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Ca^{2+}-Activated Anion Channels and Membrane Depolarizations Induced by Blue Light and Cold in Arabidopsis Seedlings[1]

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The activation of an anion channel in the plasma membrane of *Arabidopsis thaliana* hypocotyls by blue light (BL) is believed to be a signal-transducing event leading to growth inhibition. Here we report that the open probability of this particular anion channel depends on cytoplasmic Ca^{2+} ($[Ca^{2+}]_{cyt}$) within the concentration range of 1 to 10 μM, raising the possibility that BL activates the anion channel by increasing $[Ca^{2+}]_{cyt}$. Arabidopsis seedlings cytoplasmically expressing aequorin were generated to test this possibility. Aequorin luminescence did not increase during or after BL, providing evidence that Ca^{2+} does not play a second-messenger role in the activation of anion channels. However, cold shock simultaneously triggered a large increase in $[Ca^{2+}]_{cyt}$ and a 110-mV transient depolarization of the plasma membrane. A blocker of the anion channel, 5-nitro-2-(3-phenylpropylamino)-benzoic acid, blocked 61% of the cold-induced depolarization without affecting the increase in $[Ca^{2+}]_{cyt}$. These data led us to propose that cold shock opens Ca^{2+} channels at the plasma membrane, allowing an inward, depolarizing Ca^{2+} current. The resulting large increase in $[Ca^{2+}]_{cyt}$ activates the anion channel, which further depolarizes the membrane. Although an increase in $[Ca^{2+}]_{cyt}$ may activate anion channels in response to cold, it appears that BL does so via a Ca^{2+}-independent pathway.

The vigorous elongation of etiolated seedling stems is quickly inhibited by BL as part of the de-etiolation process (Cosgrove, 1994). A rapid depolarization of the plasma membrane precedes the onset of growth inhibition by only a few seconds (Spalding and Cosgrove, 1989). This electrophysiological evidence that BL alters the activity of ion channels before inhibiting hypocotyl elongation prompted a recent patch-clamp investigation of the mechanism in Arabidopsis, work that identified a BL-activated anion channel in the hypocotyl cells (Cho and Spalding, 1996). An inhibitor of this channel, NPPB, blocked the BL-induced depolarization and also rendered hypocotyl growth less sensitive to BL. An important remaining question is how BL activates this anion channel.

The possibility that BL induces changes in $[Ca^{2+}]_{cyt}$ to trigger the opening of anion channels emerges from a consideration of the algal action potential, a transient depolarization known to involve Cl^- efflux (Gaffey and Mullins, 1958; Mullins, 1962; Hope and Findlay, 1964). More recent studies of this membrane phenomenon have identified plasma membrane anion channels (Coleman, 1986; Shiina and Tazawa, 1987; Okihara et al., 1991; Thiel et al., 1993) that would be activated by the large increase in $[Ca^{2+}]_{cyt}$ known to occur during the action potential (Williamson and Ashley, 1982). The currently accepted model of the action potential is that a rapid increase in $[Ca^{2+}]_{cyt}$ activates Cl^- channels, which depolarizes the plasma membrane (Johannes et al., 1991; Tyerman, 1992). Could this mechanism be similar to that which activates anion channels in response to BL? Although the BL-induced depolarization is much slower than the action potential and is not propagated (Spalding and Cosgrove, 1989), it is possible that the $[Ca^{2+}]_{cyt}$ component of the mechanism is shared. The fact that $[Ca^{2+}]_{cyt}$ has been shown to control the activity of anion channels in plant cells (Schroeder and Hagiwara, 1989; Hedrich et al., 1990) as well as animal cells (Zygmunt and Gibbons, 1992; Scott et al., 1995) adds to the plausibility of this mechanism operating during the early phases of BL signaling.

A transient membrane depolarization induced specifically by red light in a certain species of moss involves the activation of anion channels (Ermolayeva et al., 1996) and in this regard is similar to the depolarization induced by BL in seedlings. It was shown that Ca^{2+} carries a significant portion of the depolarizing current in the moss, adding to the general impression that phytochrome signals are transduced by changes in $[Ca^{2+}]_{cyt}$ (Hepler and Wayne, 1985; Shacklock et al., 1992; Neuhaus et al., 1993; Bowler et al., 1994; Roux, 1994). Because different photomorphogenic signaling pathways may share certain elements (Chamo-

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Abbreviations: BL, blue light; $[Ca^{2+}]_{cyt}$, cytoplasmic Ca^{2+} concentration; I_o, open channel current; NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; P_o, open probability; V_m, membrane potential; V_s, surface potential.

vitz and Deng, 1996), perhaps some BL responses also involve changes in $[Ca^{2+}]_{cyt}$, as has been suggested for phototropism (Gehring et al., 1990). After one considers all of the above, the possibility that BL activates the anion channel in Arabidopsis hypocotyls by increasing $[Ca^{2+}]_{cyt}$ seems worthy of testing.

