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Anion-Channel Blockers Interfere with Auxin Responses in Dark-Grown Arabidopsis Hypocotyls[1]

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Anion channels are thought to participate in signal transduction and turgor regulation in higher plant cells. The regulation of hypocotyl cell elongation is a situation in which these channels could play important roles because it involves ionic fluxes that are implicated in turgor control and orchestrated by various signals. We have used a pharmacological approach to reveal the contribution of anion channels in the regulation of the development of hypocotyls by auxins. Auxins induce an inhibition of elongation, a disintegration of the cortical cell layers, and the formation of adventitious roots on *Arabidopsis thaliana* hypocotyls grown in the dark. Anion-channel blockers such as anthracene-9-carboxylic acid, 4,4′-diisothiocyanatostilbene-2–2′-disulfonic acid, 4-acetamido-4′-isothiocyanato-stilbene-2–2′-disulfonic acid, and R(+)-methylindazone; indanyloxyacteic acid-94, which produce little or no stimulation of hypocotyl elongation by themselves, are able to counteract the inhibition and the disintegration induced by auxins with various efficiencies. This interference appears to be specific for auxins and does not occur when hypocotyl elongation is inhibited by other growth regulators such as ethylene or cytokinins. The putative involvement of anion channels in auxin signal transduction is discussed.

Anion channels are thought to play important roles in signal transduction and turgor regulation in higher plant cells (Schroeder and Hedrich, 1989; Ward et al., 1995). Such roles have been investigated in detail in guard cells, but little is known about the roles of anion channels in other cell types. The regulation of cell elongation is another situation in which they could play important roles because it also involves ionic fluxes that are implicated in turgor control and are orchestrated by various signals. Hypocotyl development is an attractive model in which to study the regulation of cell elongation and the perception of corresponding signals. In *Arabidopsis thaliana* all of the cells forming the hypocotyl are generated in the embryo, and, therefore, after germination the growth of the hypocotyl only involves cell elongation (Gendreau et al., 1997). On the other hand, growth regulators such as ethylene (Ecker, 1995), cytokinins (Chory et al., 1994; Cary et al., 1995), brassinosteroids (Clouse, 1996), and auxins (Cleland, 1995), as well as environmental signals such as light (Elich and Chory, 1994) and mechanical stimulations (Jones and Mitchell, 1989) all contribute to the regulation of hypocotyl elongation.

In guard cells a pharmacological approach has been used successfully to demonstrate the involvement of anion channels in the stomatal closure induced by ABA (Schroeder et al., 1993; Schwartz et al., 1995). At least two different types of anion channels are present at the plasma membrane of guard cells (Keller et al., 1989; Schroeder and Hagiwara, 1989): the slow anion channel (the S type) and the rapid anion channel (the R type). The Cl^--channel blockers initially developed to inhibit Cl^- channels from muscle or epithelial cells in animals (Greger, 1990) have been used to determine the pharmacological profiles of R- and S-type channels (Marten et al., 1992, 1993; Schroeder et al., 1993; Schwartz et al., 1995). These profiles largely overlap, but the sensitivity to submicromolar concentrations of stilbene derivatives appears as a distinctive feature of R-type anion channels (Marten et al., 1993). This singular trait has been used to demonstrate that the S-type anion channel catalyzes the sustained anion efflux leading to stomatal closure in response to ABA (Schroeder et al., 1993).

In hypocotyl cells anion channels could also be involved in cell turgor decrease. In the case of guard cells, turgor decrease results in stomatal closure. We made the hypothesis that in hypocotyl cells, anion-channel activation could lead to growth limitation by diminishing the turgor pressure that represents the motor force for cell growth (Cleland, 1995). According to this hypothesis, anion-channel blockers should stimulate hypocotyl growth or prevent the inhibition of hypocotyl elongation induced by biological signals. Alternatively, hypocotyl anion channels may be involved in signal transduction. If so, anion-channel blockers should be able to prevent developmental responses to signals that use anion channels in their transduction pathways.

Recently, two different types of anion channels have been characterized in hypocotyl cells of Arabidopsis using the patch-clamp technique (Thomine et al., 1995; Cho and Spalding, 1996). Cho and Spalding (1996) have provided

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Abbreviations: 9-AC, anthracene-9-carboxylic acid; DIDS, 4,4′-diisothiocyanato-stilbene-2–2′-disulfonic acid; IAA-94, R(+)-methylindazone;indanyloxyacetic acid-94; NAA, naphthalene acetic acid; NPPB, 5-nitro-2-(3-phenylpropyl amino)-benzoic acid; SITS, 4-acetamido-4′-isothiocyanato-stilbene-2–2′-disulfonic acid.

convincing arguments that anion channels are involved in blue-light transduction in Arabidopsis hypocotyl cells. They have characterized an anion channel that can be activated by blue light in the cell-attached configuration. This channel is blocked by NPPB, an anion-channel blocker. In turn, NPPB blocks blue-light-induced depolarization of hypocotyl cells and is able to partially counteract the inhibition of hypocotyl elongation induced by blue light.

Several lines of argument suggest the involvement of anion channels in auxin transduction. Voltage-dependent anion channels are modulated by auxins in guard cells (Marten et al., 1991) and in cultured tobacco (*Nicotiana tabacum*) cells (Zimmermann et al., 1994). Keller and Van Volkenburgh (1996) have shown that the depolarization that precedes auxin-induced membrane hyperpolarization in oat (*Avena sativa*) coleoptiles is sensitive to external Cl^- and blocked by 9-AC, an anion-channel blocker. At the level of the hypocotyl, auxin is known to promote a transient increase in the elongation rate of excised segments (Cleland, 1995). Recently, Cho and Hong (1996) have shown that this short-term response is sensitive to Ca^{2+}-channel antagonists. However, in intact plants on a longer time scale, the most commonly observed response of hypocotyls to exogenous auxin is inhibition, which may correspond to supraoptimal auxin effects. In particular, Arabidopsis mutants with increased auxin levels display a reduced elongation of the hypocotyl in the dark that is associated with cortex disintegration and adventitious rhizogenesis (Boerjan et al., 1995; King et al., 1995).

