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Diacylglycerols induce both ion pumping in patch-clamped guard-cell protoplasts and opening of intact stomata

(active transport/blue light/second messengers/signal transduction)

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ABSTRACT **Stomatal guard cells in leaves regulate the apertures of microscopic pores through which photosynthetic gas exchange and water vapor loss occur. Environmental signals, including light, high humidity, and low CO_2 concentrations, open stomata by increasing the volume of guard cells. Activation of a plasma membrane H^+ pump initiates K^+ and Cl^- influx, accompanied by malate synthesis, resulting in osmotic water flow into the guard cells, a bowing apart of the guard-cell pair, and consequent stomatal opening. Physiological and electrophysiological techniques were employed to investigate the possibility that a second-messenger lipid, 1,2-diacylglycerol, is involved in the transduction of opening stimuli. The synthetic diacylglycerols 1,2-dihexanoylglycerol and 1,2-dioctanoylglycerol enhanced light-induced stomatal opening in *Commelina communis* and induced stomatal opening under darkness, whereas an isomer with no known second-messenger role, 1,3-dioctanoylglycerol, did not affect stomatal responses. 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7), an inhibitor of protein kinase C, the enzyme typically activated by 1,2-diacylglycerol in animal cells, inhibited light-stimulated stomatal opening and enhanced dark-induced stomatal closure. *N*-[(2-Methylamino)ethyl]-5-isoquinolinesulfonamide (H-8), which inhibits cyclic nucleotide-dependent protein kinases preferentially over lipid-dependent protein kinases such as protein kinase C, had little effect on stomatal apertures. Whole-cell patch clamping of guard-cell protoplasts of *Vicia faba* revealed that 1,2-dihexanoylglycerol and 1-oleoyl-2-acetylglycerol activated an ATP-dependent, voltage-independent current, suggesting activation of an electrogenic ion pump such as the H^+ pump. Diacylglycerol or functionally similar lipids may act through protein phosphorylation to provide the intracellular signals that mediate H^+-ATPase activation and stomatal opening in response to light or other opening stimuli.**

Stomatal responses to light have received detailed study. Patch clamping of guard-cell protoplasts has revealed that red light, acting through guard-cell chlorophyll, and blue light, acting through both chlorophyll and an unidentified blue light receptor, stimulate ATP-dependent outward currents (1, 2). Light also stimulates H^+ secretion from these cells (3). These data indicate that light activates a plasma membrane H^+-ATPase which establishes a negative membrane potential that opens voltage-regulated K^+ channels and drives K^+ uptake through these channels (4). Calculated energy requirements for blue light-stimulated H^+ excretion (5) suggest that neither blue light-driven photophosphorylation nor direct energization by blue light of a membrane process leading to H^+ secretion would be sufficient to account for the extent of H^+ pumping observed. In addition, blue light activation of outward current begins only after a 25- to 35-sec delay (2). These observations suggest that light and other stimuli are signals which are processed intracellularly into H^+-pump activation.

In the present research, we have investigated a potential role of a second-messenger lipid, diacylglycerol, in mediating stomatal opening. Diacylglycerol is produced endogenously via several biochemical pathways, including hydrolysis of the phosphatidylinositols and phosphatidylcholines (6). In animal systems, diacylglycerol activates a Ca^{2+}- and phospholipid-dependent kinase, protein kinase C (PKC).

Evidence is accumulating for an involvement of lipids in signal transduction in plants (7). Osmotic shock (8, 9) accelerates phospholipid turnover. In *Samanea* pulvinar motor cells, which show light-sensitive volume changes similar to those of guard cells, light increases turnover rates of phospholipids (10). To examine whether diacylglycerol is involved in the regulation of volume change in guard cells, we studied the effects of synthetic diacylglycerols and inhibitors of protein kinases on stomatal movements and guard-cell ion fluxes.

