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An anion channel in *Arabidopsis* hypocotyls activated by blue light

[depolarization/growth inhibition/5-nitro-2-(3-phenylpropylamino)-benzoic acid]

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ABSTRACT **A rapid, transient depolarization of the plasma membrane in seedling stems is one of the earliest effects of blue light detected in plants. It appears to play a role in transducing blue light into inhibition of hypocotyl (stem) elongation, and perhaps other responses. The possibility that activation of a Cl^- conductance is part of the depolarization mechanism was raised previously and addressed here. By patch clamping hypocotyl cells isolated from dark-grown (etiolated) *Arabidopsis* seedlings, blue light was found to activate an anion channel residing at the plasma membrane. An anion-channel blocker commonly known as NPPB [5-nitro-2-(3-phenylpropylamino)-benzoic acid] potently and reversibly blocked this anion channel. NPPB also blocked the blue-light-induced depolarization *in vivo* and decreased the inhibitory effect of blue light on hypocotyl elongation. These results indicate that activation of this anion channel plays a role in transducing blue light into growth inhibition.**

The variety and importance of blue-light effects on plant growth and development has made their study an exciting field for more than a century. Modern investigations of the early cellular and molecular effects of blue light have provided information about the signaling pathways that mediate some of the various responses (1). For example, blue light opens stomata after activating the H^+-ATPase of the guard cell plasma membrane (2). Also, rapid phosphorylation of a membrane protein by a pathway that includes the *NPH1* gene product is a very early step in the phototropic response of *Arabidopsis* seedlings to unilateral blue light (3). In a separate response of seedlings, high-irradiance blue light (BL) inhibits hypocotyl elongation as part of the de-etiolation process (4). The photoreceptor for this response is thought to be the flavoprotein encoded by the *HY4* gene of *Arabidopsis* (5, 6). Electrophysiological studies of the early stages of BL-induced growth inhibition have demonstrated that a transient depolarization of the plasma membrane occurs only a few seconds before rapid inhibition of hypocotyl growth begins (7). The ionic currents responsible for the depolarization apparently play a role in transducing BL into the change in growth rate (4, 7). Identification of the transporter(s) conducting the BL-induced currents would add molecular-level detail to our understanding of this transduction chain.

The resting potential of plant cells is generally maintained more negative than the equilibrium potentials of Ca^{2+} and Cl^-, and usually K^+ (8), by the electrogenic H^+-ATPase. Thus, BL could depolarize the membrane by activating channels selective for any or all of these ions. Previous work provided evidence against Ca^{2+} or K^+ being the carrier of the depolarizing current (9). Instead, activation of Cl^- channels and inhibition of the H^+-ATPase were proposed to be of primary importance.

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Channels that transport Cl^- in plants, commonly referred to as anion channels (10), are considered to play important roles in plant cell physiology (11, 12). In addition to being the depolarizing conductance driving plant action potentials (10, 13, 14), anion channels have been linked to auxin (15) and abscisic acid signaling pathways in guard cells (16, 17). Here we describe a role for anion channels in a BL signaling pathway leading to growth inhibition in etiolated *Arabidopsis* hypocotyls.

MATERIALS AND METHODS

Plant Growth. Seeds of the Landsberg erecta ecotype of *Arabidopsis thaliana* were sown on 25-mm diameter Petri plates containing 1% agar, 1 mM KCl, and 1 mM $CaCl_2$; placed in darkness at 4°C for 2 days; irradiated with red light (2 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for 0.5 h at room temperature; placed in loosely closed plastic containers kept humid by water on the bottom; and transferred to completely dark growth cabinets in a darkroom for 5 days. For the growth studies, either 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB, Calbiochem) or the maximum final concentration of solvent (0.016% dimethyl sulfoxide) was added to the agar. These seedlings were grown either in the dark for 5 days, or in the dark for 1 day and BL for 4 days. All manipulations were performed in the darkroom under green light (0.002 $\mu mol \cdot m^{-2} \cdot s^{-1}$) from by cool white fluorescent bulbs each wrapped with two layers of Roscolux #90 (Dark Yellow Green; Rosco, Port Chester, NY) and one layer of Roscolux #21 (Golden Amber) gel filter.

