□) ($n = 9$).

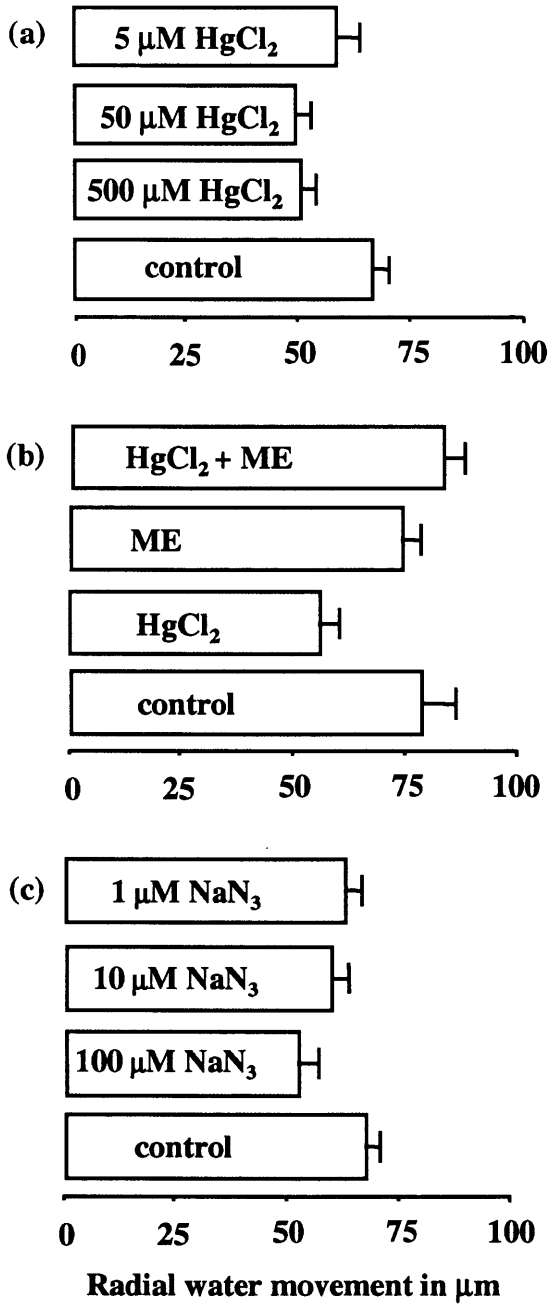

Figure 2. (*a*) Effects of the aquaporin inhibitor mercuric chloride ($HgCl_2$) at concentrations of 5 μM, 50 μM and 500 μM $HgCl_2$ on the mean lateral water movement. (*b*) Effect of 10 μM ME and of a 10-min preexposure of the tissue to ME following combined 50 μM $HgCl_2$ and 10 μM ME treatment. The effect of 10-min NaN_3 treatment at concentrations of 1 μM, 10 μM and 100 μM is presented in (*c*).

Determination of tissue water exchange ratios by deuterium NMR. ^2H NMR was performed with a JEOL (GX270 FT) NMR spectrometer (JEOL, Tokyo, Japan) according to a protocol essentially described in ref. 18. The operating frequency was 51.47 MHz, the probe temperature was 24 °C and 128 scans were performed at an acquisition time of 1.24 s per scan. As an internal reference one and the same sealed glass tube containing 1% (v/v) ^2H-trifluoroacetic acid in benzene was placed coaxially in the sample tubes. Results are expressed as a ratio between the peak of the internal reference and the peak of the sample. Sample treatments included weighing of the tissue to ensure near identical weights (±5 mg), substitution of the water with 95% ^2H_2O and homogenizing of the tissue prior to spectroscopy; the other treatment parameters remained as described above.

Results

Figure 1a demonstrates that a 10-min exposure to 1 μM rANP significantly ($P \geq 0.05$) increases radial solute movements out of xylem cells of *Tradescantia* stems. Similar increases in radial water movements are found in the presence of irPNP at a concentration of 100 ng total protein/100 μL. Increases are even more pronounced and very highly significant ($P \geq 0.001$) when the exposure times to the NPs were increased from 10 min to 30 min (fig. 1b). Furthermore, the cell-permeant cGMP analogue 8-Br-cGMP at concentrations of 100 nM and 30-min treatments also increases radial solute movements, and these increases are also highly significant ($P \geq 0.001$) (fig. 1b). An example of a xylem cell transporting brilliant blue is shown in figure 1c, whereas figure 1d details the typical cellular xylem structure as viewed with confocal laser scanning microscopy. In figure 1e we show a ^2H nuclear magnetic resonance (NMR) trace where the two peaks represent the internal reference (left) and the sample (right). The inset compares mean ratio between internal references and controls with mean ratios between internal references and 1 μM rANP treatments, suggesting that total ^2H_2O in the tissue is increased as a result of the treatment with NP.

