Ozone inhibits guard cell K^+ channels implicated in stomatal opening

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Ozone (O_3) deleteriously affects organisms ranging from humans to crop plants, yet little is understood regarding the underlying mechanisms. In plants, O_3 decreases CO_2 assimilation, but whether this could result from direct O_3 action on guard cells remained unknown. Potassium flux causes osmotically driven changes in guard cell volume that regulate apertures of associated microscopic pores through which CO_2 is supplied to the photosynthetic mesophyll tissue. We show in *Vicia faba* that O_3 inhibits (*i*) guard cell K^+ channels that mediate K^+ uptake that drives stomatal opening; (*ii*) stomatal opening in isolated epidermes; and (*iii*) stomatal opening in leaves, such that CO_2 assimilation is reduced without direct effects of O_3 on photosynthetic capacity. Direct O_3 effects on guard cells may have ecological and agronomic implications for plant productivity and for response to other environmental stressors including drought.

Ozone (O_3) in the upper atmosphere is highly beneficial as a UV filter, but O_3 of anthropogenic origin at ground level is a serious pollutant. Elevated ground-level O_3 is associated with impaired human health (1, 2), reduced primary production in forest ecosystems (3), and losses in agricultural yield estimated at $3 billion annually for the U.S. alone (4). However, the cellular targets of O_3 action remain essentially unknown. In plants, O_3 reduces rates of photosynthetic carbon fixation. Such decreases could reflect (*i*) direct effects of O_3, and/or the reactive oxygen intermediates generated on O_3 exposure ($\cdot OH$, H_2O_2 etc.), on the interior, photosynthetic mesophyll cells; or (*ii*) a decrease in CO_2 availability to the mesophyll cells, as a result of impaired function of guard cells in the plant epidermis. Guard cells control rates of CO_2 entry to the mesophyll by regulating the apertures of the stomatal pores in the leaf epidermis through which gas exchange occurs. Reduced stomatal conductance is commonly observed after O_3 exposure (5, 6), and it is possible that these reductions reflect a primary response of the guard cells to O_3 (7). Alternatively, reduced stomatal conductance could be a secondary response of the guard cells after either O_3 injury of adjacent epidermal cells (8) or alterations in the leaf environment caused by O_3 damage to the mesophyll tissue. In particular, high internal CO_2 concentrations, as result from inhibition of carbon assimilation, are a potent signal for stomatal closure (9) and have been hypothesized to be the proximate cause of the stomatal responses to O_3 (10).

Because the changes in guard cell volume that regulate stomatal apertures are driven in large part by ion uptake and loss, we hypothesized that, if O_3 does directly affect guard cells, it may do so by altering the activity of ion channels in the guard cell plasma membrane. There is indirect evidence that O_3 affects the plant plasma membrane because altered membrane permeability and ion leakage are frequently detected in leaves and in the unicellular alga *Chlorella sorokiniana* after treatment with the gas (11). Little research has been performed on the underlying mechanisms, although two studies have demonstrated that membrane vesicles isolated from leaves of O_3-treated pinto bean (*Phaseolus vulgaris* L. var Pinto) plants display altered Ca^{2+} transport (12) and a decrease in K^+-stimulated ATPase activity (13). However, all of these studies lack specificity with regard to cell type and involvement of molecularly defined transport molecules. In mammals, an effect of O_3 on ion transport in airway epithelial cells also has been suggested by indirect measurements (14, 15) but never tested by direct electrophysiological measurements of ion channel activity.

In response to stimuli that elicit stomatal opening, K^+ influx increases guard cell osmotic content, resulting in water uptake, a bowing out of the two guard cells that define each stomatal pore, and an increase in stomatal aperture. K^+ influx occurs through inwardly rectifying K^+ channels and is driven by membrane hyperpolarization caused by H^+ ATPase activation and anion channel inactivation. Conversely, stimuli that elicit stomatal closure inhibit H^+ ATPase activity and activate anion channels, resulting in membrane depolarization that both activates outwardly rectifying K^+ channels and provides the driving force for K^+ efflux (16, 17).

In the present study, we used the technique of whole cell patch clamping to directly assess the effects of O_3 on plasma membrane K^+ channels of guard cells of fava bean (*Vicia faba*), the most commonly used species for cellular studies of guard cell function. The influence of O_3 on stomatal regulation was also studied in isolated epidermal peels and whole plants.

