Evidence for $K^+$ channel control in *Vicia* guard cells coupled by G-proteins to a 7TMS receptor mimetic

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**Summary**

To explore the involvement of a class of seven-transmembrane-span (7TMS) receptors in cellular signalling, a synthetic analogue (mas7) of the amphipathic tetradecapeptide mastoparan was used to mimic hormonal stimulus in guard cells of *Vicia faba*. The ability for mas7 to substitute for an activated receptor complex was assayed by the effect on guard cell ion channel activities in the absence of any hormonal stimulus. Currents carried by inward-($I_{k_{in}}$) and outward-($I_{k_{out}}$) rectifying potassium channels were determined under voltage clamp conditions before, during, and after exposure to mas7. The dominant effect of mas7 was to inactivate $I_{k_{in}}$ within 30 sec of application. By contrast, $I_{k_{out}}$ was largely unaffected under these conditions. The effect of mas7 on $I_{k_{in}}$ was both concentration- and voltage-dependent. At any one clamp voltage, mas7 inactivation showed Michaelian behaviour, with a mean $K_0$ of $0.05 \pm 0.02 \mu M$ at $-240 \text{ mV}$. Increasing mas7 concentration also shifted the voltage for half-maximal activation of the current negative, with 0.5 $\mu M$ mas7 effecting a $-13 \pm 2 \text{ mV}$ displacement and lengthening the halftime for activation of the current by up to threefold. By contrast, the non-amphipathic analogue of mas7, masCP, had no appreciable effect on the steady-state current or its activation kinetics; nor was the poly-cation polylysine able to substitute for mas7 in its action on the $K^+$ channels. Application of the non-hydrolysable analogue of GDP, GDP-ß-S, either by iontophoresis or by diffusion from the microelectrode, effectively blocked mas7-induced inactivation of $I_{k_{in}}$. These, and additional results provide in vivo evidence for the involvement of G-protein-linked 7TMS receptors in the regulation of membrane transport in a higher plant cell.

**Introduction**

Classical concepts of cellular signalling have emerged from studies of animal cells and appear to be remarkably conserved throughout both eukaryotes and prokaryotes. Typically, signals such as hormones are coupled to a receptor protein at the external surface of the plasma membrane, with subsequent signal transduction via association of the receptor/agonist complex with a heterotrimeric guanine nucleotide binding protein (G-protein). The G-protein is activated by exchange of bound GDP for GTP and dissociates into $\alpha$ and $\beta\gamma$ subunits, which propagates the signal leading to the regulation, among others, of ion channels.

In plants, evidence to support the existence of G-proteins similar to those identified in animals is gathering. As well as higher molecular-weight heterotrimeric G-proteins, low molecular-weight monomeric GTP-binding proteins have been found, similar to the Ras, Rho and Ypt/Rab type involved in cellular ‘housekeeping’ functions (Bednarek *et al.*, 1994; Matsui *et al.*, 1989; Terryn *et al.*, 1993). Plant G-proteins identified at the gene level include GPA1 from *Arabidopsis* (Ma *et al.*, 1990), TGA1 from tomato (Ma *et al.*, 1991), both encoding $G_\alpha$ subunits, and $\beta$-subunit-like proteins from *Chlamydomonas* and *Arabidopsis* (Schloss, 1990; Weiss *et al.*, 1994). To date, G-proteins of the heterotrimeric class have been implicated in a diverse array of physiological processes crucial for plant growth and development. From responses to fungal elicitors (Vera-Estrella *et al.*, 1994), hormonal signalling (Zaina *et al.*, 1990), phototransduction (Bossen *et al.*, 1990; Romero and Lam, 1993; Warpeha *et al.*, 1991), secondary messenger regulation (Dillenschneider *et al.*, 1986), ionic homeostasis (Allan *et al.*, 1989), and control of ion channels (Fairley-Grenot and Assmann, 1991; Li and Assmann, 1993), to enzyme regulation (Legendre *et al.*, 1993) all have been shown to be influenced by G-proteins. (For a review of GTP-binding proteins in plants, see Terryn *et al.* (1993)). Evidence for G-protein function in vivo comes from a relatively small number of model cell systems, including the stomatal guard cell. Guard cells control stomatal aperture through changes in cellular turgor, processes that are driven by fluxes of potassium and other ions, and these occur in response to environmental stimuli such as light, $\text{CO}_2$ and phytohormones. By far the best characterized responses are those to auxin and abscisic acid (ABA). Perception of either hormone initiates a signalling cascade which entails changes in cytoplasmic-free $[\text{Ca}^{2+}]_c$ (Blatt *et al.*, 1990; McAinsh *et al.*, 1990) and pH (pH$_i$) (Blatt, 1992; Blatt and Armstrong, 1993) and converges on two classes of potassium channels, at least one anion conductance, and the H$^+$ATPase (Blatt and Thiel, 1993).

