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AUXIN CONTROL OF CALCIUM-MEDIATED PEROXIDASE SECRETION BY AUXIN-DEPENDENT AND AUXIN-INDEPENDENT SUGARBEET CELLS

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Key Word Index—*Beta vulgaris*; Chenopodiaceae; sugarbeet; peroxidase; secretion; calcium; suspension culture; habituation; callus.

Abstract—Peroxidase secretion by three sugarbeet cell lines in the absence of Ca^{2+} was unaffected by the addition of auxins of different chemical structures. The six auxins tested had no effect on the Ca^{2+}-promoted peroxidase secretion by habituated non-organogenic cells (HNO), but they enhanced the secretion by normal non-organogenic cells (NNO) and three of them inhibited it in the case of the habituated organogenic line. A 5-week subculture of the auxin-independent HNO callus on auxin-containing media increased the sensitivity of cells to Ca^{2+} and their responsiveness to auxins in suspension cultures. Conversely, subculture of the auxin-requiring NNO callus on auxin-deficient medium induced a decreased sensitivity to Ca^{2+} and a complete loss of response to auxins. The possible regulation of peroxidase secretion by Ca^{2+}- and auxin-receptors is discussed.

INTRODUCTION

Peroxidase (EC 1.11.1.7) release into the liquid media of *in vitro* cultured plant cells is well established [1] but it is only recently that the process has been shown to be energy dependent [2–4] and mediated by Ca^{2+} [5–7], as is the case for peroxidase exocytosis in animals [8]. Although roles in cellular growth and differentiation have been proposed for these extracellular peroxidases [1], the physiological control of their active secretion remains unknown. The involvement of phytohormones is expected [9, 10] but unexplored.

In order to investigate the possible involvement of growth regulators, the effect of six auxins of different chemical nature was tested on calcium-mediated peroxidase secretion by auxin-dependent and auxin-independent sugarbeet cells.

RESULTS

Effect of auxins in the suspension cell cultures on peroxidase secretion

As shown in earlier papers [3, 6, 7], peroxidase secretion, both in the presence and absence of Ca^{2+}, is largely dependent on the cell line. In the absence of Ca^{2+}, the release of peroxidases is rather low for all three types of cells, although somewhat greater in the HO† line. Figure 1 shows that none of the six auxins examined significantly modified this release in the three suspension cultures after a 90 min incubation. Ca^{2+} promoted this release by some 400% in the HNO cells up to more than 700% in the HO cells. The concentration used was sufficient to induce the maximum response in spinach-cell suspension cultures [2]. HNO cells, even in the presence of Ca^{2+}, were completely insensitive to auxins, at all the concentrations tested. NNO cells responded to increasing amounts of the six auxins by an increased secretion of peroxidases. BSAA and PRB-8 showed the greatest effect; AIA and IBA were the least effective. By contrast, in the HO cell suspension culture three auxins (NAA, BSAA, PRB-8) among the six studied induced a decreased peroxidase secretion.

Effect of auxin starvation in the culture medium of NNO callus and of auxin addition to the culture medium of HNO callus

Calli from the auxin-requiring NNO sugarbeet line were subcultured for 5 weeks on auxin (2, 4-D and BAP)-deficient media or in the presence of the auxins 2,4-D or IBA alone. Calli from the auxin-independent HNO line derived from hormoneless media were cultured on auxin-containing media for 5 weeks. Cells derived from these various conditions were suspended in liquid media and their Ca^{2+}-mediated peroxidase secretion was measured in the presence of the six different auxins. Table 1 shows that normal NNO callus cells, when cultured in the absence of regulators and transferred to suspension culture, secreted less peroxidase in response to Ca^{2+}. It also shows that such auxin-deprived cells no longer responded to the different auxins.

Conversely, auxin-independent HNO cells cultured on auxin-containing media for 5 weeks before their transfer to liquid medium secreted more enzymes in response to Ca^{2+} and even more under the effect of the different auxins tested (Table 2). Thus, depending on the culture

†Abbreviations: NNO, normal non-organogenic cells; HNO, habituated non-organogenic cells; HO, habituated organogenic cells; BAP, benzylaminopurine; BSAA, [benzo(b)selenienyl-3]acetic acid; 2,4-D, dichlorophenoxyacetic acid; IAA, indolylacetic acid; IBA, indolylbutric acid; NAA, naphthalene-acetic acid; PRB-8, α-chloro,β(3-chloro, σ-totyl)propionitrile.