In this study we have used anion-channel blockers to reveal the possible involvement of anion channels in the regulation of the development of hypocotyls by auxins. We describe the effects of auxin on Arabidopsis hypocotyl development in the dark, and show that anion-channel blockers such as 9-AC and DIDS, which only produce a slight stimulation of hypocotyl elongation by themselves, are able to counteract the pronounced inhibition induced by auxins. A range of anion-channel inhibitors that are able to counteract the effects of auxin with different efficiencies are tested. Finally, we show that this interference is specific for auxins and does not occur when hypocotyl elongation is inhibited by other growth regulators such as ethylene or cytokinins.

MATERIALS AND METHODS

Arabidopsis thaliana ecotype Columbia plantlets were grown on a liquid medium containing 5 mM KNO_3, 2.5 mM K_2HPO_4/KH_2PO_4, pH 6.0, 2 mM $MgSO_4$, 1 mM $Ca(NO_3)_2$, 1 mM Mes, 50 μM Fe-EDTA, Murashige and Skoog microelements (Murashige and Skoog, 1962), and 10 g/L Suc. Erlenmeyer flasks containing 30 to 40 sterilized seeds in 10 mL of medium were put on an orbitary shaker (120 revolutions/min) at 26°C in the dark. After 24 h, when the radicle was separated from the seed integuments, the medium was replaced by a medium containing the substances to be tested (auxin and/or anion-channel blockers). This procedure was designed to avoid the putative inhibitory effects of the molecules tested on seed germination. The seedlings were then placed back on the shaker and grown for 6 d at 26°C in the dark. After this period, the effects of the molecules on the plantlets were analyzed. The hypocotyls were measured, and mean lengths were calculated for 30 to 40 hypocotyls.

Data have been normalized using the mean length of the control given in the figure legend for each experiment. The SE never exceeded 5% within one experiment. However, individual experiments are represented with different symbols to give an idea of the variability between experiments. Curves fitted by eye have been superimposed on the experimental data. In some experiments the number of adventitious roots and root primordia developing on the hypocotyl has been determined under the microscope after clearing of the tissues (Beeckman and Engler, 1994). The results are expressed as mean numbers of adventitious roots or root primordia (±SE) per hypocotyl. The lengths and diameters of hypocotyl epidermal cells were determined under the microscope using a micrometer. Cells were measured in the basal region of the hypocotyl, where cell elongation was nearly complete. These measurements were taken after 5 d of culture because later than that it was no longer possible to measure cells at the bases of auxin-treated hypocotyls because of disintegration of epidermal and cortical cell layers.

The auxins 1-NAA, 2-NAA, IAA, and 2,4-D and the channel blockers 9-AC, DIDS, SITS, and niflumic acid were purchased from Sigma. IAA-94 was purchased from RBI (Natick, MA) and NPPB was purchased from Tocris Cookson (St. Louis, MO). Auxins, ACC, cytokinins, DIDS, and SITS were dissolved in water alkalinized by NaOH. The other molecules were prepared as 20 mM stock solutions in 4% DMSO. Fresh stock solutions were prepared for each experiment. Because the molecules were tested at a maximal concentration of 200 μM, the final concentration of DMSO never exceeded 0.04%. When these molecules were tested, appropriate controls were made to ensure that DMSO did not significantly alter hypocotyl development and did not interfere with auxin.

RESULTS

Micromolar Concentrations of Auxins Deeply Alter Dark-Grown Arabidopsis Hypocotyl Development

One week after germination, the dark-grown Arabidopsis hypocotyls cultured with auxins displayed three major morphological responses. The first response was a reduction in hypocotyl length by 60 to 90% over control after treatment with 1 μM 1-NAA (Fig. 1A). This reduction in length was associated with a thickening of the hypocotyl (Fig. 2, A–C). At the cellular level auxin induced a change in the cell shape (Table I): the cell length decreased by 33% and the diameter increased by 18%. The second response was a peeling of the cortical and epidermal cell layers of the hypocotyl. This decortication started near the hypocotyl-root junction and developed upward toward the cotyledons, leaving only the stele intact (Fig. 2, A–C). The third response was the induction of adventitious root primordia

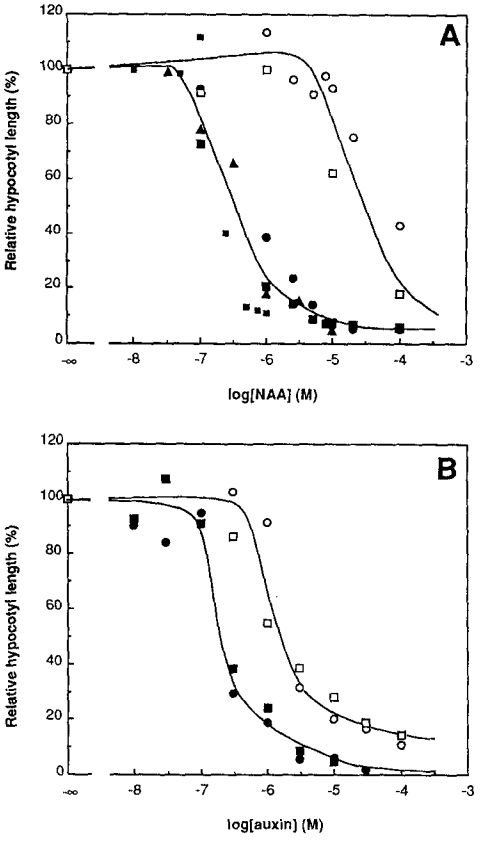

Figure 1. Inhibition of hypocotyl elongation by auxins. The relative length of the hypocotyls from plantlets grown in the dark for 7 d and transferred for the last 6 d to media containing different concentrations of auxins is presented as a function of the logarithm of auxin concentration. A, Effect of the auxin agonist 1-NAA (filled symbols) and its analog 2-NAA (open symbols) on relative hypocotyl length. The data concerning the effect of 1-NAA are the results of four independent experiments in which the mean lengths of the control hypocotyls, taken as 100%, were 16.5 mm (●), 13.4 mm (■), 12.0 mm (■), and 13.8 mm (▲). The data concerning the effect of 2-NAA are the results of two independent experiments in which the mean lengths of the control hypocotyls, taken as 100%, were 10.5 mm (○) and 13.9 mm (□). B, Effect of IAA (open symbols) and 2,4-D (filled symbols) on relative hypocotyl length. The data are the results of two independent experiments for each molecule in which the mean lengths of the control hypocotyls, taken as 100%, were 12.3 mm (●) and 11.0 mm (■) for 2,4-D, and 9.6 mm (○) and 13.2 mm (□) for IAA.