In the case of ABA, current through a class of inwardly rectifying $K^+$ channel ($I_{k_{in}}$) is reduced, contributing to a...
pattern of changes in transport that precipitates stomatal closure. This channel activates upon sufficient membrane hyperpolarization, and normally provides a major pathway for K\(^+\) influx during guard cell swelling and stomatal opening. \(k_{in}\) is sensitive to pH\(_i\) (Blatt; Blatt and Armstrong, 1993) and to changes in [Ca\(^{2+}\)], with increasing [Ca\(^{2+}\)], inactivating current (Lemtiri-Chlieh and MacRobbie, 1994). In contrast, the outwardly rectifying potassium channels \(k_{out}\) of these cells, responsible for K\(^+\) efflux during stomatal closure, appear to be remarkably insensitive to Ca\(^{2+}\) (Lemtiri-Chlieh and MacRobbie, 1994). The latter channels are highly sensitive to pH\(_i\), with acid-going pH\(_i\), serving to all but eliminate K\(^+\) channel activity, and alkalini- zations increasing the current (Blatt and Armstrong, 1993). During ABA-induced stomatal closure, \(k_{out}\) conductance is enhanced, but can be blocked by acid-going pH\(_i\) (Blatt and Armstrong, 1993), indicating a second-messenger role for cytoplasmic pH.

Both classes of K\(^+\) channels are also subject to regulation by phosphatases which may play a role in hormonal control of guard cell transport. In the case of the outward rectifier, a 1/2A-type phosphatase was implicated in channel regulation from studies with okadaic acid (Thiel and Blatt, 1994), and Luan et al. (1993) have shown that the [Ca\(^{2+}\)]-dependence of \(k_{in}\) can be affected by Ca\(^{2+}\)-dependent protein phosphatases. A phosphatase may also contribute to ABA sensing of guard cells in stomatal regulation. The gene product of abi\(_1\), one of several loci responsible for reduced ABA responsiveness and a wilty phenotype in Arabidopsis thaliana, is now known to encode a 2C-type (serine/threon ine) protein phosphatase that includes a putative Ca\(^{2+}\)- binding domain (Leung et al., 1994; Meyer et al., 1994). Thus, an effective signalling machinery is in place within the guard cell allowing a graded coupling of external signals to volume changes and stomatal aperture.

More elusive is the nature of the putative receptors that coordinate and initiate signals to propagate within this signalling network. Almost exclusively, animal G-proteins are coupled to a family of seven-transmembrane-span (7TMS) receptors (Dohlman, 1991). Such receptors link agonist binding at the external surface of the membrane to intracellular G-proteins and subsequent signal generation. A common architecture for 7TMS receptors isolated thus far is emerging, and their ubiquitous nature underlines their importance in cellular signalling cascades. Indirect evidence exists to support the concept of G-protein linked 7TMS receptors in plant signal transduction (see Terryn et al., 1993). However, to date, firm evidence linking 7TMS receptors to G-protein function and cellular physiology has been lacking.

Recently, White et al. (1993) showed that the mastoparan analogue mas7 could stimulate GTP-\(\gamma\)-S binding to pea and maize microsomal membranes. Mastoparan and its synthetic analogue mas7, are toxins commonly used as diagnostics for the existence of G-protein-coupled 7TMS receptors. Mastoparan is a tetradecapeptide and was originally isolated from wasp venom (Hirai et al., 1979). Upon transfer from aqueous to lipid environment, mastoparan undergoes a conformational change, assuming a helical formation (Higashijima et al., 1984). In this form, mastoparan has structural and functional similarities to the third intracellular loop of 7TMS receptors (Higashijima et al., 1988; Weingarten et al., 1990), a cytoplasmic domain that is thought to be important in determining G-protein interaction. Mastoparan effectively mimics this domain and stimulates GTPase activity (i.e. exchange of GDP for GTP) independent of the usual agonist/receptor interaction. This study utilizes mas7 to probe receptor/G-protein coupling in guard cells of Vicia faba. We show that mas7 effects a selective reduction of current through \(k_{in}\), but not through \(k_{out}\), and that this response can be ascribed to G-protein-mediated modulation via secondary messengers.