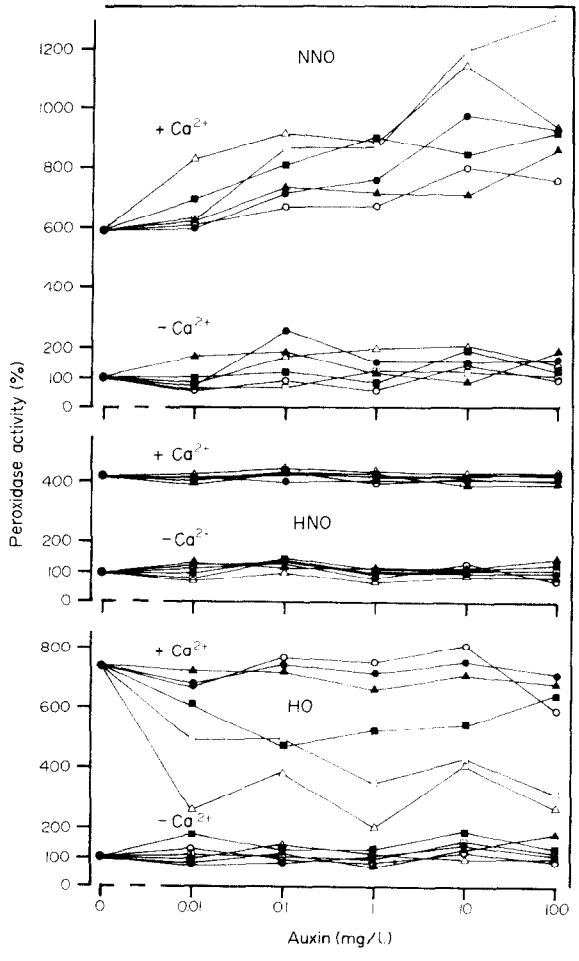

Fig. 1. Effect of increasing concentrations of six different auxins on the secretion of peroxidases by the three sugarbeet cell types (NNO, HNO, HO) in the presence or absence of 1 mM $CaCl_2$. Peroxidase activity (ΔA_{470}/g fr. wt per 10 min) was measured in the media 90 min after the addition of auxin and $CaCl_2$. Controls without Ca^{2+} and without auxin were fixed at 100%. ▲, IBA; ■, NAA; ●, 2, 4-D; Δ, PRB-8; □, BSAA; O, IAA.

conditions, normal cells can lose their sensitivity to auxins and habituated ones may acquire it. As far as the short-term effect of auxins is concerned, it appeared that there was no great difference among the six regulators tested. Suspension cultures obtained from callus grown on IBA-containing medium always exhibited a greater response to Ca^{2+} than suspension cultures from 2,4-D-containing medium. It also appears from Tables 1 and 2 that BAP increased the effect of 2,4-D on Ca^{2+}-mediated peroxidase secretion, but that 2,4-D and BAP together were less effective than IBA.

DISCUSSION

Response to calcium

The different sensitivity of the three lines of cells towards Ca^{2+} had already been reported [6, 7] and it had been hypothesized that the differences could be related to the sensitivity of the three types of cells towards exogenous auxin [3]. It is known that Ca^{2+} may interact with auxin in the control of cell elongation [11] and that membranes release Ca^{2+} in response to auxin [12]. The Ca^{2+} release, following auxin treatment, is accompanied by a decrease in membrane-associated sites for Ca^{2+} binding [13]. Conversely, Ca^{2+} could inhibit the specific auxin binding to membranes [14]. The present results demonstrate that the ability of this ion to enhance peroxidase secretion actually depends on the previous auxin treatment of the cells rather than on the line itself, since habituated cells transferred to an auxin-containing medium become more responsive to Ca^{2+}, while normal cells deprived of auxin for 5 weeks become less responsive. This could be explained by the possible existence of a Ca^{2+} receptor, the level of which would be determined by the exogenously supplied auxin during the culture of the callus. Such Ca^{2+}-binding proteins, able to displace membrane Ca^{2+}, have been shown to be present in the membrane-free supernatant of higher plant homogenates [15]. Changes in subcellular Ca^{2+} compartmentation through modulation of Ca^{2+} pumps or gates is another attractive possibility for auxin action [16], in view of the second messenger role of Ca^{2+} and the occurrence of calmodulin, a widespread Ca^{2+}-dependent regulatory protein, in plants [17]. Inhibitors of calmodulin such as phenothiazine have already been reported to reduce strongly the effect of Ca^{2+} on peroxidase secretion [2, 3]. The possible relation between auxins and calmodulin, however, still remains to be elucidated.