on the hypocotyl (Fig. 2C). The mean number of adventitious root primordia per hypocotyl increased from 4.8 in control plantlets to 15.3 in plantlets treated with 1 μM 1-NAA. These responses correspond to supraoptimal effects of auxin that do not normally occur in vivo, but are reminiscent of the phenotypes of *superroot* and *rooty* Arabidopsis mutants that display elevated endogenous levels of auxin (Boerjan et al., 1995; King et al., 1995).

The three different responses could be dissociated in time; the inhibition of elongation became clear 2 to 4 d after germination, whereas decortication and adventitious root primordia appeared only after 5 to 6 d (data not shown). All three active auxins tested (1-NAA, 2,4-D, and IAA) induced an inhibition of hypocotyl elongation (Fig. 1, A and B), a decortication of the hypocotyl, and the formation of adventitious root primordia on the hypocotyl at micromolar concentrations. The auxin inhibition concentrations for 50% displacement deduced from measurements of hypocotyl length on different auxin concentrations were 0.3, 0.2, and 3 μM for 1-NAA, 2,4-D, and IAA, respectively (Fig. 1, A and B). The response was specific for active auxins: 2-NAA, an analog of 1-NAA, induced an inhibition of hypocotyl elongation only at concentrations 2 orders of magnitude higher than those of 1-NAA (Fig. 1A). In addition to their effect on hypocotyls, all active auxins also altered root development, inhibiting primary root elongation and promoting the formation of secondary roots.

Anion-Channel Blockers Slightly Stimulate Hypocotyl Elongation and Counteract the Responses of Hypocotyls to Auxin

9-AC and DIDS have been shown to block plant anion channels in guard cells (Marten et al., 1992, 1993; Schwartz et al., 1995) and in cultured tobacco (*Nicotiana tabacum*) cells (Zimmermann et al., 1994). When applied alone, 9-AC or DIDS had little effect on hypocotyl development (Fig. 2A). At a concentration of 50 μM, 9-AC and DIDS increased the hypocotyl length by up to 28 and 46%, respectively (Fig. 3, A and B). At concentrations higher than 100 μM, they induced an inhibition of hypocotyl elongation. The stimulatory effects of DIDS and 9-AC were variable; 50 μM 9-AC or DIDS induced an increase in length from 7 to 28% and from −2 to 46%, respectively.

The hypocotyls treated with 100 μM 9-AC were thinner than control hypocotyls (Fig. 2A). This was associated with a slight increase in cell length to 107% of the control value (Table I). One hundred micromolar DIDS did not have such effects. In addition, 9-AC strongly altered root development. It inhibited the growth of the main root and prevented lateral root formation (data not shown). It also prevented completely the formation of adventitious root primordia on the hypocotyls. DIDS also inhibited root development, but much less so than 9-AC. Additionally, DIDS often induced a pronounced elongation of the petioles of the cotyledons (Fig. 2D).

In a second step, we studied the interactions between the anion-channel blockers and auxins. 9-AC or DIDS strongly attenuated the responses of Arabidopsis hypocotyls to auxin (1 μM 1-NAA) (Fig. 2, A and B). First, DIDS and 9-AC counteracted the inhibitory effect of auxin on elongation. Figure 3 shows the effect of increasing concentrations of 9-AC or DIDS on the length of hypocotyls treated by 1 μM 1-NAA. The auxin treatment reduced the hypocotyl length to about 20 to 30% of the control value. The hypocotyl length recovered between 85 and 106% or between 92 and 100% of the control value in the presence of 100 μM 9-AC or DIDS, respectively (Fig. 3). These attenuations of auxin-induced inhibition were concentration dependent, with effector concentrations for 50% response in the range of 30 to 50 μM (Fig. 3).

The effect on hypocotyl length was paralleled by the effects at the cellular level (Table I). Auxin induced a

