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Synergistic Effect of Light and Fusicoccin on Stomatal Opening[1]

Epidermal Peel and Patch Clamp Experiments

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ABSTRACT

Upon incubation of epidermal peels of *Commelina communis* in 1 millimolar KCl, a synergistic effect of light and low fusicoccin (FC) concentrations on stomatal opening is observed. In 1 millimolar KCl, stomata remain closed even in the light. However, addition of 0.1 micromolar FC results in opening up to 12 micrometers. The same FC concentration stimulates less than 5 micrometers of opening in darkness. The synergistic effect (a) decreases with increasing FC or KCl concentrations; (b) is dark-reversible; (c) like stomatal opening in high KCl concentrations (120 millimolar) is partially inhibited by the K^+ channel blocker, tetraethylammonium$^+$ (20 millimolar). In whole-cell patch-clamp experiments with guard cell protoplasts of *Vicia faba*, FC (1 or 10 micromolar) stimulates an increase in outward current that is essentially voltage independent between −100 and +60 millivolts, and occurs even when the membrane potential is held at a voltage (−60 millivolts) at which K^+ channels are inactivated. These results are indicative of FC activation of a H^+ pump. FC effects on the magnitude of inward and outward K^+ currents are not observed. Epidermal peel and patch clamp data are both consistent with the hypothesis that the plasma membrane H^+ ATPase of guard cells is a primary locus for the FC effect on stomatal apertures.

The fungal toxin FC[2] has long been known to promote stomatal opening (25, 28). This effect has been attributed primarily to the ability of FC to stimulate the plasma membrane H^+ ATPase of plant cells (16). In guard cells, activation of a H^+ pump in the plasma membrane creates an electrochemical gradient that is presumed to drive the K^+ influx necessary for guard cell swelling and stomatal opening (1, 30). K^+-selective ion channels present in the guard cell membrane (21) provide a pathway for K^+ movement across the cell membrane in response to an electrochemical driving force.

Several developments have prompted our reexamination of the mechanisms by which FC regulates stomatal apertures. A FC binding protein has been identified in guard cells and other plant tissues (8, 26). The FC binding protein is not part of the H^+ ATPase molecule, opening the possibility that FC could have sites of action in addition to the H^+ ATPase. Tracer studies and electrophysiological recording from intact guard cells (3, 4, 7) have led to the hypotheses that FC also (a) activates a carrier that mediates K^+ uptake against its electrochemical gradient, and (b) inactivates ion channels that mediate K^+ efflux, a hypothesis that can be directly tested using the electrophysiological technique of patch clamping. Finally, demonstration that the mechanism of stomatal closure is not simply the reverse of the opening process (10, 15) calls for explicit examination of FC effects on stomatal opening *versus* closure.

The data presented here illustrate a synergistic effect of light and FC on stomatal opening. No evidence was found in epidermal peel or patch clamp experiments for regulation of K^+ fluxes by FC, suggesting that the observed synergism does not involve a specific effect of FC on K^+-selective ion channels.

MATERIALS AND METHODS

Plant Material

Plants of *Commelina communis* and *Vicia faba* were raised as described previously (13, 22).

Epidermal Peel Experiments

Epidermal peel experiments assaying stomatal opening and closure (see Figs. 1–3) were performed as described previously (10, 22). KCl and FC concentrations of the incubation medium are indicated in the figures and figure legends. Each value presented in the experiments of Figures 1 through 3 is the mean of at least 60 apertures from at least three different experiments. Unless otherwise indicated, the SD did not exceed ±0.5 μm. For the experiments of Figure 4, a peel was placed in a microchamber filled with 10 mM KCl, 10 mM Mes, pH 6.1, plus chemicals of interest. The apertures of 3 to 4 individually identified stomata on a given peel were measured every 5 min. Peels were in darkness except during

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[2] Abbreviations: FC, fusicoccin; nmg, *n*-methyl-D-glucamine; TEA, tetraethylammonium; V_H, holding potential.

measurement, when the microscope light was on for less than 1 min (0.008 mmol m^{-2} s^{-1} white light). To avoid anoxia and maintain constant concentrations in the incubation solution, that solution was replaced after each measurement. Each data point in Figure 4 is the mean of at least 12 different stomatal apertures in at least four different epidermal peels.

