Nitric Oxide Block of Outward-Rectifying K^+ Channels Indicates Direct Control by Protein Nitrosylation in Guard Cells[1]

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Recent work has indicated that nitric oxide (NO) and its synthesis are important elements of signal cascades in plant pathogen defense and are a prerequisite for drought and abscisic acid responses in Arabidopsis (*Arabidopsis thaliana*) and *Vicia faba* guard cells. Nonetheless, its mechanism(s) of action has not been well defined. NO regulates inward-rectifying K^+ channels of *Vicia* guard cells through its action on Ca^{2+} release from intercellular Ca^{2+} stores, but alternative pathways are indicated for its action on the outward-rectifying K^+ channels ($I_{K,out}$), which are Ca^{2+} insensitive. We report here that NO affects $I_{K,out}$ when NO is elevated above approximately 10 to 20 nM. NO action on $I_{K,out}$ was consistent with oxidative stress and was suppressed by several reducing agents, the most effective being British anti-Lewisite (2,3-dimercapto-1-propanol). The effect of NO on the K^+ channel was mimicked by phenylarsine oxide, an oxidizing agent that cross-links vicinal thiols. Neither intracellular pH buffering nor the phosphotyrosine kinase antagonist genistein affected NO action on $I_{K,out}$, indicating that changes in cytosolic pH and tyrosine phosphorylation are unlikely to contribute to NO or phenylarsine oxide action in this instance. Instead, our results strongly suggest that NO directly modifies the K^+ channel or a closely associated regulatory protein, probably by nitrosylation of cysteine sulfhydryl groups.

The gas nitric oxide (NO) is a highly reactive, membrane-permeant free radical that is a natural constituent of all living cells and serves as a signaling molecule. In animals, NO acts indirectly through guanylate cyclase to activate cGMP-dependent cellular responses and affects the gating of Ca^{2+}-dependent K^+ channels, Ca^{2+} and Na^+ channels (Bolotina et al., 1994; Tang et al., 2001; Renganathan et al., 2002). Although actions of NO in plants have been known for almost one-half century (Bhatia and Sybenga, 1965), only recently has the gas been recognized to play a physiological role in events as diverse as photomorphogenesis (Zhang et al., 2003), cell growth (Lamattina et al., 2003; Pagnussat et al., 2003), abiotic stress (Garcia-Mata and Lamattina, 2003), and plant pathogen defense (Delledonne et al., 1998; Durner et al., 1998). In plants, NO occurs as a by-product of metabolism from NO_2 through photoconversion by carotenoids, reaction with nitrate reductases and with Gly decarboxylase (Rockel et al., 2002; Garcia-Mata and Lamattina, 2003); it is formed nonenzymatically from NO_2^- in the apoplast (Bethke et al., 2004); and it is also released by specific NO synthases (Chandok et al., 2003; Guo et al., 2003).

NO affects guard cells and their control of gas exchange and transpirational water loss through the stomata of the leaf epidermis. The gas enhances plant tolerance to drought (Garcia-Mata and Lamattina, 2003) and contributes to stomatal closure evoked by the water-stress hormone abscisic acid (ABA). NO scavengers suppress ABA action in closing stomata, NO donors promote closure in the absence of ABA (Garcia-Mata and Lamattina, 2002; Neill et al., 2002), and, in Arabidopsis (*Arabidopsis thaliana*) deficient in NO synthesis, stomata fail to close in ABA (Desikan et al., 2002). ABA closes stomata by facilitating osmotic solute loss to reduce guard cell turgor. Among its actions, ABA raises cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) and cytosolic pH (pH_i) signals that, in turn, inactivate inward-rectifying K^+ channels to prevent K^+ uptake, and activate outward-rectifying K^+ channels ($I_{K,out}$) and Cl^- (anion) channels at the plasma membrane for solute efflux (Blatt, 2000a; Hetherington, 2001; Schroeder et al., 2001).

Our recent work (Garcia-Mata et al., 2003) demonstrated that NO acts on inward-rectifying K^+ channels and anion channels by activating ryanodine-sensitive Ca^{2+} channels of intercellular Ca^{2+} stores to elevate $[Ca^{2+}]_i$ in Vicia guard cells. At these very low levels, NO had no influence on $I_{K,out}$, consistent with the Ca^{2+} insensitivity of these K^+ channels. However, NO might be expected to have additional effects on stomatal behavior at higher concentrations. Oxidative stress in plants is known to suppress stomatal closure (see Willmer and Fricker, 1996) and, in some circumstances, can suppress $I_{K,out}$ (Kohler et al., 2003) and

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promote stomatal opening (Black and Black, 1979). Indeed, NO can bond covalently with the SH residues of Cys to form *S*-nitrosothiols, and this simple reaction is the basis of many regulatory cascades (Stamler et al., 2001; Ahern et al., 2002), including vascular homeostasis and endotoxic shock in animals (Liu et al., 2004). In subsequent experiments, we observed a reversible decrease in $I_{K,out}$ with moderate elevation to submicromolar NO and have since explored the effects of NO on the $I_{K,out}$ in vivo. Our key observations are that a NO-mediated block of $I_{K,out}$ is suppressed by reducing reagents, especially British anti-Lewisite (BAL; 2,3-dimercapto-1-propanol), and is mimicked by the oxidizing reagent phenylarsine oxide (PAO) that targets viscinal SH residues. These findings indicate that the $I_{K,out}$ can become locked down under nitrosative stress, and they lead us to propose that NO action on $I_{K,out}$ is mediated by direct *S*-nitrosylation of Cys residues closely associated with the ion channel.