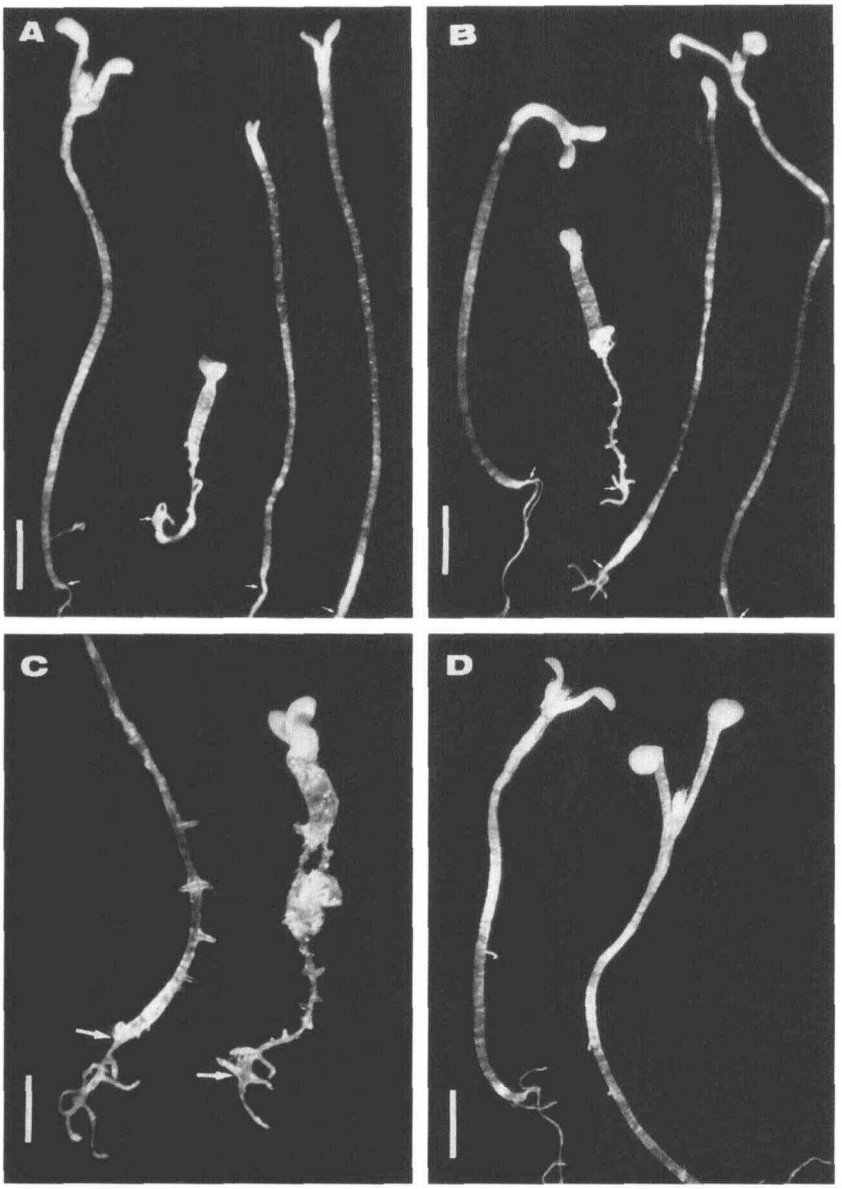

Figure 2. Effect of anion-channel blockers on the auxin-induced phenotype. The plantlets were grown in the dark for 7 d, and at the end of the 1st d, they were transferred in a medium containing the active molecules, as indicated. A, Effect of 9-AC on auxin responses. Left to right: Control, 1 μM 1-NAA, 1 μM 1-NAA plus 100 μM 9-AC, and 100 μM 9-AC. B, Effect of DIDS on auxin responses. Left to right: Control, 1 μM 1-NAA, 1 μM 1-NAA plus 100 μM DIDS, and 100 μM DIDS. C, Auxin-induced rhizogenesis in the absence and in the presence of DIDS. Left: 1 μM 1-NAA plus 100 μM DIDS; right: 1 μM 1-NAA. D, Effect of DIDS on the elongation of the petioles of the cotyledons. Left: control; right: 100 μM DIDS. The size of the scale bar is 1.6 mm in A, B, and D, and 0.8 mm in C. Arrows in A, B, and C indicate the junction between root and hypocotyl.

decrease of the cell length to 67% of the control value and an increase in cell diameter to 118% of the control value. These changes in cell enlargement were largely reversed by 9-AC and DIDS, which induced a recovery of the cell length to 92 and 96% of the control values and of the cell diameter to 94 and 103% of the control values, respectively. Another effect of 9-AC and DIDS was to prevent the decortication of hypocotyls. As illustrated in Figure 2, A through C, in the presence of 100 μM 9-AC or DIDS, the auxin-treated hypocotyls no longer underwent peeling of the epidermal and cortical cell layers.

Finally, 9-AC and DIDS displayed different effects on auxin-induced adventitious rhizogenesis. Figure 4 shows the effect of 100 μM 9-AC or DIDS on the adventitious rhizogenesis induced by 1 μM 1-NAA. 9-AC had strong effects on root development; alone, it prevented completely the formation of adventitious roots on hypocotyls (Fig. 4A) and it also counteracted the induction by auxin of adventitious primordia on hypocotyls. In contrast to 9-AC, DIDS did not prevent the formation of adventitious root primordia, nor did it counteract the inductive effect of auxin (Figs. 2C and 4B). Thus, a subset of auxin responses, the inhibition of elongation and the decortication, are prevented by two anion-channel blockers from distinct chemical families, whereas auxin-induced rhizogenesis displayed a differential sensitivity to the two inhibitors.

In contrast to the observations on hypocotyls, the effects of 1-NAA and 9-AC on the root system were additive, i.e. root growth was extremely reduced and no lateral roots were formed (Fig. 2A). DIDS, which did not strongly alter root development by itself, did not counteract auxin morphogenetic effects on the root system (i.e. the inhibition of the primary root and the increase of lateral root formation) (Fig. 2C).

For further insight into the action of the anion-channel blockers, we tested the effect of 100 μM 9-AC in the

Table I. *Effects of auxin and anion-channel blockers on the length and diameter of hypocotyl cells*

Epidermal cell sizes have been measured on four to six hypocotyls per treatment from 5-d-old dark-grown plantlets just before the onset of the auxin-induced decortication of the hypocotyl base. Numbers in parentheses = *n*. Values are means ± SE.

Treatment	Cell Length	Cell Diameter
	μm	
Control	440 ± 9 (72)	22.2 ± 0.6 (72)
1-NAA[a]	294 ± 8 (74)	26.2 ± 0.7 (76)
1-NAA 1 μM + 9-AC[b]	405 ± 9 (56)	20.9 ± 0.7 (55)
1-NAA 1 μM + DIDS [b]	421 ± 11 (65)	22.8 ± 0.7 (65)
9-AC[b]	471 ± 12 (51)	23.5 ± 1.0 (51)
DIDS[b]	438 ± 13 (63)	22.3 ± 0.8 (65)

[a] 1 μM. [b] 100 μM.

presence of different concentrations of 1-NAA. Figure 5 shows that in the presence of 100 μM 9-AC, the auxin dose-response curve for the inhibition of the elongation was shifted toward higher concentrations by a factor of about 5. Similarly, in the presence of 100 μM DIDS, three-times-higher concentrations of auxins were required to obtain an inhibition equivalent to the one induced by auxin alone. Therefore, 9-AC and DIDS lowered hypocotyl sensitivity to auxin rather than blocking completely the response to auxin.