**Results**

mas7 and K\(^+\) channel currents

To explore the effect of mas7, membrane currents were recorded under voltage clamp at regular intervals before, during and after treatments with the toxin. Figure 1 shows currents recorded from one guard cell, and details the current carried by the inwardly rectifying K\(^+\) channels \(k_{in}\). The current was apparent upon clamping the cell from a holding potential of −100 mV to potentials between −120 and −250 mV and then to a tailing potential of −30 mV (Figure 1a). The K\(^+\) current was characterized by a slow rise in clamp current magnitude, with halftimes of about 100 msec near −200 mV. Steady-state characteristics for \(k_{in}\) were determined from the trajectories, as shown in Figure 1(a), by first subtracting the instantaneous current recorded at the beginning of each voltage step from the steady-state current, measured at the end of the step. Figure 1(b) shows the results of this current subtraction and highlights \(k_{in}\) dependence on clamp voltage. Figure 1(b) also shows that the effect of mas7 was to reduce \(k_{in}\) in a concentration-dependent manner. By contrast, voltage-clamp measurements at voltages positive of −100 mV and steady-state data for whole-cell currents demonstrated that mas7 had no consistent effect on the outwardly rectifying K\(^+\) channels \(k_{out}\), nor did it affect background membrane conductance (Figure 2; see also Figure 1). For the cell shown in Figure 1, 0.1 \(\mu\)M mas7 reduced \(k_{in}\) by 46% at −250 mV, while 0.5 \(\mu\)M and 1.0 \(\mu\)M effected reductions of 69% and 80%, respectively, at this voltage. Inactivation by mas7 was Michaelian at any one voltage (Figure 3), and comparable results were obtained in all 15 other cells treated with mas7. Pooled results from all guard cells
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Figure 1. Inactivation of $I_{K,\text{lin}}$ by mas7.
Data from one Vicia guard cell bathed in 5 mM Ca$^{2+}$-MES with 10 mM KCl.
Cell parameters: surface area, $1.8 \times 10^{-5}$ cm$^2$; volume 4.9 pl; stomatal aperture, 7 $\mu$m.
(a) Current trajectories of the inward rectifier ($I_{K,\text{lin}}$) recorded before (•) and during exposure to 0.1 (■), 0.5 (○) and 1.0 (△) $\mu$M mas7. Clamp cycle voltages (above); conditioning, $-100$ mV; test ($n = 8$), $-120$ to $-250$ mV; tailing, $-30$ mV. Scale: vertical, $250$ mV or $25$ $\mu$A cm$^{-2}$; horizontal, $500$ msec. Note the absence of any increase of background (instantaneous) current at the beginning of each test clamp step.
(b) Steady-state current-voltage relations for $I_{K,\text{lin}}$ taken from currents recorded at the end of the voltage pulse scan in (a) after subtracting the instantaneous background currents recorded less than $5$ msec into the test pulse at each voltage. Data shown are for control (•), 0.1 (■), 0.5 (○) and 1.0 (△) $\mu$M mas7. Lines are calculated from the fitted conductances using equations (1) and (2) (see text).
Inset: plot of steady-state conductance as a function of clamp voltage for control (O), 0.1 (■), 0.5 (○) and 1.0 (△) $\mu$M mas7. Conductances fitted jointly to the data in (a) and (b) using equations (1) and (2) (see text) yielded a common apparent gating charge, $\delta$, of 1.43 ± 0.08. Vertical lines mark voltages for half-maximal activation, $V_{1/2}$: -163 (O), -175 (■), -177 (○) and -183 mV (△).

Figure 2. Whole-cell current response to mas7.
Steady-state current are from the same guard cell as in Figure 1, gathered using a bipolar staircase pulse protocol to scan through the accessible voltage range before (•) and during (V) exposure to 0.5 $\mu$M mas7. The dashed line indicates the background current estimated by linear extrapolation from points between $E_K$ (-71 mV) and $-120$ mV (see Blatt, 1992). Currents above (positive-going) and below (negative-going) this line correspond to $I_{K,\text{lin}}$ or in background current during challenge with mas7.

from tail current measurements, were not significantly affected by mas7 (Table 1).

The effect of mas7 on $I_{K,\text{lin}}$ activation implied a significant influence on the voltage-dependence for the steady-state current. Analysis of the currents, such as shown in Figure 1, supported this prediction. Figure 1(b, inset) shows the conductance $g_{K,\text{lin}}$ calculated from the data in Figure 1(a) and plotted as a function of clamp voltage. Conductances were determined assuming an ohmic dependence on membrane voltage so that

$$g_K = I_K/(V - E_K) \quad (1)$$

As shown, the data have been fitted jointly to the Boltzmann function

$$g_K = \frac{g_{K,\text{max}}}{1 + e^{(V_{1/2} - V)/RT}} \quad (2)$$

where $g_{K,\text{max}}$ is the conductance at saturating negative membrane voltage, $\delta$ is the apparent gating charge, $V_{1/2}$ is the characteristic half-maximal activation voltage and $F$, $R$ and $T$ have their usual meanings. Satisfactory fittings were obtained only when $V_{1/2}$ and $g_{K,\text{max}}$ were allowed to vary between mas7 treatments, and yielded a concentration-dependent shift in the voltage for half-maximal activation of $-11 \pm 2.8$ in 0.1 $\mu$M, $-13 \pm 1.8$ for 0.5 $\mu$M, and $-19 \pm 3.1$ mV for 1.0 $\mu$M mas7. Thus, mas7 not only reduced the maximal $K^+$ channel current, but effectively displaced channel gating to increasingly negative voltages. Qualitatively similar results were obtained on a cell-by-cell basis...
Figure 3. Inactivation of $I_{K,\text{in}}$ as a function of mas7 concentration at a clamp voltage of $-240 \text{ mV}$.

Data points are means ± SE for all 15 cells challenged with mas7 alone (Θ), and following cytoplasmic loading with nucleotides from electrodes containing 5-10 mM GDP-β-S (γ, $n=13$) and ADP-β-S (□, $n=5$). Data for mas7 treatments (Θ) were fitted to a Michaelis-Menten function, giving an apparent $K_M$ of $0.05 \pm 0.02 \mu M$ and maximum inactivation of 80%. Roughly equivalent findings were obtained when the maximum inactivation was constrained to a value of 100%.

from all 15 other V. faba guard cells challenged with mas7 (data not shown).

Uncoupling $K^+$ channel inactivation with GDP-β-S

There are principally three possible explanations for the effect of mas7 on $I_{K,\text{in}}$. The first explanation is that mas7 indeed acts as a receptor-mimetic and, by stimulating GDP/GTP exchange of a G-protein, initiates a signalling cascade that culminates with the inactivation of $I_{K,\text{in}}$. The second explanation invokes a direct interaction of mas7, by virtue of its high density of positive charges, with the mouth of the $K^+$ channel to effect a block of $I_{K,\text{in}}$. Finally, mas7 might act independently of any G-protein activation to influence downstream signalling elements that affect $K^+$ channel gating. Each explanation makes a number of experimentally testable predictions.

