Control of Guard Cell Ion Channels by Hydrogen Peroxide and Abscisic Acid Indicates Their Action through Alternate Signaling Pathways[1]

Barbara Köhler[2], Adrian Hills, and Michael R. Blatt*

Laboratory of Plant Physiology and Biophysics, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

Recent evidence has implicated the action of reactive oxygen species (ROS), notably hydrogen peroxide (H_2O_2), in abscisic acid (ABA) signaling of guard cells. ABA is known to evoke increases in cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$), dependent on flux through Ca^{2+} channels in the plasma membrane and release from intracellular Ca^{2+} stores (Grabov and Blatt, 1998; Hamilton et al., 2000; Pei et al., 2000), which inactivates inward-rectifying K^+ channels ($I_{K,in}$) and activates anion channels to bias the plasma membrane for solute efflux and stomatal closure (MacRobbie, 1997; Blatt, 2000; Schroeder et al., 2001). ABA also activates outward-rectifying K^+ channels ($I_{K,out}$) through a parallel rise in cytosolic pH (see Blatt, 2000, and refs. therein). H_2O_2 was suggested as an intermediate early in ABA signal transduction because when added externally it, too, triggers stomatal closure and is known to activate Ca^{2+} channels and elevate $[Ca^{2+}]_i$ in many plant cells (Price et al., 1994; Pei et al., 2000; Murata et al., 2001; Schroeder et al., 2001; Zhang et al., 2001b). ROS production is augmented by exogenous ABA and its block by diphenylene iodonium and the abi1 mutant (dominant-negative) protein phosphatase suppresses stomatal closure in Arabidopsis (Pei et al., 2000; Murata et al., 2001; Zhang et al., 2001b).

These observations aside, little attention has focused on the K^+ channels that ultimately mediate the solute flux to drive stomatal closure. We expected H_2O_2 to trigger the same pattern of response, activating the Ca^{2+} channels, inactivating $I_{K,in}$, and activating $I_{K,out}$, assuming that it transmitted the ABA signal. However, our results underscored both qualitative and quantitative differences between H_2O_2 and ABA actions, leading us to question the validity of arguments for H_2O_2 as a second messenger in this case.

H_2O_2 MIMICS ABA ACTIVATION OF CA^{2+} CHANNELS

The hyperpolarization-activated Ca^{2+} channel in the plasma membrane of *Vicia faba* guard cells was activated by micromolar concentrations of H_2O_2 (Fig. 1) under the same conditions we used previously to characterize its response to ABA and protein phosphorylation (Hamilton et al., 2000; Köhler and Blatt, 2002). At a voltage of −150 mV, activation by H_2O_2 occurred both in the cell-attached configuration (Fig. 1, A and B) and with isolated inside-out patches (not shown). One hundred micromolar H_2O_2 enhanced channel activity (NP_o) more than 100-fold (Fig. 1C), with NP_o rising from 0.002 ± 0.001 to 0.14 ± 0.08 in cell attached, and from 0.0001 ± 0.00001 to 0.15 ± 0.06 in inside-out recordings. H_2O_2 is freely permeable across biological membranes (Heldt and Fluegge, 1992), so the similar effects on the Ca^{2+} channel in attached and isolated patches suggests the dominant site of action is on, or closely associated with the channel protein itself.

Qualitatively, H_2O_2 action on the Ca^{2+} channel was comparable with that of ABA and phosphatase antagonists (Hamilton et al., 2000; Köhler and Blatt, 2002), arising from increases in open probability and a recruitment of "cryptic" channels (compare with Köhler and Blatt, 2002). In the presence of 500 μM H_2O_2, the open probability (P_o) increased from 0.0004 ± 0.0002 to 0.03 ± 0.01 and the number of channels rose 6 ± 2-fold ($n = 6$). Analysis of open and closed lifetime distributions from isolated patches with a single channel in H_2O_2 indicated open (τ_o, 0.8 ± 0.03 and 3.5 ± 0.4 ms) and closed (τ_c, 1.1 ± 0.3, 6 ± 3, and 223 ± 39 ms) lifetimes comparable with those obtained previously in ABA (Hamilton et al., 2001; Köhler and Blatt, 2002). Like ABA, H_2O_2 had no measurable effect on single channel amplitude (Hamilton et al., 2000; see also Fig. 1D). These results and the channel characteristics indicate that H_2O_2 and ABA activate the same Ca^{2+} channel and in a similar manner.