MATERIALS AND METHODS

Plant Material. Plants of *Commelina communis* and *Vicia faba* were grown from seeds in controlled environmental chambers. *C. communis* was raised with 16 hr of white light (0.4 mmol·m^{-2}·sec^{-1}) per day at a constant temperature of 25°C. *V. faba* was raised with a 10-hr photoperiod of white light (0.15 mmol·m^{-2}·sec^{-1}) and day/night temperatures of 19°C and 17°C, respectively.

Chemicals. 1,2-Dihexanoylglycerol (1,2-DG6), 1,2-dioctanoylglycerol (1,2-DG8) and 1-oleoyl-2-acetylglycerol (OAG) are synthetic diacylglycerols that activate PKC (11, 12); 1,3-dioctanoylglycerol (1,3-DG8), however, is an isomer that does not significantly activate PKC (13), and has no known second-messenger role (12). 1-(5-Isoquinolinylsulfonyl)-2-methylpiperazine (H-7) preferentially inhibits PKC and *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide (H-8) preferentially inhibits cyclic nucleotide-dependent protein kinases (14). 1,2-DG8, 1,2-DG6, 1,3-DG8, OAG, and H-7 were purchased from Sigma. H-8 was purchased from Seikagaku America (St. Petersburg, FL). Stock solutions of synthetic diacylglycerols were dissolved in ethanol, whereas H-7 and H-8 were dissolved in dimethyl sulfoxide.

Stomatal Opening and Closure Experiments. The youngest fully expanded leaves from ≈1-month-old plants of *C. communis* were used. Opening experiments were initiated with closed stomata from leaves kept in darkness, and closure experiments with open stomata from leaves kept in white light (1.0 mmol·m^{-2}·sec^{-1}) for 2 hr. The abaxial epidermis

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Abbreviations: DG6, dihexanoylglycerol; DG8, dioctanoylglycerol; InsP_3, inositol 1,4,5-trisphosphate; OAG, 1-oleoyl-2-acetylglycerol; PKC, protein kinase C; V_m, membrane potential.

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was peeled and floated on solutions containing 45 mM KCl, 10 mM Mes, and 5 mM KOH (pH 6.1) or, in addition, solvent or test chemical dissolved in solvent. Final concentrations of ethanol and dimethyl sulfoxide did not exceed 0.4% and 0.6%, respectively. For closure experiments, 0.1 mM $CaCl_2$ was added to the medium to accelerate closure (15). Synthetic diacylglycerols and ethanol were added every 30 min, since diacylglycerol is metabolized within 30 min (16, 17) by some animal cells. Peels for opening experiments were kept for 2 hr either under white light (0.3 or 0.5 mmol·m^{-2}·sec^{-1}) or in darkness. Peels for closure experiments were kept in darkness for 0.5 hr. Following incubation, 40 stomatal apertures per treatment were measured with an ocular micrometer. At least three separate experiments were performed for each treatment, and the average and twice the standard error of the combined measurements are illustrated.

Patch-Clamp Experiments. Guard-cell protoplasts were isolated from the youngest expanded leaves of 2- to 4-week-old plants of *V. faba* according to established procedures (3, 18). Patch electrodes were pulled from Kimax-51 glass capillaries and filled with a solution consisting of 50 mM *N*-methylglucamine glutamate, 10 mM Hepes, 3 mM NaOH, 5 mM $MgCl_2$, 100 μM EGTA, 3 mM Na_2ATP (pH 7.4) and mannitol to raise osmolarity to 500 mosmol·kg^{-1}. Protoplasts were placed in a bath solution consisting of 50 mM *N*-methylglucamine glutamate, 10 mM Hepes, 3 mM NaOH, 5 mM $MgCl_2$ (pH 7.15), and D-mannitol to raise osmolarity to 600 mosmol·kg^{-1}. Ca^{2+} contribution from the solution in which the protoplasts were stored resulted in a final Ca^{2+} concentration of 10 μM in the bath. Patch clamping was performed under red light (0.05 mmol·m^{-2}·sec^{-1}) obtained by placing Roscolux 19 and Cinemoid no. 5A filters in the microscope light path (12-V, 60-W bulb; Rainin, Woburn, MA). Phosphate was not included in the pipette solution, thus minimizing ionic currents stimulated by red light (1). When the resistance of the seal between the electrode and the cell membrane was >1 GΩ and capacitance of the cell was >4 pF (whole-cell mode), the voltage across the cell membrane (V_m) was clamped to 0 mV, and whole-cell current was filtered at 2 kHz and recorded using a Hitachi 561 chart recorder. Only positive (outward) currents were observed under these conditions. Test chemicals were added to the medium bathing protoplasts, with final concentrations reported. Blue light (0.05 mmol·m^{-2}·sec^{-1}) was obtained by filtering light from a fiber-optic light source (model QHI-85, EKE bulb; Ca-Pra, Watertown, MA) through Rohm and Haas 2424 blue Plexiglas (maximum transmittance at 470 nm; half-bandwidth, 100 nm). Experiments were performed at room temperature (21–23°C).