In addition to 9-AC and DIDS, we tested a range of anion-channel inhibitors for their efficiency in counteracting hypocotyl responses to auxins: SITS, another stilbene derivative that blocks the R-type anion channel of guard cells (Marten et al., 1993), and IAA-94, NPPB, and niflumic acid, which block both the S- and R-type anion channels of guard cells (Marten et al., 1992; Schroeder et al., 1993; Schwartz et al., 1995). Like 9-AC and DIDS, SITS alone increased the hypocotyl length by up to 40% at the concentration of 50 μM (Fig. 6A). And, like DIDS, SITS often induced an abnormal elongation of the petiole of the cotyledons. Therefore, this seems to be a property shared by several stilbene derivatives. IAA-94 had only a slight effect on hypocotyl length in the concentration range tested (Fig. 6B). Niflumic acid and NPPB induced a strong reduction of hypocotyl elongation; the hypocotyl length was reduced to between 17 and 65% of the control value by 30 μM NPPB and to between 35 and 53% of the control value by 100 μM niflumic acid (Fig. 6, C and D).

Among these anion-channel blockers, only SITS and IAA-94 attenuated partially auxin-induced responses. Hypocotyls inhibited to 20% of the length of the control by 1 μM 1-NAA recovered to up to 80 or 60% of the control length in the presence of 100 μM SITS or IAA-94, respectively (Fig. 6, A and B). SITS and IAA-94 also counteracted auxin-induced decortication but were less efficient than 9-AC and DIDS. Finally, niflumic acid and NPPB did not prevent the inhibition of hypocotyl elongation induced by auxin at any of the concentrations tested (Fig. 6, C and D). However, 100 μM niflumic acid counteracted auxin-induced decortication, but also had a strong inhibitory effect at this concentration.

Anion-Channel Blockers Counteract Auxin Responses but Not Responses to Cytokinins or Ethylene

Figure 7A shows that 9-AC and DIDS are also able to counteract the effects of 2,4-D (0.1 μM) or IAA (3 μM) on hypocotyl elongation. They also counteracted the decortication induced by IAA and 2,4-D, and their effects on IAA-induced or 2,4-D-induced adventitious rhizogenesis were qualitatively similar to what we described for 1-NAA. Therefore, the effect of anion-channel inhibitors is not limited to 1-NAA, because they also interfere with a stronger auxin, 2,4-D, and with the natural auxin, IAA.

To determine whether the anion-channel blockers could counteract the inhibition of hypocotyl elongation induced by other growth regulators, we tested their effect on the responses to ethylene and cytokinins. We used ACC, an

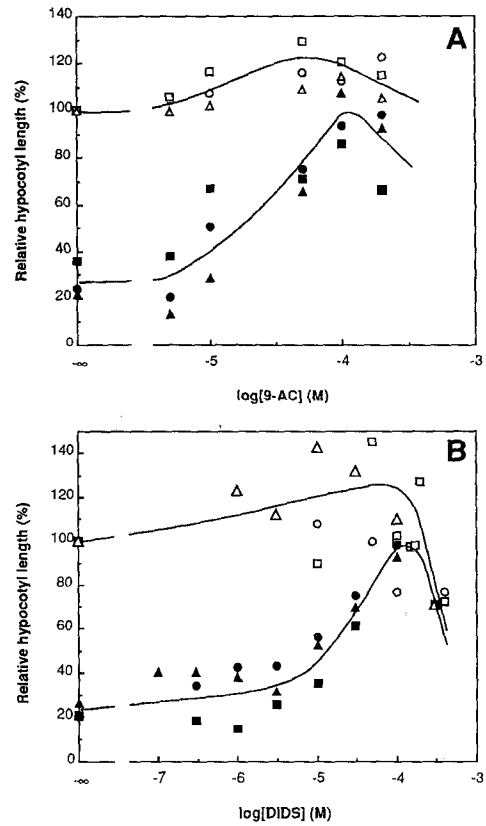

Figure 3. Interference of 9-AC and DIDS with auxin induced inhibition of hypocotyl elongation in dark-grown Arabidopsis plantlets. The relative length of the hypocotyls from plantlets grown in the dark for 7 d, transferred for the last 6 d on media containing different concentrations of anion-channel blockers in the absence (open symbols) or in the presence (filled symbols) of 1 μM 1-NAA is presented as a function of the anion-channel blocker concentration. A, Dose-response curve to 9-AC in the absence or in the presence of 1-NAA. The data are the results of six independent experiments. The lengths of the untreated control hypocotyls, taken as 100%, were 15.1 mm (○), 12.0 mm (□), 14.0 mm (△), 11.9 mm (●), 11.4 mm (■), and 9.6 mm (▲). B, Dose-response curve to DIDS in the absence and in the presence of 1-NAA. The data are the results of six independent experiments. The mean lengths of the untreated control hypocotyls, taken as 100%, were 14.2 mm (○), 11.5 mm (□), 10.7 mm (△), 11.9 mm (●), 11.5 mm (■), and 11.8 mm (▲).