RESULTS

NO Inactivates $I_{K,out}$

Although our previous work indicated that $I_{K,out}$ is not appreciably sensitive to NO application at rates up to 10 nM/min, we found that exposures to higher levels of NO resulted in a reduction in the amplitude of this K^+ current. Figure 1 (inset) shows current traces and steady-state current-voltage (*I-V*) curves from one Vicia guard cell recorded before (○), during (■), and after (●) exposure to 50 nM/min NO. Clamp voltage steps from −100 mV to voltages between −90 and +50 mV yielded current typical of $I_{K,out}$, showing a time-dependent activation. NO exposure of 2 min reduced the current to less than 10% of the control at all clamp voltages. Washing NO from the bath for 10 min led to a gradual recovery and, in this cell, an overshoot of the current relative to the control that we ascribe to secondary effects of NO (see "Discussion"). Similar results were obtained in 15 other experiments with this level of NO (see also Fig. 2). In every case, exposures to NO suppressed $I_{K,out}$ within 2 to 5 min of the treatments, and washout with fresh solution minus NO led to complete recovery over periods of 5 to 10 min (recovery half-time [$t_{1/2}$], 6 ± 2 min). We noted a very sharp dependence in $I_{K,out}$ to the level of NO exposure. Figure 2 summarizes data from 117 separate experiments with NO exposure levels between 1 nM/min and 500 nM/min. Plotted as the mean of the current complement (=relative inactivation [block]), these data were well fitted to the Hill equation (Hill, 1910) with an apparent Hill coefficient of 4.6 ± 0.4 and K_i of 19.5 ± 0.5 nM/min. Thus, the effect of NO showed a remarkably high degree of cooperativity and was essentially complete at NO levels of 30 nM/min and above.

NO did not appear to affect K^+ channel gating, as evident from two observations. First, no indication

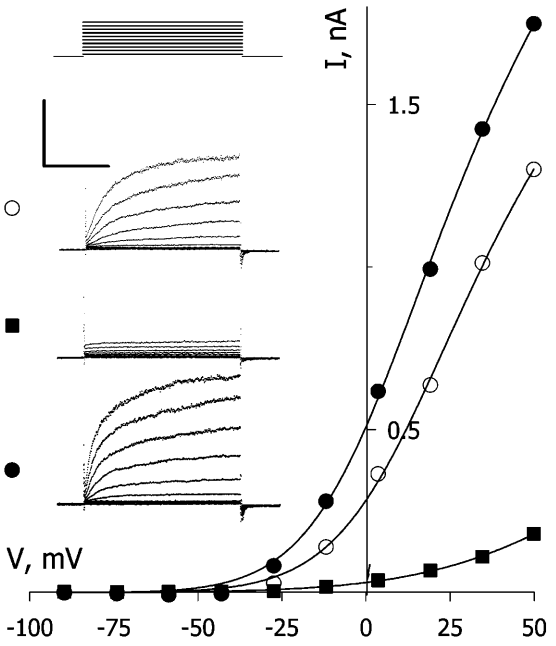

Figure 1. NO inactivates $I_{K,out}$. Steady-state current-voltage curves derived from voltage clamp recordings (insets) from an intact *Vicia* guard cell before (○), during exposure to 50 nM/min NO (■), and 8 min after washing in buffer without NO (●). Curves are corrected for instantaneous current recorded at each voltage. Inset, Corresponding current traces cross-referenced by symbol with the voltage protocol shown above (conditioning voltage, −100 mV; test voltages, −90 to +50 mV; tailing voltage, −100 mV). Scale, Horizontal, 2 s; vertical, 1 nA.

could be found for a change in gating kinetics. Using NO levels that gave partial suppression of $I_{K,out}$, we found no significant difference in half-times for current activation compared with current activation kinetics before NO treatments from the same guard cells. Activation half-times at +50 mV before treatments were 291 ± 23 ms and during exposures to 20 nM/min NO were 315 ± 38 ms (n = 11). Second, fitting the steady-state *I-V* curves from these same experiments to a Boltzmann function showed no appreciable change in the voltage sensitivity for gating. Visually satisfactory and statistically best fittings were obtained with joint fittings (±NO) to the equation

$$I_{K,out} = G_{max}(V - E_K)/1 + e^{-\delta F(V - V_{1/2})/RT}. \quad (1)$$

in which only the maximum conductance (G_{max}) varied between curves. Here R, T, and F have their usual meanings. The remaining parameters, the gating charge coefficient (δ), K^+ equilibrium voltage (E_K), and the voltage giving half-maximal activation ($V_{1/2}$), were held in common between data sets. Joint fittings in which $V_{1/2}$ also varied between curves gave statistically equivalent results and yielded values for $V_{1/2}$ of 11 ± 4 mV before, and 16 ± 5 mV during NO treatments. Thus, the voltage dependence for activation was unaffected by the presence of NO.

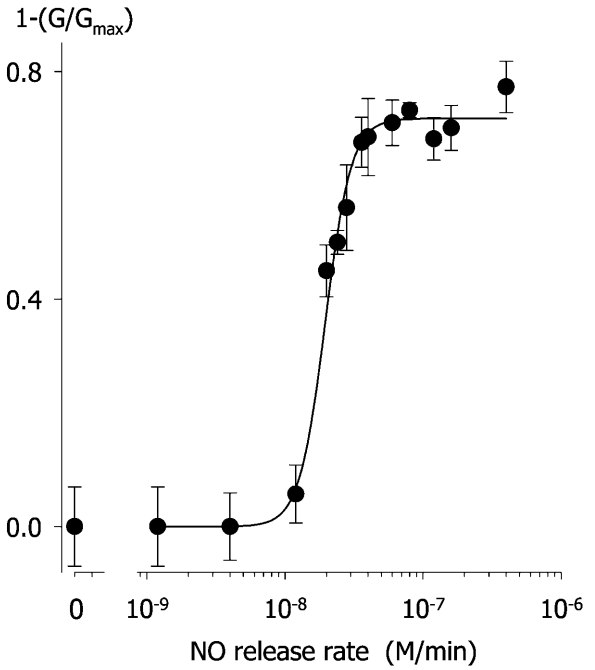

Figure 2. NO inactivation of $I_{K,out}$ is strongly cooperative. $I_{K,out}$ steady-state currents derived as in Figure 1 at +50 mV before and during NO treatments. Data are given as means ± SE of the conductance complement (=relative block) from 117 experiments (>5 experiments per data point) and are plotted as a function of NO exposure. The solid curve is the best fit to the Hill equation

$$1 - G/G_{max} = [NO]^n/(K_{1/2}^n + [NO]^n) \quad (2)$$

yielding a cooperativity coefficient of 4.6 ± 0.4 and an apparent $K_{1/2}$ of 19.5 ± 0.4 nM/min with a maximum block of 74% ± 5%.