In the first case, preventing GDP/GTP exchange and G-protein activation should effectively block the mas7-evoked signal and, hence, $I_{K,\text{in}}$ inactivation. Equally, non-amphipathic analogues of mas7 would be expected to lack physiological activity. As a positive test for coupling via G-protein activation, the non-hydrolysable analogue of GDP, GDP-β-S, was used which could be expected to lock putative G-protein intermediates in the inactivated state by irreversible binding to the $G_\alpha$ subunit. It was reasoned that by curtailing $G_\alpha$ activation, any mas7 signal transmitted in this manner would also be blocked. Guard cells were loaded with GDP-β-S, either by passive diffusion into the cytoplasm from the voltage-following barrel of the standard, double-barrelled microelectrodes or by iontophoretic injection of the nucleotide. In the latter case guard cells were impaled with four-barrelled microelectrodes and two barrels were used for injection, one containing nucleotide in a 10 mM K+-acetate carrier electrolyte.

Figure 5 summarizes the results from one guard cell loaded with GDP-β-S by iontophoresis. Current trajectories were recorded under voltage-clamp before and during injection of GDP-β-S, and during subsequent exposure to 0.5 μM mas7. Prior to injection, GDP-β-S was retained in the microelectrode by applying a 0.25 nA backing current, and nucleotide injection was effected with a 0.25 nA current over 2 min. It is apparent from the current trajectories (Figure 5a) and the steady-state current-voltage relations (Figure 5b) that injection of GDP-β-S itself had no measurable effect on $I_{K,\text{in}}$. However, after loading the cell with GDP-β-S, $I_{K,\text{in}}$ was insensitive to mas7 treatment. Comparable results were obtained in all 13 guard cells loaded with GDP-β-S prior to mas7 application. For seven guard cells loaded by diffusion, partial and temporary declines in $I_{K,\text{in}}$ were observed when mas7 was added in the first 15 min following impalements. In each case, however, washing
mas7 from the bath resulted in complete recovery of $I_{k_{in}}$ and later challenges with mas7 showed the current to develop complete insensitivity to the toxin. The protective effect of GDP-$\beta$-S is in stark contrast to the effect of mas7 alone, to which $I_{k_{in}}$ showed a dramatic reduction with virtually no recovery in every case (see Figures 1-3). We noted that GDP-$\beta$-S alone effected an increase in the halftime for $I_{k_{in}}$ deactivation (Table 1), although it had no significant effect on activation halftimes (Figure 4).

However, cytoplasmatic loading with GDP-$\beta$-S tempered the effect of mas7 on the kinetics for $I_{k_{in}}$ activation (Figure 4). By contrast, the current retained its sensitivity to mas7 in five guard cells preloaded with ADP-$\beta$-S, demonstrating the nucleotide specificity of the response (Figure 6). A calculation of the nucleotide load for the experiment in Figure 5 gave a value of 1 mM, given a cytoplasmatic volume of 0.5 pl and assuming a transport number of 0.03 for GDP-$\beta$-S in the carrier electrolyte. This value represents a theoretical maximum and assumes, in the transport number, a charge-weighted equivalence in mobilities between GDP-$\beta$-S, $K^+$ and the acetate anion. Hence, the true nucleotide load was probably closer to 100 $\mu$M.

masCP, TEA and $K^+$ channel block

Although the ability of GDP-$\beta$-S to protect $I_{k_{in}}$ from mas7 inactivation argues in favour of G-protein-dependent channel modulation, the results do not rule out additional direct effects of the oligopeptide on the channels. If mas7 effected channel block directly, interacting at the channel mouth (Obermeyer et al., 1994), then similarly charged oligopeptide analogues of mas7 would be expected to block $I_{k_{in}}$, irrespective of any amphipathicity. As a check against
charge interactions between the K⁺ channels and mas7, guard cells were also challenged with masCP. MasCP is an analogue of mas7 and like mas7, comprises 14 amino acids but with an additional positive charge (Leu substituted at position 6 with Lys). As a result, masCP is predicted not to form an amphipathic helix at the lipid interface and it does not mimic the third cytoplasmic loop of the 7TMS receptors (Higashijima et al., 1998). In vitro masCP, unlike mas7, is ineffective at promoting GTP-γ-S binding to plant membrane fractions (White et al., 1993). Thus, masCP was expected to lack physiological activity in G-protein activation, but might substitute for mas7 in direct, charge-dependent channel block.

When masCP was tested over the same concentration range as mas7, masCP failed to evoke any consistent change in \( k_{\text{in}} \). Indeed, in three of 15 cells tested \( k_{\text{in}} \) was enhanced 10–15% in the presence of 0.5 μM masCP compared with currents recorded from the same cells prior to treatments. Figure 7(a) shows the effect of 0.1 μM masCP—a concentration close to the \( K_i \) for mas7—on the current–voltage relations of one Vicia guard cell.

Guard cells were also challenged with mas7 in the presence of TEA which blocks K⁺ channels by binding within the mouth of the channel pore. We reasoned that if mas7 interacted with the K⁺ channel by entering the channel mouth to block the K⁺ current, then TEA might compete with mas7 for binding and prevent inactivation. So in these experiments, the guard cells were first exposed to 10 mM TEA and mas7 was added subsequently in the presence of TEA. Channel current was assayed for the effect of mas7 after first washing the peptide and then TEA from the bath.