Protoplast Preparation. Protoplasts were prepared under normal laboratory light from 1-mm hypocotyl sections cut from a 5-mm region subtending the apical hook except for those used in the light-activation experiments in which case all steps were performed in the darkroom. Sections from 40–50 seedlings were infiltrated with 0.3 M sorbitol, 10 mM KCl, 1 mM $CaCl_2$, and 5 mM Mes (pH 5.2) with 1,3-bis[tris(hydroxymethyl)methylamino] propane (BTP) plus 2.5 $mg \cdot ml^{-1}$ Cellulysin (Calbiochem), 1 $mg \cdot ml^{-1}$ Pectinase (Sigma), and 2.5 $mg \cdot ml^{-1}$ BSA (Sigma) by applying vacuum three times for 20 s each. The tissue segments were incubated for 2 h and then washed twice with the above solution minus the enzymes. Protoplasts were released by resuspending the segments in the recording solution (see below).

Patch Clamping. Borosillicate glass capillaries (Sutter Instrument, Novato, CA) were pulled and fire polished with a horizontal puller (P87; Sutter Instruments). The resulting patch pipets were filled with 130 mM CsCl, 2 mM $MgCl_2$, 4 mM MgATP, 0.2 mM guanosine 5′-[γ-thio]triphosphate, 2 mM EGTA, 1.56 mM $CaCl_2$ (free Ca^{2+} = 1 μM), 4 mM Mes, and 10 mM Hepes (pH 7.2) with BTP. The 0.5 ml recording

Abbreviations: BL, high-irradiance blue light; V_m, membrane potential; NPPB, 5-nitro-2-(3- phenylpropylamino)-benzoic acid; BTP, 1,3-bis[tris(hydroxymethyl)methylamino] propane; V_s, surface potential; I_o, current through an open channel.

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chamber contained 30 mM CsCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, 50 mM sorbitol, and 10 mM Mes (pH 5.7) with BTP (all from Sigma) and could be perfused at a rate of several milliliters per min with a solution selected by a switchable manifold. Activity coefficients from Robinson and Stokes (18) were used to calculate equilibrium potentials. Junction potentials measured by the method described in (19) did not exceed 1.3 mV and were therefore ignored.

Single-channel currents were recorded using an Axopatch 200A patch-clamp amplifier plus headstage (Axon Instruments, Foster City, CA), a data recorder (VR-10B; Instrutech, Great Neck, NY), and a VCR. The data were replayed from the VCR tape, low-pass filtered at 500 Hz with a tunable 8-pole Bessel filter (902LPF; Frequency Devices, Haverhill, MA), and digitized at 1 kHz (except where stated otherwise) by a computer equipped with a DigiData 1200 A/D board (Axon Instruments), which also commanded the pipet voltage.

The preparation was lit by green light (0.3 $\mu mol \cdot m^{-2} \cdot s^{-1}$) produced by placing a filter made of three layers of green (Roscolux #90) and one layer of amber (Roscolux #21) gels in the path of the 50-W illuminator of the inverted microscope (Diaphot-TMD; Nikon). BL (80 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was delivered to a patch-clamped protoplast by placing one layer of Roscolux #85 (Deep Blue) in the path of the illuminator before removing the green filter. Red light (90 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was produced by placing four layers of Roscolux #26 (Light Red) in the light path. All irradiances were measured with an IL1700 radiometer equipped with an SED 033 quantum sensor (International Light, Newburyport, MA).

Surface Potential (V_s) Measurements. Changes in membrane potential of hypocotyl cells can be measured with agar surface-contact electrodes which report a voltage called surface potential, V_s (7). A glass capillary filled with 1 M KCl and tipped with a drop of 1% agar containing 1 mM KCl and 1 mM $CaCl_2$ contacted the apical 2 mm of an intact seedling growing on agar containing the same salts. A reference electrode contacted the agar substrate near the base of the seedling. The basal half of the hypocotyl was shaded with aluminum foil or black electrical tape. A 450-W xenon arc lamp, located outside of the darkroom, produced light that passed through an interference filter having peak transmittance at 450 nm and a bandwidth of 50 nm (model 71260; Oriel, Stratford, CT). This BL (85 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was delivered to the seedling by a liquid light guide which passed through the wall into the darkroom. A computer equipped with an A/D board and running a custom program administered the light treatment via a shutter and digitized V_s after it was low-pass filtered at 5 Hz. A detailed description and demonstration of this apparatus has been published (20).