NO Action Is Not Mediated by pH_i

In guard cells, $I_{K,out}$ rises steeply with pH_i above 7.3, and driving pH_i to values near 7.0 virtually eliminates the current (Grabov and Blatt, 1997). Indeed, control of the $I_{K,out}$ by ABA is mediated through a rise in pH_i (Blatt and Armstrong, 1993). Because oxidative and related stresses are known to affect pH_i, (Jackson, 1991; Low and Merida, 1996), we suppressed changes in pH_i by passive loading to buffer the cytosol near pH_i 7.5 to test its possible role in NO-mediated inactivation of the channel. Guard cells were impaled with microelectrodes containing 200 mM K^+-HEPES, pH 7.5, conditions that previously were found to prevent ABA-mediated increases in pH_i and its enhancement of $I_{K,out}$ (Blatt and Armstrong, 1993). Figure 3 shows the results from one guard cell exposed to NO after 15-min buffer loading from the microelectrode. As before, NO treatment led to a rapid block of $I_{K,out}$, which recovered subsequently on extended washing of NO from the bath. Despite the pH buffer, we noted an increase in the leak current (see legends, Figs. 3 and 4) consistent with the effect of NO in promoting Cl^- channel activity through its action on $[Ca^{2+}]_i$ (Garcia-Mata et al., 2003). Similar results were obtained in five independent experiments, yielding values for G_{max} (see Eq. 1) of 0.73 ± 0.08 and 0.04 ± 0.03 before and during NO treatment, respectively. In no case was there any indication that buffering pH_i suppressed the NO-evoked decrease in $I_{K,out}$. Although we cannot categorically rule out an effect on pH_i from these experiments, the data do suggest that changes in pH_i are not the primary mechanism leading to the inactivation of $I_{K,out}$ by NO.

Reducing Reagents Protect $I_{K,out}$ from NO Inactivation

In addition to its action on Ca^{2+} signaling, NO can modify the thiol group of Cys residues directly by reversible *S*-nitrosylation (Stamler et al., 2001; Ahern et al., 2002). As a test for possible redox-mediated inactivation of $I_{K,out}$ by NO, we applied the reductants dithiothreitol (DTT) and BAL. Both DTT and BAL are membrane permeant and capable of reducing Cys thiol adducts. In control experiments, neither reagent affected $I_{K,out}$ at concentrations as high as 1 mM (DTT treatment did lead to a small, but not very significant, increase relative to the control; see Fig. 5). However, after NO exposures, both DTT and BAL dramatically accelerated the recovery of $I_{K,out}$, reversing the effect of

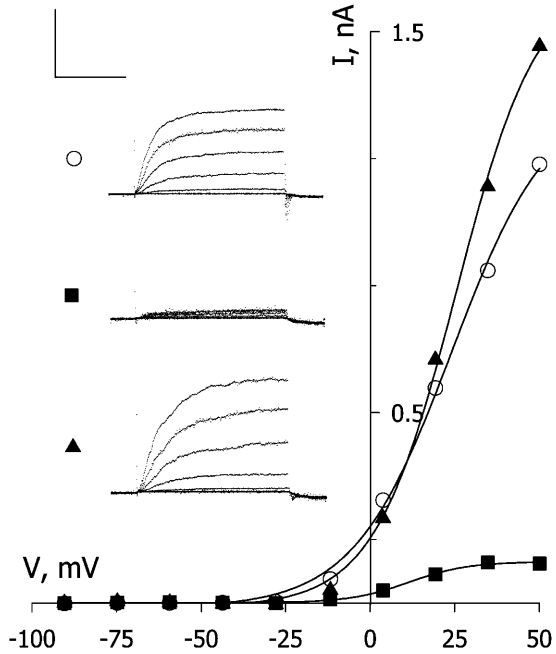

Figure 3. Buffering pH_i does not suppress NO-evoked inactivation of $I_{K,out}$. Steady-state current-voltage curves derived from voltage clamp recordings (inset) from an intact *Vicia* guard cell before (○), during 3-min exposure to 50 nM NO (■), and 8 min after washing in buffer without NO (▲). Curves are corrected for instantaneous current recorded at each voltage. The guard cell was first loaded with 200 mM K^+-HEPES, pH 7.5, from the microelectrode for 10 min. Inset, Corresponding current traces cross-referenced by symbol. Voltage protocol as in Figure 1. Scale, Horizontal, 2 s; vertical, 1 nA. Note the inward (anion) current in the final clamp steps of each cycle during and, to a lesser extent, after NO treatment.

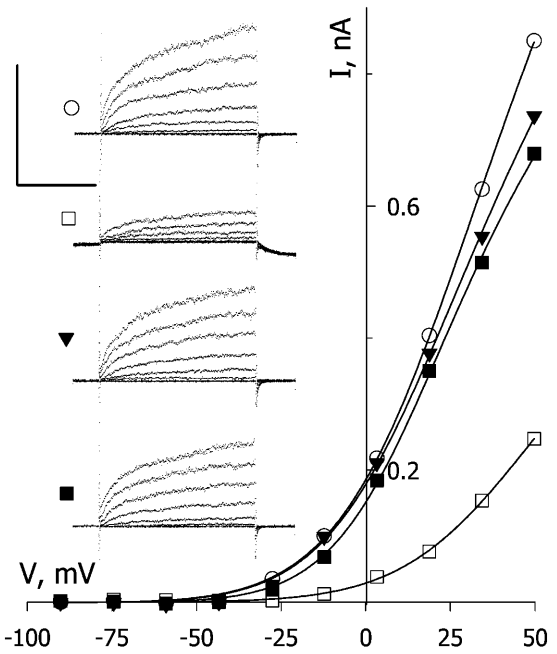

Figure 4. The vicinal dicysteine reducing agent BAL restores $I_{K,out}$ and protects against subsequent NO treatment. Steady-state current-voltage curves derived from voltage clamp recordings (inset) from an intact *Vicia* guard cell before (○) and during 2-min exposure to 50 nM/min NO (□). The guard cell was then washed with 0.3 mM BAL for 2 min (▼). After washing with buffer solution alone (BAL) for 6 min, the cell was again challenged with 50 nM NO, this time for 8 min (■), but without any appreciable effect on $I_{K,out}$. Voltage protocol as in Figure 1. Scale, Horizontal, 2 s; vertical, 1 nA.