One method of measuring changes in $[Ca^{2+}]_{cyt}$ is to engineer plants to express aequorin, a jellyfish protein that emits photons at a rate dependent on $[Ca^{2+}]_{cyt}$. The aequorin may be targeted to the cytosol (Knight et al., 1991, 1992, 1996; Sedbrook et al., 1996) or to specific organelles such as the chloroplast (Johnson et al., 1995) or the tonoplast (Knight et al., 1996). We produced transgenic Arabidopsis plants expressing cytosolic aequorin and used them to address the possible role of $[Ca^{2+}]_{cyt}$ in the BL-induced activation of anion channels. In the process, we learned more about how cold shock rapidly alters ion transport at the plasma membrane.

MATERIALS AND METHODS

Stable Transformation of Arabidopsis with Aequorin

Arabidopsis thaliana (Columbia ecotype) was stably transformed with a cauliflower mosaic virus 35S:aequorin:cauliflower mosaic virus 3′ gene construct in the binary plasmid pMAQ2 (the kind gift of Marc Knight [University of Oxford, Oxford, UK]; Knight et al., 1991). The pMAQ2 plasmid was transferred into *Agrobacterium tumefaciens* strain GV3101(pMP90) (Koncz and Schell, 1986) by electroporation (Mattanovich et al., 1989), and a Kan^R, $Gent^R$ colony with the appropriate DNA restriction digest pattern was used to transform root explants by the procedure of Valvekens et al. (1988). Kanamycin-resistant transformed lines were obtained and analyzed both for aequorin expression and for a Kan^R segregation ratio indicative of a single locus of transgene insertion. In vivo expression of the aequorin photoprotein was assessed by luminometer measurement of cold- and wind-induced luminescence from 4- to 10-d-old seedlings after overnight reconstitution with 2 μM coelenterazine (Molecular Probes, Eugene, OR) as described by Knight et al. (1991). Line CS behaved as expected for a single locus of insertion and showed both strong aequorin expression and Kan^R. A homozygous progeny line, C2:6, was used for the work described here.

Reconstitution of Active Aequorin

A TLC sprayer unit (Alltech, Deerfield, IL) was used to deliver a fine mist of 5 μM coelenterazine (Molecular Probes) plus 0.05% Triton-X (Sigma) to 3- to 4-d-old etiolated Arabidopsis seedlings expressing aequorin, grown on Petri plates containing 1% agar, 1 mM KCl, and 1 mM $CaCl_2$. Each dish received five to seven sprayings of coelenterazine before the seedlings were placed in complete darkness overnight.

Luminometer Measurements

Blocks of agar containing 40 to 60 etiolated seedlings, grown for 4 to 5 d as previously described (Cho and Spalding, 1996), were cut and placed into luminometer cuvettes and allowed to recover for at least 2 h unless otherwise specified. All experiments were performed in a darkroom lit by 0.002 μmol m^{-2} s^{-1} green safelight. BL (450 nm, 50-nm bandwidth, 100 μmol m^{-2} s^{-1}) was produced and delivered to a group of seedlings in a cuvette as previously reported (Cho and Spalding, 1996). The seedlings were quickly (within 2–4 s) placed in a luminometer (Pico-lite, United Technologies, Packard, Downers Grove, IL) to measure aequorin luminescence. Touch stimulus was applied by inserting a thin plastic needle into the injection port of the luminometer and gently agitating. Wind was applied by injecting 20 mL of room temperature air into the injection port with a syringe. Cold shock was administered by injecting 1 mL of 0°C distilled water or a 1 mM KCl plus 1 mM $CaCl_2$ solution.

For treatment with 20 μM NPPB (Calbiochem), seedlings were inverted to submerge the apical half of the hypocotyls in NPPB plus 0.1% Tween 20 for 1 h, and then they were removed from solution and placed upright to recover for 2 h. Control seedlings (H_2O plus Tween 20) were treated identically. After the recovery period, agar blocks containing exactly 40 seedlings were removed from the Petri plates and placed upright into luminometer cuvette tubes before experimental treatments were performed.