RESULTS

Stomatal Opening Regulated by Synthetic Diacylglycerols. Initial apertures and the extent of opening or closure of stomata varied somewhat, possibly because of variation in seeds or slight seasonal changes in growing conditions. 1,2-DG6 and 1,2-DG8 enhanced stomatal opening under white light and inhibited stomatal closure induced by darkness (Fig. 1). 1,2-DG6 was an effective substitute for light, with 25 μM sufficient to stimulate stomatal opening under darkness (Fig. 2). In a separate experiment, 1,2-DG8 (50 μM) also induced significant stomatal opening in darkness (data not shown). In contrast, 1,3-DG8 concentrations as high as 50 μM did not affect stomatal apertures (Fig. 2).

H-7 (50 μM) inhibited light-stimulated stomatal opening and promoted stomatal closure (200 μM) in darkness (Fig. 3). H-8 was a weak inhibitor of stomatal opening and had no effect on stomatal closure (Fig. 3).

Patch Clamping. 1,2-DG6 and OAG stimulated outward currents from guard-cell protoplasts of *V. faba* (Fig. 4). Because of possible alterations in membrane responses during protoplast isolation, only data from those protoplasts that later responded to blue pulse irradiation, which induces net outward current (2) from the same type of cells [consistent with previous electrophysiological responses of intact guard cells to light (19)], were used for illustrations and analyses. 1,2-DG6 (25 μM) and OAG (20 μM) increased outward current in 62% and 86%, respectively, of all cells that subsequently responded to blue light. The average current increase was 1.2 ± 0.33 pA (average ± standard error, n = 8) for 1,2-DG6, 2.1 ± 0.62 pA (n = 10) for OAG, and 2.2 ± 0.36 pA (n = 22) for blue light (30 sec, 0.05 mmol·m^{-2}·sec^{-1}). Currents as large as 3.0 pA, 6.5 pA, and 7.5 pA were stimulated by 1,2-DG6, OAG, and blue light, respectively. The average current induced by 20 μM OAG was comparable in magnitude to the average current at the peak of blue light response. These blue light-stimulated currents were, on average, somewhat smaller than those previously reported from the whole-cell configuration (2), and weak stomatal responses to blue light were also observed in the intact leaves of these low-light-grown plants (S.M.A., unpublished data). Ethanol, the solvent used to dissolve the synthetic diacylglycerols, occasionally increased the current in 4 out of 13