Patch Clamp Experiments

Guard cell protoplasts were isolated from the youngest fully expanded leaves of 3- to 4-week-old plants of *V. faba* according to an established protocol (12). Patch clamping was performed in the whole-cell configuration using an Axopatch 1B amplifier (Axon Instruments, Foster City, CA). Whole-cell current was filtered at 2 kHz and continuously recorded using a Hitachi 561 chart recorder. Current responses to rapid voltage stimuli were also digitized and stored on disk (PDP11/73 computer, Indec Systems, Capitola, CA) for later analysis. In all experiments, seal resistance was monitored frequently during the course of the experiment. Those experiments in which large spontaneous changes in seal resistance occurred during the course of the experiment were discarded.

Experimental Procedure

Two sets of solutions were used (Table I).

nmg-Glutamate Solutions

After attainment of the whole cell configuration, membrane potential was held at 0 mV ($E_{nmg\text{-}glu}$), except when voltage protocols (see below) were administered. When membrane current and seal resistance were stable, as determined from the chart recorder trace (typically about 5 min after achieving the whole cell configuration), the effect on membrane current of changes in membrane potential was determined by stepping membrane voltage from −100 to +60 mV in +20-mV steps, each of which lasted for 500 ms. FC was then added to the bath to a final concentration of 1 μM. FC promoted a gradual increase in outward current. After this increase had plateaued, as could be ascertained from the chart recorder trace, a second set of voltage steps was administered, and the current responses recorded. Steady-state current (I) at the end of each 500-ms voltage step was subsequently computed as the average of the last 20 data points recorded at that voltage, and plotted as a function of membrane potential (V). These I/V plots were constructed from the data obtained before and after FC exposure. Subtracting the "after FC" I/V curve from the "before FC" I/V curve resulted in a plot of the FC-stimulated current as a function of voltage ("delta I/V curve").

Table I. *Solutions Used in Patch Clamp Experiments*

Ion	External Solution	Internal Solution	Calculated Reversal Potential
	mM		*mV*
nmg-glutamate solutions			
n-Methyl-glucamine$^+$	50	50	0
Glutamate$^-$	50	50	0
Na^+	3	3	0
Mg^{2+}	5	8	−6
Cl^-	12	10	−5
Ca^{2+}	1	1×10^{-7}	≫ + 100
H^+	$1 \times 10^{-7.15}$	$1 \times 10^{-7.4}$	+15
K^+-Glutamate solutions			
K^+	12.7	110	−55
Glutamate$^-$	10	100	+59
Mg^{2+}	2	2	0
Cl^-	6	4	−10
Ca^{2+}	1	2×10^{-9}	≫ + 100
H^+	$1 \times 10^{-7.5}$	$1 \times 10^{-7.5}$	0

K^+ Glutamate Solutions

In these experiments, membrane potential was held at −60 mV, *i.e.* close to the reversal potential for K^+, except when voltage protocols were administered. As in the nmg-glutamate experiments, current-voltage relationships were determined before and after administration of FC (10 μM from a 10-mM stock solution). Two different voltage protocols were used. The first protocol consisted of stepping the membrane potential in 20-mV increments from −100 to +60 mV, with each step lasting 100 ms. Subsequent analysis consisted of determining the magnitude of membrane current (average of 20 data points) prevailing 10 to 12 ms into the voltage step; during this time interval, changes in baseline current could be measured with minimal interference from slowly activating K^+ currents. By determining this I/V relationship before and after FC exposure, it was possible to ascertain the voltage-dependence of the FC-stimulated current, just as was done for the nmg-glutamate solutions where K^+ was absent.

The second protocol, also administered before and after FC exposure, consisted of stepping the membrane potential from −160 to +60 mV in 20-mV increments, with each step lasting 2000 ms. These voltage steps were of sufficient duration that voltage-regulated K^+ currents were fully activated. In subsequent analysis, any instantaneously or continuously activated (*e.g.* FC-stimulated) current was subtracted from each data trace. The I/V relationship of the remaining (time-activated K^+) current was then determined as the average of the last 20 data points at each voltage, and plotted as a function of membrane potential. By subtracting the after FC I/V curve from the before FC I/V curve, a plot was obtained of the effect of FC on K^+ current at each potential.