NO within the period required for full solution exchange in the chamber (BAL recovery $t_{1/2}$, 21 ± 6 s; DTT recovery $t_{1/2}$, 27 ± 8 s; see also Fig. 5).

Remarkably, we found that prior treatments with BAL also protected $I_{K,out}$ from subsequent exposures to NO, even over 10-min periods of continuous superfusion. Figure 4 shows currents and steady-state *I-V* curves obtained from one guard cell exposed to 50 nM/min NO before and after BAL treatment. Following the control voltage clamp recording (○), the guard cell was challenged with NO for 2 min (□) before washing with 0.3 mM BAL for 3 min (▲). After washing with buffer solution alone for 6 min, the cell was again challenged with 50 nM/min NO, this time for 8 min (■), but without any appreciable effect on $I_{K,out}$. Virtually identical results were obtained in five other experiments with BAL (Fig. 5). By contrast, repeated treatments with NO invariably led to a suppression of $I_{K,out}$ that was quantitatively comparable to the effect of the first exposure (Fig. 5). Similar experiments with DTT showed a limited, but statistically significant, suppression of NO action on $I_{K,out}$ (Fig. 5) We cannot discount the possibility that a residue of BAL, especially, might remain in the lipid phase of the membrane to suppress NO action. It is more difficult, however, to explain the absence of any NO action, even after 10-min continuous superfusion with NO. In all events, the ability for both reductants to reverse NO-evoked inactivation of $I_{K,out}$ suggests that NO targets SH residues of Cys associated with the K^+ channel, either on the cytosolic side of the membrane or within the lipid bilayer.

Membrane-Permeant SH Oxidants Inactivate $I_{K,out}$

On the assumption that NO modifies one or more free Cys SH groups, we used several redox reagents with known chemistry to explore a role for Cys thiols in NO action. A summary of these results is shown in Figure 6. *N*-ethylmaleimide (NEM) is a highly reactive SH reagent that covalently and irreversibly alkylates free Cys thiols; like NEM, iodoacetamide (IodAA) alkylates free Cys thiols; and 5,5′-dithiobis-2-nitrozoic acid (DTNB) is an oxidizing reagent that acts by formation of a disulfide bond between itself and free Cys SH groups to give dithiobenzoate complexes with proteins. NEM is moderately membrane permeant, but neither IodAA nor DTNB penetrates membranes readily (Broillet and Firestein, 1996; Broillet, 2000). We

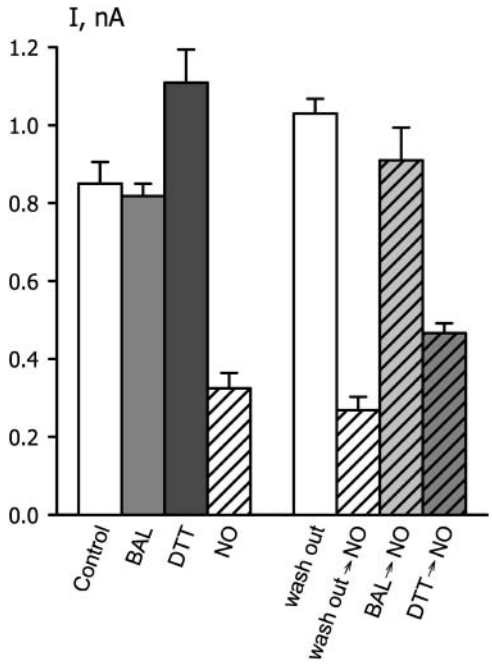

Figure 5. Reducing agents restore $I_{K,out}$ and protect against subsequent NO treatment. Summary of steady-state $I_{K,out}$ determined at +50 mV before and after exposure to 50 nM/min NO and/or 0.3 mM BAL and DTT. Data are means ± SE of 5 to 15 independent experiments in each case. Bars on left are data from single treatments only or none (control); bars on right are data from guard cells exposed first to 50 nM/min NO and the gas then washed out (1) with buffer alone for 10 min (=washout); (2) with buffer alone for 10 min before a second exposure to 50 nM/min NO (=washout→NO); (3) with 0.3 mM BAL for 2 min and then with buffer alone for 6 to 8 min before a second exposure to 50 nM/min NO (=BAL→NO); and (4) with 0.3 mM DTT for 2 min and then with buffer alone for 6 to 8 min before a second exposure to 50 nM min^{-1} NO (=DTT→NO). Measurements were taken in buffer only (no shading), BAL (light shading), DTT (dark shading), and NO (diagonal lines).