Sensitivity towards auxin

HNO cells, which do not require the presence of exogenous auxin for their growth, were insensitive to the six auxins tested during secretion experiments. NNO cells, which require such a supply, exhibited a greater Ca^{2+}-mediated secretion in the presence of auxins. The effectiveness of each of the six auxins tested in promoting the effect of Ca^{2+} on peroxidase secretion was different in each case. It seemed to be dependent on the cell line and on the hormonal composition of the culture medium. HO cells, which contain a lower level of IAA [18] and are inhibited in their bud-forming capacity in the presence of exogenously supplied auxins (data not shown), secrete less peroxidase when three of the six auxins (the three most active on this material?) are added to calcium (Fig. 1). Tables 1 and 2 show that none of the observed responses corresponded to inherent properties of the cell lines. Long-term (5 weeks) pretreatment of HNO and NNO calli with and without auxin respectively reversed the short-term (90 min) secretory reactions to auxins in the presence of calcium. Thus, in addition to the putative role of the auxin modulation of a calcium receptor, the problem can also be posed in terms of auxin receptors induced by auxins themselves in the course of the culture of the callus. It is already known that auxin induces an increase in micro-viscosity of plasma membranes. The mean width of membranes after auxin treatment is significantly decreased. This ultrastructural effect is specific for active auxins [19]. It is known that in animals the presence of hormones may affect the number and the properties of hormone receptors [20].

EXPERIMENTAL

Tissue cultures. Cell suspensions from normal auxin-

Table 1. Effect of auxins in the callus culture media on the short-term effects of auxins (100 mg/l.) and calcium (1 mM) on peroxidase secretion (ΔA_{470}/g fr. wt per 10 min) in 5-week-old NNO cell suspension cultures

Suspension media (90 min)	Callus culture media (5 weeks)			
	Original 2,4-D (0.01 mg/l.) BAP (0.01 mg/l.)	0	2,4-D (0.01 mg/l.)	IBA (0.01 mg/l.)
0	0.043 ± 0.003	0.026 ± 0.005	0.031 ± 0.004	0.022 ± 0.004
0 + Ca^{2+}	0.219 ± 0.011	0.067 ± 0.006	0.176 ± 0.012	0.340 ± 0.048
NAA	0.028 ± 0.013	0.009 ± 0.003	0.030 ± 0.007	0.033 ± 0.006
NAA + Ca^{2+}	0.329 ± 0.026	0.054 ± 0.010	0.255 ± 0.033	0.573 ± 0.038
2,4-D	0.034 ± 0.007	0.018 ± 0.007	0.024 ± 0.010	0.040 ± 0.004
2,4-D + Ca^{2+}	0.300 ± 0.015	0.045 ± 0.008	0.201 ± 0.011	0.427 ± 0.033
IBA	0.022 ± 0.003	0.023 ± 0.004	0.017 ± 0.009	0.030 ± 0.005
IBA + Ca^{2+}	0.295 ± 0.016	0.044 ± 0.005	0.185 ± 0.003	0.389 ± 0.010
BSAA	0.037 ± 0.002	0.019 ± 0.004	0.013 ± 0.005	0.033 ± 0.003
BSAA + Ca^{2+}	0.379 ± 0.015	0.055 ± 0.007	0.209 ± 0.003	0.390 ± 0.012
PRB-8	0.024 ± 0.009	0.015 ± 0.005	0.030 ± 0.007	0.033 ± 0.006
PRB-8 + Ca^{2+}	0.302 ± 0.020	0.067 ± 0.008	0.184 ± 0.008	0.329 ± 0.012
IAA	0.025 ± 0.011	0.017 ± 0.001	0.012 ± 0.006	0.037 ± 0.003
IAA + Ca^{2+}	0.259 ± 0.020	0.041 ± 0.017	0.210 ± 0.004	0.353 ± 0.026