Although differences between species cannot be ruled out, the characteristics of the Ca^{2+} channels in *V. faba* and Arabidopsis are similar (Hamilton et al., 2000, 2001; Pei et al., 2000), thus implying that the responses to H_2O_2 and ABA may be general phenomena. The

[1] This work was supported by the Biotechnology and Biological Science Research Council (grant nos. P09640, C10234, and P09561).

[2] Present address: Universität Potsdam, Institut für Biochemie und Biologie, Karl-Liebknecht-Strasse 25, 14476 Golm, Germany.

* Corresponding author; e-mail m.blatt@bio.gla.ac.uk; fax 44–141–330–4447.

www.plantphysiol.org/cgi/doi/10.1104/pp.016014.

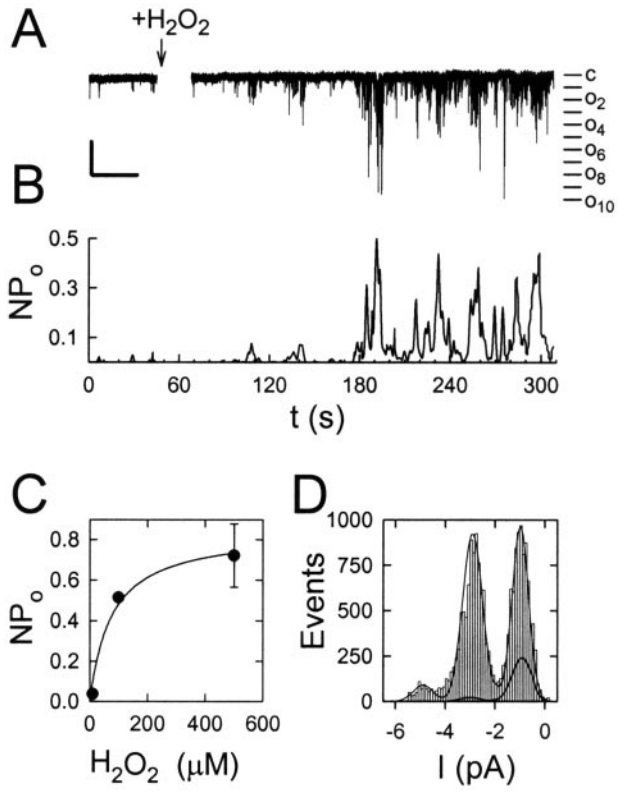

Figure 1. H_2O_2 activates guard cell Ca^{2+} channels. A, Current recorded from a cell-attached patch with 30 mM Ba^{2+}-HEPES (bath and pipette). Clamp voltage, −150 mV; arrow, 10 μM H_2O_2 addition. At least 10 channels are evident in the presence of H_2O_2 (open levels, right). Scale: vertical, 5 pA; horizontal, 30 s. B, Channel activity (NP_o = apparent channel no. X open probability) calculated from overlapping 5-s segments for the data in A. C, NP_o increases with H_2O_2 concentration. Results pooled from nine independent (cell-attached and inside-out) experiments. One millimolar ATP was added to the bath solution for inside-out recordings (Köhler and Blatt, 2002). Data normalized as NP_o ratio [=$NP_o(+H_2O_2)/NP_o(-H_2O_2)$] are fitted to a simple Michaelian function (solid curve [$K_{1/2}$] = 76 ± 28 μM). D. H_2O_2 does not change the single-channel amplitude. Amplitude histogram derived from a representative experiment with an inside-out patch in 10 μM H_2O_2. Clamp voltage, −150 mV; single-channel amplitude, 1.9 ± 0.4 pA. Gaussian fittings to amplitudes before (lower curve, amplitudes not shown for clarity) and after H_2O_2 addition. For details of experimental materials and methods, see Köhler and Blatt (2002).