Figure 2a–c shows the effects of the water channel (or aquaporin) inhibitor $HgCl_2$ in the presence and absence of ME (a and b) and NaN_3 (c) on lateral solute movements. $HgCl_2$ markedly reduces radial water movements below control values at concentrations of 50 μM ($P \geq 0.005$) and 500 μM ($P \geq 0.005$), whereas the effect of 5 μM $HgCl_2$ is not significant ($P \geq 0.4$) (fig. 2a). The lowest tested $HgCl_2$ concentration that yields significant inhibition is 50 μM, and this concentration was therefore used in subsequent experiments.

Since the sulphydryl-reducing agent ME is known to reverse the effects of mercurials on aquaporins in some systems, experiments were designed to test the effect of ME in our preparation. It appears that 10

μM ME on its own does not significantly affect radial solute movement as compared with control ($P \geq 0.3$). However, when the tissue is first pretreated with 10 μM ME for 10 min prior to $HgCl_2$ exposure, protection from the mercurial-dependent decrease in radial water movement is observed (fig. 2b).

A side-effect of the mercurials is their capacity to lower the membrane potential much in the way NaN_3 does. Therefore a number of NaN_3 concentrations were tested to establish a putative role of the membrane potential in radial solute movement as well as to compare NaN_3 and $HgCl_2$ effects. In our preparation consistent significant reductions below control levels were observed in response to NaN_3 at concentrations of 100 μM ($P \geq 0.018$) (fig. 2c), whereas concentrations of 10 μM and 1 μM do not induce significant effects.

Selected tissue samples used in the experiments reported in figure 2 were also stained with neutral red, an indicator of plant cell viability, to test the effects of different treatment conditions on viability. It was found that none of the treatments induce cell death within the experimental time frame (data not shown).

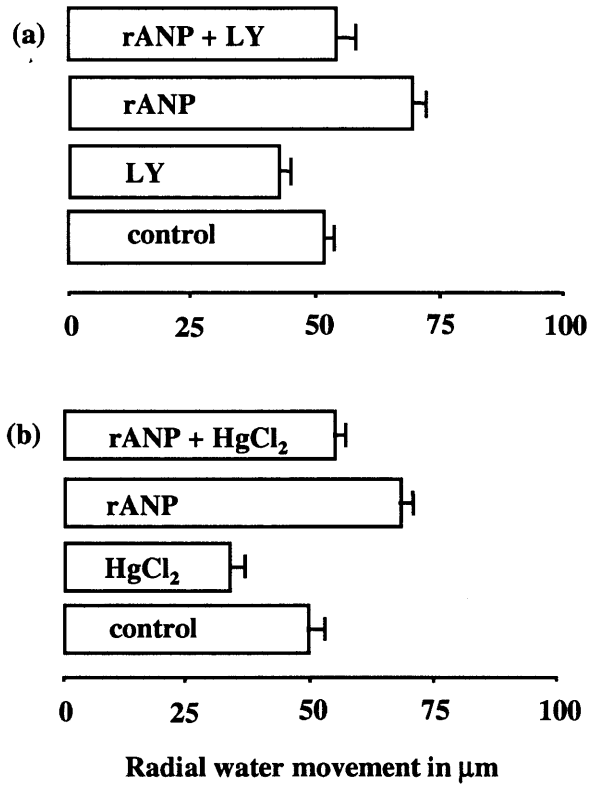

Figure 3. The effects of the guanylate cyclase inhibitor LY 83583 at a concentration of 20 μM (a) and 50 mM $HgCl_2$ (*b*) on mean lateral water movement in the presence and absence of 1 μM rANP.

Since the addition of the second messenger cGMP increases radial water movements (fig. 1b) the following experiment addresses two questions. First, does LY 83583, an inhibitor of guanylate cyclase, affect water movements and second, are rANP-dependent increases in water movement cGMP-dependent? It is observed that in the presence of 20 μM LY 83583 lateral water movement is significantly ($P \geq 0.02$) decreased as compared with control values, thus indicating a role for cGMP in this process (fig. 3a). When 20 μM LY 83583 and 1 μM rANP are added together, the levels reached were similar to those of the control but significantly below the levels of rANP in the absence of LY 83583, thus indicating that at least a partial rANP response is not dependent on LY 83583-inhibited guanylate cyclases (fig. 3a).