RESULTS

Epidermal Peel Experiments

In 1 mM KCl, low concentrations of FC combined with exposure to white light synergistically stimulated stomatal opening (Figs. 1A and 2). With increasing FC concentrations, stomatal opening occurred even in darkness and the synergistic effect diminished (Fig. 1A). The synergistic effect of light and 0.1 μM FC also diminished as KCl concentrations were increased (Fig. 1B).

Stomata that were either opened by light on the intact leaf (Fig. 2) or opened in isolated peels by light and 0.1 μM FC (data not shown) could close to a significant extent upon imposition of darkness, indicating that 0.1 μM FC did not prevent ion efflux, and then reopen upon exposure to white

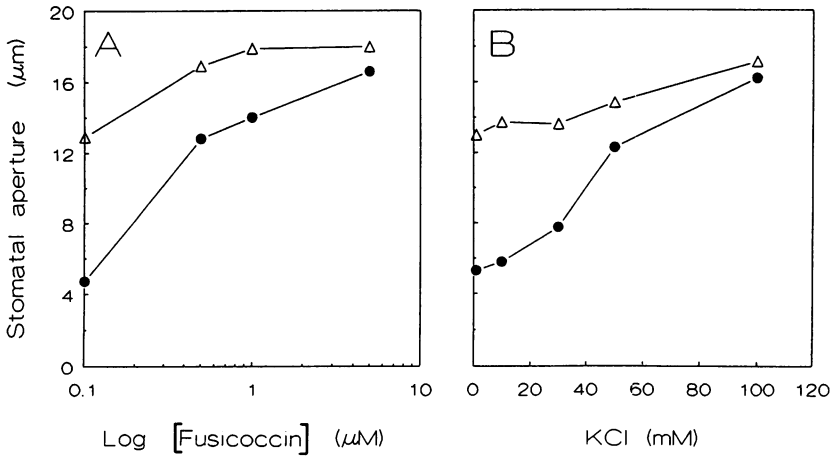

Figure 1. Stomatal opening in FC or KCl. A, The effect of different FC concentrations in the presence of 1 mM KCl on stomatal opening in *C. communis* in light (△) or darkness (●). B, The effect of different KCl concentrations in the presence of 0.1 μM FC on stomatal opening in light (△) or darkness (●). White light intensity was 0.120 mmol m^{-2} s^{-1}.

light (Fig. 2). These results indicate that the stomata remained functional after treatment with this low FC concentration. In the absence of FC, stomata in 1 mM KCl closed in darkness and remained closed even in the light (Fig. 2). The K^+ channel blocker, TEA, was equally effective in inhibiting stomatal opening stimulated by either 0.1 μM FC + 1 mM KCl, or 120 mM KCl (Fig. 3).

In the absence of FC, stomatal closure was only slightly accelerated by the H^+ ATPase inhibitor, vanadate (Fig. 4A; see also ref. 22), suggesting that physiologically relevant closing stimuli had already minimized pump activity. In contrast, 50 mM TEACl significantly slowed stomatal closure (Fig. 4A). Although 1 μM FC prevented stomatal closure (Fig. 4B), this effect could be completely eliminated by inclusion of 1 mM vanadate in the incubation solution (Fig. 4B), despite little direct effect of vanadate on stomatal closure (Fig. 4A). Vanadate would presumably have had no effect on the TEA response, but this could not be confirmed because vanadate fails to penetrate guard cells in the presence of high anion concentrations (22).