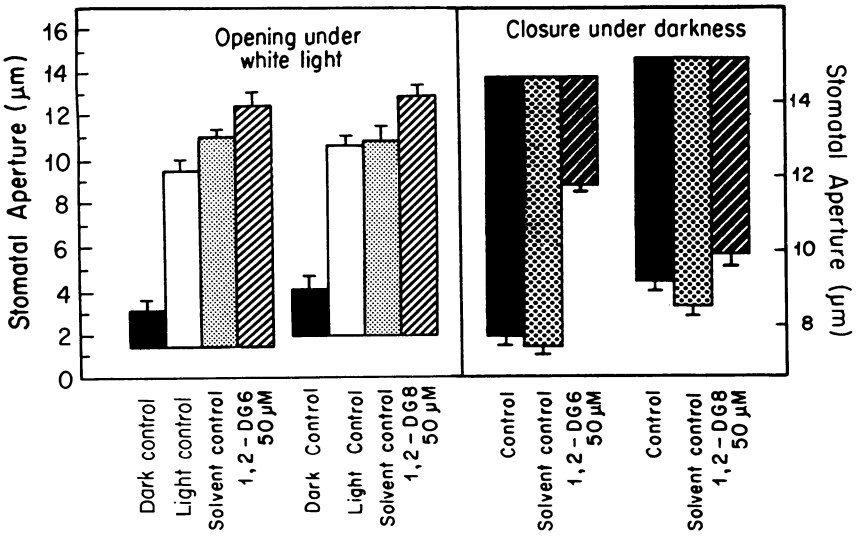

FIG. 1. 1,2-DG6 and 1,2-DG8 promote stomatal opening in *C. communis* under white light (0.5 mmol·m^{-2}·sec^{-1}) and inhibit closure under darkness. Bars start from initial apertures and end at final apertures above or below which bars indicating two standard errors are shown.

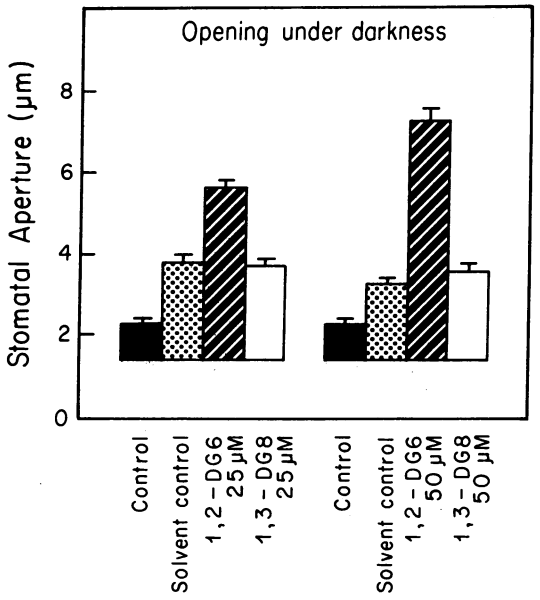

FIG. 2. 1,2-DG6 but not 1,3-DG8 promotes stomatal opening under darkness in *C. communis*. Other specifications are as in Fig. 1.

cells that responded to blue light, but the magnitude of this effect was small (0.7 ± 0.18 pA, $n = 4$). This effect and the slight stimulation of stomatal opening by ethanol (Figs. 1 and 2) may reflect ethanol activation of phospholipase C (20), which cleaves phospholipids, producing diacylglycerol.

The current elicited by 20 μM OAG (Fig. 5*A*) was constant at ≈2.8 pA for V_m ranging from −120 to +40 mV, and increased slightly only at the high V_m of +80 mV. A similar voltage independence of the OAG effect was observed in four other cells (Fig. 5*A* legend, data not shown). Substitution of MgATP for Na_2ATP did not alter the magnitudes of the currents elicited by 1,2-DG6 and OAG (Fig. 4). However, elimination of ATP from the patch-pipette solution prevented the diacylglycerol effect ($n = 5$, Fig. 5*B*).

DISCUSSION

Diacylglycerols Regulate Stomatal Apertures. The data reported here suggest a role for diacylglycerol in the regulation of volume changes in guard cells, with consequent effects on stomatal apertures. Synthetic 1,2-diacylglycerols promoted stomatal opening and inhibited stomatal closure, whereas 1,3-DG8, an isomer that does not have second-messenger activity in animal cells, had no such effects. Our data admit the possibility that diacylglycerol or functionally analogous lipids could mediate any or all of the stimuli that elicit stomatal opening. The effects of light and diacylglycerol were not simply additive (compare Figs. 1 and 2), as might be expected for two independent regulators of stomatal function. Instead, addition of diacylglycerol could substitute for light in promoting stomatal opening. Since both red and blue light activate electrogenic ion pumping in guard cells (1, 2), the possibility that diacylglycerol acts on ion transport mechanisms at the guard-cell plasma membrane was investigated.