Since we have shown that radial water movements are modulated by both $HgCl_2$ and NPs, the following series of experiments (fig. 3b) addressed the question of the interdependence of the two responses. It is observed that in the presence of 50 μM $HgCl_2$, which on its own significantly decreases lateral water movements ($P = 0.005$), 1 μM rANP still exerts an effect (fig. 3b). However, while treatments with rANP lead to significantly bigger values in the absence as compared with the presence of water channel inhibitors ($P = 0.000$), the rANP-induced levels in the presence of the mercurial are not significantly increased over those of the control. It is therefore concluded that rANP-induced effects are at least in part dependent on the presence of functional water channels, whereas a direct effect of $HgCl_2$ on rANP cannot be excluded entirely.

Discussion

In this study we show that rANP, irPNP and the second messenger cGMP promote lateral water movements out of single xylem cells of *Tradescantia* shoots. All three compounds have previously been shown also to promote stomatal guard cell opening, and the current results are extended evidence for the role of an NP hormone system operating in plants. In particular, we propose that the NP hormone system has an important role in the regulation of water and solute movements out of the xylem. The ^2H NMR data not only affirm a role for rANP in fluid exchange but also validate our observations with dye movement experiments.

Several mechanisms could conceivably be (co)operating to achieve NP-dependent solute movements, including the modulation of ion channel activities in the conductive tissue itself or the mesophyll cells adjacent to it. The channel activities in turn may be modulated by signal transduction pathways involving up- or down-regulation of guanylate cyclases in response to NP-

binding to specific receptors in these cells. The guanylate cyclase inhibitor LY 83583 is used as a tool in NP research in animals and plant research [e.g. 12, 14] even though there is some uncertainty about its precise mode of action. However, in plants LY 83583 has been reported to inhibit different cGMP-dependent processes such as GA-dependent gene induction in barley aleurone layers [12] and rANP- or kinetin-dependent stomatal opening [14]. Our results of increased radial water movement in response to the cell-permeant cGMP analogue as well as the LY 83583-dependent reduction of rANP-induced water movements are both consistent with a role of cGMP in this process.

Furthermore, a mechanism has recently been reported in animal systems [19] which links NPs to changes in water channel activities. Such changes in water channel activity are in some instances modulated by cyclic nucleotides [20], so that NP-dependent upregulation of guanylate cyclase activity leading to increases in cGMP levels could then directly influence intercellular water movement. This hypothesis is supported first by the structural and functional evidence for plant water channels [20–25] and second by the susceptibility of NP effects to the water channel inhibitor $HgCl_2$. Mercurial derivatives are used extensively in water channel studies [e.g. 23–25], and the reversible Hg^+ effect has been attributed to the interaction with the cysteine-189 residue of the pore protein since a homologous protein with a substitution of this cysteine residue confers mercury insensitivity [26]. While the reversibility of the Hg^+ effect with the reducing agents ME or dithiothreitol is a well-established fact [e.g. 27] and explained by physical removal of the Hg^+ from the binding site, we show here that ME can actually prevent inhibition possibly by directly binding to $HgCl_2$. This preventive effect is taken as a strong indication for the specificity of the Hg^+ effect on water channels. While other putative effects of mercurials such as lowering of the respiration rate and a concomitant decrease of membrane potential [25] cannot be excluded and lowering the respiration rate with NaN_3 does indeed also affect lateral solute movements (fig. 2c), we ascertained that the NaN_3 effect was not reversed by ME (result not shown). The absence of an ME effect on NaN_3-inhibited lateral water movements thus considerably strengthens the argument for a mercurial-specific effect on water channels.

It is noteworthy that $HgCl_2$-induced reduction of hydraulic conductivity has recently been reported in the tomato root system [27], and this reduction and its reversibility with ME have been interpreted as evidence for channel-mediated water pathways [27]. While there is little doubt of the inhibitory effect of $HgCl_2$ on rANP-induced increases in radial water movements, we still observe a significant increase as compared with the $HgCl_2$ reduced levels in the absence of the NP. This would suggest that, first, the water channels are not the exclusive but one of several targets of NP action and, second, that other targets may include the modulation of specific ion channels. A third possibility would be a direct effect of $HgCl_2$ on rANP that in turn might diminish the activity of the NP. These hypotheses are currently being tested.

Acknowledgements. We thank Gail Dyson for her assistance with the ESMS and NMR and Made Pharmawati for preparing the irPNP. I.N.S. is in receipt of an AUSAID scholarship, and C.A.G. receives grant-aided support from the Australian Research Council.

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