To treat with NPPB, seedlings were inverted to submerge the apical half of the hypocotyl in different concentrations of it and 0.1% Tween 20 for 1 h. Controls were treated with the 0.016% dimethyl sulfoxide and Tween 20. After re-orientation and at least 2 h of recovery, electrodes were connected. V_s was allowed to stabilize for 5–10 min before a treatment began.

Growth Studies. After growth in BL for 4 days in the presence of NPPB, hypocotyl length was measured to the nearest 36 μm with a dissecting microscope fitted with a calibrated ocular micrometer. Dark-grown hypocotyls were measured to the nearest millimeter with a ruler. The BL used in these experiments was produced by two F20T12/BB bulbs (Phillips Lighting, Somerset, NJ). These specialty bulbs produced 65 $\mu mol \cdot m^{-2} \cdot s^{-1}$ of BL, 94% of which was between 400 and 500 nm as determined by a spectroradiometer (Li-Cor, Lincoln, NB).

RESULTS

BL-Induced Depolarization in *Arabidopsis* Seedlings. Membrane potential (V_m) was measured by impaling hypocotyls of etiolated *Arabidopsis* seedlings with an intracellular microelectrode. Although the data are not shown, BL induced a transient depolarization similar in kinetics and magnitude to that observed in the hypocotyls of etiolated cucumber (7), pea, zucchini, soybean, and radish seedlings (21).

Anion Channels at the Plasma Membrane. The patch-clamp technique was used to determine whether anion channels are present at the plasma membrane of *Arabidopsis* hypocotyl cells as a first test of their involvement in this BL response. Solutions based on CsCl were used to observe anion-channel currents without interference from the ubiquitous K^+ channels, which Cs^+ does not significantly permeate (22). Fig. 1*A* shows single-channel recordings from cell-free, outside–out patches of plasma membrane. When the voltage was clamped at values more negative than the equilibrium potential for Cl^- (E_{Cl}), channel openings of long duration resulting in inward currents were observed. The amplitude of the unitary currents (I_o) increased with increasingly negative voltage but the probability of channel opening did not. A plot of I_o versus V_m is shown in Fig. 1*B*. A linear fit of the data between −30 mV and +20 mV (solid line in Fig. 1*B*) intercepted the voltage axis within 1 mV of E_{Cl}, demonstrating that the currents in Fig. 1*A* were carried by Cl^- and not Cs^+. When other Cl^- gradients obtained, making E_{Cl} +21 mV or +10 mV, the observed reversal potentials were +23 mV and +4 mV, respectively (data not