Materials and Methods

Growth Conditions. Broad bean (*V. faba*) plants were grown from seeds in 3 parts Metro-mix 360 to 1 part Perlite and were watered 3–4 times per week with ¼ strength Hoagland solution. Plants used for epidermal peel and patch-clamp experiments were grown in environmental growth chambers under a 10-hr light/14-hr dark cycle with a light intensity of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ and a temperature of 25/23°C. Plants used in the gas exchange experiments were grown in a greenhouse under natural illumination with 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplemental illumination between 8 a.m. and 8 p.m. The youngest fully expanded leaves from 3- to 4-week-old plants were used in all experiments.

Ozone Exposure. Protoplasts were isolated from three fully expanded leaves as described (18, 19). The protoplasts were exposed to O_3 in a 300 mM K^+-phosphate buffer (pH 5.7) containing 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 40 mM K_2SO_4 to a final osmolality of 440 $mOsm \cdot kg^{-1}$. Ozone was introduced into the buffer, which was determined not to scavenge O_3, by bubbling 930 $ml \cdot liter^{-1}$ O_3 (in O_2) through the buffer for 30 min. The buffer was treated with O_3 before cell exposure. Ozone concentration in the buffer was determined spectrophotometrically by using the indigo method (20). In the first experiment (Fig. 1 *A* and *B*), the protoplasts were pretreated with the O_3-containing buffer (33–354 $\mu l \cdot liter^{-1}$) for <5 min and were immediately transferred to bath solution I (see below) for patch clamping. In the second experiment (Fig. 1 *C* and *D*), O_3-

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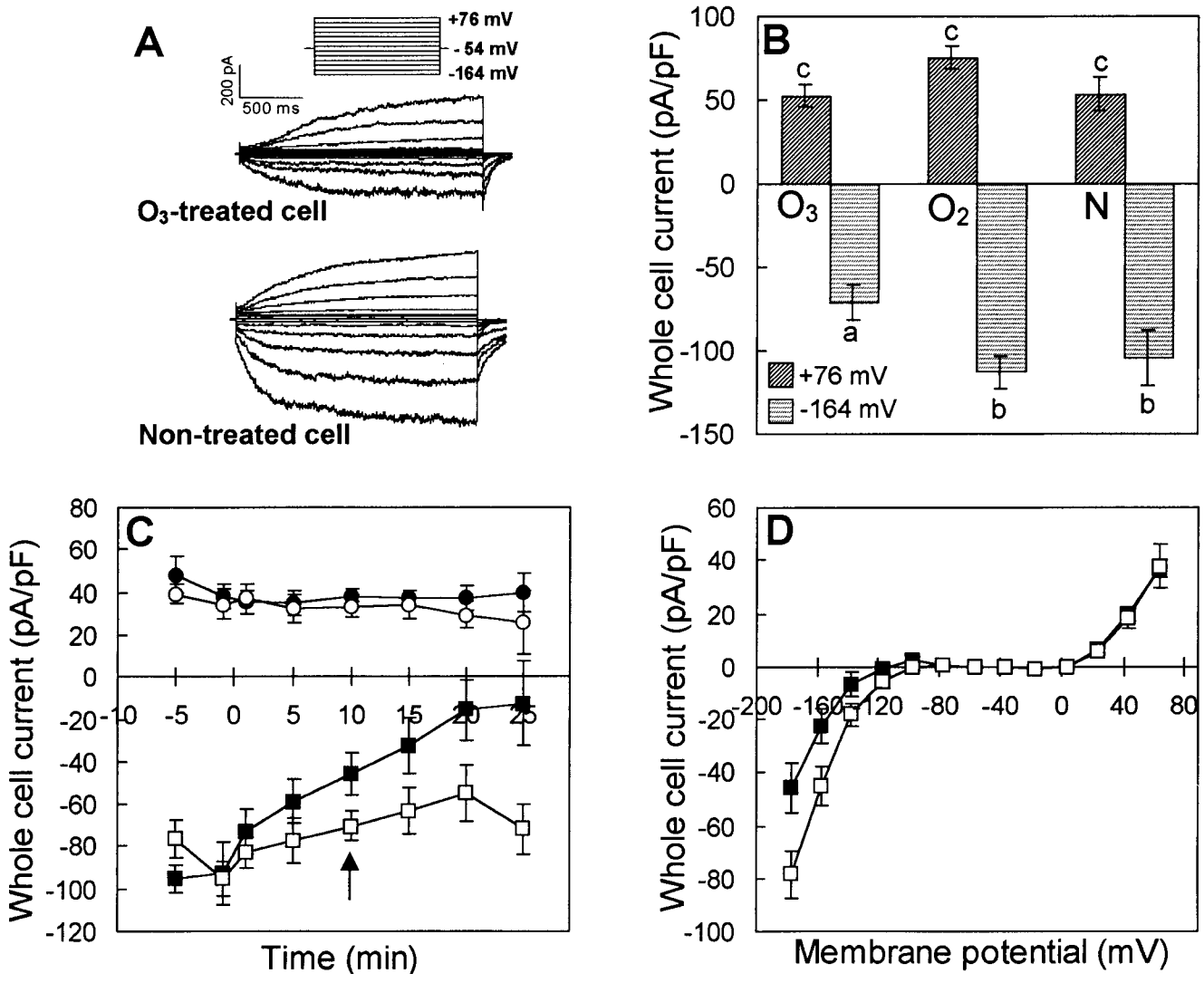