Ca^{2+} channels of both species are strongly voltage dependent, activating negative of −100 mV, and both are permeable to Ba^{2+} as well as Ca^{2+}. We found that H_2O_2 activated the *V. faba* Ca^{2+} channel with a similar concentration dependence ($K_{1/2}$ = 76 ± 28 μM H_2O_2) and with a delay (2 ± 0.5 min, n = 9) that was independent of the H_2O_2 concentration between 10 and 500 μM both in cell-attached and inside-out configurations (Fig. 1; compare with Pei et al., 2000).

H_2O_2 DOES NOT MIMIC ABA ACTIVATION OF $I_{K,out}$

Zhang et al. (2001a) reported that $I_{K,in}$ in *V. faba* guard cells is suppressed by exogenous H_2O_2. Significantly, they worked at a concentration of 10 μM H_2O_2—well below the $K_{1/2}$ for the Ca^{2+} channel—but did not pursue the observation further. To quantify the effects of H_2O_2 on $I_{K,in}$ and $I_{K,out}$, we carried out voltage-clamp experiments with intact guard cells as described previously (Blatt and Armstrong, 1993; Grabov and Blatt, 1998, 1999). We found that (Fig. 2), like ABA, H_2O_2 treatments suppressed $I_{K,in}$, shifting its activation to more negative voltages. However, unlike ABA, H_2O_2 also depressed $I_{K,out}$ and the effect on both K^+ channels was irreversible. The response of $I_{K,out}$ and $I_{K,in}$ to H_2O_2 occurred with halftimes of 6 ± 2 and 4 ± 0.5 min, respectively, for concentrations from 1 to 50 μM (Fig. 3, A and B). Furthermore, we observed quantitatively equivalent results, even when exposures were restricted to 30 to