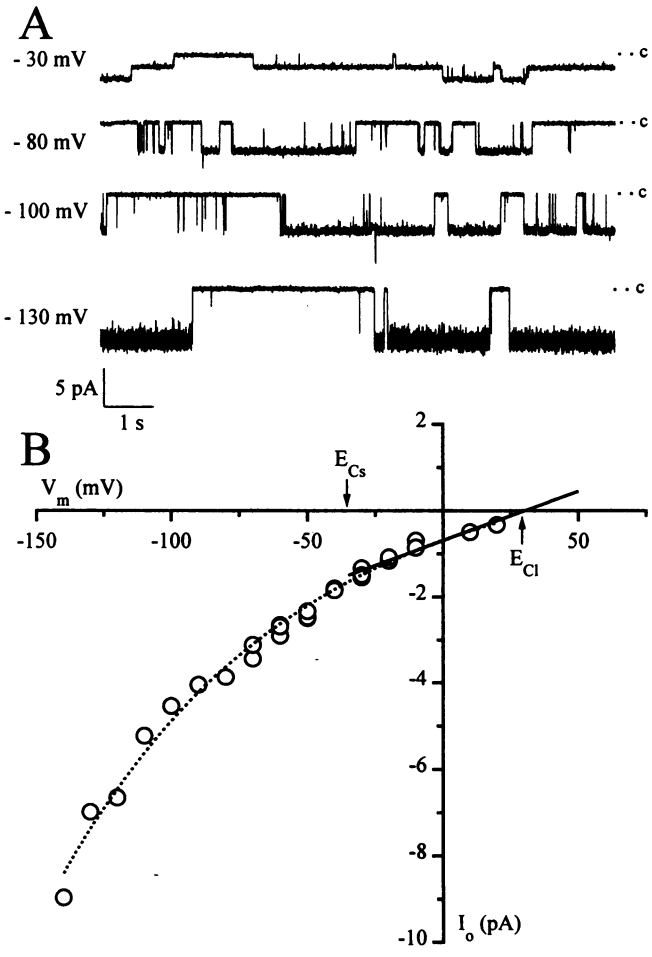

FIG. 1. Current-voltage relationship of an *Arabidopsis* anion channel. (*A*) Single-channel currents across outside-out patches of plasma membrane. V_m is shown at the left of each trace. The closed-state current is denoted by "c." (*B*) The dependence of the current through the open channel (I_o) on V_m. Data from three separate patches were fit with a sigmoidal curve (dashed line). The data between −30 mV and +20 mV were fit by linear regression. E_{Cl} and E_{Cs} are the equilibrium potentials for Cl^- and Cs^+, respectively.

shown). The unitary slope conductance at voltages near the reversal potential was 23 pS, and double that between −100 mV and −30 mV.

Anion Channel Activation by BL. The effect of BL on the activity of this channel was investigated by preparing and patch-clamping protoplasts under green safelight. Fig. 2*A* shows a recording of channel activity in a cell-attached patch clamped at a voltage 100 mV more negative than the (unknown) resting V_m of the protoplast. Channel activity was low during ≈5 min in darkness (not shown). An increase in activity developed following the onset of BL, beginning within a few seconds and reaching a point at which three channels were open simultaneously ≈2.5 min after the BL treatment ended. The BL-induced shift from occupancy of the closed state (c) to occupancy of the open state (o) is shown by all-points histograms (Fig. 2*B*) summarizing 1-min periods immediately before, immediately after, and 2 min after the onset of BL (data from Fig. 2*A*). An increase in open probability of this channel in response to BL was observed in each of the three independent cases when a cell-attached patch displaying anion-channel activity was irradiated. The response was specific for BL as red light did not increase the open probability of anion channels in two independent experiments, one of which is shown in Fig. 2*C*. The open probability of the channel in Fig. 2*C* was 0.33 during 100 s in darkness, not all of which is shown, and 0.25 during the ensuing 100 s of red light. Changes in open probability of this order are not unusual in patches that received no treatment, so the decrease following red light is probably not significant. Longer-term effects of red or blue light could not be assessed because of the difficulty in maintaining seals and channel activity in cell-attached patches for more than a few minutes.

Block of the Anion Channel and BL-Responses by NPPB. NPPB is a blocker of animal and plant anion channels (23, 24). Its effect on the anion channel of *Arabidopsis* hypocotyl cells is shown in the continuous recording of channel currents across an outside–out patch in Fig. 3. Channel activity was initially high, with two channels open simultaneously much of the time. Perfusing the recording chamber with 20 μM NPPB completely inhibited channel activity. The channels remained inhibited until

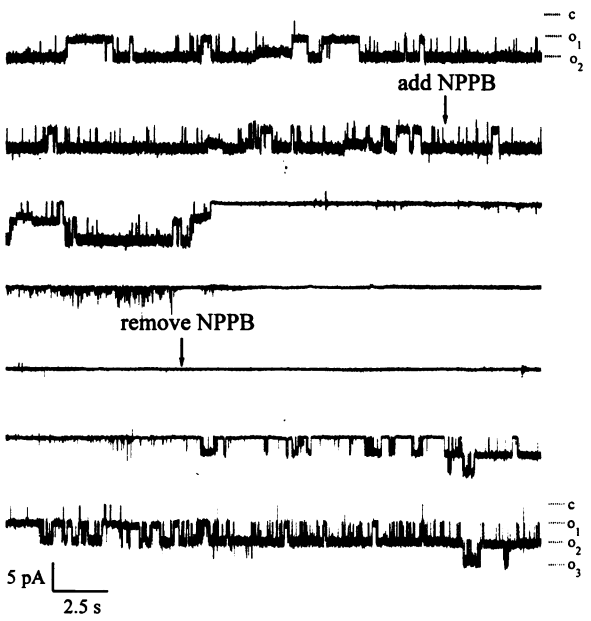

FIG. 3. Block of the anion channel by NPPB. Continuous recording of channel activity in an outside–out patch demonstrated that perfusion of the bath with 20 μM NPPB completely and reversibly inhibited the anion channel. V_m was −80 mV. The closed-state and open-state currents are denoted by "c" and "o," respectively.