Fig. 1. Ozone exposure of *V. faba* guard cell protoplasts reduces inward K^+ currents. (*A*) Representative whole cell currents recorded from one O_3-treated and one nontreated cell using 100 mM K^+ bath and pipette solutions (I). Currents were identified as K^+ currents based on their reversal potentials (data not shown) and their characteristic time-activation and voltage-dependence (24). Seal resistance for the cells in *A* was 2.0 GΩ for the O_3-treated cell and 1.2 GΩ for the nontreated cell. (*B*) Average voltage-activated whole cell K^+ currents, normalized for cell size, measured at membrane potentials of +76 mV and −164 mV. For both *A* and *B*, protoplasts were exposed to 33–354 μl·liter^{-1} O_3 ($n = 21$) for <5 min in a 300 mM K^+-phosphate buffer (see *Material and Methods*). Controls were protoplasts exposed to O_2-treated phosphate buffer (O_2, $n = 14$) or to nontreated phosphate buffer (N, $n = 11$). Protoplasts were transferred to bath solution I for immediate patch clamping after exposure, and whole cell recordings were performed between 8 and 50 min after exposure, depending on the time required to obtain a whole cell seal. Seal resistance ranged from 0.9 to 4 GΩ for the 46 cells of this experiment and did not differ significantly between treatments (data not shown). Ozone induced a significant ($P < 0.01$) reduction in inward currents compared with O_2- and nontreated protoplasts; there was no significant treatment effect on the outward currents. (*C*) Voltage-activated whole cell currents measured before and after treatment (treatment at time 0) at membrane potentials of +63 mV for O_3-treated (●) or nontreated (○) protoplasts and at −177 mV for O_3-treated (■) or nontreated (□) protoplasts. The effects on inward K^+ current of O_3, time, and O_3 treatment by time are significant at the $P < 0.05$ level. (*D*) Current-voltage relationships from whole cell measurements recorded 10 min (indicated by arrow in *C*) after adding O_3-treated (■, $n = 6$) or nontreated buffer (□, $n = 8$) directly to the bath solution (phosphate buffer described above). Ozone significantly inhibited inward K^+ current at voltages ≤−117 mV. For both *C* and *D*, the whole cell configuration was obtained with a 10 mM K^+ bath solution (II) and a 100 mM K^+ pipette solution (II). The bath was thereafter perfused with the phosphate buffer described in *Materials and Methods* for 3 min, and 200 μl of O_3-treated or nontreated phosphate buffer was added to the 1-ml bath dish ≈5 min after the end of perfusion. The initial O_3 concentration in the dish ranged from 10 to 30 μl·liter^{-1} and in the presence of the protoplasts the concentration dropped by 75% within 10 s.

containing buffer was added during the patch clamp experiment to protoplasts bathed in the phosphate buffer described above. The initial concentration after adding O_3 to the dish ranged from 10 to 30 μl·liter^{-1} O_3. The concentration dropped by 75% within 10 s. Because O_3 is so unstable and remained in solution for brief periods of time, it was not possible to obtain a repeatable concentration of the gas in each of these experiments. Despite this variation, similar inhibition of the inwardly rectifying K^+ currents (I_{Kin}) was observed throughout the range of O_3 concentrations. It has been observed by others (see ref. 11 for review and references) that relatively high concentrations of O_3 are required to elicit a response when cells or biochemicals are exposed to the gas *in vitro*. This may relate to the solubility of the gas and the scavenging potential of the cells.

