Boron and Calcium Sites Involved in Indole-3-Acetic Acid Transport in Sunflower Hypocotyl Segments

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ABSTRACT

Sunflower (Helianthus annuus L. cv Russian Mammoth) hypocotyl segments deficient in either B or Ca exhibited a higher rate of potassium leakage, compared to nondeficient segments. Potassium leakage, used here as an indication of membrane integrity, was completely reversed by the addition of H$_3$BO$_3$ or Ca(NO$_3$)$_2$ to the incubation medium of the B-deficient or Ca-deficient hypocotyl segments, respectively. This role of B and Ca in membrane integrity, which may be important in the entry and exit of auxin in cells, is identified as the first site of action for each of these two essential elements in the basipetal secretion of auxin. A second site for B is postulated because auxin transport was not restored, even when K$^+$ leakage has been completely reversed to the nondeficient level, when B-deficient hypocotyls were incubated in B solution. This lack of reversibility of auxin transport implied that the incubation for 2 h in B solution was not enough to restore the auxin transport process. However, since the transfer of B-deficient seedlings to B solutions prevented further deterioration of auxin transport, these observations suggest that: (a) either an intact seedling, or a longer period of incubation of the hypocotyl in B solution, is required for the synthesis or maintenance of the functional second site for B; (b) B is probably essential in the synthesis of a ligand, which may or may not be needed to bind B, but which is essential in the basipetal transport of auxin. The second site for Ca in auxin transport, is indicated by the complete reversal of its inhibition in Ca-deficient hypocotyl, when incubated in Ca solution. The second site for Ca is thought to be directly involved in the secretion of auxin, in which Ca probably plays the role of a second messenger, as in stimulus-response coupling. The two sites for Ca can be distinguished from each other by their cation specificity. The requirement for Ca in the first site can be substituted by other divalent cations, while the second site is highly specific for Ca.

In the preceding article (17) it was shown that sunflower hypocotyl segments from seedlings deficient in either B or Ca had (a) a higher rate of K$^+$ leakage, (b) a higher rate of respiration, and (c) a lower rate of basipetal auxin transport than hypocotyls from control, nondeficient seedlings. Although similar processes were being affected by the deficiency of either element, separate sites of action for B and for Ca were postulated since the only instance wherein any of the inhibited process can be reversed was by the transfer of the seedling to a solution containing the deficient element. Thus, transfer of the B-deficient seedlings to solutions containing Ca, did not modify the seedling response. Furthermore, seedlings deficient in both B and Ca showed greater effect on the above processes, compared to seedlings deficient only in one element.

In the present communication, the sites for each element are further characterized and differentiated by their reversibility upon incubation of the deficient hypocotyl segments in B or Ca solutions.

MATERIALS AND METHODS

Hypocotyl segments from 5 to 8 d old sunflower (Helianthus annuus L. cv Russian mammoth) seedlings were used in the experiments. The conditions for growing the seedlings were described in the preceding article (17). Basically, the seeds were germinated between paper towels moistened with distilled H$_2$O and after 1 d the seeds with good radicle break were transferred to one-fourth strength Hoagland solution A (6) containing 0.023 mM H$_3$BO$_3$ (0.25 µg/ml B). After the 4th d, the seedlings were transferred to fresh Hoagland solution, or to solutions lacking B, Ca, or both elements.

Auxin transport was measured in 5 mm hypocotyl segments with donor agar (1%) containing 10 µM [1-¹⁴C]IAA (13.5 mCi/mM) at the apical end, and a blank agar receiver at the basal end. At the end of the transport period, the receiver agar was dried on filter paper and the radioactivity determined by liquid scintillation counting.

Potassium leakage from the hypocotyl segments was measured in segments identical to the ones used in auxin transport. The segments were incubated in 10 ml 0.1 mM KCl placed on a mechanical shaker. At the end of the incubation period, the increase in K$^+$ in the medium was determined using a K$^+$-specific electrode. All treatments were replicated at least three times (16).

RESULTS

Reversibility of Potassium Leakage in Hypocotyl Deficient in either Boron or Calcium. A certain amount of K$^+$ leaches out of the hypocotyl segments even when incubated in 0.1 mM KCl. The rate of K$^+$ leakage was higher in the B- or Ca-deficient hypocotyl segments compared with the control, nondeficient hypocotyls (17). The presence of H$_3$BO$_3$ or Ca(NO$_3$)$_2$ in the incubation medium of the B- or Ca-deficient hypocotyls, respectively, reduced the rate of K$^+$ leakage. Boric acid at 0.046 to 0.46 mM caused a reduction of K$^+$ leakage to the level of the nondeficient hypocotyl (Fig. 1A). In four out of six trials, incubation of the B-deficient hypocotyls in 4.6 mM H$_3$BO$_3$ caused a reduction of K$^+$ leakage to levels lower than the control, nondeficient hypocotyls. Increasing H$_3$BO$_3$ to 46 mM caused K$^+$ leakage to revert back towards increasing rates.