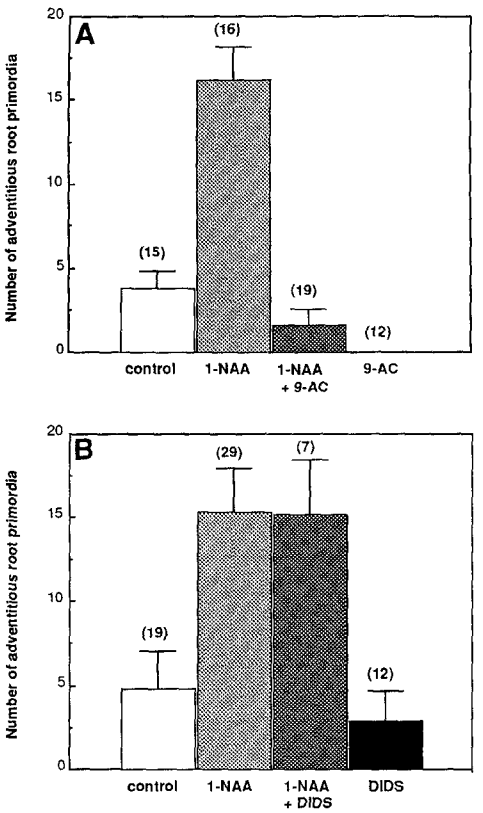

Figure 4. Effect of anion-channel blockers on auxin-induced adventitious rhizogenesis. The number of adventitious roots and adventitious root primordia developing on the hypocotyls was determined under a microscope after clearing of the plantlets grown in the dark for 7 d and treated by 1-NAA, 9-AC, and DIDS, as indicated. 1-NAA was applied at 1 μM; 9-AC and DIDS were applied at 100 μM. The results are expressed as mean numbers of adventitious roots or root primordia (± SE) per hypocotyl. The numbers above the bars indicate the total number of plantlets analyzed. A, Effect of 9-AC on adventitious rhizogenesis on the hypocotyl. The data are the results of four experiments. The medium contained 0.04% DMSO. B, Effect of DIDS on adventitious rhizogenesis on the hypocotyl. The data are the results of five experiments for control and the 1-NAA treatment and of two experiments for the DIDS and DIDS plus 1-NAA treatments.

endogenous precursor of ethylene, to induce the ethylene response on hypocotyls. At the concentration of 3 μM, ACC induced an inhibition of root elongation and a reduction of hypocotyl length to about 60% of the control value, which was associated with thickening. Figure 7B illustrates that in these conditions neither 9-AC nor DIDS at the concentration of 100 μM attenuated the reduction in hypocotyl length or the thickening induced by 3 μM ACC; neither did they prevent the inhibition of root growth.

We tested two cytokinins, BA and kinetin. These cytokinins did not reduce the hypocotyl length at concentrations lower than 10 μM, and a concentration of 100 μM BA was required to obtain a reduction in hypocotyl length to 20% of the control value (equivalent to the inhibition induced by 1 μM 1-NAA). Kinetin was even less efficient. After a treatment with 100 μM BA for 7 d, the plantlets were still healthy, their hypocotyls were thicker than those of controls, and their cotyledons were expanded (Chory et al., 1994). Figure 7C shows that 9-AC and DIDS did not counteract the inhibition induced by 100 μM BA and did not prevent the other effects of BA on plantlets. Similar results were obtained with a lower concentration of BA (30 μM), which induced a moderate inhibition of hypocotyl growth (60%), and with kinetin (data not shown). Therefore, the anion-channel inhibitors that we have tested interfere specifically with auxin responses and not with responses to other growth factors.

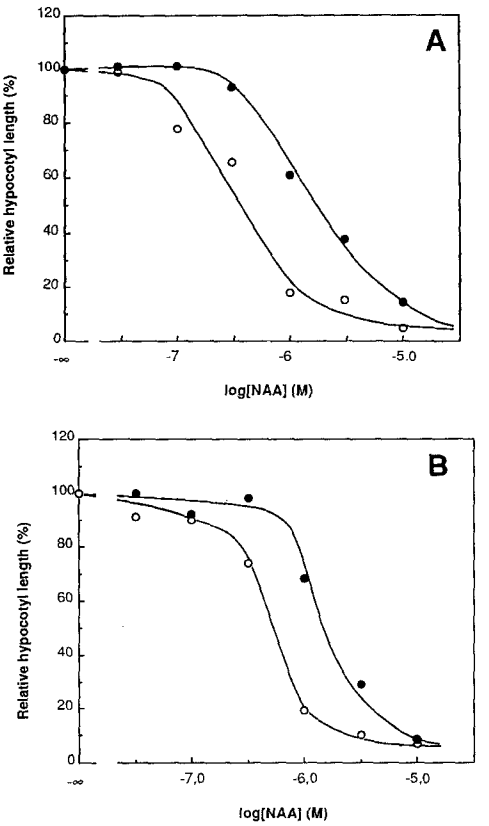

Figure 5. Shift of the dose-response curve for auxin-induced inhibition of hypocotyl elongation by 9-AC and DIDS. The relative length of the hypocotyls from plantlets grown in the dark for 7 d, transferred for the last 6 d to media containing different concentrations of 1-NAA in the absence (○) or presence (●) of 100 μM anion-channel blocker, is presented as a function of 1-NAA concentration. A, Dose-response curve to auxin in the absence or presence of 9-AC. The results of one representative experiment of two are presented. Note that in this case, the data were normalized to the mean hypocotyl lengths of untreated controls (13.8 mm) in the absence of 9-AC, and to the hypocotyl length of the plantlets treated by 100 μM 9-AC (15.6 mm), when auxin was added together with 100 μM 9-AC. B, Dose-response curve to auxin in the absence or in the presence of DIDS. The results of one representative experiment of two are presented. Note that in this case, the data were normalized to the mean hypocotyl lengths of untreated control (13.0 mm) in the absence of DIDS, and to the hypocotyl length of the plantlets treated by 100 μM DIDS (14.8 mm), when auxin was added together with 100 μM DIDS.