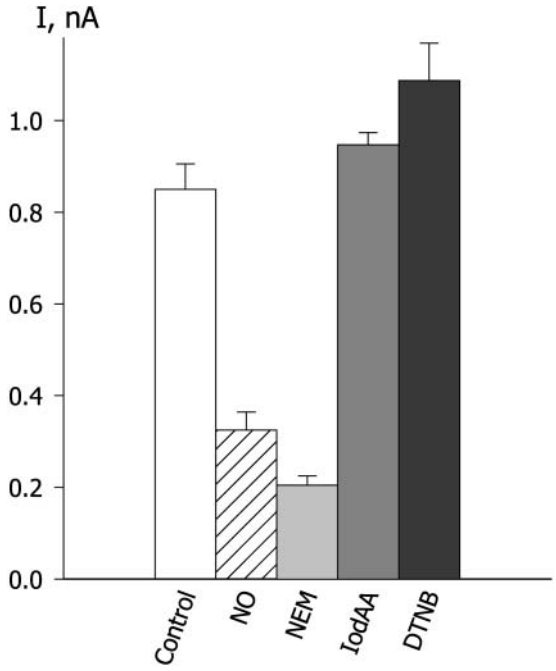

Figure 6. Membrane-impermeant SH-modifying agents IodAA and DTNB are ineffective in suppressing $I_{K,out}$. Summary of steady-state $I_{K,out}$ determined at +50 mV before (control) and after exposure to 50 nM/min NO, to 1 mM of the membrane-permeant *N*-ethylmaleimide (NEM), to 1 mM IodAA, and to 0.2 mM DTNB. Data are means ± SE of 5 to 15 independent experiments in each case.

found that exposure to 1 mM NEM led to rapid and complete inactivation of $I_{K,out}$ and the current could not be recovered, even after extensive washing with fresh solution (minus NEM) for periods of up to 20 min. By contrast, exposures to 0.2 mM DTNB and 1 mM IodAA had little effect on $I_{K,out}$, although the guard cells were continuously superfused with these solutions for periods of 6 to 12 min.

PAO Mimics NO Suppression of $I_{K,out}$

The organic arsenical PAO is a redox reagent that preferentially reacts with closely spaced thiol groups and forms stable ring complexes. In mammalian tissues, PAO targets protein Tyr phosphatases (Carballo et al., 1999) through its ability to oxidatively bridge critical Cys, and it also affects other protein-signaling intermediates, including human syntaxin 1A (Arien et al., 2003) and Rho GTPases (Gerhard et al., 2003). In guard cells, PAO has been reported to counter the effects of ABA, dark, and H_2O_2 in closing stomata and to promote stomatal opening, at least in part through its effect on tonoplast ion transport (MacRobbie, 2002), and to affect ABA-mediated gene expression (Heimovaaradijkstra et al., 1996).

Because the actions of PAO on stomatal aperture are also consistent with a suppression of $I_{K,out}$, we challenged guard cells with PAO during voltage clamp experiments. Figure 7 shows measurements from one guard cell exposed to 10 μM PAO. Like NO, exposure to PAO treatment led to a rapid block of $I_{K,out}$. Unlike NO, the effect was not reversed on washing PAO from the bath. However, $I_{K,out}$ activity was restored fully within 3 min of washing with BAL (Fig. 7; see also Fig. 8A), consistent with the ability of BAL to reduce arsenical bridged dicysteines, and recovered partially with DTT treatments (see Fig. 8A). Similar results were obtained in 11 other experiments with BAL and four experiments with DTT. PAO has been suggested to act through a protein Tyr phosphatase pathway in its action on guard cells (MacRobbie, 2002). Therefore, we also tested genestein, a protein Tyr phosphatase antagonist, for its ability to suppress NO action on the K^+ channel. Our reasoning was that, if PAO-sensitive Tyr dephosphorylation was also essential for control of $I_{K,out}$ by NO, then antagonism of the corresponding kinase might counteract the effects of NO. However, genestein treatments showed no influence on NO-evoked block of $I_{K,out}$ (Fig. 8B).

As with NO, we found a steep dependence of $I_{K,out}$ on PAO concentration (Fig. 9). Plotted as the mean of the current complement (=relative inactivation), these data were well fitted to the Hill equation (Hill, 1910), with an apparent Hill coefficient of 2.3 ± 0.5 and K_i of 3.2 ± 0.4 μM. Thus, the effect of PAO showed a degree of cooperativity precisely one-half that of NO and was fully reversed by BAL, consistent with the ability of PAO and BAL to target vicinal Cys pairs.

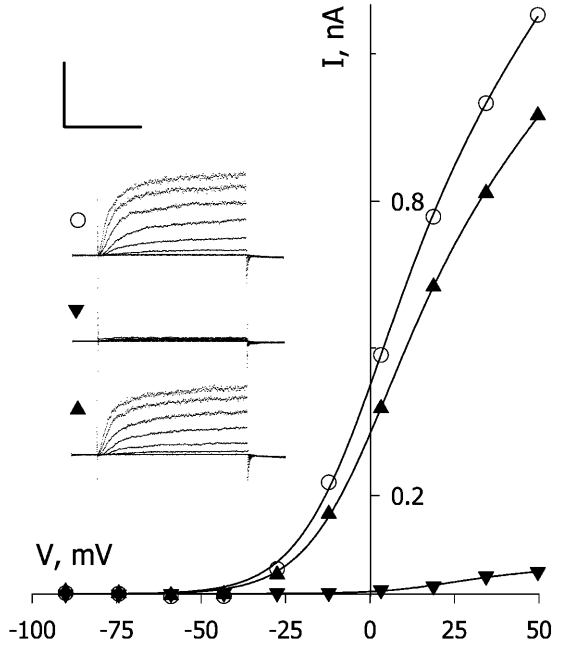

Figure 7. Dicysteine cross-linker PAO inactivates $I_{K,out}$. Steady-state current-voltage curves derived from voltage clamp recordings (inset) from an intact *Vicia* guard cell before (○), during 5-min exposure to 10 μM PAO (▼), and after 3 min washing in buffer with 0.3 mM BAL (▲). Curves are corrected for instantaneous current recorded at each voltage. Inset, Corresponding current traces cross-referenced by symbol. Voltage protocol as in Figure 1. Scale, Horizontal, 2 s; vertical, 1 nA.