Table 2. Effect of auxins in the callus culture media on the short-term effect of auxins (100 mg/l.) and calcium (1 mM) on peroxidase secretion (ΔA_{470}/g fr. wt per 10 min) in 5-week-old HNO cell suspension cultures

Suspension media (90 min)	Callus culture media (5 weeks)			
	2,4-D (0.01 mg/l.) BAP (0.01 mg/l.)	Original 0	2,4-D(0.01 mg/l.)	IBA (0.01 mg/l.)
0	0.010 ± 0.004	0.013 ± 0.005	0.010 ± 0.001	0.012 ± 0.001
0 + Ca^{2+}	0.139 ± 0.006	0.087 ± 0.003	0.109 ± 0.015	0.223 ± 0.010
NAA	0.007 ± 0.003	0.013 ± 0.003	0.008 ± 0.002	0.020 ± 0.004
NAA + Ca^{2+}	0.184 ± 0.007	0.084 ± 0.001	0.129 ± 0.012	0.296 ± 0.015
2,4-D	0.009 ± 0.004	0.016 ± 0.005	0.008 ± 0.002	0.021 ± 0.003
2,4-D + Ca^{2+}	0.168 ± 0.021	0.089 ± 0.003	0.130 ± 0.017	0.295 ± 0.020
IBA	0.015 ± 0.004	0.017 ± 0.006	0.010 ± 0.001	0.019 ± 0.001
IBA + Ca^{2+}	0.172 ± 0.009	0.084 ± 0.001	0.128 ± 0.004	0.362 ± 0.028
BSAA	0.010 ± 0.005	0.018 ± 0.005	0.003 ± 0.001	0.019 ± 0.002
BSAA + Ca^{2+}	0.182 ± 0.006	0.084 ± 0.002	0.123 ± 0.010	0.220 ± 0.004
PRB-8	0.008 ± 0.002	0.016 ± 0.006	0.008 ± 0.001	0.018 ± 0.004
PRB-8 + Ca^{2+}	0.165 ± 0.006	0.082 ± 0.001	0.110 ± 0.013	0.174 ± 0.013
IAA	0.015 ± 0.001	0.011 ± 0.002	0.005 ± 0.002	0.019 ± 0.004
IAA + Ca^{2+}	0.167 ± 0.011	0.086 ± 0.002	0.109 ± 0.007	0.275 ± 0.022

dependent (NNO), habituated organogenic (HO) and non-organogenic (HNO) auxin-independent calli of sugarbeet (*Beta vulgaris* L. altissima) were used. Experimental conditions for obtaining and maintaining these tissues in stock cultures have been reported elsewhere [21]. Inocula of such calli were taken during the exponential phase of growth and transferred into 150 ml flasks containing 50 ml of the medium used for callus growth, without agar. BAP (10 μg/l.) together with 2,4-D (10 μg/l.) or IBA (10 μg/l.) alone were added to the media containing normal or habituated cells. The flasks were continuously shaken under a 16 hr photoperiod with fluorescent white light (3000 lx) at 20°. After 5 weeks of culture, the cells were

transferred to fresh media all deprived of growth regulators, and assayed for peroxidase release 16 hr later.

Peroxidase release. For each assay, cells (*ca* 200 mg fr. wt) were resuspended in 2 ml buffer containing 50 mM *N*-morpholino-3-propane-sulphonic acid adjusted to pH 6.2 with tris-hydroxymethylaminomethane and 20 mM KCl. Each aliquot was left for 15 min without stirring. $CaCl_2$ (1 mM), 1BA, NAA, 2,4-D, PRB 8 (see ref. [22]), BSAA (see ref. [23]), and IAA were added to cell suspensions at the concns specified in each case using small vols. of stock solns. During the assays, the cell suspensions were gently stirred at fixed intervals.

Peroxidase activity present in the medium after 90 min was assayed by taking 50 μl samples. The assay was performed at 20° in 2.5 ml of 40 mM Pi buffer (pH 6.1) containing 8 mM guaiacol and 2 mM H_2O_2. The increase in absorbance at 470 nm was read after 10 min.

All experiments were repeated at least three times.

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