The Influence of Growth Regulators on Membrane Permeability in Cultures of Winter Wheat Cells

Maria Filek[a,*], Jolanta Biesaga-Kościelniak[a], Izabela Marcińska[a], Ivana Macháčková[b], and Jan Krekule[b]

[a] Department of Plant Physiology, Polish Academy of Sciences, 30-239 Kraków, Podłużna 3, Poland. Fax: +48 12 425 33 20. E-mail: mariafilek@excite.com
[b] Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 135, 160502 Praha 6, Czech Republic

* Author for correspondence and reprint requests

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The effect of plant growth substances (IAA, 2,4-D, zeatin, kinetin, zearalenone) were studied on membrane properties of the cells of embryogenic (E) and non-embryogenic (NE) calli derived from immature inflorescences (inf) or embryos (emb) of winter wheat. Calli initiated from inflorescences show higher permeability. The ion leakage from cells of E calli was higher than from cells of NE calli. Growth regulators were used in concentrations of 2–30 mg/l (about 10–140 μM). All tested growth substances increased ion leakage from NE emb cells, IAA, zeatin and kinetin being most effective. In NE inf cells the effect of growth substances was similar as in NE emb, but much weaker. In E cells of both types (inf and emb) growth substances decreased ion leakage. Changes in the leakage of potassium and calcium ions were similar to those in total ion leakage. The uptake of labelled auxins (IAA and 2,4-D) was higher in NE cells (especially in NE inf) than in E cells. The endogenous level of IAA was higher in E cells than in NE cells and in inf cells than in emb cells. The importance of auxin in determining permeability of cell membranes is discussed.

Key words: Growth Regulators, Cells, Permeability

Introduction

Selectively permeable lipoprotein membranes are essential for maintaining the internal environment of cells. By controlling which molecules enter and leave the cell, the plasma membrane helps to conserve important resources and keeps the cell in osmotic equilibrium with extra-cellular fluid. Energy-dependent protein "pumps" embedded in the plasma membrane establish differences in ion concentrations and electrical charge between the inside and outside of the cell (Ohirogge and Browse, 1995).

Membrane permeability strongly depends on the physical state of the lipids. The influence of changes in the lipid composition on bilayer permeability has been studied in detail by van Bilsen and Hoekstra (1993). Phase transition and lateral phase separation of the membrane have been proposed as primary molecular events resulting in membrane leakiness (McKersie *et al.*, 1988; Raison and Orr, 1990; Hoekstra *et al.*, 1992). Changes in lipid contents due to the influence of stress factors and developmental processes indicate the significant role of a specific membrane structure in the physiological processes (Wilson and Stillwell, 1991; Mérillon *et al.*, 1993; Surjus and Durand, 1996; Ishikawa and Wagatsuma, 1998). Our earlier results showed some differences in the lipid composition of non-embryogenic and embryogenic cells and after phytohormone (kinetin, IAA) treatment (Laggner *et al.*, 2003). Stillwell *et al.* (1984, 1985) indicated the role of kinetin, BAP and zeatin in permeability changes of the liposome system and the influence of these phytohormones on physicochemical properties of the liposome membrane. Our zeta potential investigation recently described (Filek *et al.*, 2002) showed that growth regulators (kinetin, 2,4-D, zearalenone) can affect membrane electric properties and charge.

The aim of the present study was to investigate the influence of phytohormones on membrane permeability in embryogenic and non-embryogenic callus tissue (callus tissues of winter wheat forming or not forming embryos – see Materials and Methods). It was found earlier that winter wheat plants regenerated from calli derived from

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immature inflorescences headed and flowered without vernalization. On the contrary, those regenerated from young embryos failed to flower if not exposed to cold first. As plant growth regulators are the main factors determining regeneration potential of plant tissue cultures (Michalczuk *et al.*, 1992; Piccarelli *et al.*, 2001; An *et al.*, 2001), it could be anticipated that growth regulators are responsible for these physiological differences (Hobbie, 1998; Leyser, 2004). The plasmalemma is considered to be one of the primary sites of interaction between chemicals and plant cells (Wilson and Stillwell, 1991). Variation in the membrane stability results in an increased permeability and leakage of electrolytes. Changes of the membrane permeability brought about by treatment with plant growth regulators were measured in winter wheat callus cells initiated from either of two: inflorescences or embryos. Treated calli of both types were either in the non-embryogenic or in the embryogenic state. The effect of following phytohormones was observed: 2,4-D and IAA (auxins), kinetin and zeatin (cytokins) and zearalenone (an estrogenic substance shown to have a hormone-like character).