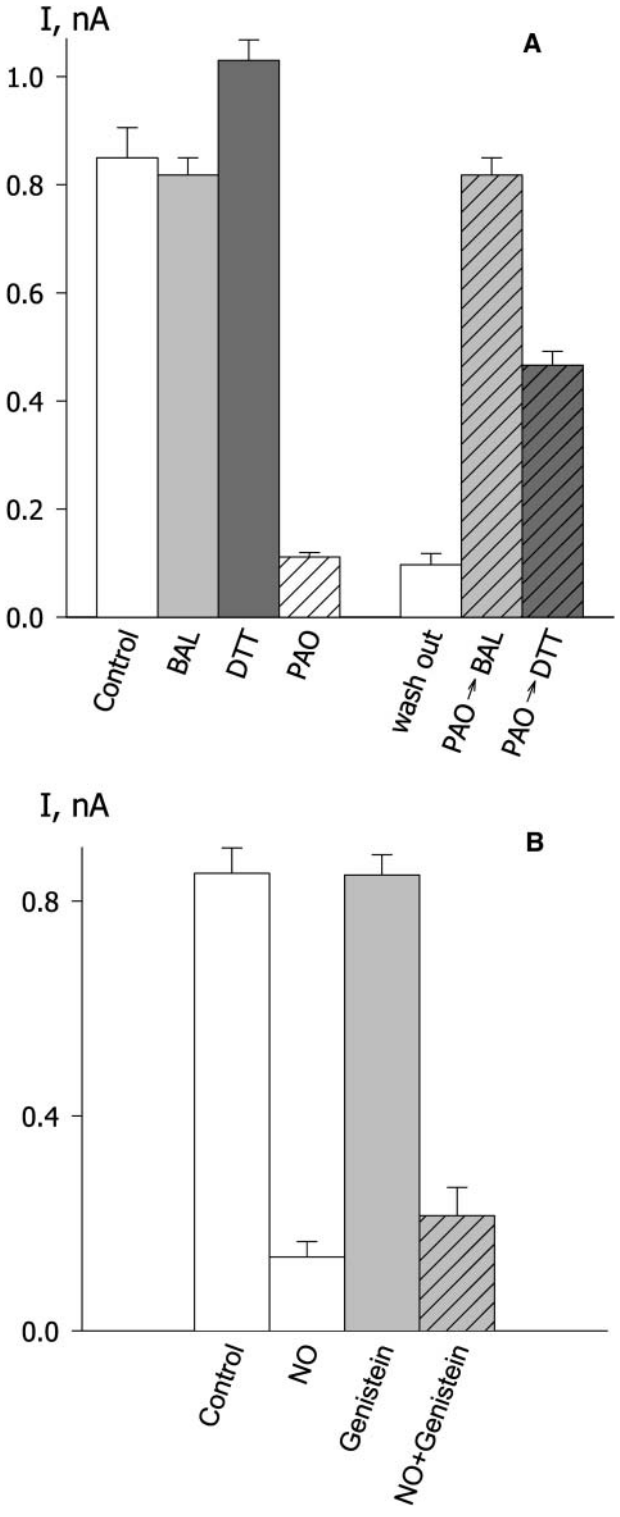

Figure 8. Reducing agents restore $I_{K,out}$ and protect against subsequent PAO treatment, but the kinase antagonist genestein is ineffective. A, Summary of steady-state $I_{K,out}$ determined at +50 mV before and after exposure to 10 μM PAO and/or 0.3 mM BAL and DTT. Data are means ± SE of 5 to 12 independent experiments in each case. Bars on left are data from single treatments only or none (control); bars on right are data from guard cells exposed first to 10 μM PAO and the oxidizing reagent then washed out (1) with buffer alone for 10 min (=washout); (2) with 0.3 mM BAL for 2 min and then with buffer alone for 6 to 8 min before a second exposure to PAO (=PAO→BAL); and (3) with 0.3 mM DTT for 2 min and then with buffer alone for 6 to 8 min before a second exposure to PAO (=PAO→DTT). Measurements taken in buffer only (no shading), BAL (light shading), DTT (dark shading), PAO (diagonal lines). B, Summary of steady-state $I_{K,out}$ determined at +50 mV before and after exposure to 10 μM genestein and to 50 nM min^{-1} NO with and without concurrent treatment with 10 μM genestein.

DISCUSSION

NO is now widely recognized to contribute to cellular signaling in plants, especially in response to environmental stress and pathogen attack. It has been widely implicated in Ca^{2+}-dependent responses (Delledonne et al., 1998; Durner et al., 1998; Klessig et al., 2000; Desikan et al., 2002; Garcia-Mata and Lamattina, 2003) and, in guard cells, NO was recently demonstrated to promote Ca^{2+} release from endomembrane stores thereby potentiating evoked Ca^{2+}-induced Ca^{2+} release and elevation of $[Ca^{2+}]_i$ (Garcia-Mata et al., 2003). Nonetheless, other actions of NO can be anticipated that are unlinked to Ca^{2+}. As a free radical, NO is highly reactive in redox exchange reactions and, thus, is capable of targeting a wide variety of proteins. In animals, NO reacts preferentially with exposed Cys thiols to form *S*-nitrosyl adducts that affect the function of a number of cellular functions, including ion transport (Stamler et al., 1992, 2001; Broillet and Firestein, 1996; Broillet, 2000; Ahern et al., 2002; Sun et al., 2003). With the exception of its influence on mitochondrial respiration (Chiandussi et al., 2002; Zottini et al., 2002), however, in plants *S*-nitrosylation of proteins has attracted much less attention. Our results now implicate NO-mediated *S*-nitrosylation in regulation of the $I_{K,out}$ of *Vicia* stomatal guard cells and indicate target site(s) for NO action that are on or closely associated with the channel protein at the inner face of the membrane.