Simultaneous Recording of V_S and $[Ca^{2+}]_{cyt}$

These experiments were conducted in total darkness. One-second pulses of BL (450 nm, 50-nm bandwidth, 100 μmol m^{-2} s^{-1}) separated by 1 s of darkness were delivered to groups of 40 to 60 seedlings via a liquid light guide using a custom, computer-controlled apparatus that was previously described (Spalding, 1995). The change in V_s of a single seedling from the group was monitored using an agar-tipped surface contact electrode as described by Cho and Spalding (1996). A photomultiplier tube (model no. PM2710, International Light, Newburyport, MA) equipped with a programmable shutter (Uniblitz VS-25, Vincent Associates, Rochester, NY) was placed next to a group of aequorin-expressing seedlings and detected photon emission. During each 1 s of darkness, a shutter driver (T122, Vincent Associates) opened the shutter attached to the photomultiplier tube for 0.8 s, during which time the output of the photomultiplier tube (aequorin luminescence) was low-pass-filtered at 3 or 5 Hz and digitized at 15 Hz. Cold shock was administered by either gently dropping 0°C distilled H_2O directly on top of the seedlings or applying a 1-s stream of cold air from an inverted compressed air canister (Preval power unit, Precision Value, Yonkers, NY) 15 cm above the seedlings. Both methods of cold shock yielded similar results. Control experiments using either room temperature distilled H_2O or air elicited no change in $[Ca^{2+}]_{cyt}$.

Patch Clamping

Protoplasts of 5-d-old etiolated Arabidopsis seedlings were prepared as previously described (Cho and Spalding, 1996). Kimax-51 glass capillaries (Kimble Products, Vineland, NJ) were pulled and fire-polished with a horizontal puller (P87, Sutter Instruments, Novato, CA). The resulting patch pipettes were filled with 120 mM CsCl, 5 mM Hepes, 1 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 5.7) with Bis Tris propane. The 0.5-mL recording chamber initially was filled with a sealing solution consisting of 15 mM CsCl, 5 mM Hepes, 10 mM $CaCl_2$, and 55 mM sorbitol (pH 7.2). After a giga-ohm seal was obtained, the sealing solution was rapidly exchanged with one containing 10 μM Ca^{2+} (1.94 mM $CaCl_2$ and 2 mM EGTA), 200 mM CsCl, 5 mM Hepes, 2 mM $MgCl_2$, and 4 mM Mg-ATP (pH 7.2) and the patch excised. Solutions having either 1 or 0.1 μM free Ca^{2+} were obtained by combining 1.48 or 0.4 mM $CaCl_2$ with 2 mM EGTA. The bath solution could be perfused at a rate of several milliliters per minute with a selected solution released by a switchable manifold. Single-channel currents were recorded and analyzed using hardware, software, and methods previously described (Cho and Spalding, 1996). All chemicals were obtained from Sigma.

RESULTS

Anion Channel Activity Depends on $[Ca^{2+}]_{cyt}$

Anion channels can be studied in cell membranes by patch-clamping in the presence of CsCl. Use of this impermeant cation increases the likelihood that any channels observed conduct anions. Under these conditions, the predominant channel active in the plasma membrane of Arabidopsis hypocotyl cells is the anion channel previously found to be activated by BL (Cho and Spalding, 1996). We used similar conditions in patch-clamp experiments designed to determine whether the activity of this channel