Diacylglycerol-Stimulated Current Results from Ion Pumping. 1,2-DG6 and OAG induced outward currents from patch-clamped guard-cell protoplasts that were comparable in magnitude to the peak of blue light-induced current measured here. *In vivo*, diacylglycerol may produce a larger response since cytoplasmic components critical for maximal transport activity appear to be washed out or inactivated during equilibration of the cytoplasm with the pipette solution (21). Thus, blue light-stimulated currents from guard-cell protoplasts partially permeabilized in the "slow whole-cell" configuration were 5-fold greater than those obtained from permeabilized protoplasts in the full whole-cell configuration (4).

Three lines of investigation indicated that the diacylglycerol-stimulated outward current resulted from the activation of an electrogenic pump, as opposed to the opening of ion channels. First, with reversal potentials (E_j) for Ca^{2+}, H^+, Cl^-, Na^+, and Mg^{2+} of 59, 15, 0, −28, and 0 mV, respectively, a diacylglycerol-stimulated outward (positive) current when V_m was held at 0 mV could only passively be carried by Na^+. Na^+ channels have not been identified in guard cells, but Na^+ is permeant through K^+ channels, albeit at only one-eighth the rate of K^+ (4). We therefore substituted MgATP for Na_2ATP in the pipette solution; Mg^{2+} is not typically permeant through K^+ channels (22). This substitution shifted E_{Na^+} to 0 mV and $E_{Mg^{2+}}$ to 6 mV yet did not affect the magnitudes of the 1,2-DG6 or OAG effects (Fig. 4 and data not shown). These results indicate that the diacylglycerol-stimulated current cannot result from passive ion flux but, rather, requires the involvement of active transport.

Second, we constructed current–voltage curves of the effect of OAG. Over the range of voltage tested, H^+-pump

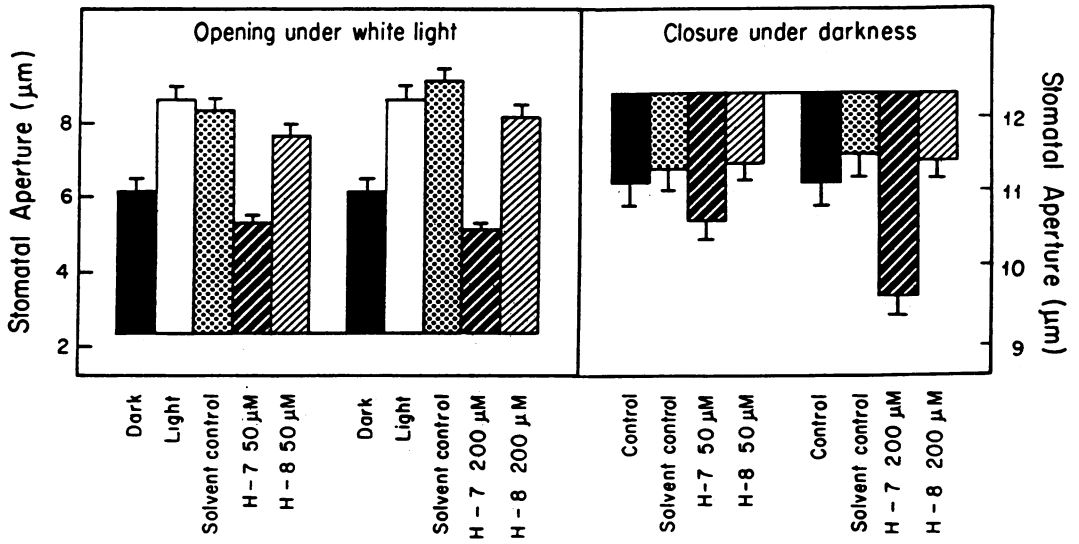

FIG. 3. H-7 inhibits stomatal opening in *C. communis* under white light (0.3 mmol·m^{-2}·sec^{-1}) and enhances closure under darkness. Other specifications are as in Fig. 1.