The epidermal peels were exposed to air with or without 1.0 μl·liter^{-1} O_3 for 3 hr in a custom-made Petri dish with a gas inlet and outlet. The flow through the Petri dish during exposure was 400 ml·liter^{-1}. Whole plants were treated with 0, 0.10, or 0.18

μl·liter^{-1} O_3 in continuous stirred tank reactors (21) located in a charcoal-filtered greenhouse.

Patch Clamp Recording. Two different sets of solutions were used for the patch-clamp recordings presented in Fig. 1 *A* and *B* (bath and pipette solution I) and *C* and *D* (bath and pipette solution II). Bath solution I contained 100 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Mes, 5 mM Hepes at pH 5.6 (adjusted with Tris), and sorbitol to a final osmolality of 460 mOsm·kg^{-1}. Pipette solution I contained 80 mM K^+ glutamate, 20 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes, 2 mM EGTA, and 2 mM Mg-ATP (Sigma) (added daily) at pH 7.8 (KOH) and adjusted to 480 mOsm·kg^{-1} with sorbitol. Bath solution II contained 10 mM K-glutamate, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM Mes, 0.25–0.50 mM *N*-methyl glucamine (pH 5.5), and mannitol to a final osmolality of 480 mOsm·kg^{-1}. This solution was used initially in experiments presented in Fig. 1 *C* and *D* because it promoted formation of a high resistance (GΩ) seal. Before O_3 exposure of the cells, this solution was exchanged by bath perfusion and was replaced with the phosphate buffer previously described. Pipette solution II contained 100 mM K-glutamate, 2 mM $MgCl_2$, 0.2 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate, 4 mM Mg-ATP, 20 mM Hepes, and mannitol to a final osmolality of 520 mOsm·kg^{-1}.

Whole-cell currents were measured in response to 1.8-s voltage pulses decremented in −20-mV steps from +63 mV to −177 mV, using an Axopatch-1B patch-clamp amplifier and a TL-1 DMA Interface (both from Axon Instruments, Foster City, CA). Between measurements, the holding potential was kept for 10.25 s at −54 mV and −67 mV, respectively, in the experiments presented in Fig. 1 *A* and *B* and Fig. 1 *C* and *D*. A schematic of the voltage stimulus is given in Fig. 1*A*. The sampling rate was 1 ms. Currents were filtered at a −3dB frequency of 500 Hz by the lowpass Bessel filter of the patch-clamp amplifier. Data analyses were accomplished by using PCLAMP 6.0.2 software (Axon Instruments). Whole-cell currents were corrected for time-independent or leak currents and were normalized—i.e., expressed in pA/pF—to eliminate differences caused by cell-to-cell variations in surface area (22). Potentials reported have been corrected for liquid junction potentials (23).

Epidermal Peel Assay. In stomatal opening experiments, abaxial epidermal peels were floated on 35 mM KCl during 3 hr of exposure to air with or without 1.0 μl·liter^{-1} O_3. The plants used for isolation of epidermal peels were kept in the dark overnight before each experiment to ensure that stomata were closed. The isolated epidermal peels were simultaneously exposed to light (200 μmol·m^{-2}·s^{-1}) in the presence or absence of O_3. In stomatal closure experiments, abaxial epidermal peels were floated on 35 mM KCl/0.5 mM $CaCl_2$ for 3 hr during exposure to air in the presence or absence of 1.0 μl·liter^{-1} O_3 under continuous light (200 μmol·m^{-2}·s^{-1}). Before the isolation of the epidermal peels for the closure experiments, the abaxial side of detached leaves was exposed to light (200 μmol·m^{-2}·s^{-1}) for 2 hr to ensure open stomata. Widths of stomatal apertures were determined with an ocular micrometer and a light microscope (Leitz Dialux 20).

Gas Exchange Measurements. Stomatal conductance, assimilation, and A/C_i curves (carbon assimilation as a function of the intercellular concentration of CO_2) were measured with a Li-Cor 6400 gas exchange system (Li-Cor, Lincoln, NE). Conditions in the gas exchange cuvette during measurements were 30% relative humidity, 350 μl·liter^{-1} CO_2 and 800 μmol·m^{-2}·s^{-1} light intensity provided by a red/blue light source (Li-Cor).