Materials and Methods

Winter wheat cv Kamila *in vitro* cultures were initiated from 10–15 mm immature inflorescences (inf) and 1.5–2 mm zygotic embryos (emb) of greenhouse-grown plants. Explants were sterilised in 70% EtOH for 3 min, 3.1% NaOCl (60% commercial bleach with 5.25% NaOCl) for 15 min and rinsed three times with sterile water. Non-embryogenic calli (NE) were obtained by culturing of explants for 3 months in darkness at 27 °C on the Murashige-Skoog medium supplemented with 30 g/l sucrose, 2 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) and 6 g/l agar. Calli were transferred onto the fresh medium every three weeks. For induction of embryogenesis (E), calli were transferred onto the same medium devoid of 2,4-D.

For the electric conductivity measurements about 5 g of calli samples were washed twice and subsequently incubated in 0.6 M mannitol solution (control) or the same solution with various concentrations (2–30 mg/l) of growth regulators: 2,4-D (2,4-dichlorophenoxyacetic acid), IAA (indole-3-acetic acid), kinetin (6-furfurylaminopurine), zeatin [6-(4-hydroxy-3-methylbut-2-enylamino)purine] and zearalenone [6-(10-hydroxy-6-oxo-*trans*-1-undecyl)-2,4-dihydroxybenzoic acid lactone]. Growth regulators were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Electrolyte leakage was determined by conductometry after incubating of the calli in the incubation medium for 1 h and after 24 h from the moment calli were boiled at 100 °C for 15 min (total leakage). Values of ion leakage were calculated as the membrane permeability (ion leakage/g fresh mass after 1 h incubation) and as the relative permeability (membrane permeability/total leakage)$_{\text{growth regulators}}$/(membrane permeability/total leakage)$_{\text{control}}$.

Leakage of K^+ and Ca^{2+} ions was measured in electrolyte solution after 1 h incubation of calli in water or water solution of phytohormones at a concentration of 10 mg/l. Ca^{2+} and K^+ ion concentration was detected by the method of flame atomic absorption (Perkin Elmer Model PE-3100) at λ = 422.7 nm and λ = 766.5 nm, respectively.

Accumulation of [^{14}C]-2,4-D and [^{14}C]-IAA in NE and E cells was measured after 2 h of incubation of these calli in the solutions of both auxins (2–10 mg/l, the specific radioactivity of both auxins was 380 MBq mmol^{-1}). Then calli were washed in 0.6 M mannitol solution and homogenized in Bray solution. About 5 μl of H_2O_2 to discolour (samples were generally colourless) and 20 μl of acetic acid (to luminescence quenching) were added. The radioactivity was detected by a scintillation counter (Beckman LS 5801).

Endogenous levels of IAA were analysed by the HPLC method. The tissue (0.5 g) was homogenized in liquid N_2 and extracted twice with 80% methanol containing 100 mg/l butylated hydroxytoluene (BHT). [^{3}H]-IAA (Amersham, specific activity 2.7 TBq mmol^{-1}, applied activity 3 kBq) was used as internal standard. After centrifugation (4 °C, 15 min, 10,000 × *g*) the supernatant was evaporated to the water phase, the same volume of 0.5 M K_2HPO_4 was added and the extract partitioned against diethyl ether. Ether was discarded and the extract was applied on a polyvinylpyrrolidone column and then washed with 10 ml 0.1 M K_2HPO_4. The eluate was acidified with 0.3 M H_3PO_4 to pH 2.7 and partitioned against diethyl ether. The ether phase was evaporated, dissolved in 300 μl of the mobile HPLC phase and the IAA content was determined using HPLC with a fluorimetric detector as described by Eder *et al.* (1988).