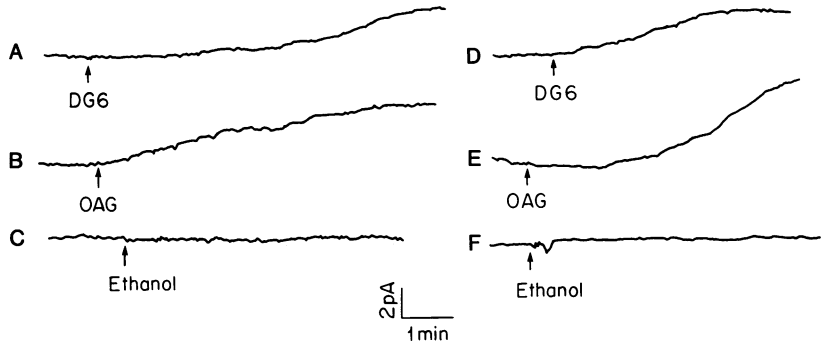

FIG. 4. 1,2-DG6 and OAG induce net outward currents from guard-cell protoplasts of *V. faba* patch-clamped in the whole-cell configuration. Traces A–C were obtained with 3 mM Na_2ATP, and traces D–F with 3 mM MgATP in the pipette solution. Ethanol occasionally induced current, but the magnitude was small (see text).

activity of intact guard cells of *V. faba* has been reported to be essentially independent of voltage (23). We observed a similar voltage independence of the lipid-stimulated current (Fig. 5*A*). This voltage independence contrasts with the clear voltage regulation of the two major channel types thus far reported in guard cells, K^+ channels, and anion channels, over this same voltage range (4, 24, 25).

Third, we found that the current could be evoked only when ATP was provided in the patch-pipette solution (Fig. 5*B*), consistent with the notion that ATP was utilized as an energy source for the observed ion transport activity. Additionally, the ATP requirement of the current development may result from activation by phosphorylation of the pump itself or of a regulatory protein.

These data point to a role of diacylglycerol as an activator of an electrogenic ion pump at the guard-cell plasma membrane, for which the most likely candidate is the H^+-ATPase, although a facultative H^+/K^+ transporter is not ruled out. These results are consistent with observed functions of diacylglycerol in animal cell types (26, 27), where diacylglycerol activates a plasma membrane Na^+/H^+ exchanger, with volume regulation as one proposed function (26).

Synthetic Diacylglycerols Inhibit Stomatal Closure. The synthetic diacylglycerols tested not only promoted stomatal opening but inhibited stomatal closure. These results are unlike those obtained with hypoxia and carbonylcyanide *m*-chlorophenylhydrazone, which inhibit both opening and closure (28), indicating that diacylglycerol does not affect stomatal apertures through nonspecific effects such as reduction of ATP levels or inhibition of cellular metabolism. However, physiological processes leading to stomatal closure are not simply the reverse of opening processes (15, 28), suggesting possible sites of diacylglycerol action [e.g., inhibition of K^+ efflux (29)] in addition to the stimulation of ion pumping reported here.

Diacylglycerol and Inositol 1,4,5-Trisphosphate ($InsP_3$). Phosphatidylinositol 4,5-bisphosphate, one source of diacylglycerol, also produces $InsP_3$ upon hydrolysis. Exogenous $InsP_3$ increases cytoplasmic Ca^{2+} in guard cells, and both $InsP_3$ and Ca^{2+} close stomata (30). However, $InsP_3$ does not affect the outwardly rectifying K^+ channel (31) that presumably plays a major role in stomatal closure (4); $InsP_3$ inhibits only the inwardly rectifying K^+ channel (31). Additional physiological studies linking second messengers to ion transport activities and assays of endogenous levels of $InsP_3$ and diacylglycerol are required to further define the roles of these second messengers in guard cells. Diacylglycerol can be formed from phosphatidylinositol 4-phosphate, phosphatidylinositol, or phosphatidylcholine without concomitant production of $InsP_3$, and temporal differences in levels of $InsP_3$ and diacylglycerol have been noted (6). Steady-state stomatal aperture, which reflects a balance between opening and closing processes (15, 28), may be determined by the relative quantities and actions of diacylglycerol and $InsP_3$ present under a given environmental condition.

