CMLS, Cell. Mol. Life Sci. 54 (1998) 272–276
1420-682X/98/030272-05 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Research Articles

Stomatal guard cell responses to kinetin and natriuretic peptides are cGMP-dependent

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Received 1 October 1997; received after revision 2 December 1997; accepted 6 January 1998

Abstract. Immunological evidence suggests that plants contain natriuretic peptides (NPs) and furthermore (3-[^{125}I]iodotyrosol^{28}) rat atrial NP (rANP) binds specifically to plant membranes. rANP and immunoaffinity-purified plant NP analogues also promote concentration-dependent stomatal opening. Here we report that kinetin, a synthetic cytokinin, and rANP induce stomatal opening in *Tradescantia albiflora* and that the 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. Furthermore, kinetin- and rANP-induced stomatal opening is reversibly inhibited by two inhibitors of guanylate cyclase, LY 83583 and methylene blue. Stomatal opening is also induced in a concentration-dependent manner by the cell-permeant cyclic guanosine-3′,5′-monophosphate (cGMP) analogue 8-Br-cGMP, and this effect is prevented by the stomatal closure promoting plant hormone abscisic acid (ABA). We conclude that in guard cells kinetin and rANP pathways operate via guanylate cyclase upregulation, and we propose that ABA-induced closure is not cGMP-dependent.

Key words. Natriuretic peptide; kinetin; abscisic acid; cGMP; stomata; guard cells.

Natriuretic peptide hormones (NPs) are strongly implicated in the regulation of salt and water balance in vertebrates. Na^+ reabsorption occurs predominantly via apical amiloride-sensitive Na^+ channels and basolateral Na^+, K^+-ATPases in renal tubular cells [1]. 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 cGMP levels. A third receptor NPR-C, does not contain a guanylate cyclase domain, but is probably linked to a cyclic adenosine-3′,5′-monophosphate (cAMP)-dependent pathway; NPR-C also functions as 'clearance' receptor by internalizing and metabolizing NPs (for review see ref. 2).

Several peptides of the NP family mediate inhibition of the apical Na^+ channels [1] and deactivation of Na^+, K^+-ATPases [3] and ANPs stimulate [4] or inhibit [5] 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 [7].

In plants it has been shown that antibodies to ANPs recognize a putative plant ANP analogue [8, 9] and that NPs isolated from plants show high degrees of similarity to vertebrate ANPs [9]. Furthermore, it was reported that rat ANP (rANP) binds specifically to isolated leaf membranes from *Tradescantia albiflora*, suggesting the presence of a low-affinity rANP-binding site [10]. It was also demonstrated that rANP causes stomatal opening in a concentration-dependent manner in *T. albiflora*

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[10], and it was speculated that rANP would do so by stimulating K^+ influx much like the classical nonpeptide hormones auxin and kinetin.

Recent reports suggest that cGMP is a second messenger in plant signal transduction and is involved in light sensing [11], phytochrome signalling (for review see ref. 12) as well as in gibberellic acid (GA)-induced gene expression in barley aleurone [13]. 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 [14]. Since NPs signal via cGMP and affect cation transport in animal cells, we are interested to further characterize NP responses in plants and in particular stomatal guard cells.

Materials and methods

Materials. Kinetin (6-furfurylaminopurine), methylene blue (3,7-bis[dimethylamino]phenazothionium chloride) and 8-bromo-cyclic-guanosine-3′,5′-monophosphate (8-Br-cGMP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LY 83583 (LY; 6-anilinoquinoline-5,8-quinone) was obtained from Calbiochem (La Jolla, CA, USA) 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 synthesized by Auspep (Parkville 3052, Australia).

Reduction and S-carboxymethylation of rANP. The reaction was performed essentially as described previously [15]; 100 µg of rANP was 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 was reflushed 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 against 1% acetic acid or H_2O, and the protein concentration was determined [16]. Molecular masses of the resulting molecules 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 minute. Spectra were acquired using a cone voltage of 70 V.