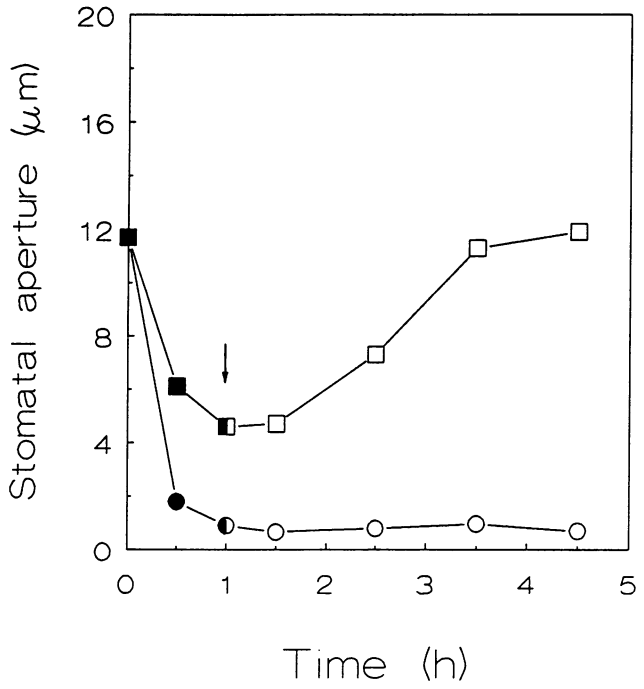

Figure 2. The effect of darkness and the transition to white light (0.120 mmol m^{-2} s^{-1}) on stomatal apertures of *C. communis* in 1 mM KCl (●,○) or in 1 mM KCl plus 0.1 μM FC (■,□). The time of transition from darkness (closed symbols) to light (open symbols) is indicated by the arrow.

Patch Clamp Experiments

Guard cell protoplasts patch clamped in 50 mM nmg-glutamate solutions showed clear activation of an outward current by addition of 1.0 μM FC (final concentration) to the bath solution (Fig. 5A). The current was essentially voltage independent over the range from −100 to +60 mV (Fig. 5B).

FC-stimulated current could also be observed when guard

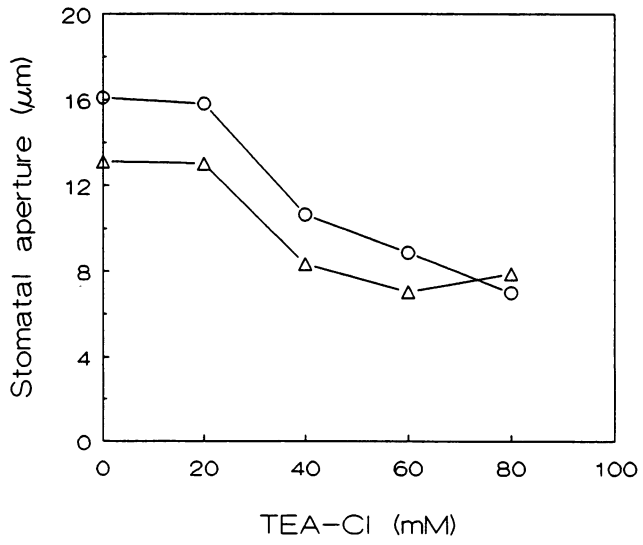

Figure 3. TEA inhibition of stomatal opening in *C. communis* in white light (0.120 mmol m^{-2} s^{-1}), and 1 mM KCl + 0.1 μM FC (△) or 120 mM KCl (○).

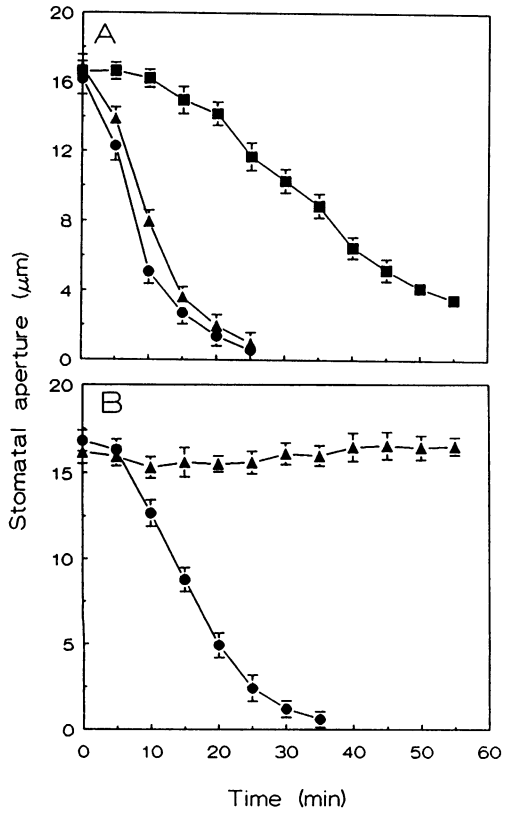

Figure 4. The rate of dark-induced stomatal closure in *C. communis*. A, 1 mM KCl (▲); 1 mM KCl + 1 mM vanadate (●); 1 mM KCl + 50 mM TEA (■). B, 1 μM FC (▲); 1 μM FC + 1 mM vanadate (●).