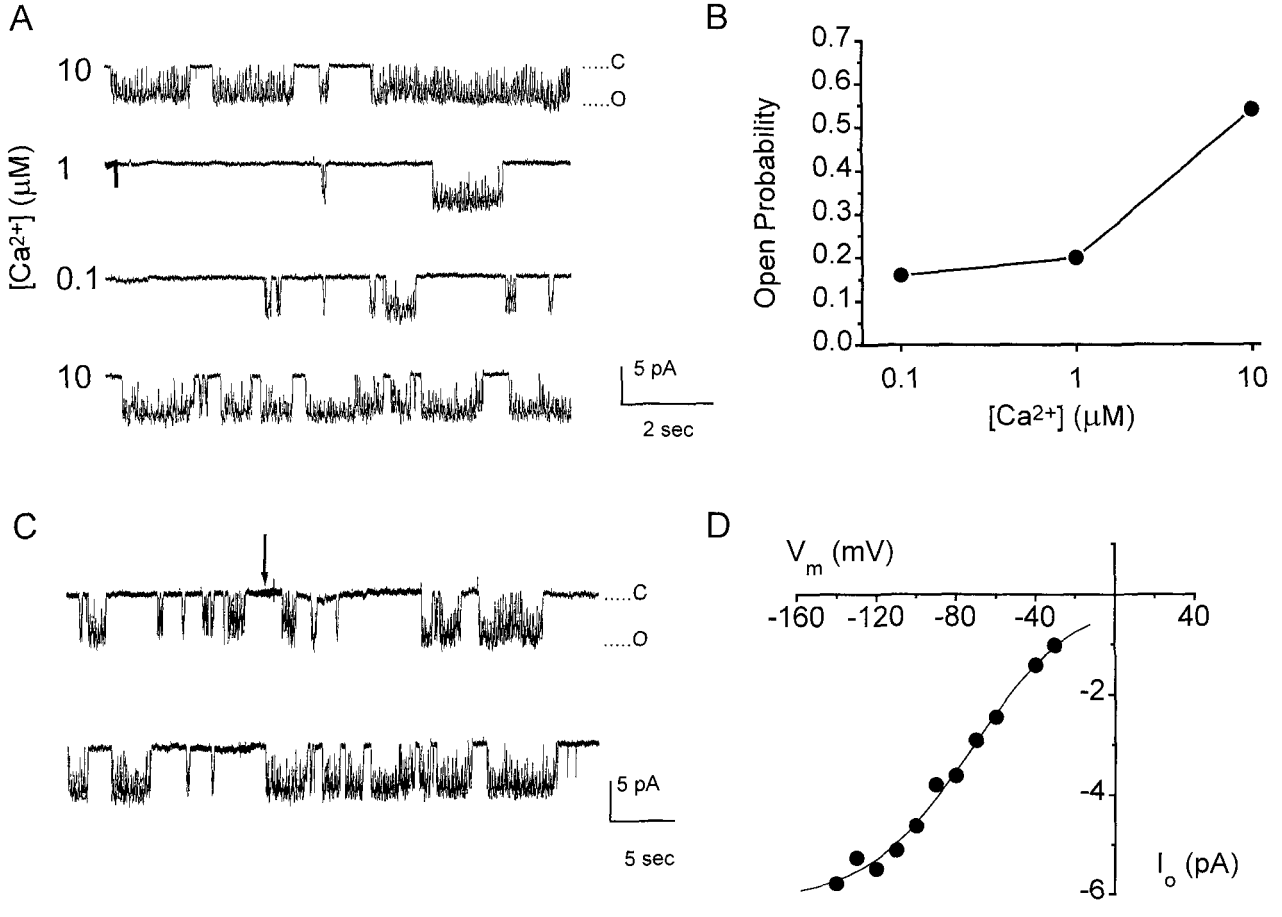

Figure 1. Anion channel activity depends on $[Ca^{2+}]_{cyt}$. A, Twenty-second recordings of anion channel activity from an inside-out patch bathed by 10, 1, and 0.1 μM Ca^{2+}. V_m was held at −110 mV. The closed and open state currents are represented by "C" and "O", respectively. Data were filtered at 250 Hz and digitized at 500 Hz. B, Dependence of anion channel P_o on $[Ca^{2+}]_{cyt}$. Each data point was calculated using between 1.8 and 4.7 min of recording obtained at each Ca^{2+} concentration shown. C, Change in P_o of the anion channel in response to an increase in $[Ca^{2+}]_{cyt}$. Continuous recording of anion channel activity from the same inside-out patch shown in A. The $[Ca^{2+}]$ in the bath was increased from 0.1 to 10 μM at the arrow and full exchange was expected 10 to 15 s later. Data were filtered at 250 Hz and digitized at 500 Hz. D, Current-voltage relationship of the anion channel in A. Data represent open-channel currents at each of the indicated clamp potentials. A sigmoidal curve was fit to these data.

depends on Ca^{2+}. We viewed this as a first test of the hypothesis that BL activates the anion channel in planta via an increase in $[Ca^{2+}]_{cyt}$. Figure 1A displays 20-s recordings of current through a single channel residing in an inside-out patch of membrane excised from an etiolated hypocotyl protoplast. Channel activity depended on the Ca^{2+} concentration at the cytosolic face of the patch, being greatest at 10 μM and lowest at 0.1 μM. Returning $[Ca^{2+}]_{cyt}$ to 10 μM after several minutes at the lower concentrations restored high channel activity (Fig. 1A, bottom trace). Analysis of the total recordings made at the various Ca^{2+} concentrations allowed the construction of the plot in Figure 1B. It shows that the P_o of the anion channel was 3-fold higher at 10 than at 1 μM, with very little difference in P_o evident between 1 and 0.1 μM. The total recording time was at least 1.8 min for each concentration of Ca^{2+} used in the experiment. Figure 1C displays a continuous recording of anion channel activity from the same patch. A recording solution containing 0.1 μM Ca^{2+} was exchanged with one having 10 μM Ca^{2+} at the arrow, and full solution exchange was expected 10 to 15 s later. The change in P_o did not occur immediately upon increasing the Ca^{2+} concentration. Instead, tens of seconds elapsed between the time the bathing solution was fully exchanged and when changes in P_o were evident. Despite this time delay, the effect of Ca^{2+} was reversible and reproducible. Figure 1D displays a plot of I_o versus V_m for this anion channel. These data were obtained by clamping the membrane at the indicated potentials and measuring the open-channel current of the major conductance state. The slope of the I_o-V_m curve in Figure 1D revealed a conductance of 52 pS for the Ca^{2+}-dependent channel, similar to the 46 pS displayed by the BL-activated anion channel studied in the same cells but with slightly lower Cl^- concentrations (Cho and Spalding, 1996). An extrapolation of the sigmoidal fit of the data would intersect the voltage axis close to the predicted equilibrium potential for Cl^-. A more rigorous determination of this channel's selectivity was made difficult by its multiple conductance states and the infrequent openings at positive voltages under these conditions. Further evidence that the channel in Figure 1 is the same type as that previously found to be activated by BL is its long open times, on the order of seconds, and the presence of subconductance states. Three independent experiments showed results qualitatively similar to that illustrated in Figure 1A.