A key observation favoring this hypothesis is that NO-mediated inactivation of $I_{K,out}$ was reversible and its recovery accelerated by treatments with membrane-permeant reducing reagents (Figs. 4 and 5). *S*-Nitrosylated thiols are readily targeted by reductants such as DTT. Thus, the fact that $I_{K,out}$ inactivation was reversed rapidly by DTT, as well as BAL, argues for this simplest of oxidative modifications by NO. The hypothesis finds additional support in the parallel actions of other oxidizing reagents on $I_{K,out}$ (Figs. 6–9). Both the alkylating reagent NEM and the SH cross-linking arsenical PAO mimicked the action of NO. Significantly, the membrane-impermeant Cys-modifying reagents IodAA and DTNB had little or no effect on the K^+ current, leading us to propose that the primary sites for oxidative modification are not accessible at the external face of the membrane. Similar lines of evidence have been drawn for direct activation of olfactory cyclic nucleotide-gated channels (Broillet and Firestein, 1996; Broillet, 2000), and inactivation of Ca^{2+} channels (Summers et al., 1999; Sun et al., 2001) by NO.

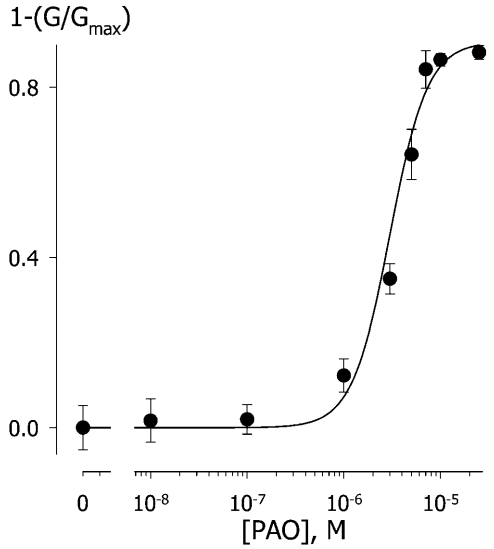

Figure 9. PAO inactivation of $I_{K,out}$ is cooperative. $I_{K,out}$ steady-state currents derived as in Figure 1 at +50 mV before and during PAO treatments. Data are given as means ± SE of the conductance complement (= relative inactivation) from 47 experiments (>5 experiments per data point) and are plotted as a function of PAO concentration. The solid curve is the best fit to the Hill equation (Eq. 2) yielding a cooperativity coefficient of 2.3 ± 0.5 and an apparent $K_{1/2}$ of 3.2 ± 0.4 μM with a maximum block of 91% ± 6%.

Equally important, we found that buffering pH_i was ineffective in countering the inactivation of $I_{K,out}$ by NO (Fig. 3). $I_{K,out}$ in guard cells is insensitive to changes in $[Ca^{2+}]_i$, but its activity is strongly dependent on pH_i (for review, see Assmann and Shimazaki, 1999; Blatt, 2000b; Hetherington, 2001; Schroeder et al., 2001; Webb et al., 2001). Activation of $I_{K,out}$ at alkaline pH_i shows cooperativity consistent with two H^+-binding sites with an apparent pK_a near 7.4 (Grabov and Blatt, 1997). Thus, the inactivation of $I_{K,out}$ might be understood if NO transiently reduced pH_i to values near 7.0. As the simplest test for an effect of NO-evoked changes in pH_i, we buffered the cytosol by diffusional loading from microelectrodes filled with 200 mM HEPES. This approach was shown to raise pH buffer capacity 4-fold, sufficient to prevent ABA-evoked increases in pH_i and $I_{K,out}$ (Blatt and Armstrong, 1993). That NO treatments inactivated $I_{K,out}$ under these conditions therefore argues strongly against any role for pH_i as an intermediate.

It is significant that *S*-nitrosylation of Cys thiols is reversible in vivo. NO is thought to undergo oxidation to form nitrous anhydride (N_2O_3), which, in turn, can donate NO either to a Cys thiol of a protein or to a small organic thiol such as glutathione. Thus, protein thiol groups can be either primary or secondary targets for *S*-nitrosylation, and denitrosylation may occur through a similar exchange process (Sun et al., 2001; Ahern et al., 2002). In fact, the mechanisms for denitrosylation in many cases have yet to be fully understood (Stamler et al., 2001), but there is evidence that specific enzymes govern levels of *S*-nitrosylation in vivo (Liu et al., 2001), and peroxidase and transaminase activities are implicated in denitrosylation (Abu-Soud and Hazen, 2000; Lai et al., 2001; Mannick and Schonhoff, 2004). *S*-Nitrosylation does confer precisely regulated posttranslational modification and probably affects the function of many proteins in much the same way as protein (de)phosphorylation (Stamler et al., 1997, 2001; Hess et al., 2001; Ahern et al., 2002). One emerging pattern is of covalent linkage to Cys in an acid-base pocket that supports (de)nitrosylation reactions similar to hemoglobin (Stamler et al., 1997; Hess et al., 2001) that confers specificity. Indeed, the action of NO on ryanodine-sensitive Ca^{2+} channels in skeletal muscle results from addition/removal of NO at a single Cys moiety out of the >40 Cys residues in the channel protein (Sun et al., 2001).

By contrast with NO, inactivation of $I_{K,out}$ by PAO was not reversible on washout within the time frame of these experiments but, like NO, its action was readily reversed by treatments with the dithiol BAL (Figs. 7 and 8). PAO preferentially reacts with closely spaced (vicinal) thiol groups in proteins to form stable, sulfur-metal ring complexes, and its targets include protein Tyr phosphatases (Carballo et al., 1999), vesicle trafficking, and associated proteins (Arien et al., 2003; Gerhard et al., 2003), as well as inducible NO synthase (Oda et al., 2000). A feature of PAO complexes is their lability to reduction by BAL. Thus, the efficacy of this reducing agent in rescuing $I_{K,out}$ suggests the presence of critical, paired Cys thiols that are exposed to oxidation by both NO and PAO within the cell. In support of this argument, we found that inactivation of $I_{K,out}$ by PAO was best fitted with a binding function and cooperativity index (Hill coefficient) precisely one-half that for NO-mediated inactivation, as if PAO oxidized paired Cys overlapping with the targets of NO action. However, these results do not identify the protein target(s) in either case.