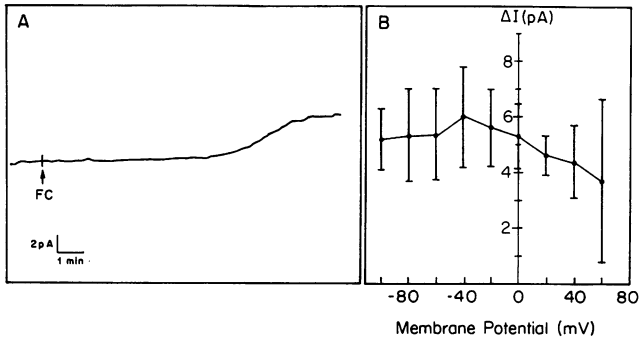

Figure 5. A, Addition of 1 μM FC to the bath solution (arrow) stimulates the appearance of a slowly developing outward current from a guard cell protoplast of *V. faba* patch clamped in the whole-cell configuration (V_H = 0 mV) in nmg-glutamate solutions. Current responses to the rapid voltage steps used to obtain data points for the results depicted in panel B are masked from the trace. B, Voltage independence of the current stimulated by 1 μM FC. Results are means ± 1 SE from cells 1 to 4 of Table II. See "Materials and Methods" for detailed descriptions of solutions and voltage stimuli.

cell protoplasts were patch clamped in K^+ glutamate solutions (Fig. 6). In these experiments, membrane potential was held at −60 mV, close to the equilibrium potential for K^+. Ten micromolar FC was employed in these experiments, which resulted in a larger, more rapidly activating current response than did 1 μM FC (Table II). Neither 1.0 nor 10.0 μM FC caused significant changes in seal resistance (Table II). Despite variability that resulted from cell-to-cell variation in the magnitude of the FC effect (Table II), the FC-stimulated current again showed no voltage dependence over the voltage range assayed (Fig. 6B).

K^+ current at different voltages was also monitored before and after exposure of protoplasts to 10 μM FC (Fig. 7, A–D). Currents were identified as K^+ currents by their dependence on K^+ (data not shown), their activation kinetics, and their voltage dependence (*cf.* 21). Small changes in K^+ current observed in the presence of FC (Fig. 8) do not appear significant, given that K^+ current magnitude at large negative voltage could be greater than −200 pA.

DISCUSSION

The results presented here demonstrate a synergistic effect of light and low FC concentrations on stomatal opening. Higher concentrations of FC, as have been used in many studies, cause stomatal opening even in darkness, thus obscuring the synergism (Fig. 1A). The synergistic effects of light and FC also diminish as external KCl concentrations are increased (Fig. 1B). This result would be expected if the primary effect of FC on ion fluxes was to create an electrical gradient for K^+ influx by activation of the H^+ pump; as external K^+ concentrations are increased, the electrochemical gradient for K^+ is shifted in the direction of passive influx, regardless of pump activity.

Patch clamp results are also consistent with FC activation of a H^+ ATPase. Similar to results reported previously (23),

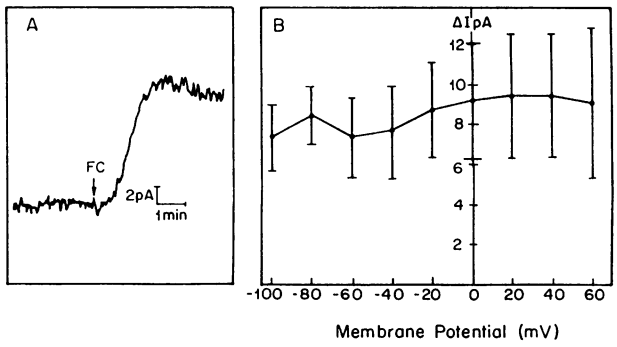

Figure 6. A, Addition of 10 μM FC to the bath solution (arrow) stimulates an 8 to 10 pA outward current from a guard cell protoplast of *V. faba* patch clamped in the whole-cell configuration (V_H = −60 mV) in K^+-glutamate solutions. Current responses to the rapid voltage steps used to obtain data points for the results depicted in panel B are masked from the trace. B, Voltage independence of the current stimulated by 10 μM FC. Results are means ± 1 SE from cells 5 to 7 of Table II. See "Materials and Methods" for detailed descriptions of solutions and voltage stimuli.