Stomatal aperture measurements. *T. albiflora* was grown in garden soil in a growth chamber in incandescent light (12 h/day) at a constant temperature of 22 °C. In each experiment segments from one single leaf (>2 mm $\times$ 5 mm) were cut, rinsed and submerged at 20–25°C in a buffer [10 mM Pipes (pH 6.3), 50 mM KCl, 1 mM $MgCl_2$ and 100 mM $CaCl_2$], in microtitre plate wells. Leaf

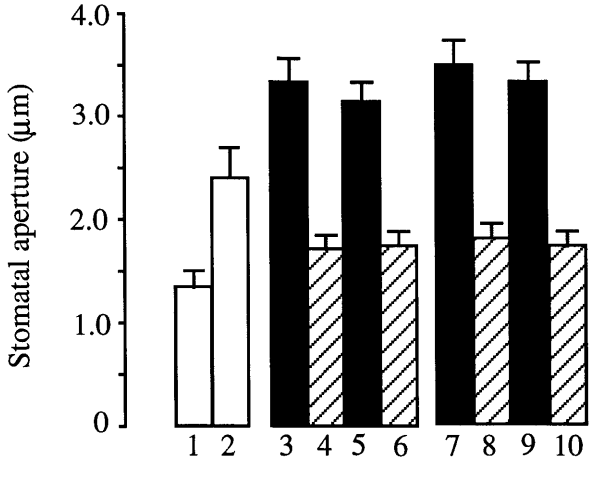

Figure 1. Mean stomatal aperture in micrometres in response to $\geq$30 min exposure to kinetin and rANP in the presence and absence of the guanylate cyclase inhibitors MB and LY. Column 1 represents the mean preillumination stomatal aperture, and column 2 shows the effect of light on the aperture. Responses to 1 µM kinetin (columns 3 and 7), 1 µM kinetin and 10 µM MB (column 4), 1 µM kinetin and 20 µM LY (column 8), 1 µM rANP (columns 5 and 9), 1 µM rANP and 10 µM MB (column 6), and 1 µM rANP and 20 µM LY (column 7) are compared with control preillumination (column 1) and postillumination (column 2) apertures. Each bar represents the mean of $>$60 stomatal measurements from three leaf segments. The error bars show standard errors (SE).

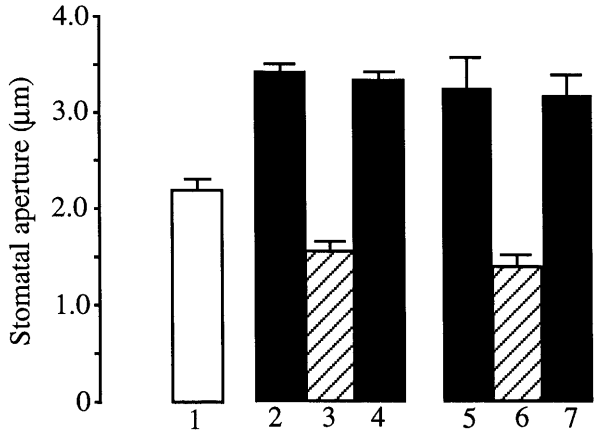

Figure 2. Reversibility of MB and LY effects on kinetin-induced stomatal opening. Column 1 represents the illuminated control aperture, columns 2 and 5 are the mean aperture induced by 1 µM kinetin, and columns 3 and 6 show the aperture in response to 1 µM kinetin in the presence of 10 µM MB (3) and 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 three 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 three volumes of LY free buffer. Bars represent the mean of $>$60 stomatal measurements from three leaf segments. The error bars show standard errors (SE).

segments were treated at 20–25 °C under incandescent light ($\lambda = 430$ nm at 35 W m^{-2}) for ≥ 30 min. Pore widths of >20 stomata from three separate segments for each treatment were measured under the microscope with a calibrated ocular micrometer, and the results were analysed using one-way analysis of variance (ANOVA).

Results

Figure 1 shows that exposure of guard cells to the 30-min illumination regime significantly ($P < 0.05$) increases mean stomatal aperture (columns 1 and 2) and that this increase is further enhanced both in the presence of 1 μM kinetin (columns 3 and 7) and 1 μM rANP (columns 5 and 9). These kinetin- or rANP-induced increases in aperture are prevented by 10 μM MB or 20 μM LY. In the presence of MB or LY and despite the presence of opening-promoting hormones kinetin or rANP, the mean aperture was consistently ($n = 5$) reduced to levels significantly ($P < 0.02$) smaller than in the illuminated control (column 2). MB and LY each on their own do not induce significant aperture changes compared with preillumination levels (result not shown).

Figure 2 demonstrates that the inhibitory effect of MB (column 3) and LY (column 6) on kinetin-induced stomatal opening is completely reversible after three 5-min rinses with three volumes of inhibitor-free buffer followed by exposure to 1 μM kinetin (columns 4 and 7). Complete reversibility after inhibitor treatment and subsequent washout was also achieved in experiments with 1 μM rANP (result not shown).