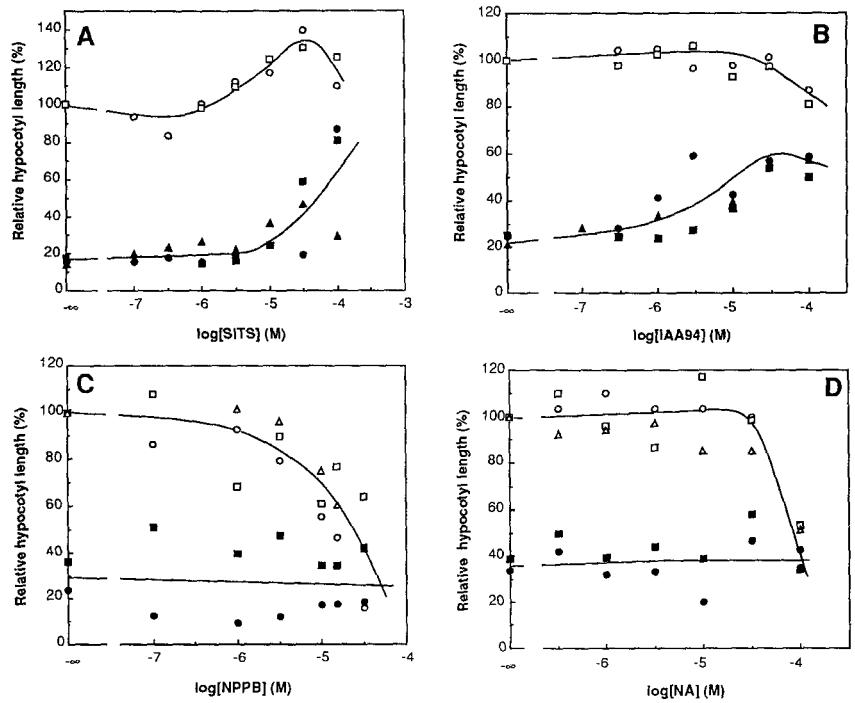

Figure 6. Interference of various anion-channel blockers with auxin-induced inhibition of hypocotyl elongation. The relative length of the hypocotyls from plantlets grown in the dark for 7 d, transferred for the last 6 d to media containing different concentrations of anion-channel blockers in the absence (open symbols) or presence (filled symbols) of 1 μM 1-NAA, is presented as a function of blocker concentration. A, Dose-response curves to SITS. The data are the results of three independent experiments. The mean lengths of the untreated control hypocotyls, taken as 100%, were 12.7 mm (○, ●), 12.5 mm (□, ■), and 9.3 mm (▲). B, Dose-response curves to IAA-94. The data are the results of three independent experiments. The mean lengths of the untreated control hypocotyls, taken as 100%, were 10.6 mm (○, ●), 13.8 mm (□, ■), and 8.9 mm (▲). C, Dose-response curves to NPPB. The data are the results of five independent experiments. The mean lengths of the untreated control hypocotyls, taken as 100%, were 12.4 mm (○), 13.1 mm (□), 11.7 mm (△), 10.9 mm (●), and 11.4 mm (■). D, Dose-response curves for niflumic acid. The data are the results of two independent experiments. The mean lengths of the untreated control hypocotyls, taken as 100%, were 11.5 mm (○, ●), 12.3 mm (□), and 14.9 mm (△, ■).

DISCUSSION

In this study we show that several molecules known as anion-channel blockers are able to prevent or attenuate the alterations of hypocotyl development induced by auxins. The anion-channel inhibitors attenuate the reduction of hypocotyl length induced by 1 μM 1-NAA with the following efficiency sequence: 9-AC = DIDS > SITS ≥ IAA94 ≫ niflumic acid = NPPB. The most efficient inhibitors, 9-AC, DIDS, and SITS, are also able to increase the hypocotyl length by 10 to 40% by themselves. However, the three main responses of hypocotyl to auxins (growth inhibition, decortication, and adventitious root formation) are not counteracted by all inhibitors. Whereas 9-AC prevents all three responses, DIDS prevents the inhibition of elongation and the decortication but not the adventitious root formation. The anion-channel blockers interfere specifically with auxin responses and not with responses to other hormonal signals, and act specifically at the level of the hypocotyl and not at other organs. This set of results suggests that the molecules that have been tested interact with one or several components involved in the control of hypocotyl elongation and/or in the perception or transduction of the auxin signal.

Morphological Effects of Anion-Channel Blockers and Anion-Channel Inhibition

The first question with regard to these results is the nature of the target of the inhibitors. All of the molecules tested have been characterized as anion-channel inhibitors in animal (Greger, 1990) and plant cells, and the active concentrations are in the range of concentrations that block anion channels in plants (Marten et al., 1992, 1993; Schwartz et al., 1995; S. Zimmermann, J.-M. Frachisse, S. Thomine, H. Barbier-Brygoo, and J. Guern, unpublished data). The other argument in favor of the hypothesis that the target of the molecules used is an anion channel is that they belong to distinct chemical families: 9-AC is a polycyclic molecule derived from anthracene, DIDS and SITS are stilbene derivatives, and IAA-94 has a different molecular structure. Thus, the main common feature of these molecules is that they have anion-channel inhibitor activity.

Two types of anion channel have already been characterized at the plasma membrane of Arabidopsis hypocotyl cells: a voltage-dependent anion channel (Thomine et al., 1995) and a blue-light-stimulated anion channel (Cho and Spalding, 1996). The voltage-dependent anion channel is

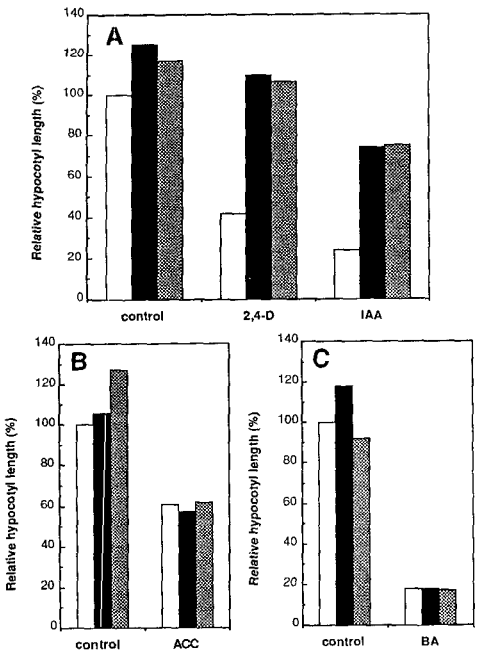

Figure 7. Auxin specificity of anion-channel blocker action. The lengths of the hypocotyls from plantlets grown in the dark for 7 d and treated by 2,4-D, IAA, ACC, BA, 9-AC, and DIDS as indicated were measured. A, Effects of 9-AC and DIDS on 2,4-D and IAA-induced inhibition of hypocotyl elongation. 2,4-D was applied at 0.1 μM, IAA at 3 μM, and 9-AC and DIDS at 100 μM. The results of one representative experiment out of three are presented. The mean length of the untreated control, taken as 100%, was 11.4 mm. B, Effect of 9-AC and DIDS on ACC-induced inhibition of hypocotyl elongation. ACC was applied at 3 μM, and 9-AC and DIDS at 100 μM. The results of one representative experiment of two are presented. The mean length of the untreated control, taken as 100%, was 12.9 mm. C, Effect of 9-AC and DIDS on BA-induced inhibition of hypocotyl elongation. The results of one representative experiment of two are presented. BA, 9-AC, and DIDS were applied at 100 μM. The mean length of the untreated control, taken as 100%, was 11.6 mm. Open bars, Control; black bars, 9-AC; and shaded bars, DIDS.