Table II. *Characteristics of the FC-Stimulated Current in Guard Cell Protoplasts of Vicia faba*

nmg-glutamate solutions were used in the 1 μM FC experiments and K^+ glutamate solutions were used in the 10 μM FC experiments. Membrane potential was held (V_H) at 0 mV ($E_{nmg\text{-}glutamate}$) for the 1 μM FC experiments and −60 mV (the equilibrium potential for K^+) for the 10 μM FC experiments.

Cell No.	Seal Resistance		Time to Response Initiation[a]	Additional Time to Maximum Response	FC-Stimulated Current at V_H
	Before FC	At maximum FC response			
	GΩ		min		pA
1 μM FC					
1	5.5	4.1	10.3	7.3	7.7
2	2.0	2.1	7.1	3.3	3.7
3	3.2	3.1	9.7	8.2	6.9
4	5.5	5.9	7.0	3.0	3.0
10 μM FC					
5	2.6	2.7	5.0	3.3	3.7
6	6.8	8.2	0.6	2.7	13.5
7	3.6	4.2	5.4	3.3	10.4
8	1.6	1.6	0.3	1.3	6.0
9	4.1	3.2	1.7	11.2	10.5
10	2.9	2.4	4.9	11.5	17.5

[a] Initiation time was defined as the time at which a unidirectional increase in outward current of 0.5 pA was observed. The time to initiation subsumes the time required for diffusion of FC to the cell and any lag in the cellular response.

FC (1 or 10 μM) activated a gradually developing outward current (Figs. 5A and 6A). This current could be observed when the membrane potential was held at −60 mV, close to the equilibrium potential for K^+ and a voltage at which K^+ channels are inactivated (21), and therefore could not be attributed to altered flux through K^+ channels. The dependence in guard cell protoplasts of *V. faba* of the FC-activated current on ATP, its dissipation by the protonophore, carbonyl cyanide,*m*-chlorophenyl hydrazone, and its inhibition by vanadate (23) are all arguments in favor of this current resulting from H^+ ATPase activation. The magnitude of this current on a surface area basis is comparable to that observed for ion carriers in animal systems (17). We also demonstrate here (Figs. 5B and 6B) that the FC-stimulated current was essentially voltage independent over the range from −100 to +60 mV, as has also been observed for the pump current of intact guard cells of *V. faba,* in experiments in which pump current was calculated as the difference in current before and after administration of sodium cyanide plus salicylhydroxamic acid (2, 3). This voltage independence contrasts sharply with the voltage dependence of K^+ and anion channels over the same voltage range (11, 20, 21). Channels selectively permeable to Mg^{2+}, Ca^{2+}, and H^+ have not been reported in guard cells, but in other systems these channels also show significant regulation by voltage (6, 9, 18). For these reasons, in concert with the wealth of other data showing that FC stimulates net H^+ extrusion from guard cells (19, 24), it is most reasonable to conclude that the observed increase in outward current results from FC activation of a H^+ ATPase at the guard-cell plasma membrane.

In previous studies, FC enhanced K^+ ($^{86}Rb^+$) influx by a mechanism that was only weakly inhibited by 10 mM TEA, as might be expected for a nonchannel pathway of K^+ uptake (7). However, we observed that TEA was equally effective in inhibiting stomatal opening, regardless of whether opening was induced by high KCl concentrations (where K^+ uptake via ion channels would be expected) or by 0.1 μM FC and white light, but that TEA concentrations greater than 20 mM had to be used to achieve an effect in either case. Our results suggest that in both cases, K^+ was entering via ion channels that were weakly blocked by TEA. Previous observations (22) that 1-mM concentrations of the H^+ ATPase inhibitor vanadate can eliminate stomatal opening stimulated by 0.1 μM FC and light also provide no evidence for FC-activated K^+ uptake.