the NPPB was washed out. Control experiments that are not shown demonstrated that the solvent (0.1% ethanol) had no effect on channel activity. This potent blocker of the anion channel also blocked the BL-induced depolarization in a concentration-dependent fashion (Fig. 4). Half-maximal inhibition was achieved with 4 μM NPPB. Evidence that NPPB did not have large secondary or nonspecific effects on cellular ionic relations was provided by microelectrode measurements of resting V_m in the hypocotyls of intact seedlings. The value recorded for seed-

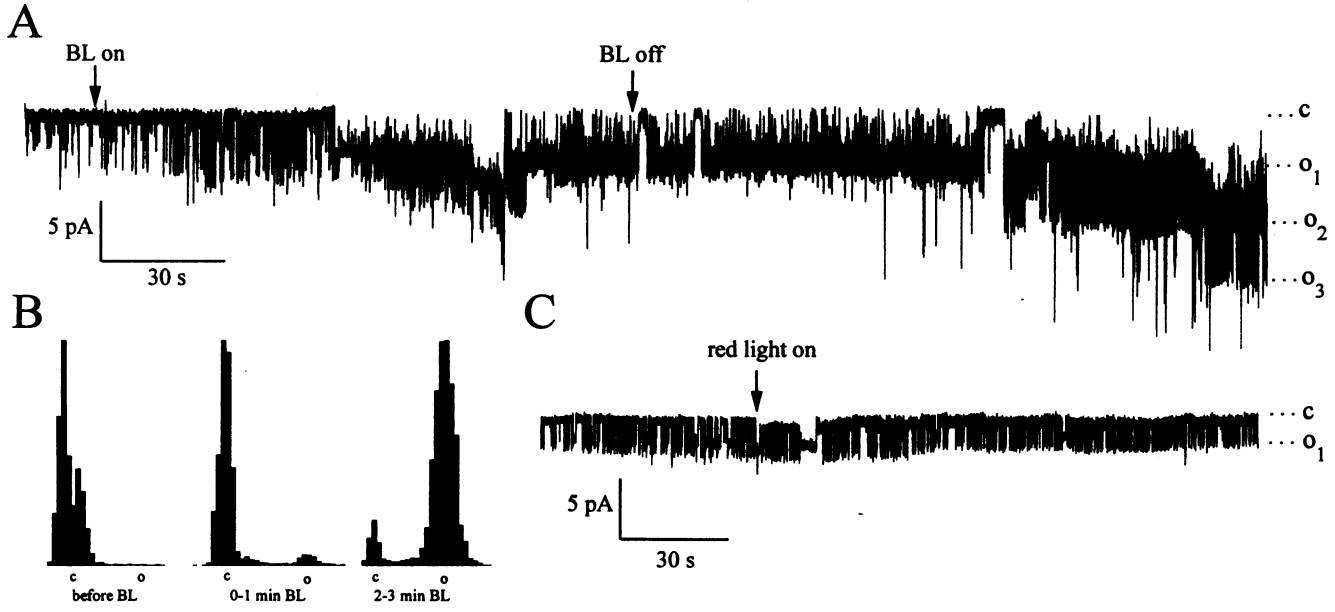

FIG. 2. Activation of anion channels by BL. (*A*) BL-activated individual anion channels in a cell-attached patch. The pipet potential was clamped at 100 mV to hyperpolarize the membrane beyond its resting voltage. (*B*) All-points frequency histograms display the shift of a channel from predominantly closed during the 1 min immediately before BL to predominantly open 2 min after the onset of BL. Each histogram contains 6145 points representing 1 min of recording. (*C*) Red light did not increase the activity of anion channels in a separate cell-attached patch that was hyperpolarized by 80 mV relative to the resting potential of the protoplast. The ionic conditions were as stated in the *Materials and Methods* except MgATP and guanosine 5′-[γ-thio]triphosphate were omitted from the pipet. The data were filtered at 100 Hz and digitized at 200 Hz. The closed-state and open-state currents are denoted by "c" and "o," respectively.