Figure 7(b) shows the effect of TEA on the current–voltage relations of a single Vicia guard cell. TEA quickly and effectively reduced \( k_{\text{in}} \). Experiments with TEA alone indicated that wash-out was equally rapid (see also Blatt, 1992), taking only 1–2 min for \( k_{\text{in}} \) to recover fully (results not shown). This is in contrast to the inactivation of \( k_{\text{in}} \) by mas7, which was largely irreversible (see Figure 1 and accompanying discussion). Application of mas7 and TEA together affected no further reduction of \( k_{\text{in}} \) compared with TEA alone. However, upon washing out mas7 and then TEA from the bath, the current did not recover (Figure 7b). Thus, mas7 could effect inactivation of \( k_{\text{in}} \) even when the channel mouth was occluded by TEA. Again, quantitatively similar results were obtained in six other cells. These, and the results with masCP argue strongly against a direct interaction between the K⁺ channels and mas7.

**Discriminating mas7/G-protein from non-specific interactions in K⁺ channel control**

The third explanation for mas7 action on \( k_{\text{in}} \) entails non-specific interactions with other signalling elements that control the K⁺ channels. Mastoparan and related analogues are known to affect Ca²⁺ binding with calmodulin (Ohki et al., 1991) and to alter the activities of C-type phospholipases (Drobak and Watkins, 1994; Wallace and Carter, 1989). These actions are typically evident only at concentrations around 10 μM and above. So, the very low \( K_i \) for mas7 inactivation of \( k_{\text{in}} \)—and ability of GDP-β-S to protect \( k_{\text{in}} \) from mas7 inactivation—argues against such indirect effects as the dominant mode of mas7 action. None the less, the data cannot rule out additional secondary effects, and we note that some Ca²⁺–dependent protein kinases are sensitive to polycations even at nanomolar concentrations (see Roberts and Harmon, 1992).

As a check against secondary interactions of these kinds, guard cells were exposed to low molecular-weight (3–5 kDa) polylysine alone, or to mas7 together with neomycin sulphate, an inhibitor of phospholipase C (Faddis and Brown, 1993; Legendre et al., 1993). Figure 8 shows the
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Figure 8. Insensitivity of I_K, in to polylysine.
Data from a single Vicia guard cell bathed in 5 mM Ca²⁺-MES with 10 mM KCl and challenged with 1 μM polylysine (3-5 kDa MW). Cell parameters: surface area, 2.3 x 10⁻⁵ cm²; volume 5.5 pl; stomatal aperture, 11 μm.
(a) Current trajectories for I_K, in before addition (○), in the presence of 1 μM polylysine (□) and after washing polylysine from the bath (■). Clamp voltages: conditioning, -100 mV; test (n = 8), -120 to -240 mV; tailing, -30 mV. Scale: vertical, 250 mV or 25 μA cm⁻²; horizontal, 500 msec.
(b) Current-voltage relations for I_K, in recorded at the end of the voltage pulse scan in (a) after subtracting the instantaneous background currents recorded less than 5 msec into the test pulse at each clamp voltage. Data cross-referenced to (a) by symbol.

How is the mas7 signal transduced?
Calcium and pH have established second messenger roles in guard cells and, while the experiments with neomycin sulphate would argue against a pivotal function of phospholipase C- and inositol trisphosphate-mediated release of intracellular Ca²⁺, alternative pathways for Ca²⁺ entry to the cytoplasm have been mooted (MacRobbie, 1992). Less still is known of the origins for changes in pH, although it is clear that physiological stimuli such as auxin and ABA influence the cytoplasmic-free [H⁺]. Increasing [Ca²⁺] (Schroeder and Hagiwara, 1989) and alkaline-going pH, both inactivate I_K, in (Blatt and Armstrong, 1993), and either of these changes could mediate its inactivation by mas7. Thus, the question arises whether changes in either pH or [Ca²⁺], could transduce the signal downstream of a mas7/G-protein interaction. To investigate the role of these second messengers downstream of the mas7 stimulus, experiments were designed to buffer changes in [Ca²⁺], and pH. Again, we reasoned that buffering any mas7-evoked changes should curtail any signal transmission via these ionic intermediates.

To buffer pH, guard cells were treated with butyrate which imposes an acid load, effectively clamping pH to a more acid value. Previous studies had shown that acid loading stimulates I_K, in but otherwise has no effect on its competence to respond to exogenous stimuli (Blatt and Armstrong, 1993). Thus, if mas7 inactivated I_K, in through pH alkalization, preloading with butyrate would be expected to relieve its effect on I_K, in. Figure 9(a) shows the response of I_K, in in one guard cell to 10 mM butyrate and to subsequent addition of 0.5 μM mas7 against this background of butyrate. Acid-loading with butyrate effected an increase in I_K, in when compared with the control. However, addition of mas7 led to inactivation of the current none the less, both compared with the control and with the butyrate treatment, and the inactivation of I_K, in was roughly equivalent on a relative basis when compared with guard cells challenged with mas7 alone (see Figure 1). Similar results were obtained in experiments with five other guard cells, giving a mean relative inactivation of I_K, in at -250 mV of 0.50 ± 0.18 in butyrate compared with the value of 0.54 with mas7 alone (see Figure 3).