Blatt and Clint (4) reported that 10 μM FC inactivated outwardly rectifying K^+ channels, thereby reducing K^+ efflux from guard cells, and presumably inhibiting stomatal closure. In 10 μM FC plus 1 mM of the H^+ ATPase inhibitor vanadate, dark-induced stomata closure was incomplete; however, these conditions also promote partial opening of closed stomata (data not shown), so that these results cannot be taken as evidence for a specific FC effect on channels mediating K^+ efflux and stomatal closure. Closure inhibited by 1 μM FC (Fig. 4B) could be competely restored by addition of 1 mM of the H^+ ATPase inhibitor vanadate. The closing observed in the presence of 1 μM FC plus vanadate contrasts sharply with the inhibition of closure caused by the K^+ channel blocker TEA (Fig. 4), suggesting that FC does not act as a significant blocker or inactivator of outward K^+ channels. An independent, nonspecific effect of vanadate that promotes stomatal closure is not likely because general inhibitors of cell metabolism have consistently been observed to have the reverse effect, *i.e.* closure is inhibited (10, 29). The most straightforward interpretation of these results is that in the presence of

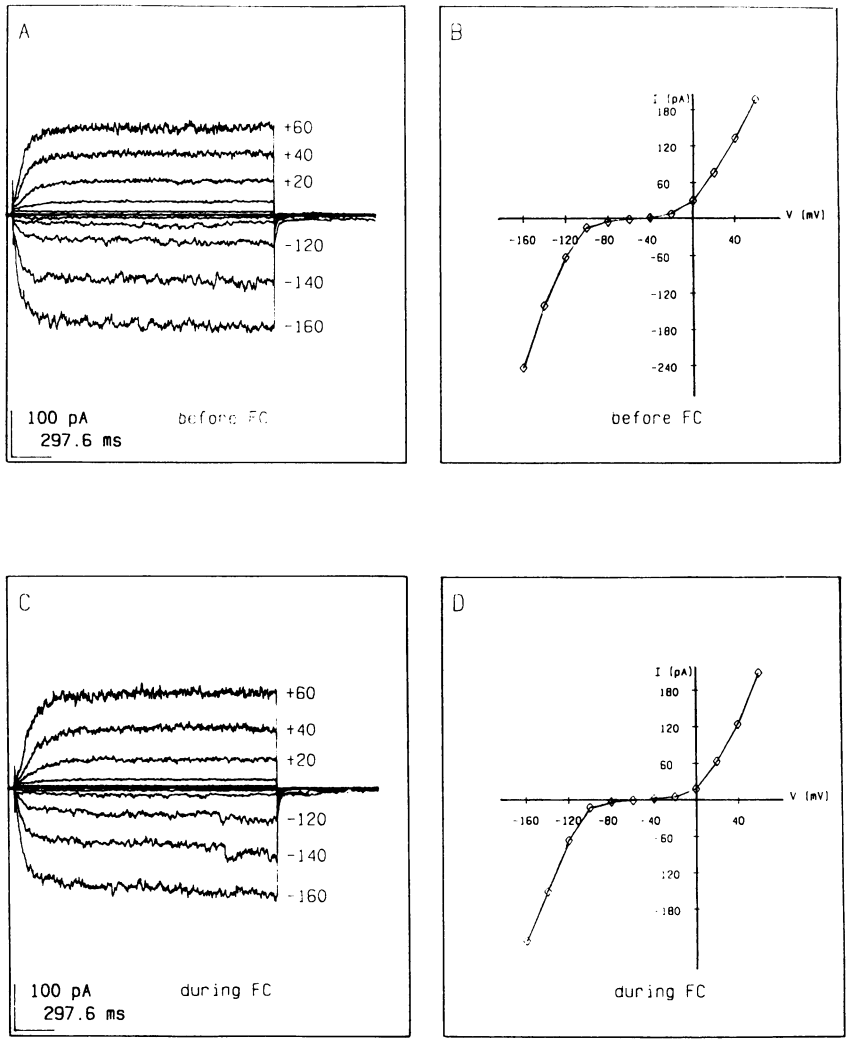

Figure 7. Absence of an effect of 10 μM FC on magnitude (A, C) and voltage-dependence (B, D) of K^+ current from the same guard cell protoplast of *V. faba* as in Figure 6A. Membrane potential was held at −60 mV and stepped for 2-s durations to the voltages indicated to the right of the current responses. Voltage stimuli were given before FC addition (A, B) and after the FC response of baseline current (Fig. 6) had plateaued (C, D).

1 or 10 μM FC, the H^+ ATPase is so strongly activated that an electrochemical gradient remains against passive K^+ efflux even when closing stimuli are imposed. Stomatal opening synergistically stimulated by 0.1 μM FC and white light could be reversibly decreased by darkness (Fig. 2), clearly indicating that FC at this concentration did not function by blocking K^+ efflux channels.