Statistical Analysis. The patch clamp data were analyzed by analysis of covariance (ANCOVA) using O_3 concentration and time between treatment and measurement of whole cell current as covariates in the first experiment (Fig. 1*B*). In the second experiment (Fig. 1 *C* and *D*), O_3 concentration was used as the covariate in a multiple analysis of covariance (MANCOVA). Multiple analysis of variance (MANOVA) was used to analyze the data from the epidermal peel and gas exchange experiments.

Table 1. Change in the extent of opening or closure of stomata in epidermal peels of *V. faba* on O_3 exposure (1.0 μl·liter^{-1} for up to 3 hr)

	Stomatal opening: Mean widths, μm ± SE		Stomatal closure: Mean widths; μm, ± SE	
Time, hr	O_3	Air	O_3	Air
0	0.5 ± 0.2*	0.5 ± 0.2*	8.1 ± 0.5*	8.1 ± 0.5*
1	–		7.3 ± 0.7	8.1 ± 0.4
1.5	6.4 ± 0.6	7.4 ± 0.7	–	–
2	–	–	6.3 ± 0.6	7.1 ± 0.6
3	8.4 ± 0.5†	10.6 ± 0.6	6.1 ± 0.6	7.0 ± 0.6

Ten stomatal apertures were measured on three epidermal peels for each treatment/time point in each of the six replicates of both opening and closure experiments (total $n = 180$).

*The 0-hr time point represents a set of peels measured before the initiation of treatments.

†Significant difference ($P < 0.01$) between O_3 and air treatment.

Results and Discussion

Two types of whole-cell patch clamp experiments were performed. In the first set of experiments (Fig. 1 *A* and *B*), protoplasts were pretreated with O_3 and subsequently subjected to whole-cell patch-clamp analysis. In the second set of experiments, protoplasts were exposed to O_3 after attainment of the whole-cell patch-clamp configuration, such that current responses before and after O_3 exposure could be evaluated on the same cell (Fig. 1 *C* and *D*). In both types of experiments, O_3 significantly ($P < 0.05$) reduced the magnitude of inward K^+ currents but had no significant effect on the magnitude of the outward K^+ currents. The reduction in inward K^+ current over time was significantly greater in O_3-treated than in control cells. The current-voltage relationship of the inward K^+ currents was negatively shifted by O_3 exposure whereas the characteristic voltage dependence of the channels was maintained (Fig. 1*D*). No appreciable effects were observed either on the kinetics of current activation (Fig. 1*A*; data not shown) or on ion selectivity, as ascertained by tail current analyses (data not shown).

These results are noteworthy for three reasons. First, they demonstrate specific inhibition of an ion channel by O_3, as has not been previously demonstrated for any biological system. Second, they demonstrate that guard cells are affected by O_3 directly. Third, because the inward K^+ channels were inhibited, these results lead to the hypothesis that O_3 acts not, or not exclusively, by promoting stomatal closure, as has been widely assumed (6), but rather that O_3 is a potent inhibitor of stomatal opening. These two different loci of action have significantly different agricultural and ecological consequences (see *General Discussion*).

To test the hypothesis derived from the patch clamp experiments that O_3 significantly inhibits stomatal opening, we measured stomatal apertures in isolated epidermal peels of *V. faba* stripped from the mesophyll tissue and exposed to O_3. Stomatal opening in response to light was significantly inhibited during a 3-hr O_3 exposure (Table 1). Although stomatal opening was inhibited by only 20%, these results have been confirmed in two other species, *Arabidopsis thaliana* and *Nicotiana tabacum*. Ozone exposure (0.30 μl·liter^{-1} for 3.5 hr) resulted in 20 and 31% inhibition of light-induced stomatal opening in epidermal peels of *A. thaliana* and *N. tabacum*, respectively, with both