Likewise, the high rate of K$^+$ leakage in the Ca-deficient hypocotyl was reversed by the addition of Ca to the incubation medium (Fig. 1B). Concentrations of 0.1 to 1.0 mM Ca(NO$_3$)$_2$
FIG. 1. The reversibility of K⁺ leakage in B- or Ca-deficient hypocotyl segments. After 4 d in complete solution the seedlings were transferred to solutions deficient in B or Ca for 2 d. Three consecutive 5 mm hypocotyl segments were obtained from each of eight seedlings and incubated for 3 h in 10 ml 0.1 mM KCl alone or in the presence of various concentrations of B or Ca. The change in K⁺ content in the segment medium was determined using a K⁺-specific electrode. SE of three replicates is indicated by the vertical bars.

FIG. 2. Reversibility of K⁺ leakage in hypocotyls deficient in B and Ca. Hypocotyl segments from 5 d old seedlings, subjected to the absence of B and Ca on the last day, were incubated for 3 h in 10 ml 0.1 mM KCl with 0.46 mM H₂BO₃ or 1.25 mM Ca(NO₃)₂ or both. A K⁺-specific electrode was used to measure the changes in K⁺ in the incubation medium. SE of three replicates is indicated by the vertical bars.

effectively reduced K⁺ leakage to the level of the control nondeficient hypocotyl incubated in 0.1 mM KCl alone. Increasing the concentration of Ca(NO₃)₂ up to 37.5 mM led to further reductions in K⁺ leakage without apparent adverse effect.

Potassium Leakage in Hypocotyls Deficient in B and Ca. Hypocotyl segments deficient in both B and Ca exhibited higher rates of K⁺ leakage compared to hypocotyls deficient in either B or Ca (17). Incubation of B- and Ca-deficient hypocotyl segments in solution containing both 0.46 mM H₂BO₃ and 1.25 mM Ca(NO₃)₂ completely reversed the high rate of K⁺ leakage to the level of the nondeficient hypocotyl segments incubated in water alone (Fig. 2). When these doubly deficient hypocotyl segments were incubated in single solutions of either H₂BO₃ or Ca(NO₃)₂, only partial reversal of K⁺ leakage was obtained. This suggests very strongly that there is a B site, independent of a Ca site, and that both sites influence membrane permeability in the sunflower hypocotyl.

Incubation of the control nondeficient hypocotyl segments in single solution or combination of 0.46 mM H₂BO₃ and 1.25 mM Ca(NO₃)₂ did not significantly change the rate of K⁺ leakage relative to segments incubated in water alone.

Degree of Deficiency and Reversibility of Auxin Transport. To determine the effect of the severity of the deficiency on the reversibility of auxin transport, the seedlings were kept in the deficient medium for various lengths of time up to 4 d. The hypocotyl segments harvested each day were incubated for 3 h in water alone, 1.84 mM H₂BO₃ or 1.25 mM Ca(NO₃)₂ before the determination of auxin transport was made.

The hypocotyl segments did not show significant inhibition of auxin transport until 48 h after transfer of the seedlings to the deficient solution. Incubation of the segments in 1.84 mM H₂BO₃ showed a slight but significant increase in auxin transport, compared to identical hypocotyls incubated in water (Fig. 3A). At 72 and 96 h after transfer of the seedlings to the B-deficient solution, no significant differences in auxin transport were observed between hypocotyls incubated in water, B or Ca solutions.

The relative degree of reversal of auxin transport was much greater in Ca-deficient hypocotyls incubated in Ca than that of B-deficient hypocotyls incubated in B solutions. At all stages tested, incubation of the Ca-deficient hypocotyls in 1.25 mM Ca(NO₃)₂ caused a significant increase in auxin transport, compared to identical segments incubated in water (Fig. 3B). Auxin transport was inhibited by about 30% during the first 48 h of the seedlings in the Ca-deficient solution; however, a 2 h incubation of the segments in the Ca solution caused auxin transport to recover to the same level as that in hypocotyls that were never subjected to Ca deficiency. The reversal of auxin transport to the nondeficient level was not complete at 72 and 96 h in the Ca-deficient solution; however, the increase in auxin transport due to incubaion in Ca solution was still significantly higher compared to identical hypocotyls incubated in water.