In patch clamp experiments as well, no consistent effects of FC on K^+ current activation or steady-state I_{K^+}/V relationships were noted, even when employing 10 μM FC (Figs. 7 and 8). Patch clamp measurements of K^+ currents were made when FC stimulation of pump current was already maximal (typically 5–9 min after FC [10 μM] administration, see Table II), indicating that FC had penetrated the protoplasts and activated a physiological response. The half-time for the reported FC effect on outward K^+ channels was 8 to 9 min, with 84% inhibition observed after 20 min (4). In three additional cells tested (Table II, cells 8–10), outward K^+ current at 20 min post-FC administration had increased in one cell by 19% and decreased in two other cells by 23 and 32%. The latter two cells also showed decreases in inward K^+ current, suggesting that a nonspecific "washout" of currents was occurring (14). These variable results fail to confirm significant inhibition of outward K^+ current by FC.

The slowly developing and irreversible nature of the reported FC effect on K^+ channels (4) may indicate not a specific effect of FC on K^+ channels, but perhaps an effect that is mediated by a FC-induced alteration of the general metabolic state or energy status of the guard cell. The effects of FC on carrier-mediated K^+ uptake and K^+ channels were primarily obtained from leaves near the end of their growing season, in which little primary pump activity could be observed (4, 7); an alternative explanation is that regulatory mechanisms for K^+ uptake and release in guard cells change with plant age.

In conclusion, results from both epidermal peel and patch clamp experiments indicate that the synergistic effect of light and low FC concentrations on stomatal apertures does not involve FC regulation of K^+ channels. One alternative is that FC and light each activate the H^+ pump via a different pathway, and the combined effects are synergistic. A second possibility is that FC stimulation of $2H^+ + malate^{2-}$ formation relieves a substrate limitation of pump activity (5); increased malate formation occurs in the presence of as little as 0.05 μM FC (5, 27). Further *in vivo* and *in vitro* experiments will be required to test such possibilities.

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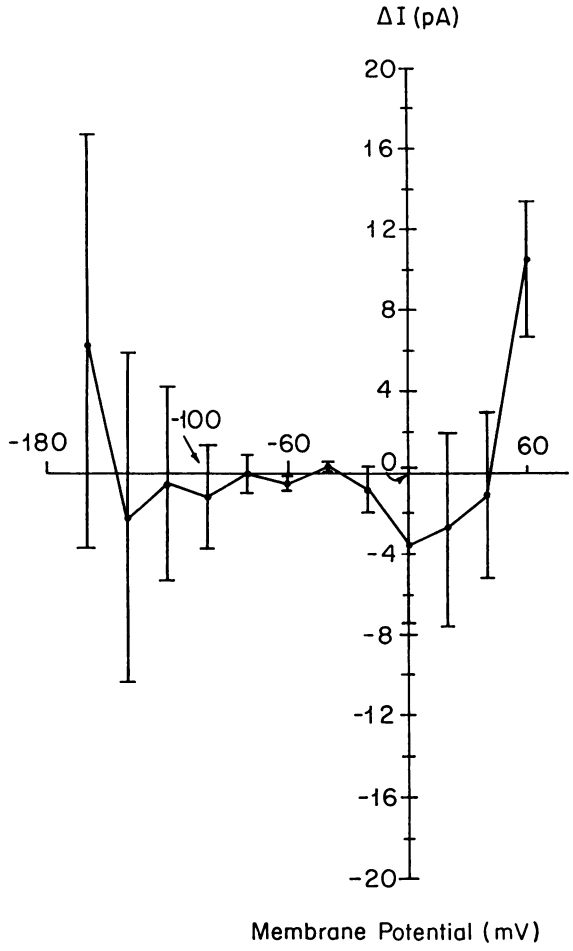

Figure 8. Absence of an effect of 10 μM FC on the magnitude of steady-state K^+ current in the same guard cell protoplasts of *V. faba* as in Figure 6B. At each voltage, steady-state current before FC addition was subtracted from the steady-state current prevailing after FC addition (*e.g.* Fig. 7D). Results are averages ± 1 SE from cells 5 to 7 of Table II.

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