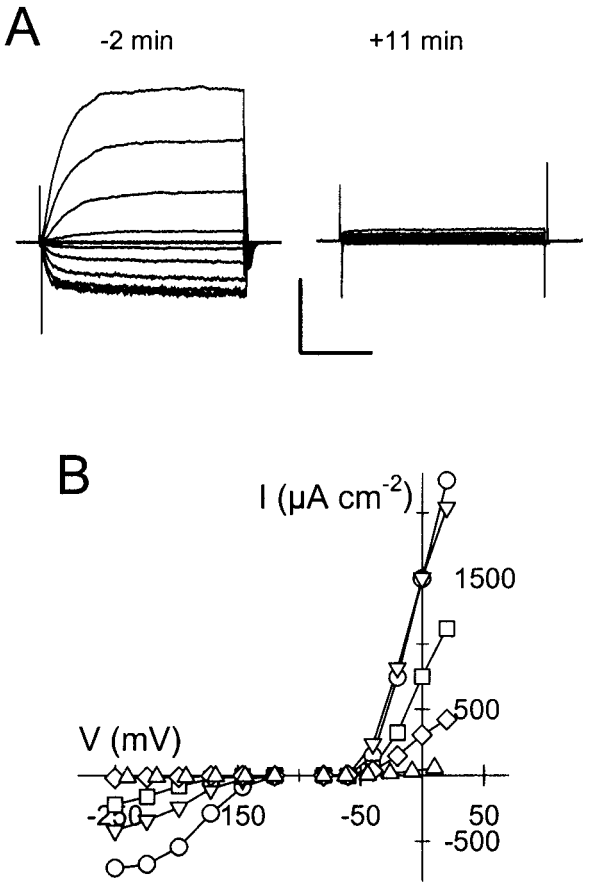

Figure 2. H_2O_2 suppresses both $I_{K,in}$ and $I_{K,out}$. Data from an intact *V. faba* guard cell under a two-electrode voltage clamp bathed in 10 mM KCl and 5 mM Ca^{2+}-MES, pH 6.1. A, Current response 2 min before and 11 min after 1-min exposure to 10 μM H_2O_2. Three-second clamp voltage steps (12) to voltages between +30 and −250 mV from a holding voltage of −100 mV. Scale: horizontal, 1 s; vertical, 100 μA cm^{-2}. B, Current-voltage curves derived from A and additional data of the same cell before (○) and 1 (▽), 3 (□), 6 (◇), and 11 (Δ) min after adding H_2O_2. K^+ channel currents obtained by subtracting instantaneous from steady-state current at each voltage. Data for $I_{K,in}$ and $I_{K,out}$ fitted jointly to common Boltzmann functions (solid curves). For details, see Grabov and Blatt (1999) and Blatt and Armstrong (1993).

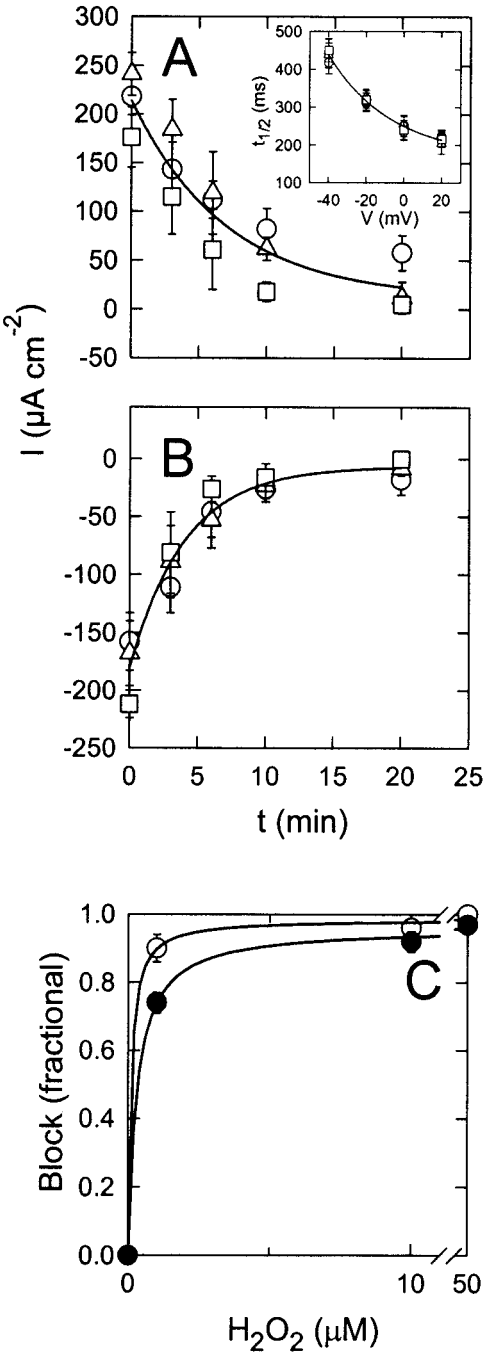

Figure 3. *V. faba* guard cell K^+ channels are roughly 100-fold more sensitive to H_2O_2 than the Ca^{2+} channel. A, Time for block of $I_{K,out}$ independent of H_2O_2 concentrations above 1 μM. Current at 0 mV determined as in Figure 2 and plotted as time after adding 1 ($n = 3$, ○), 10 ($n = 4$, Δ), and 50 ($n = 3$, □) μM H_2O_2 for 2 min. Solid curve, Fitting to single exponential decay ($t_{1/2}$, 6 ± 2 min). Inset, Halftimes for $I_{K,out}$ activation in the presence of 1 ($n = 3$, ○), 10 ($n = 4$, Δ), and 50 ($n = 3$, □) μM H_2O_2. Data fitted empirically to a single exponential decay function. B, Time for block of $I_{K,in}$ recorded at −200 mV, as in A. Solid curve, Fitting to single exponential decay ($t_{1/2}$, 4 ± 0.5 min). C, Block by H_2O_2 of $I_{K,in}$ (○) at −200 mV and $I_{K,out}$ (●) at 0 mV fitted to Michaelian functions. $K_{1/2}$:$I_{K,in}$, 0.1 ± 0.4 μM; $I_{K,out}$, 0.3 ± 0.2 μM.