The incubation of the Ca-deficient hypocotyls in 1.84 mM H₂BO₃ did not significantly affect the rate of auxin transport (Fig. 3B), but incubation of B-deficient hypocotyls in 1.25 mM Ca(NO₃)₂ caused a slight but significant increase in auxin transport (Fig. 3A).

Further experiments were conducted to determine the effect of wide ranges of H₂BO₃ or Ca(NO₃)₂ concentrations on the reversibility of auxin transport. Figure 4 shows the response of B- and Ca-deficient hypocotyl segments relative to hypocotyls of nondeficient seedlings incubated in water alone. In 17 out of 20 trials, the incubation of B-deficient hypocotyl in 0.046 to 0.46 mM H₂BO₃ caused only a slight but significant increase in auxin transport.
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as the concentration of Ca in the incubation medium was increased. Auxin transport was also increased in control nondeficient hypocotyls when incubated in Ca(NO₃)₂ solutions higher than 1.25 mM, which was the concentration of Ca in the seedling root medium.

Hyphocotyls Deficient in Boron and Calcium. Auxin transport declined much faster in hypocotyls deficient in both B and Ca, compared to hypocotyls deficient in either element (17). Figure 6 shows a decline in auxin transport by 20 to 30% after 24 h in Hoagland solution deficient in both Ca and B. Incubation of the doubly deficient hypocotyl segments in 0.46 mM H₃BO₃ gave equivocal results. The data in Figure 6 shows no significant increase in auxin transport relative to identical hypocotyls incubated in water; a significant promotion of auxin transport was obtained in a similar experiment (data not shown).

The incubation of hypocotyl segments deficient in both B and Ca in 1.25 mM Ca(NO₃)₂ caused auxin transport to increase to levels as high as that in nondeficient hypocotyls incubated in water (Fig. 6). The presence of both B and Ca in the incubation medium caused the highest increase in auxin transport, although in most cases, the increase was not significantly different from incubation in Ca solution alone.

Incubation of control, nondeficient hypocotyl segments in 0.46 mM H₃BO₃ or 1.25 mM Ca(NO₃)₂, or both, did not significantly affect auxin transport, compared to identical segments incubated in water (Fig. 6).

**DISCUSSION**

The foregoing results indicate that the essential elements boron and calcium probably occupy several sites of action, directly or indirectly involved in the basipetal transport of auxin. We believe there are at least two sites for each element and the succeeding discussion will elaborate this viewpoint. We prefer to identify these sites as the “first” and “second,” rather than “primary” and “secondary” to avoid connotation of greater significance of one site over the other. Actually, as will be seen later, the second sites for each element are more directly involved with auxin transport, and the first sites probably only indirectly involved.

First Sites for Boron and Calcium. The basipetal transport of auxin through tissues and organs involves the entry of this hormone at the apical plasmalemma of each cell followed by an active secretion at the basal plasma membrane. It is known that factors affecting the morphology of membrane structure (5, 7) also affect its permeability, and very likely the transport of auxin. Deficiency of either B or Ca is known to change membrane transport, compared to identical segments incubated in water alone. However, none of the B concentrations used caused auxin transport in B-deficient hypocotyls to increase to the same level as hypocotyls of nondeficient seedlings incubated in water alone (Fig. 4).

Incubation of Ca-deficient hypocotyl segments in increasing concentrations of Ca caused corresponding increases in auxin transport. Incubation in 1.25 to 37.5 mM Ca(NO₃)₂ caused auxin transport in Ca-deficient hypocotyl to exceed that of nondeficient hypocotyl incubated in water alone (Fig. 4).

The response of B-deficient hypocotyl to incubation in Ca solution (Fig. 3A) was tested further by incubation of the segments in a wider range of Ca concentrations. Figure 5 shows significant increases in auxin transport in B-deficient hypocotyls...
permeability in plant cells (10, 18). This is indeed the case with the B- or C-deficient hypocotyl segments, as shown by the increase in K⁺ leakage (Fig. 1, A and B; ref. 17). The membrane site affected by the lack of B appears to be different from the site affected by the lack of Ca, although deficiency of either element is manifested by an increase in K⁺ leakage. The presence of a distinct site for each element, rather than both elements affecting one site, is supported by the following results: (a) hypocotyl segments deficient in either B or Ca showed the increase in K⁺ leakage under conditions where the other element, B or Ca, is present in concentrations similar to those of the control, nondeficient hypocotyls, (b) the leakage involved under deficient only present control membrane to the operation of the second site. We propose to identify these B- or Ca-requiring entities that control membrane permeability of the plasmalemma, as the first site for each element.