Figure 3 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 3063 (fig. 3b) is circularized due to a disulphide bond formed between the cysteines in positions 7 and 23 (fig. 3a). Successful reduction and S-carboxymethylation has linearizes (L) the molecule (fig. 3a) and increases the molecular mass to 3181 (fig. 3b). While the N form of the molecule promotes significant ($P < 0.02$) stomatal opening at 1 μM rANP, the L form shows no biological activity at that same concentration (fig. 3c).

The effect of the cell-permeant cGMP analogue 8-Br-cGMP on stomatal opening is demonstrated in figure 4. Results are expressed as percentage increase over untreated control and demonstrate that the effect of cGMP is concentration dependent (fig. 4). Maximal opening was reached with the highest-tested concentration of 100 μM 8-Br-cGMP, whereas concentrations ≤ 100 nM did not lead to significant aperture changes ($P > 0.7$). Furthermore, cGMP-dependent stomatal opening is completely prevented by 1 μM abscisic acid (ABA), a mevalonic acid-derived plant hormone (fig. 4) which among other functions promotes stomatal closure [17, 18].

Discussion

The data (fig. 1) show that the synthetic plant hormone kinetin opens stomata in *T. albiflora* and confirm induction of opening by the animal peptide hormone rANP [10]. While the classic plant hormones auxin and cytokinins, which have been shown to promote stomatal

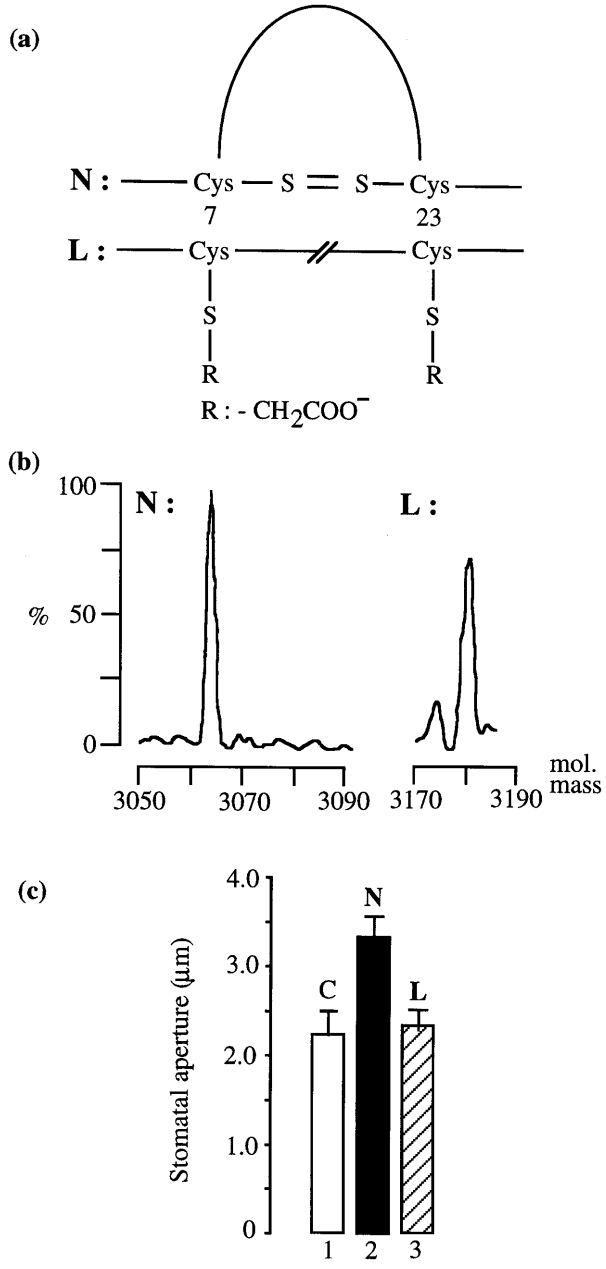

Figure 3. Dependence of the biological activity on the secondary structure of the rANP molecule. Schematic representation of the molecule in its native (N) form and in its linearized form (L) after S-carboxymethylation (*a*). Electrospray mass spectra determining the molecular masses of the N and L forms 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*).

opening [19, 20], are relatively small molecules (MW < 400), auxin being synthesized from tryptophan or indol and kinetin being an adenine derivative [18], rANP is the first peptide hormone that has been reported to affect stomatal guard cells and conceivably the first bona fide peptide hormone with biological activity in plants [10]. However, there is now growing evidence that plant signal peptides such as systemin [21] and PNPs, plant immunoanalogues of ANP [22], may turn out to be true plant peptide hormones which regulate plant development and responses to environmental stimuli at concentrations as low as $<10^{-8}$ M. If so, traditional concepts of plant responses and the assumption of their fundamental difference from animal-type responses may be severely challenged.