How might NO action be mediated, then? The inactivation of $I_{K,out}$ is sensitive to NO over a remarkably narrow range of concentrations. We found that raising NO generation to levels only marginally above 20 nM/min gave almost complete inactivation, consistent with at least a 4-fold cooperativity for the effect. No appreciable change was evident in the relaxation kinetics for the current, nor in its voltage sensitivity in the presence of NO. These are characteristics most easily explained with a reduction in the number of functional channels at the membrane. Voltage-gated ion channels, presumably including $I_{K,out}$, operate as tetramers with each subunit contributing to both gating and permeation (Doyle et al., 1998; Yellen, 2002; Jiang et al., 2003; Very and Sentenac, 2003). So one interpretation is that NO targets a single pair of Cys within the K^+ channel protein that are critical for channel activation, and oxidizing these Cys thiols in two of the four subunits is sufficient to block channel opening. However, at present, other interpretations that do not entail a direct action on the K^+ channel are equally plausible. We note that NO washout (Fig. 1; see also Fig. 5) occasionally led

to an overshoot in $I_{K,out}$, consistent with additional secondary actions on other regulatory factors. In this context, it is of interest, for example, that the Arabidopsis ABI1 protein phosphatase is similarly sensitive to micromolar PAO (K_i, 3 μM), although in vitro its inactivation shows no cooperativity (Meinhard and Grill, 2001). The dominant-negative mutant of this protein phosphatase also suppresses the activity of $I_{K,out}$ in tobacco guard cells (Armstrong et al., 1995).

An equally important question is of the physiological function for NO sensitivity of $I_{K,out}$. At present, our knowledge of the roles for NO in plants is expanding rapidly, so a definitive answer is not possible at this time. However, we can offer two conjectures. (1) On the assumption that NO action is not specifically targeted to $I_{K,out}$, its inactivation may represent a broad-range response to oxidative stress. The K^+ channel is known to be sensitive to concentrations of H_2O_2 only marginally higher than the steady-state levels of NO achieved in these experiments and, like NO, H_2O_2 inactivates the current (Kohler et al., 2003). This effect is consistent with previous reports that oxidative stress in plants can suppress stomatal closure (see Willmer and Fricker, 1996) and even promote stomatal opening (Black and Black, 1979). (2) On the assumption that NO action is targeted to $I_{K,out}$, its inactivation may reflect an imbalance between nitrosylation and denitrosylation (Liu et al., 2001) superimposed by the exogenous addition of NO in these experiments. From this explanation, it would follow that the K^+ channel activity is finely tuned to the balance of *S*-nitrosylation in vivo. Indeed, these two explanations are not mutually exclusive, but a resolution of this question is not yet possible.

MATERIALS AND METHODS

Plant Material and Electrophysiology

Epidermal peels were prepared from *Vicia faba* grown under a 16-h light/8-h dark and 21°C/14°C cycle (Blatt and Armstrong, 1993). All operations were carried out on a Zeiss Axiovert microscope (Zeiss, Jena, Germany) with 63× LWD DIC optics. Epidermal peels were fixed in the experimental chamber with an optically clear, pressure-sensitive adhesive (50/50 medical adhesive; Dow Corning, Brussels) and were bathed in 5 mM Ca^{2+}-MES, pH 6.1 [MES titrated to its pK_a with $Ca(OH)_2$], with 10 mM KCl. Measurements were carried out in continuously flowing solution at 20 chamber volumes/min (Grabov and Blatt, 1998).

Microelectrodes

Recordings were obtained with two-barrelled microelectrodes coated with paraffin wax to reduce electrode capacitance (Blatt and Armstrong, 1993). Current-passing and voltage-recording barrels were filled with 200 mM K^+ acetate, pH 7.5, to minimize salt leakage and salt loading artifacts associated with the Cl^- anion without imposing a pH load. In some experiments, the electrodes were filled with 200 mM K^+-HEPES, pH 7.5, to suppress any changes in pH_i. Connections to amplifier headstages were via 1 M KCl Ag|AgCl half-cells, and a matching half-cell and 1 M KCl-agar bridge served as the reference (bath) electrode.

NO Release

NO was generated in solution from *S*-nitroso-*N*-acetyl-penacillamine (SNAP), which spontaneously releases NO in a pseudo first-order reaction with a half-time in solution of approximately 5 h (Hou et al., 1999). NO generation was assayed by the Griess reaction (Zhang et al., 2003) in perfusion buffer and indicated that 10 μM SNAP releases 2.5 to 3.0 μM NO over 2 h, equivalent to approximately 2 nM NO min^{-2} μM^{-1} SNAP in standing solution (not shown). Because the K^+ channel measurements were carried out in continuously flowing solution, this figure represents a lower estimate of the rate of NO generation.

Electrical and Numerical Analysis

Mechanical and electrical design has been described previously (Blatt and Armstrong, 1993). Voltage clamp control, data acquisition, and analysis were carried out using Henry II software (Y-Science, Glasgow, UK; available for academic use by download at http://www.gla.ac.uk/ibls/BMB/mrb/lppbh.htm). Currents were normally filtered with a low-pass Butterworth filter (cutoff frequency, 1 kHz) and sampled at 2 kHz. $I_{K,out}$ was determined and activation half-times were taken from two-step voltage clamp protcols after subtracting instantaneous currents at each voltage. Where appropriate, analyses were carried out by nonlinear, least-squares fittings using a Marquardt-Levenberg alorithm (Marquardt, 1963). Results are reported as means ± SE and taken to be significant at $P < 0.05$.

Chemicals and Solutions

SNAP was dissolved in 1:1 ethanol:water, and PAO was dissolved in DMSO before >1,000-fold dilution for use. Ethanol and DMSO alone at this concentration had no effect (Grabov and Blatt, 1998; Hamilton et al., 2000). All other compounds were used directly. All reagents were from Sigma (Poole, UK) or Calbiochem (Darmstadt, Germany).

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