Second Active Site for Boron. A second B-dependent site more directly involved in auxin transport is postulated. This is based on the finding that incubation of B-deficient hypocotyl segments in B solution, had very little effect in reversing auxin transport to the control, nondeficient level (Fig. 4), although K⁺ leakage was completely reversed (Fig. 1A). The ineffectiveness of the 2 h incubation in B solution in reversing the inhibition of auxin transport, suggests that B may or may not be an integral component of the second site. If B is an integral component of the second site, then probably there is a missing ligand in B-deficient cells which is needed to bind B. If B is not an integral component of the second site, then this element is probably required in the synthesis of a ligand, or formation of a structure, essential in the operation of the auxin transport carrier.

The transfer of B-deficient seedlings to solutions with B, prevented the further decline in auxin transport (17), suggesting that an intact seedling, or probably a longer period of incubation of the hypocotyl segment in B solution, is required for the formation of the functional second site for B.

The small significant increase in auxin transport, resulting from the incubation of B-deficient hypocotyls in B solution (Figs. 3A and 4), is probably the result of a greater membrane integrity, as shown by the reversal of K⁺ leakage (Fig. 1A). Therefore, this increase in auxin transport is most likely due to the first site for B, rather than the second site.

Second Active Site for Calcium. Unlike the second active site for B, the second site for Ca, like its first site, requires the presence of Ca during the time of auxin secretion. A simple incubation of the Ca-deficient hypocotyl segment in Ca solution increased auxin transport to the same level as that of hypocotyl segments never subjected to Ca deficiency (Fig. 3B). The absolute response to Ca depends on its concentration in the incubation medium (Fig. 4; Ref. 2). Even the control, nondeficient hypocotyl segments responded positively to incubation in Ca, when its concentration was higher than that present in the seedling root solution (Fig. 5; Ref. 2). This may be construed as indicating that our control, nondeficient seedlings may actually be Ca-deficient since the control root solution contained only 1.25 mM Ca(NO₃)₂ instead of 5 mM in full strength Hoagland solution. However, it was shown earlier that increasing the concentration above 1 mM Ca in the root solution did not result in an increase of the rate of auxin transport (2).

The incubation of B-deficient hypocotyl in Ca solutions resulted in higher rates of auxin transport compared with incubation in B solutions (Figs. 3, A and B, and 5). This effect could not be due to an interaction of the two elements, such as Ca making B more available since addition of B itself has very little effect.

We believe the increase in auxin transport resulting from incubation of hypocotyl segments in Ca solutions, regardless of whether they were deficient in B or Ca, or nondeficient (Figs. 3 and 5) is probably the manifestation of the direct role of Ca in the secretion of IAA. We consider this the second active site for Ca. One of the major roles of Ca in animal cells is in the secretion of many kinds of substances, from inorganic ions, proteins and enzymes, to neurotransmitters and other hormones (Refs. 1, 12, 14 for animal cells; 9, 15 for plant cells).

The first Ca site cannot be distinguished from the second Ca site by their response to Ca concentration in the incubation medium (compare Figs. 1B and 4). However, the possibility that two sites exist is indicated by their cation specificity. It is widely known that the role of Ca in the first site controlling membrane permeability, can be taken over to a certain extent by other polyvalent cations (11, 19). Thus, K⁺ leakage was significantly reduced in the sunflower hypocotyl segment incubated in solutions of Sr and to a certain extent Mg; these cations, however, do not have significant effects on auxin transport (Table I; Ref. 2).

The exact mechanism or role of Ca in the secretion of auxin and such substances as hydrolases in the barley aleurone cells (9), or peroxidases in spinach cell suspension (15), is not known. Several lines of investigation are being pursued by workers using animal cells (4). There is evidence for the possible involvement of the Ca-binding protein calmodulin in the phosphorylation of proteins involved in secretion (3). This could explain the specificity observed with the effectiveness of μM Ca in the cytoplasm in the presence of mM Mg. The polyphosphoinositides have also been implicated in the secretion of substances (8). In most of the above studies, the model involves the presence of the secretory substance in vesicles and their secretion mechanism by exocytosis. There is no evidence or reason to suppose that the transport of auxin involves vesicle exocytosis. However, a recent treatise also questions the present consensus regarding the significance of vesicles and exocytosis as a means of secretion mechanism.
(13).

In summary, the behavior of hypocotyl segments deficient in B, Ca, or both, in terms of K⁺ leakage and auxin transport, together with the reversibility of these processes, made us deduce that each element, B and Ca, has at least two sites of action important in the transport of auxin. The first site for each element controls general membrane permeability. The second site for B is probably involved in the synthesis of a ligand which could be an integral component of the auxin transport protein, or an entity associated with it. Calcium in the second site is hypothesized to play the role of a second messenger in the operation of the auxin transport carrier, as in the mechanism of stimulus-secretion coupling found in animal cells.

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