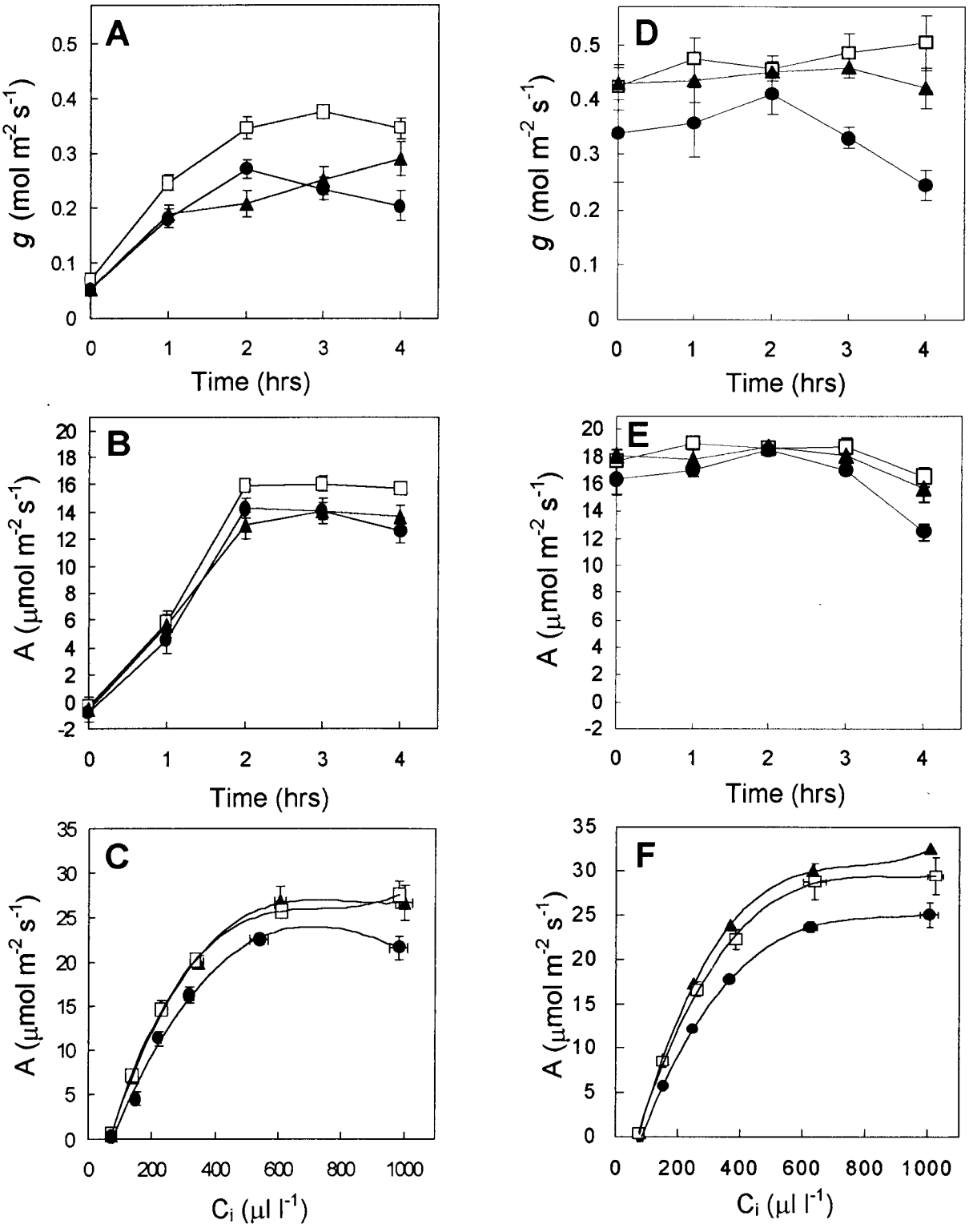

Fig. 2. Stomatal conductance (*A* and *D*) and assimilation (*B* and *E*) measured on the first fully expanded leaves of *V. faba* plants during exposure to charcoal-filtered air containing 0 (□), 0.10 (▲), or 0.18 (●) μl·liter^{-1} O_3 for 4 hr in continuous stirred tank reactors (24). (*A–C*) The plants were kept in the dark overnight and until the start of exposure to allow observation of the influence of O_3 on light-induced stomatal opening (natural illumination supplemented with 150 μmol·m^{-2}·s^{-1}). Both O_3 treatments resulted in significant ($P < 0.01$) reductions in stomatal conductance and assimilation. (*D–F*) The plants were kept in the light (natural illumination supplemented with 150 μmol·m^{-2}·s^{-1}) for 4 hr before exposure to ensure open stomata at start of exposure. Only the highest O_3 treatment resulted in significant ($P < 0.01$) reduction of stomatal conductance (after 3 and 4 hr) and assimilation (after 4 hr). In each replicate experiment, gas exchange was measured on five individual plants for each treatment/time point. (*C* and *F*) Assimilation (A) versus intercellular CO_2 concentration (C_i) measured within 60 min after the end of O_3 exposure, on the first fully expanded leaf of two individual plants for each treatment. The average A/C_i curve for the plants exposed to 0.10 μl·liter^{-1} O_3 was not significantly different from that of plants exposed to charcoal-filtered air whereas plants exposed to 0.18 μl·liter^{-1} O_3 had significantly lower A/C_i curves ($P < 0.05$). For all panels, the average of three experiments is shown.