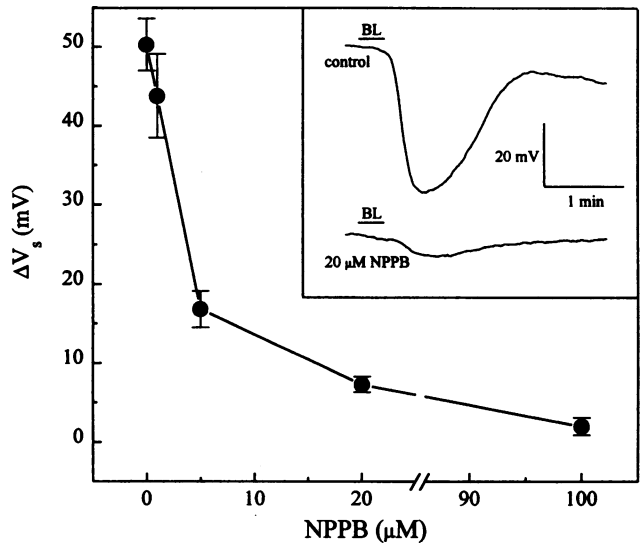

FIG. 4. Block of the BL-induced depolarization by NPPB. A 20-s pulse of BL was delivered to intact, etiolated seedlings treated with or without NPPB. Values for the change in membrane potential measured with surface electrodes are the means ± SE of 6–18 measurements per treatment. (*Inset*) Representative electrical response from a control seedling and one treated with 20 μM NPPB. The bars next to each trace show the timing of the BL pulse.

lings treated with 20 μM NPPB (−146 ± 3 mV; $n = 4$) was not significantly different ($P = 0.05$) from the control value of -155 ± 4 mV ($n = 4$). Thus, NPPB did not block the BL-induced depolarization by depolarizing the membrane prior to the BL pulse.

Seedlings were grown in BL on a series of NPPB concentrations for 4 days to determine if blocking the anion channels reduced the inhibitory effect of BL on growth. Fig. 5 shows that the hypocotyls of seedlings grown in the presence of 40 μM NPPB were 57% longer than those grown in its absence. The concentration of NPPB that gave half of the maximal increase in growth was ≈11 μM, roughly comparable to that which half-inhibited the depolarization. NPPB did not enhance the growth of hypocotyls maintained in darkness (Fig. 5), indicating that it did not act as a general growth stimulant. Nor did NPPB affect the timing of germination, so its effect on hypocotyl length was not due to a change in growing period. Thus, NPPB appeared to alleviate some of the growth inhibition due to BL.

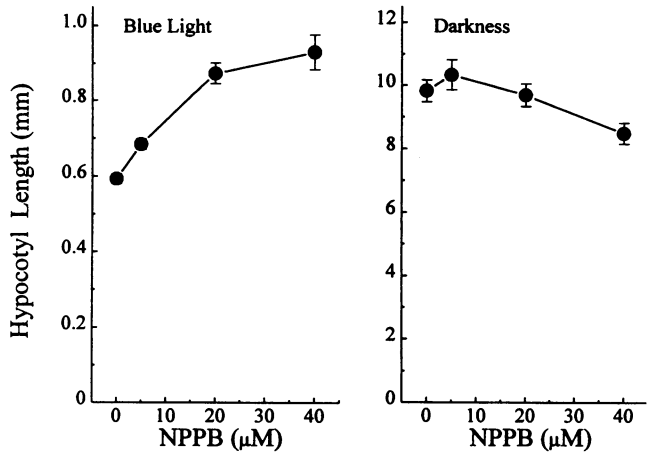

FIG. 5. Effect of NPPB on hypocotyl length of seedlings grown in BL and darkness. Data are the means ± SE of between 58 and 147 measurements per treatment pooled from two to three trials. Error bars smaller than the symbols are not shown.