60 s and H_2O_2 was then washed from the bath (Fig. 3, A and B). One micromolar H_2O_2 was sufficient for near-maximal effect on both K^+ channels (Fig. 3C). Finally, H_2O_2 did not have a significant effect on the halftimes for activation at any concentration tested ($I_{K,out}$, Fig. 3A, inset; $I_{K,in}$, not shown), suggesting an effect mediated by a change in the number of functional channels rather than by alterations in their gating kinetics.

It is not surprising that the guard cell K^+ channels are sensitive to ROS because, like many proteins, they can be expected to harbor reactive groups (e.g. sulfhydryl bonds; for KAT1 of Arabidopsis, see Anderson et al., 1992). Previous data have shown effects of O_3 on *V. faba* guard cell K^+ channels (Torsethaugen et al., 1999) and ROS action in vivo (Wang et al., 1997) and after heterologous expression (Duprat et al., 1995) is known for other K^+ channels. In fact, a direct action of H_2O_2 to render the K^+ channels nonfunctional seems the simplest explanation in this case because the effects were complete without change in activation kinetics and at concentrations roughly 100-fold lower than were effective in activating the Ca^{2+} channel. Although at present we cannot rule out a rise in $[Ca^{2+}]_i$ at these very low concentrations, H_2O_2 action solely through $[Ca^{2+}]_i$ is inconsistent with the response of $I_{K,out}$, which is known to be Ca^{2+} insensitive (Hosoi et al., 1988; Blatt and Armstrong, 1993; Lemtiri-Chlieh and MacRobbie, 1994; Grabov and Blatt, 1999). At first sight, it is surprising that H_2O_2 should suppress $I_{K,out}$ because H_2O_2 induces stomatal closure in epidermal strips of Arabidopsis and *V. faba* (Pei et al., 2000; Zhang et al., 2001b) and, therefore, might be expected to stimulate K^+ loss from the cells. However, other pathways for K^+ efflux have been reported (Thiel et al., 1992; Pei et al., 1998) and their response to H_2O_2 is unknown (see also Duprat et al., 1995).

Most important, the finding that H_2O_2 inhibited the K^+ outward rectifier in guard cells shows that H_2O_2 does not mimic ABA action on guard cell ion channels as it acts on the K^+ outward rectifier in a manner entirely contrary to that of ABA. This observation brings into question previous evidence based on exogenous applications for a role of H_2O_2 as a second messenger in ABA signaling of stomata. We pose this question now also in a general sense. Although it may be argued that H_2O_2 could act differently inside and outside the guard cell, the ROS is freely permeable across biological membranes (Heldt and Fluegge, 1992). Therefore, any such differences in action would require localized and exceedingly tight coupling between the sites for H_2O_2 generation and action at the inner surface of the plasma membrane. Other differences in action between ABA and H_2O_2 are known. For example, Allen et al. (2000) reported that H_2O_2 triggered Ca^{2+} oscillations with a "Ca^{2+} fingerprint" but not an "ABA fingerprint" in the Arabidopsis det3 mutant, suggesting that the signaling cascades are different, although they might share

components. No doubt, ABA and oxidative stress responses are linked (Guan et al., 2000; Pei et al., 2000; Zhang et al., 2001b), but through a network of signaling pathways that have evolved to deal with the common situation of combined stress inputs (Knight and Knight, 2001).