Changes in $[Ca^{2+}]_{cyt}$ in Response to BL, Touch, Wind, and Cold Shock

The Ca^{2+} dependence of the anion channel demonstrated in Figure 1 leads to the question of whether BL causes an increase in $[Ca^{2+}]_{cyt}$. This was tested using transgenic seedlings cytoplasmically expressing aequorin. Figure 2 shows results of experiments in which a group of 40 to 60 seedlings was irradiated with a 20-s pulse of BL (100 μmol m^{-2} s^{-1}) and quickly (2–4 s) placed into a luminometer for monitoring the emission of photons by aequorin. Superimposed on this trace is a recording of a depolarization typical of etiolated Arabidopsis hypocotyls treated

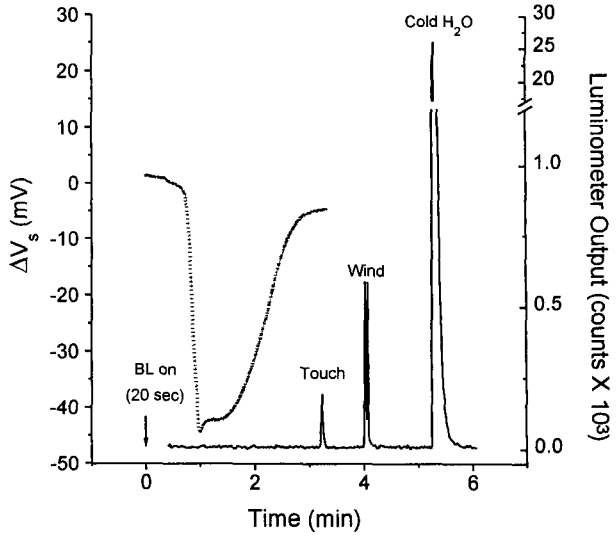

Figure 2. Changes in $[Ca^{2+}]_{cyt}$ in response to BL, touch, wind, and cold-shock stimulation. Continuous recording showing aequorin luminescence (right axis) corresponding to $[Ca^{2+}]_{cyt}$ in response to a 20-s pulse of BL (100 μmol m^{-2} s^{-1}), touch, wind, and cold shock. Superimposed on this figure is a depolarization (mV, left axis) typical of Arabidopsis hypocotyls in response to a similar fluence BL.

with a similar fluence BL but measured separately. Although increases in $[Ca^{2+}]_{cyt}$ similar to those reported in tobacco seedlings (Knight et al., 1991, 1992, 1996) were detected in response to touch, wind, and cold shock, no change was observed immediately after the BL pulse or up to 3.5 min following it. Even when the most rapid phase of the BL-induced depolarization should have been occurring, no change in $[Ca^{2+}]_{cyt}$ above baseline levels was detected. The results in Figure 2 are representative of four independent experiments.

Although no change in $[Ca^{2+}]_{cyt}$ was detected in response to the 20-s pulse of BL, important changes in $[Ca^{2+}]_{cyt}$ that may have occurred during the light treatment or during the 2 to 4 s between BL exposure and placement of the seedlings in the luminometer would not have been detected. To test for such early changes in $[Ca^{2+}]_{cyt}$, aequorin luminescence was measured in a group of seedlings during the BL response using a custom apparatus that used computer-controlled shutters (Spalding, 1995) and a photomultiplier tube. Aequorin luminescence was recorded during each dark period of a continuous train of 1-s BL pulses delivered to a group of seedlings. The change in V_s of one seedling of the group was simultaneously measured. Figure 3A shows that the pulsed BL induced a depolarization, but no change in $[Ca^{2+}]_{cyt}$ could be detected before, during, or after the depolarization. Figure 3B shows that this custom apparatus readily recorded the large change in $[Ca^{2+}]_{cyt}$ triggered by cold shock, previously reported to be 1.8 to 2.4 μM (Knight et al., 1993, 1996), and revealed the striking result that a rapid membrane depolarization occurred simultaneously with it. The temporal resolution of our apparatus (55 ms) was insufficient to determine which began first. Similar results were obtained in 12 independent experiments, as well as in