Potential Pathways of Diacylglycerol Action. In animal systems, diacylglycerol commonly activates PKC, consistent with our observation that H-7, preferentially an inhibitor of C-type protein kinases, but not H-8, preferentially an inhibitor of cyclic nucleotide-dependent protein kinases (14), inhibits stomatal opening and enhances stomatal closure (Fig. 3). PKC, *per se*, has not been isolated from plant tissue to date; lipid-regulated kinases have been identified in plants but these have not been purified to homogeneity (32). Such enzymes may possess sufficient structural similarity to PKC that they are inhibited by H-7 but not H-8, accounting for our

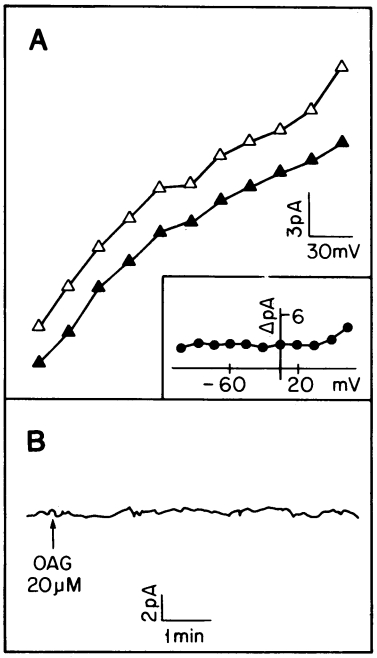

FIG. 5. (*A*) Voltage independence of the OAG effect in guard-cell protoplasts of *V. faba* patch-clamped in the whole-cell configuration. Membrane potential was stepped for 500 msec from 0 mV to voltages that ranged in 20-mV increments from −120 mV to +80 mV. Steady-state current was measured at each potential and was plotted as a function of the potential to which the membrane was clamped (△, 8 min after addition of 20 μM OAG; ▲, before addition of OAG). *Inset* illustrates the constant difference current stimulated by OAG (●). One response from two similar replicates is shown; three experiments utilizing a slightly different voltage protocol (0.2-mV steps in V_m, each of which lasted for 100 μsec) gave comparable results. (*B*) Absence of a response to OAG when the solution filling the patch pipette lacked ATP.

observations, or our data may reflect the presence of PKC in these specialized plant cells.

A second possibility is that it may not be diacylglycerol itself, but rather a metabolite of diacylglycerol, such as a free fatty acid (33), that actually plays a second-messenger role. A third possibility is that exogenous addition of diacylglycerol activates a kinase which *in vivo* is primarily activated by a different metabolite. Mammalian PKC is activated by diacylglycerol but can also be activated by oleoic acid and lysophosphatidylcholine (34). It may be relevant that plant cDNAs bearing sequence homology to animal protein-serine/threonine kinase genes encode proteins whose catalytic domains are similar to animal PKC but whose regulatory domains are divergent (35), suggesting that the lipid specificity of plant kinases differs from that of PKC. Lysophospholipids (33) are attractive candidates for endogenous regulators in guard cells, as these lipids have been shown to activate both ATP hydrolysis and H^+ extrusion in oat membrane vesicles (36, 37).

In pea epicotyls, blue light regulates phosphorylation of a 120-kDa plasma membrane protein (38). Similarly, a 120-kDa protein is phosphorylated in the presence of platelet-activating factor, a derivative of phosphatidylcholine that stimulates plasma membrane ATPase activity (39). The functional roles of the 120-kDa proteins remain to be elucidated, but one possibility is that their phosphorylation is a step on the pathway to H^+-ATPase activation. Our results, showing that both diacylglycerol and light elicit electrogenic pumping in guard cells, may indicate light-induced production of lipid second messengers and subsequent stimulation of a kinase responsible for H^+-ATPase activation.

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