DISCUSSION

Channels conducting inward currents across the plasma membrane of etiolated hypocotyl protoplasts were activated by BL. Although identification of channels by thermodynamic criteria is not possible in the cell-attached mode, the following points support our conclusion that the channel activated by BL (Fig. 2*A*) is the same as the anion channel identified in excised patches (Fig. 1). (*i*) Under the imposed conditions, only an efflux of anions or the inward movement of Cs^+ could be responsible for the inward currents shown in Fig. 2. The former is much more probable than the latter as Cs^+ is a very impermeant ion (22) and we never observed inward Cs^+ currents across excised patches. (*ii*) The BL-activated channel and the anion channel displayed similar kinetic characteristics. Neither was strongly voltage-dependent, both frequently adopted an open state that persisted on the order of a second, and they displayed roughly similar conductances and subconductance states. These features plus the block by NPPB are characteristics shared reasonably well with the S-type anion channel of *Vicia* guard cells (19, 23, 25, 26).

The depolarization induced by BL in intact seedlings was inhibited by NPPB in the same concentration range that blocked the anion channel (Fig. 4). The inhibitory effect of NPPB on the depolarization was probably not due to a general deleterious effect of the compound because seedling growth, which is very sensitive to perturbances, was promoted by NPPB in BL and relatively unaffected in the dark (Fig. 5). While these results indicate that activation of the anion channel by BL is responsible for the depolarization, we cannot rule out the possibility that an NPPB-sensitive conductance other than the anion channel characterized here also contributes to it.

The absence of a significant effect of NPPB on the resting V_m, also evidence that the drug did not exert large nonspecific effects on membrane transport, indicates that the anion channel is a minor contributor to the normal steady-state conductance of the membrane. This result plus the direct demonstration of BL-induced channel activation (Fig. 2) and the pharmacological experiments (Figs. 3 and 4) lead us to conclude that the anion channel is rapidly activated by BL from a relatively quiescent state, resulting in an efflux of anions from the cell and membrane depolarization. A similar mechanism was recently suggested to mediate a depolarization induced by white light in the epidermal cells of pea leaves (27).

The time course of the channel activation was slightly slower than that of the depolarization. This may well be related to the fact that even gentle handling of intact seedlings diminishes and slows the BL-induced depolarization unless a recovery period of some tens of minutes is allowed. Another feature of the time course was that the channels appeared to remain activated for longer than BL was present. For the membrane to repolarize during a period of high anion-channel activity, compensatory changes in the activities of some other transporters must occur.

Previously published evidence indicated that inhibition of the plasma membrane H^+-ATPase (proton pump) contributed to the BL-induced depolarization in cucumber hypocotyls (9). This seems at odds with the effective inhibition of the depolarization by a specific blocker of the anion channel. More direct evidence of proton-pump inhibition should be obtained before it is fully accepted as a component of this BL response.

Our interpretation of the data presented here is that NPPB renders growth less sensitive to BL by reducing activation of the anion channel. Although NPPB increased hypocotyl length by 57% in BL, those treated seedlings were greatly inhibited relative to dark-grown controls. This is probably due, at least in part, to the phytochrome system which also transduces BL into growth inhibition and is presumably unimpaired by NPPB. There may also exist other BL-specific pathways that inhibit growth in an NPPB-independent fashion.

BL-induced growth inhibition is due to a reduction in the rate of relaxation of turgor-derived stresses in the cell wall (4). How could the activation of anion channels lead so quickly to a reduction in wall stress relaxation? The relationship between pH and wall extensibility provides a basis for speculation. Cell walls extend more readily at acidic pH, possibly because of the pH dependence of wall proteins known as expansins (28). Alkalinization of the cell wall and, hence, growth inhibition could occur if an efflux of anions resulting from channel activation provided substrate for a H^+-anion symporter at the plasma membrane. The existence of such a transport activity in hypocotyls is plausible because the widely expressed *Arabidopsis* gene *CHL1* encodes a H^+-NO_3^- symporter (29), which may transport Cl^- as well (30). A H^+-anion symporter would also provide for the necessarily active return of anions to the cytoplasm (31).

Growth inhibition by this hypothetical mechanism would persist for as long as the anion channels remained activated. If this was the only mechanism by which BL inhibited growth, one may expect NPPB to more effectively lengthen hypocotyls. Perhaps BL induces two types of growth inhibition: a rapid, transient type resulting directly from the activation of anion channels, and a more slowly developing persistent inhibition that does not involve anion channels and is therefore unaffected by NPPB. The rapid growth inhibition is well established (4), and a growth inhibition that develops hours after a pulse of BL has been reported (32).

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