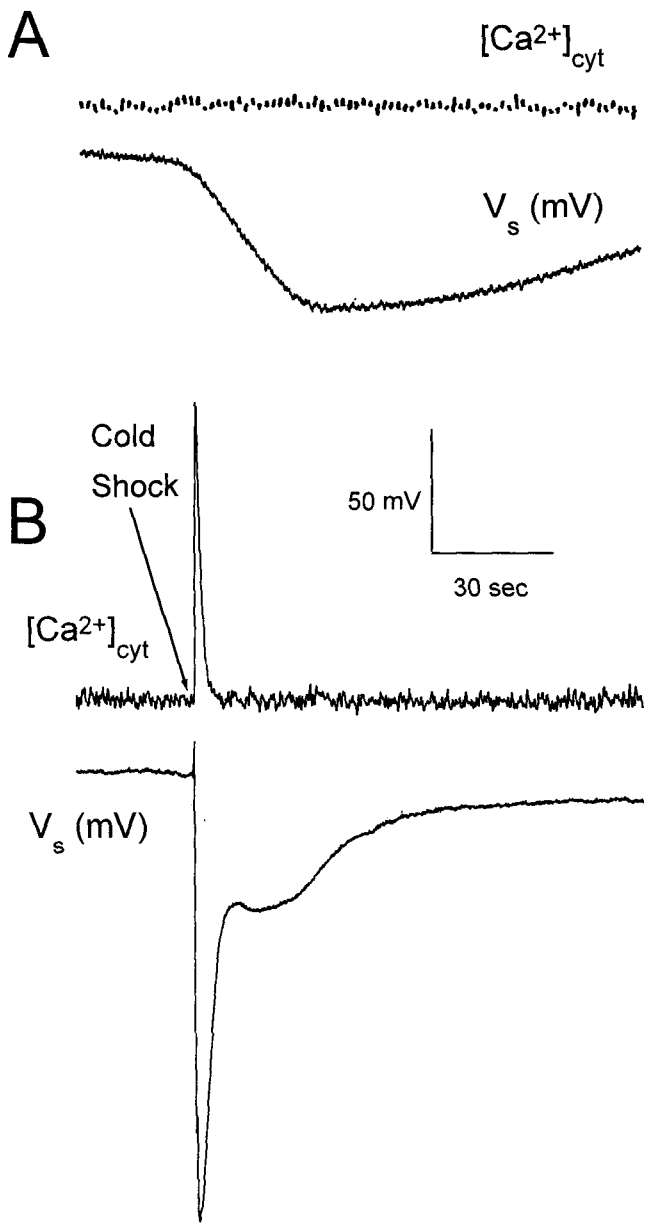

Figure 3. Simultaneous recording of $[Ca^{2+}]_{cyt}$ and changes in V_m in response to BL and cold shock. A, Dual recording of $[Ca^{2+}]_{cyt}$ (top trace) and changes in V_m made with surface contact electrodes (V_s; bottom trace) during a train of 1-s pulses of BL that started 1 s after the beginning of this trace. B, Changes in $[Ca^{2+}]_{cyt}$ and V_s in response to cold shock (arrow) in groups of seedlings previously exposed to BL. The time and voltage scales apply to both A and B.

cases in which the V_m of hypocotyl cells was measured directly with intracellular microelectrodes (data not shown). Room temperature H_2O or compressed air caused no change in $[Ca^{2+}]_{cyt}$ or V_s, indicating that it was the temperature change per se that triggered both the Ca^{2+} and electric change (data not shown). Touch and wind stimuli elicited small increases in $[Ca^{2+}]_{cyt}$ (not shown) but their effects on V_m were not determined because of the difficulty of maintaining an electrical recording during these disturbances.

Effect of NPPB on BL and Cold-Shock Responses

NPPB is a blocker of animal and plant anion channels (Marten et al., 1992; Schroeder et al., 1993). The anion channel activated by BL in hypocotyl cells of etiolated Arabidopsis seedlings is potently and reversibly blocked by NPPB (Cho and Spalding, 1996). Consistent with our previous studies, pretreatment of the seedlings with 20 μM NPPB reduced the BL-induced depolarization by 85% relative to controls as shown in Figure 4. NPPB blocked 61% of the cold-induced depolarization, indicating that it is partly due to an anion current (Fig. 4). The NPPB-insensitive portion is probably the result of an inward Ca^{2+} current that depolarizes the membrane and simultaneously increases $[Ca^{2+}]_{cyt}$. To test the possibility that an anion channel-dependent change in V_m triggered the opening of Ca^{2+} channels, seedlings expressing aequorin were treated with NPPB and given cold shock. Figure 5 shows that NPPB did not affect the magnitude of the cold-induced change in $[Ca^{2+}]_{cyt}$, nor was its time course affected (data not shown). Thus, the cold-induced depolarization appears to result from a combination of inward Ca^{2+} and anion currents, the former not dependent on the latter.