Since rANP signals via cGMP in animals [2], experiments were designed to find evidence for cGMP-dependent signalling by kinetin and rANP in stomatal guard cells. Both guanylate cyclase inhibitors MB [4, 23 and references therein] and LY [13, 24 and references therein] completely prevent the effects of kinetin and rANP as well as the effect of the illumination regime. This does indicate that the promotion of opening by both hormones and light requires a functional guanylate cyclase, and we are now testing if any of the guard cell K^+ channels responsible for the main cation movements which regulate guard cell turgor (therefore aperture) are responsive to cGMP. Indeed KAT1 in *A. thaliana* is the precedent for such a cGMP-dependent K^+ channel in plants [14]. Since kinetin and rANP both promote stomatal opening and are both dependent on cGMP signalling, we speculated that antagonistic hormones such as ABA which promote stomatal closure [17] or prevent opening might use cGMP-independent signal pathways or possibly operate via guanylate cyclase downregulation. Our finding that cGMP-induced opening is prevented by ABA (fig. 4) is indeed strongly indicative of such a cGMP-independent mode of ABA action. Another system where two antagonistic hormones regulate and integrate different processes (e.g. α-amylase expression) is the barley aleurone layer. In this tissue GA but not ABA increases cGMP levels, and LY inhibits GA- but not ABA-stimulated accumulation of specific and inducible mRNAs [14], thus suggesting that ABA works in a way which is either cGMP-independent or at least does not require guanylate cyclase upregulation.

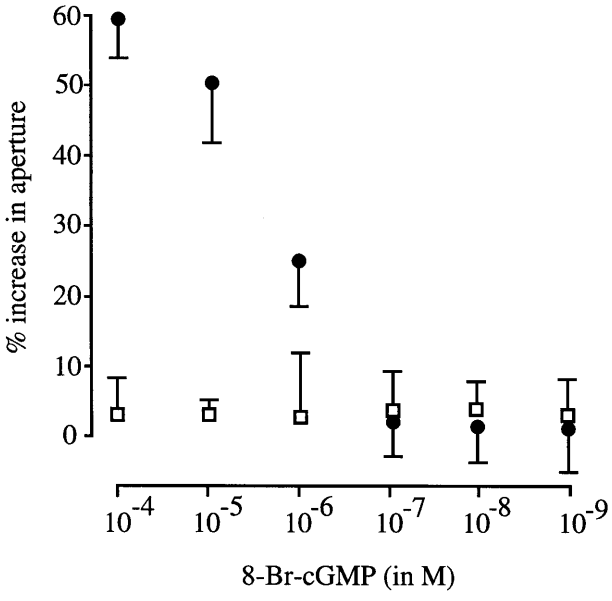

Figure 4. Mean stomatal aperture increase (%) in response to 30-min exposure to decreasing (100 μM, 10 μM, 1 μM, 0.10 μM and 0.01 μM) concentrations of 8-Br-cGMP in the presence (□) or absence (●) of 1 μM 13 ABA. Means and standard errors were calculated from >60 measurements.

The effects of both inhibitors of soluble guanylate cyclase MB and LY were completely reversed upon washout (fig. 2), indicating that these inhibitors do not exert irreversible adverse effects on stomatal functioning in the experimental system. This finding, together with the observation that 8-Br-cGMP-induced stomatal opening is not significantly inhibited by MB and LY (result not shown), indicates that the action of the inhibitors is a specific rather than a cytotoxic effect on, for example, K^+ channels.

The peptide hormone rANP is a 28-amino acid molecule that forms a loop due to a disulphide bridge between cysteines in positions 7 and 23. In addition, the molecule contains six charged amino acids (five arginines and one aspartic acid). In order to prove that the observed effects of rANP in plants are not solely due to its charges but reside in the native conformation, we have linearized the molecule and ascertained that its biological activity was lost, thereby excluding a nonspecific charge effect residing in the primary structure only. Such a loss of biological activity after irreversible reduction of the molecule has previously been demonstrated in animal systems [14] and is taken as evidence of a receptor-mediated effect which requires interaction with a ligand of specific conformation.

In summary, we conclude that kinetin- and rANP-modulated stomatal movements are cGMP-dependent, and we speculate that plant kinetin and plant natriuretic peptide receptors are associated with guanylate cyclase domains. We also propose that ABA-dependent stom-

atal closure is not mediated by increases in cGMP levels and is probably cGMP-independent.

Acknowledgements. We thank G. Dyson for her assistance with the ESMS. M.P. is in receipt of an AUSAID scholarship, and C.A.G. receives grant-aided support from the Australian Research Council.

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