In conclusion, we question the role of H_2O_2 as a critical second messenger regulating guard cell ion channels in response to ABA. The Ca^{2+} channel is a target for both ABA and H_2O_2 signal processing, but in our view it serves as a focal point integrating signal transduction pathways and, thus, links these several pathways, among others, to membrane voltage (Gradmann et al., 1993; Grabov and Blatt, 1998, 1999), NAD(P) H and the cellular redox state (Murata et al., 2001), and protein phosphorylation (Köhler and Blatt, 2002). Our data suggest that the ABA and H_2O_2 pathways diverge further downstream in their actions on the K^+ channels and, thus, on stomatal control.

Received October 10, 2002; returned for revision October 22, 2002; accepted October 22, 2002.

LITERATURE CITED

Allen GJ, Chu SP, Schumacher K, Shimazaki CT, Vafeados D, Kemper A, Hawke SD, Tallman G, Tsien RY, Harper JF et al. (2000) Science **289:** 2338–2342

Anderson JA, Huprikar SS, Kochian LV, Lucas WJ, Gaber RF (1992) Proc Natl Acad Sci USA **89:** 3736–3740

Blatt MR (2000) Annu Rev Cell Dev Biol **16:** 221–241

Blatt MR, Armstrong F (1993) Planta **191:** 330–341

Duprat F, Guillemare E, Romey G, Fink M, Lesage F, Lazdunski M, Honore E (1995) Proc Nat Acad Sci USA **92:** 11796–11800

Grabov A, Blatt MR (1998) Proc Natl Acad Sci USA **95:** 4778–4783

Grabov A, Blatt MR (1999) Plant Physiol **119:** 277–287

Gradmann D, Blatt MR, Thiel G (1993) J Membr Biol **136:** 327–332

Guan LQM, Zhao J, Scandalios JG (2000) Plant J **22:** 87–95

Hamilton DWA, Hills A, Blatt MR (2001) FEBS Lett **491:** 99–103

Hamilton DWA, Hills A, Köhler B, Blatt MR (2000) Proc Natl Acad Sci USA **97:** 4967–4972

Heldt H-W, Fluegge UI (1992) *In* AK Tobin, ed, Plant Organelles. Cambridge University Press, Cambridge, UK, pp 21–47

Hosoi S, Iino M, Shimazaki K (1988) Plant Cell Physiol **29:** 907–911

Knight H, Knight MR (2001) Trends Plant Sci **6:** 262–267

Köhler B, Blatt MR (2002) Plant J **32:** 185–194

Lemtiri-Chlieh F, MacRobbie EAC (1994) J Membr Biol **137:** 99–107

MacRobbie EAC (1997) J Exp Bot **48:** 515–528

Murata Y, Pei ZM, Mori IC, Schroeder J (2001) Plant Cell **13:** 2513–2523

Pei ZM, Baizabal Aguirre VM, Allen GJ, Schroeder JI (1998) Proc Natl Acad Sci USA **95:** 6548–6553

Pei ZM, MurataY, Benning G, Thomine S, Klusener B, Allen GJ, Grill E, Schroeder JI (2000) Nature **406:** 731–734

Price AH, Taylor A, Ripley SJ, Griffiths A, Trewavas AJ, Knight MR (1994) Plant Cell **6:** 1301–1310

Schroeder JI, Allen GJ, Hugouvieux V, Kwak JM, Waner D (2001) Ann Rev Plant Physiol Mol Biol **52:** 627–658

Thiel G, MacRobbie EAC, Blatt MR (1992) J Membr Biol **126:** 1–18

Torsethaugen G, Pell EJ, Assmann SM (1999) Proc Natl Acad Sci USA **96:** 13577–13582

Wang ZW, Nara M, Wang YX, Kotlikoff MI (1997) J Gen Physiol **110:** 35–44

Zhang X, Miao YC, An GY, Zhou Y, Shangguan ZP, Gao JF, Song CP (2001a) Cell Res **11:** 195–202

Zhang X, Zhang L, Dong FC, Gao JF, Galbraith DW, Song CP (2001b) Plant Physiol **126:** 1438–1448