Other researchers have used $LaCl_3$ to block Ca^{2+} fluxes across the plasma membrane (Knight et al., 1992, 1996). Consistent with the above interpretation of the membrane response to cold, 10 mM $LaCl_3$ blocked essentially all of the cold-induced increase in $[Ca^{2+}]_{cyt}$ as well as the accompanying rapid membrane depolarization (not shown). However, experiments to be reported elsewhere have raised strong doubts about the specificity of $LaCl_3$ for Ca^{2+} channels, making its inhibitory effects difficult to interpret.

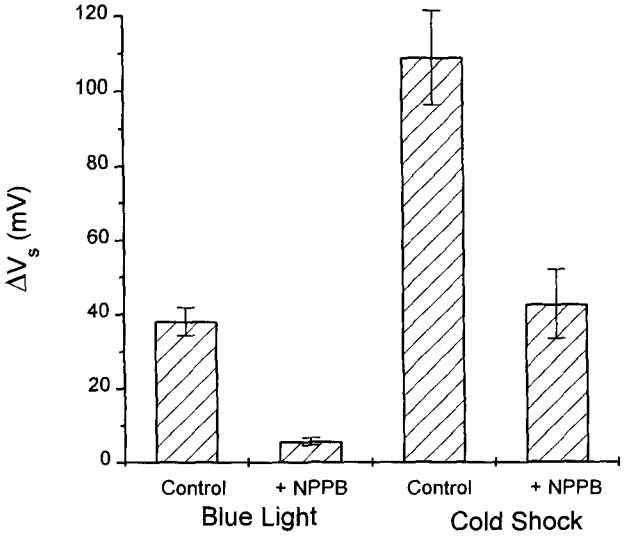

Figure 4. Effects of NPPB on the depolarizations induced by BL and cold shock. Magnitude of membrane depolarizations induced by BL and cold shock in the presence or absence of 20 μM NPPB. Depolarizations were measured with surface electrodes and are reported as means ± SE of 9 to 12 independent experiments.

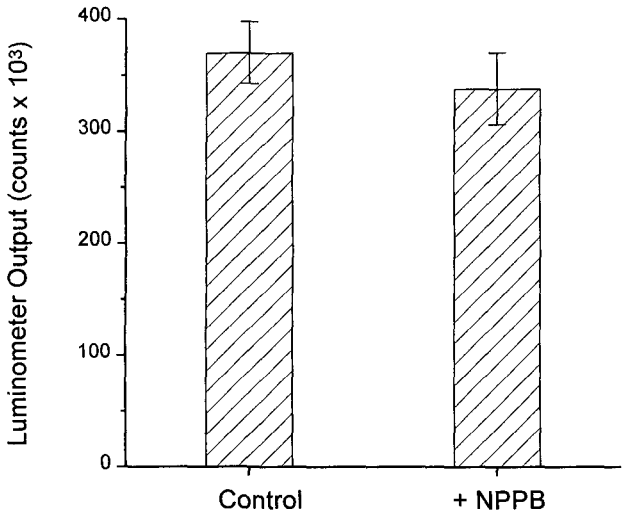

Figure 5. NPPB does not affect the cold-induced increase in $[Ca^{2+}]_{cyt}$. Changes in $[Ca^{2+}]_{cyt}$ in response to cold shock are reported as peak counts per second (±SE) from 21 to 23 groups of 40 to 60 seedlings each.

DISCUSSION

The anion channel identified in Figure 1 was strongly activated by Ca^{2+} only when its concentration at the cytosolic face of a membrane patch was increased into the micromolar range, the range into which cold shock is known to elevate $[Ca^{2+}]_{cyt}$ (Knight et al., 1993, 1996). Our methods of detecting aequorin luminescence were capable of measuring the much smaller increase in $[Ca^{2+}]_{cyt}$ elicited by touch, and yet we did not detect any change in response to BL (Figs. 2 and 3). Therefore, any BL-induced increase in $[Ca^{2+}]_{cyt}$ must be below the detection level of our two measuring methods. Such small changes in $[Ca^{2+}]_{cyt}$ would not be expected to affect the activity of the anion channel, according to the Ca^{2+} dependence of P_o shown in Figure 1. Thus, BL does not appear to activate anion channels by increasing $[Ca^{2+}]_{cyt}$. This is consistent with the previous observation that the magnitude of the BL-induced depolarization was unaffected by chelating extracellular Ca^{2+} with EGTA (Spalding and Cosgrove, 1992).