To buffer cytoplasmic-free [Ca²⁺], three guard cells were preloaded with 50 mM EGTA, introduced by diffusion from the voltage-following barrels of two-barrelled microelectrodes (Blatt et al., 1990). Loading EGTA had a negligible effect on I_K, in in each case (see also Blatt et al., 1990). Figure 9(b) shows that a comparable degree of inactivation was observed in I_K, in on adding 0.5 μM mas7, even after prior loading with EGTA for 20 min (compare with Figure 1), and the current failed to recover on washing mas7 from the bath.

Discussion
While inward-rectifying K⁺ channels in guard cells of V. faba had previously been shown to be subject to regulation by
putative G-proteins (Fairley-Grenot and Assmann, 1991), their nature and potential receptor coupling has remained unknown. Our data now strongly suggest that in vivo the K⁺ channels are coupled through G-proteins to a class of 7TMS-like receptors. Three principal observations bear on this conclusion: (i) mas7 inactivation of the K⁺ channels was specific to \( I_{\text{K,in}} \), the toxin being without consistent or significant effect on \( I_{\text{K,out}} \) or background membrane conductance (Figures 1 and 2); (ii) cytoplasmic loading with GDP-\( \beta \)-S, which could be expected to uncouple putative G-protein signals by irreversibly binding and inactivating \( G_{\alpha} \), was effective in protecting \( I_{\text{K,in}} \) against mas7-evoked inactivation and could not be substituted with equivalent ADP-\( \beta \)-S loads (Figures 5 and 6); (iii) an inactive mas7 analogue, masCP, and polylysine were without effect on the current while pretreatments with TEA, neomycin sulphate and cytoplasmic loadings with the Ca²⁺ buffer EGTA failed to prevent the inactivation of \( I_{\text{K,in}} \) in subsequent mas7 exposures (Figures 7-9). The first point rules out any non-specific detergent-like action of mas7 on the plasma membrane (cf. Okumura et al., 1981; Weidman and Winter, 1994); such effects would be visible as increases in 'leak' current against the very low background membrane conductance near the free-running potential (typically 10–20 \( \mu \)S cm⁻² or 1–3 GΩ, comparable with background conductances of patch recordings from plant membranes). The third point argues against the predominance of secondary interactions of mas7 with downstream elements, including Ca²⁺-dependent protein kinases (Roberts and Harmon, 1992), phospholipase C and Ca²⁺-related events (Drobak and Watkins, 1994; Ohki et al., 1991; Wallace and Carter, 1989). However, the strongest argument for a mas7/G-protein-mediated pathway is the specificity for and GDP-\( \beta \)-S antagonism of K⁺ channel inactivation (Figures 5 and 6).

Indeed, these results underline the parallels found in the signalling strategies utilized by both animals and plants. Thus far, studies which have investigated the roles of G-proteins in guard cell signalling have generally concentrated on putative targets, and not on possible receptor types. Although we cannot provide conclusive evidence that the receptor has seven transmembrane domains, and can only allude to the nature of the region interacting with the G-protein(s), evidence from animal cells suggest that the 7TMS receptor class is one which is predominantly linked to heterotrimeric G-proteins. To date, there is only one report of a receptor with a single TMS domain (Okamoto et al., 1990), which none the less retained the crucial regions thought to be important for G-protein interaction. Our data imply that even if the functional region, which mas7 mimics, is not part of 7TMS receptor per se, it must be adequate both for interaction with the G-protein, and for subsequent signal transduction in the absence of agonist.

**Nature of \( G_{\alpha} \) with which mas7 interacts**

In animal cells, mas7 is thought to interact preferentially with members of the \( G_{\alpha}/G_{\beta} \) family of heterotrimeric G-proteins (Higashijima et al., 1988, 1990; Weingarten et al., 1990), and mastoparan has been shown to have a lower sensitivity for \( G_{\text{ras}} \) than for \( G_{\text{ras}}/G_{\beta} \). Mas7 is thought to stimulate \( G_{\beta} \), which would result in a decrease of Ca²⁺ via phospholipase C-dependent IP₃ hydrolysis. Previously, \( G_{\beta} \) as well as \( G_{\text{ras}}/G_{\alpha} \) classes had been implicated in control of \( I_{\text{K,in}} \) on the basis of sensitivity to pertussis toxin (PTX) and cholera toxin (CTX) (Fairley-Grenot and Assmann, 1991). Both toxins inactivate \( I_{\text{K,in}} \), and it was suggested, therefore, that at least two classes of \( G_{\alpha} \) might control the K⁺ channels, the first inactivated by PTX (\( G_{\alpha} \) and...
like) and the second stimulated by CTX (Gαi-like). Following this model, mas7 would be predicted to stimulate a Gαi-type protein, causing a decrease in Ca^{2+} and thereby enhancing I_{K, in}. Our results suggest that this is not the case. As yet, interactions determining receptor/Gα subunit specificity are not well defined, especially in plant cells. Therefore the question remains open as to the nature of the native Gα with which mas7 interacts. The two heterotrimeric Gα subunits so far identified in plants have been assigned to the Gαi class on the basis of sequence homology (Ma et al., 1990, 1991). However, the situation is further complicated by the lack of PTX-ribosylation sites in the two cloned Gα subunits. Thus, it is to be anticipated that the structure/function relationships determined by individual G-proteins may not be conserved between plants and animals, and only tentative suggestions can be made at this time as to the nature of the Gα with which mas7 interacts.