reductions significant at the $P \leq 0.05$ level (S. R. Weightman, E.J.P., and S.M.A., unpublished work). The importance of these results is two-fold. First, the data support the hypothesis derived from the electrophysiological measurements that O_3 inhibits stomatal opening. Second, consistent with the patch clamp results, they demonstrate that intact guard cells are indeed directly affected by O_3. Although this possibility had been previously inferred (25), no direct evidence had ever been presented to support it. The same concentration of O_3 did not stimulate significant differences in stomatal closure at any of the individual time points measured. Guard cells retained competence to drive stomatal closure under these circumstances, as evidenced by stomatal closure when control peels were transferred to darkness under otherwise identical conditions [final aperture of 1.8 ± 0.3 μm after 3 hr of dark incubation (data not shown)]. Greater stomatal closure of O_3-treated epidermal peels was statistically significant when data from all individual time points were pooled (not shown), suggesting that there could be a small O_3 effect. Our inability to detect effects of O_3 on outward K^+ channels could have resulted because the impact was minimal and variability in electrophysiological recordings precluded detection. Alternatively, O_3 induction of stomatal closure might have resulted from effects on transporters other than K^+ channels. For example, opening of anion channels has been proposed to play a central role in stomatal closure by both directly mediating anion loss and by depolarizing the membrane, which then drives loss of K^+ (26, 27).Taken together, the results of the patch clamp and epidermal peel experiments led to two hypotheses concerning stomatal function in the intact plant. First, we hypothesized that, if leaves were treated with O_3 under conditions in which stomata initially were closed, subsequent stomatal opening would be impaired. Second, we hypothesized that O_3-inhibition of stomatal opening and thus of CO_2 influx could lead to reductions in photosynthetic rate even in the absence of any direct effects of O_3 on mesophyll photosynthesis. To test these hypotheses, whole *V. faba* plants were exposed to 0, 0.10, or 0.18 μl·liter^{-1} O_3 for 4 hr. Exposures were initiated under darkness, a condition in which stomata are closed and stomatal conductance is low. Subsequent illumination stimulated stomatal opening, resulting in increases in stomatal conductance, as measured through nondestructive gas exchange analysis. Both 0.10 and 0.18 μl·liter^{-1} O_3 significantly inhibited the increase in stomatal conductance and decreased rates of mesophyll carbon assimilation relative to control plants (Fig. 2*A* and *B*). At the end of the exposure, photosynthetic capability of the mesophyll tissue was assessed by A/C_i curves (Fig. 2*C*). Because C_i is experimentally manipulated in these protocols, the role of stomatal response in controlling photosynthetic rates is "eliminated." The A/C_i curves revealed that, at the lower O_3 concentration, there was no direct effect of O_3 on carbon assimilation; at a given C_i, assimilation rates were identical in O_3-treated and control plants. Therefore, the reduced rates of carbon assimilation seen under those conditions could only be attributed to a reduced availability of the substrate CO_2 to the mesophyll, as a result of the O_3-inhibition of stomatal opening. At the higher O_3 concentration, a direct effect of O_3 on mesophyll photosynthesis could be observed as well, detected as a depression in the A/C_i curve; these results agree with previous reports (28–31). The same O_3 treatments then were applied to plants with initially open stomata (Fig. 2 *D–F*). Only when the photosynthetic apparatus was directly affected by the O_3 treatment (at 0.18 μl·liter^{-1} O_3) as indicated by the A/Ci curve (Fig. 2*F*), did significant stomatal closure and reduction in assimilation occur (Fig. 2 *D* and *E*). At the lower O_3 dose (0.10 μl·liter^{-1} O_3), stomata did not close significantly in response to O_3, despite the fact that this dose sufficed to inhibit stomatal opening (Fig. 2*A*), and photosynthesis was unaffected. These results further support the conclusion that the process of stomatal opening is more sensitive to O_3 than is the induction of stomatal closure.