One caveat is that larger changes in Ca^{2+} may have occurred in response to BL but only in a subset of the hypocotyl cells, and this limited but physiologically important change was not detected by our methods. Arguing against this is the previously published finding that both cortical and epidermal cells, including trichomes, respond to BL with a similar depolarization (Spalding and Cosgrove, 1992). Also, our measurements reflected the collective responses of at least 40 seedlings, an effort to boost the signal from any BL-responding cells. Another caveat that must be considered is that the anion channel may have been made less sensitive to Ca^{2+} by excision of the patch. If a substantial decrease in Ca^{2+} sensitivity accompanied patch excision, perhaps due to the loss of a key regulatory element such as calmodulin (Bethke and Jones, 1994), we would be incorrect to expect that only large, micromolar changes in Ca^{2+} would affect the activity of the channel in vivo.

In addition to providing evidence against a role for Ca^{2+} in the BL-induced depolarization, the present work revealed some details about the electrophysiological response to cold shock. The very rapid, transient depolarization reported here (Fig. 3B) was similar to that previously measured in cucumber roots by Minorsky and Spanswick (1989). They presented evidence that the depolarization was at least in part due to an inward Ca^{2+} current across the plasma membrane. Our observation that the membrane depolarized simultaneously with an increase in $[Ca^{2+}]_{cyt}$ (Fig. 3B) is fully consistent with those previously published results and the identification of extracellular Ca^{2+} as the main source of the cold-induced Ca^{2+} increase (Knight et al., 1992, 1996). The fact that blocking the anion channel with NPPB did not affect the cold-induced increase in $[Ca^{2+}]_{cyt}$ (Fig. 5) indicates that a depolarization initiated by anion channel activation may not be the signal that opens the Ca^{2+} channels. Although Ca^{2+} channels may be depolarization-activated (Thuleau et al., 1994), their initial opening in response to cold appears to be more directly a result of the temperature change than of the depolarization caused by anion channel activation.

The most straightforward interpretation of the available data is that cold shock activates Ca^{2+}-permeable channels at the plasma membrane (Knight et al., 1992). The resulting inward Ca^{2+} current depolarizes the plasma membrane (Minorsky and Spanswick, 1989) while also increasing $[Ca^{2+}]_{cyt}$. As $[Ca^{2+}]_{cyt}$ increases into the micromolar range, anion channel activity increases (Fig. 1), which furthers the depolarization. This explains why a portion of the depolarization is sensitive to NPPB (Fig. 4), whereas the increase in $[Ca^{2+}]_{cyt}$ is not (Fig. 5). In fact, this proposed mechanism for the cold-induced depolarization is similar to that proposed for the moss phytochrome-mediated depolarization (Ermolayeva et al., 1996) and the algal action potential (Johannes et al., 1991; Tyerman, 1992). Whether the cold-induced depolarization is propagated similarly to action potentials has not been determined; Arabidopsis seedlings would not be the best experimental preparation to address this point.

The change in anion-channel P_o in response to changes in $[Ca^{2+}]_{cyt}$ was not immediate; it took some tens of seconds to develop (Fig. 1C). This suggests that $[Ca^{2+}]_{cyt}$ does not directly affect anion channel activity but does so through intermediaries such as Ca^{2+}-dependent kinases and/or phosphatases (Schmidt et al., 1995; Pei et al., 1997). The relatively sluggish response of the channel to $[Ca^{2+}]_{cyt}$ may seem at odds with the rapidity of the in vivo response (Fig. 3B), but perhaps is not surprising considering that patches of membrane detached from the cell may have lacked components that tighten the coupling to the channels.

Our results indicate that the BL-induced depolarization is initiated by a Ca^{2+}-independent mechanism. Recent molecular studies of the HY4 photoreceptor have indicated that redox reactions may be important early steps in BL signaling (Lin et al., 1995). Changes in redox state are also known to activate ion channels in Arabidopsis leaves (Spalding et al., 1992) and could be an important aspect of the mechanism by which BL activates anion channels. Anion-channel activity could also be influenced through phos-

phorylation events (Schmidt et al., 1995; Pei et al., 1997), which are known to be important in the early stages of the BL signal transduction pathway leading to phototropism (Short and Briggs, 1994; Liscum and Briggs, 1995; Salomon et al., 1996).

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