An alternative explanation for the apparent Ca^{2+}-like) and the second stimulated by CTX (Gαi-like). Following this model, mas7 would be predicted to stimulate a Gαi-type protein, causing a decrease in Ca^{2+} and thereby enhancing I_{K, in}. Our results suggest that this is not the case. As yet, interactions determining receptor/Gα subunit specificity are not well defined, especially in plant cells. Therefore the question remains open as to the nature of the native Gα with which mas7 interacts. The two heterotrimeric Gα subunits so far identified in plants have been assigned to the Gαi class on the basis of sequence homology (Ma et al., 1990, 1991). However, the situation is further complicated by the lack of PTX-ribosylation sites in the two cloned Gα subunits. Thus, it is to be anticipated that the structure/function relationships determined by individual G-proteins may not be conserved between plants and animals, and only tentative suggestions can be made at this time as to the nature of the Gα with which mas7 interacts.

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### Downstream coupling to I_{K, in}

One of the most striking effects of mas7 on I_{K, in} was on the activation time constant, \( t_{1/2} \) (Figure 4) and a corresponding shift in the half-activation voltage (Figure 1). This effect is consistent with a reduction in the open probability of the channel (\( P_o \)) and cannot be explained simply in terms of changes in conductance or numbers of available channels. Indeed, Wu and Assmann (1994) have recently described the effects of GDP-\( \beta \)-S and GTP-\( \gamma \)-S, at the single channel level, on the kinetics of I_{K, in} activation and deactivation. They found that the effects of the nucleotides were wholly attributable to alterations in \( P_o \) rather than any change in channel conductance or permeation. It is of interest that a similar behaviour was found in the effect of mastoparan on the maxi Ca^{2+}-activated K^+ channel of bovine chromaffin cells. Glavinovic et al. (1992) resolved the effect of mastoparan at the single channel level and found that the peptide reduced \( P_o \) in a concentration-dependent manner. However, the effect of mastoparan was complex, having a more pronounced effect on certain defined subcomponents of opening.

Although it is apparent that mas7 can inactivate I_{K, in} in a manner analogous to that observed with hormones such as abscisic acid and auxin, the pathway of signal transduction is not clear from these studies. Given the effect of mas7 on the kinetics of the channel opening, and the fact that \( pHi \) and [Ca^{2+}]_i also effect changes in gating of I_{K, in} (Blatt, 1992; Schroeder and Hagiwara, 1989), it was somewhat surprising that an unequivocal role could not be found for either. Our results suggest that alkaline-going changes in \( pHi \) are unlikely to contribute to mas7-induced signalling. Even when the cytoplasmic pH of the guard cells was clamped with butyrate, mas7 treatments resulted in the inactivation of I_{K, in}. Nor could I_{K, in} inactivation be prevented by buffering changes in [Ca^{2+}]_i. In this latter instance a simple interpretation is more difficult, however. A similar approach for buffering changes in guard cell [Ca^{2+}]_i has been adopted previously with success (Blatt et al., 1990). None the less, EGTA is known to be poor in chelating rapid changes in [Ca^{2+}]_i, and is often unable to contend with the rapidity of agonist-induced Ca^{2+} transients. There is evidence for a mastoparan-evoked, IP_3-gated calcium spike in animal cells (Mousli et al., 1990; Okano et al., 1995), and mastoparan also stimulates IP_3 production in Chlamydomonas reinhardtii (Quarmby et al., 1992), suggesting that its effect on calcium release is not confined to animal cells.

The lack of putative ionic signalling intermediates raises the tantalizing possibility that mas7 signal transduction might be membrane delimited, such that no cytoplasmic signalling intermediates are required for interaction between a G-protein subunit and the K^+ channel (see Brown, 1993, for a review). Recent evidence suggests that a membrane-delimited pathway for G-proteins in K^+ channel coupling does exist in guard cells (Wu and Assmann, 1994). Indeed, there are parallels between the mechanism of inactivation of I_{K, in} by mas7 and the effect of guanine nucleotides on the channel. A membrane-delimited pathway of G-protein/channel coupling would be unlikely to necessitate gross changes in [Ca^{2+}]_i, or \( pHi \), as localized changes might be sufficient to effect transduction. However, the evidence at present does not confirm any direct physical coupling between the G-protein and the channel.

Yet another interpretation of mas7 action on I_{K, in}, and one which could support coupling via G_{phospholipase A_2} invokes mas7 stimulation of phospholipase A_2. The resulting generation of lipid messengers could explain K^+ channel modulation without recourse to the ionic messengers. In animal cells one of the few assigned signalling roles of G_{phospholipase A_2} along with the control of K^+ channels, is to activate a cytoplasmic phospholipase A_2 (PLA_2; Kim et al., 1989; Kurachi et al., 1989; Reuveny et al., 1994). Stimulation of PLA_2 would
generate lysolipids and fatty acids such as arachidonic acid. Indeed, arachidonic acid and other fatty acids have been recently implicated in the modulation of guard cell K+ channels (Lee et al., 1994), and certain species of lysolipids may exert similar control on the channels (Armstrong and Blatt, unpublished observations). Furthermore, in this context mastoparan has been shown to stimulate PLA2 in Zucchini hypocotyls in relation to auxin-induced H+ATPase activity and growth. Regulation of all PLA2 isozymes thus far identified has shown a strong dependence on [Ca2+]i, but a recent report suggests that the enzymatic activity can show [Ca2+]i-independence (Nalefski et al., 1994). It may therefore be significant that, in the guard cells, mas7 appears to regulate the K+ channels independent of changes in cytoplasmic-free [Ca2+] and pH.