General Discussion

The experiments reported here show that O_3 directly affects guard cells themselves, targeting inward K^+ channels, and that O_3 inhibits stomatal opening. These results challenge the prevailing dogma that O_3, either directly or indirectly, promotes stomatal closure. Moreover, it has been thought that, if stomata are closed at the time of O_3 exposure, there will be minimal adverse effects because the gas will be unable to penetrate to the interior photosynthetic mesophyll (32, 33). However, our results do not support this theory, showing instead that O_3 exposure initiated while stomata are closed can depress subsequent carbon assimilation via O_3 action solely on the guard cells. Our whole plant experiments were conducted with concentrations of O_3 that can be found in polluted atmospheres (34); this is particularly true for the treatment with 0.10 μl·liter^{-1} O_3. These studies, therefore, have important agronomic and ecological ramifications, as illustrated by the following three examples. First, stomatal closure during a period of drought may be less readily reversed in O_3-exposed plants. This is particularly relevant because the highest O_3 concentrations are sometimes associated with times of drought: e.g., as was reported in Great Britain during significant droughts in 1976 and 1982 (35). In fact, when ash trees (*Fraxinus excelsior* L.) received long term O_3 exposures during periodic drought exposures, there was some evidence that stomatal conductance might recover more slowly during rewatering in O_3-treated than control plants (36). Second, in major agricultural regions with high light environments and significant O_3 exposure—e.g., the South Coast Air Basin of California, which has the most extreme O_3 levels in the U.S. (37)—midday stomatal closure ("mid-day depression") often occurs because of the low ambient humidity that results from the high light, high temperature conditions of midday. Because the generation of O_3 in photochemical smog depends on high solar irradiation (34), O_3 inhibition of stomatal opening could significantly retard stomatal reopening in the afternoon after this mid-day depression and consequently reduce crop yield. Third, the presence of nighttime O_3 has been documented in rural locations due to long distant transport from urban sources (34) and at high elevation sites, including sites of ecological importance such as Shenandoah National Park in Virginia (38, 39). Although it has been reported that there can be an adverse impact on biomass accumulation when O_3 exposure of plants occurs exclusively in the dark (39, 40), this impact was previously explained by the modest stomatal conductance that could be detected under darkness and the concomitant O_3 uptake that would be expected. Our results suggest that inability of closed stomata to open completely after a dark exposure could be of equal or greater importance in reducing plant productivity.

One possible mechanism for the observed O_3 effect on guard cell ion currents is direct oxidation of the channel proteins. It is plausible that the exposed amino acids of the inward K^+ channels are more susceptible to oxidation compared with those of the outward K^+ channels. The inwardly rectifying K^+ channel of guard cells, which is shown as the target of O_3 action in this study, has been cloned from the model plant species *A. thaliana* (41, 42). One next step will be to assess whether alterations, either in ion channel amino acids particularly susceptible to oxidation or in levels of cellular reductants (43, 44, 45), may improve rates of guard cell recovery after the termination of an O_3 event. It will be of interest to determine whether other guard cell ion transporters are also targets of O_3 action, although the inhibition by O_3 of stomatal opening, but not induction of stomatal closure under our conditions, implicates O_3 targeting of an "opening-

specific'' component, for which the inwardly rectifying K^+ channels is the best example. Alternatively, elevation of cytosolic Ca^{2+} levels after O_3 exposure is another plausible mechanism to explain the electrophysiological responses documented here. Cytosolic Ca^{2+} has been implicated as a signaling molecule in guard cell responses to other oxidative stresses (46) and has been reported to inhibit the inward K^+ channel (47). Interestingly, an increase in cytosolic free Ca^{2+} levels also has been reported after exposure of human tracheal epithelial cells to O_3 (48). Our identification of a specific ion channel as a target for O_3 action may prompt comparable studies in mammalian systems, leading to improved understanding of and treatment for the disease etiologies exacerbated by O_3. Finally, as implied by our whole plant experiments, genetic alterations in channel properties or cellular signaling events that would lead to accelerated rates of recovery of stomatal function after O_3 exposure could improve plant productivity in geographic regions with significant O_3 exposure.

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