insensitive to 9-AC and DIDS (Thomine et al., 1997) and therefore appears very unlikely to be the channel involved in the responses that we describe here. The blue-light-stimulated anion channel is very sensitive to NPPB (Cho and Spalding, 1996). Whether it is blocked by the other inhibitors has not been investigated yet, but its high sensitivity to NPPB argues against its involvement in the physiological response shown in the present work. Therefore, the two anion channels already characterized in Arabidopsis hypocotyls have a pharmacological signature distinct from what could be expected from the effects described here at the whole-plant level. This calls for a more extensive description of the diversity of anion-channel types in hypocotyl cells, including the possibility that the anion channel involved could reside on the vacuolar membrane.

In this study we focused our attention on interference with the hypocotyl responses to auxins that are shared by most of the blockers tested. However, a striking feature of the action of the anion-channel blockers is that they induce a wide variety of responses in different organs of the plantlet. One possible explanation for these effects that are not common to several types of inhibitor molecules is that they could reflect nonspecific interactions with targets that are not anion channels. In fact, examples of nonspecific inhibition of plant ion channels by molecules developed to block animal ion channels have already been described. Antagonists of animal L-type Ca^{2+} channels are potent inhibitors of plant K^+-outward rectifiers (Terry et al., 1992; Thomine et al., 1994), and, more recently, anion-channel blockers have been shown to interact with plant K^+-outward rectifiers (Garrill et al., 1996). An alternative explanation for the variety of effects of anion-channel blockers is that it reveals the existence of a variety of anion channels exhibiting distinct pharmacological profiles and controlling various developmental responses of the plant.

Anion-Channel Blockers and Auxin Signaling

Our results argue for interference of anion-channel blockers with components involved in auxin signaling rather than those involved with the basic machinery of elongation: (a) the inhibitors not only restore normal hypocotyl elongation, they also prevent the decortication; (b) 9-AC and DIDS decrease the sensitivity of the hypocotyls to auxin rather than completely preventing auxin-induced inhibition of the elongation; and (c) the interference of anion-channel inhibitors with growth-inhibition responses is specific for auxins. The inhibition of elongation induced by all three auxins tested, 1-NAA, 2,4-D, and IAA, is prevented by 9-AC and DIDS, but the inhibition of elongation induced by ethylene and cytokinins is not.

The interaction with auxin can occur at various points: auxin transport, auxin perception, and auxin signal transduction. First, let us consider the hypothesis of an interaction with auxin transport. Greger (1990) has emphasized that most of the Cl^--channel inhibitors are also able to interact with anion transporters independently of the chemical family to which they belong. In our case, an interaction between the molecules used and the auxin-efflux carrier that catalyzes the excretion of the auxin anion (Lomax et al., 1995) could be suspected. If this hypothesis is true, naphthylphthalamic acid, a potent inhibitor of the auxin-efflux carrier, should mimic the effect of anion-channel blockers on hypocotyl growth and on auxin responses. We could show that naphthylphthalamic acid at concentrations between 0.1 and 10 μM does not counteract hypocotyl responses to auxin, and even enhances the sensitivity to auxins (S. Thomine and F. Lelièvre, unpublished data). Therefore, the target of the anion-channel blockers involved in hypocotyl elongation is not the auxin-efflux transporter. However, an interaction with the auxin-influx carrier, about which little is currently known (Lomax et al., 1995), cannot be excluded.

Alternatively, the interference may occur at the perception site for auxins. Indeed, the observed decrease in auxin sensitivity in the presence of 9-AC and DIDS (Fig. 5) may follow from a competition between auxin and

blocker molecules at a common binding site. Such a competition between auxin and other anionic compounds has been hypothesized to occur at the extracellular face of the R-type anion channel of guard cells (Lohse and Hedrich, 1995).

Finally, the anion-channel blockers may disturb the auxin transduction pathway. This could also account for the observed shift in auxin sensitivity. If this is the case, it is very unlikely that anion-channel blockers act through the inhibition of auxin-induced ethylene synthesis, because the 80% inhibition of elongation induced by auxin cannot be mediated by ethylene alone, which does not inhibit hypocotyl elongation by more than 40% at saturating concentrations of ACC (Fig. 7). Further analysis of this hypothesis would require a study using ethylene biosynthesis inhibitors and/or ethylene biosynthesis and response mutants.

We favor the hypothesis that molecules such as 9-AC, DIDS, and SITS act on an anion channel involved in early auxin signal transduction. The anion-channel blockers that counteract auxin-induced inhibition of hypocotyl elongation slightly stimulate the hypocotyl elongation in the absence of auxin. This increased length might reflect an interaction of anion-channel blockers with response to endogenous auxin, or it could be explained by a constitutive low activity of anion channels. This latter idea would be in agreement with what is already known of anion-channel modulation by auxins. Indeed, in guard cells (Marten et al., 1991) and tobacco suspension-cultured cells (Zimmermann et al., 1994), auxin does not strictly activate the anion channels but, rather, modulates their voltage-dependent regulation in a way that facilitates their activation by small depolarizations. A channel displaying both such auxin regulation and pharmacological properties fitting with the profile determined in this study has yet to be identified in hypocotyl cells. Studies using characterized auxin mutants (Hobbie et al., 1994) should help to localize the targets for anion-channel blockers within the auxin signal transduction cascade.

Finally, even if the mechanisms by which the tested inhibitors act are unknown, these molecules have a striking ability to counteract auxin responses and might prove to be useful tools for exploring auxin-signaling pathways. These molecules could be used to design genetic screens to select for new mutants impaired in auxin transduction. Ultimately, this approach may lead to the identification of the target(s) of these inhibitors that might be a component of auxin-signaling pathways.

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