**Experimental procedures**

**Plant culture and experimental protocol**

Plants of Vicia faba var. bunyard were grown in 10 cm² pots at 16–20°C with a photoperiod of 16 h light: 8 h dark under artificial lighting, and watered with Hoagland's solution (Blatt, 1987b). For experiments, plants approximately 4–6 weeks old were used, with only the youngest newly expanded leaves chosen. Leaves were harvested just before each experiment. Epidermal strips were taken from the abaxial surface and mounted on a glass chamber that had been precoated with an optically clear, pressure-sensitive, silicone adhesive (No. 355 medical adhesive, Dow Coming, Midland, MI, USA). Measurements were conducted with the strips submerged in rapidly flowing solutions (10 ml min⁻¹ ~ 20 chamber volumes min⁻¹). Ambient temperatures during experiments were 20°C–22°C.

All operations were carried out on a Zeiss IM inverted microscope (Zeiss, Oberochen, Germany) fitted with Nomarski Differential Interference Contrast optics. Surface areas and volumes of impaled cells were calculated assuming a cylindrical geometry (Blatt, 1987a). The orthogonal dimensions (diameter, length) of the cells were measured with a calibrated eye-piece micrometer. Cell dimensions typically varied over 10–14 lM (diameter) and 35–45 lM (length). Estimated surface areas were thus 1.9 ± 10⁻⁵, 3.1 ± 10⁻⁵ cm² and cell volumes were 2.7–7.8 pl.

Standard bath medium was prepared with 5 mM 2-(N-morpholino)ethanesulfonic acid (MES, pKa = 6.1). The buffer was titrated to its pKₐ (=6.15) with Ca(OH)₂ (final [Ca²⁺] = 1 mM). Potassium chloride (KCl), tetraethylammonium chloride (TEA), EGTA, ADP·β-S and GDP-β-S were from Sigma Chemical Co. (St Louis, USA). Mas7 and masCP were included as required in a solution of the same buffers and salts via a 5 ml syringe placed at the side of the chamber. Exposures in these cases were carried out under stopped-flow conditions. Trials were carried out without peptide additions showed that stopped flow alone had no appreciable effect on the K⁺ currents.

**Microelectrodes**

Recordings were obtained using two- and four-barrelled microelectrodes (Blatt, 1987a, 1991; Blatt and Slayman, 1987) coated with paraffin to reduce electrode capacitance. Current-passing and voltage-recording barrels were filled with 200 mM K⁺-acetate (pH 7.5) to minimize salt leakage and self-loading artefacts associated with the Cl⁻ anion (Blatt, 1987a; Blatt and Slayman, 1983). For injections of non-hydrolysable nucleotides, four-barrelled microelectrodes were used, of which one barrel was filled with 100 lM or 1 mM nucleotide and backfilled with 10 mM K⁺-acetate. Current through this barrel was balanced by filling a second barrel with 50 mM K⁺-acetate. The remaining two barrels (for current-passing and voltage-following) were filled with 200 mM K⁺-acetate, as above. In all cases, connection to the amplifier head-stage was via a 1 M KCl/Ag-AgCl halfcell. A matching halfcell and 1 M KCl-agar bridge served as the reference (bath) electrode.

**Electrical**

Mechanical, electrical and software design have been described in detail previously (Blatt, 1987a, 1987b, 1990, 1991). Membrane currents were recorded with a voltage clamp under microprocessor control using a WyeScience AD/DA converter, voltage clamp and μP amplifier system (WyeScience, Wye, Kent, UK). Steady-state current-voltage (I-V) relations were determined by clamping cells to a bipolar staircase of command voltages (Blatt, 1987b). Steps alternated positive and negative from the resting potential, Vᵣ, typically a total of 20 bipolar pulse pairs, and were separated by equivalent periods when the membrane was clamped to Vᵣ. The current signal was filtered using a six pole Butterworth filter at 1 or 3 kHz (~3 dB) before sampling, and currents and voltages were recorded during the final 10 msec of each pulse.

For time-dependent characteristics, current and voltage were sampled continuously at 1 kHz while the clamped potential was driven through one to eight cycles of four programmable pulse steps. In all cases the holding potential was set to Vᵣ at the start of the clamp cycle. No attempt was made to compensate for the series resistance (Rₛ) to ground. Estimates for Rₛ indicated that it was unlikely to pose a serious problem in measurements of clamp potential (Blatt, 1990, 1992), despite the often high resistivity of the bathing media (=2.5 kΩ cm for 5 mM Ca²⁺-MES with 0.1 mM KCl).

**Numerical analysis**

Data analysis was carried out by non-linear, least-squares (Marquardt, 1983) and, where appropriate, results are reported as the mean ± standard error of (n) observations.

**Chemicals and solutions**

The pH buffer, MES, butyric acid, TEA, EGTA, ADP·β-S and GDP-β-S were from Sigma Chemical Co. (St Louis, USA). Mas7 and masCP were gifts of Dr F.A. Milliner, University of Leeds. Otherwise, all chemicals were analytical grade from BDH Ltd (Poole, Dorset, UK).

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