Growth regulator treatment [mg/l] ([μM])		Ion leakage [μS/g fresh mass]			
		Inflorescences		Embryos	
		NE	E	NE	E
0		138 ± 3	170 ± 5	118 ± 3	125 ± 3
2,4-D	5 (20)	138 ± 4	140 ± 5	150 ± 5	105 ± 4
	30 (140)	148 ± 5	135 ± 5	158 ± 6	110 ± 4
IAA	5 (20)	161 ± 4	129 ± 4	185 ± 5	105 ± 4
	30 (140)	165 ± 5	124 ± 5	195 ± 4	111 ± 4
Kinetin	5 (20)	159 ± 5	116 ± 5	180 ± 6	108 ± 3
	30 (140)	165 ± 2	121 ± 4	185 ± 5	103 ± 3
Zeatin	5 (20)	145 ± 4	135 ± 5	180 ± 5	100 ± 4
	30 (140)	150 ± 6	120 ± 5	185 ± 5	100 ± 4
Zearale-	5 (15)	145 ± 6	145 ± 6	145 ± 5	115 ± 4
none	30 (100)	125 ± 5	120 ± 5	119 ± 5	95 ± 4

Table I. Influence of growth regulators treatment (1 h incubation) on ion leakage of non-embryogenic (NE) and embryogenic (E) winter wheat calli initiated from inflorescences and embryos. 0 – control, without growth regulators. Values represent averages from 5 replicates ± SD.

Results

Membrane permeability of winter wheat calli tissue initiated from inflorescences was higher than the one obtained from embryos (Table I). Additionally, E cells exhibited higher ion leakage in comparison to NE cells. All investigated phytohormones depending on their concentration increased ion leakage from NE emb. Zearalenoene increased the permeability in NE emb at a lower concentration, whereas at higher concentrations it had no effect or slightly decreased the permeability. For NE inf, IAA and kinetin increased the ion leakage at all applied concentrations; 2,4-D and zeatin only at the highest level and zearalenone acted similarly as in NE emb (Table I). In the case of E tissues, a decrease in membrane permeability after phytohormone treatment took place, but it was more visible in the E inf cells.

The same character of changes of permeability in NE and E cells was observed for calculated relative ion leakage (Fig. 1). Growth regulator treatment generally increased NE emb permeability by 5–12% and of NE inf by 2.5–7%. Among the investigated regulators, IAA and kinetin were most effective. Zeatin and 2,4-D effects were less significant whereas zearalenone blocked ion flux at higher concentrations. For E calli, a decrease in ion leakage by 7–12% for inf and by 2.5–7% for emb after phytohormone treatment was observed. The effectiveness was in the following order:

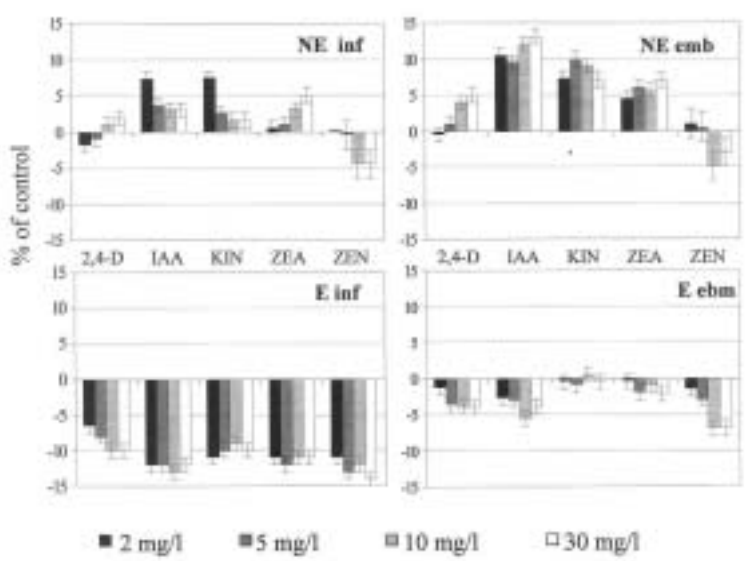

Fig. 1. Relative ion leakage (in %) calculated as the ratio of ion leakage to total leakage in the presence of growth regulators divided by ion leakage to total leakage without growth regulators (control). For details see Materials and Methods. KIN, kinetin; ZEA, zeatin; ZEN, zearalenone. Values represent averages from 5 replicates ± SE.

Table II. Influence of growth regulators on potassium and calcium ion efflux from non-embryogenic (NE) and embryogenic (E) tissue culture of winter wheat initiated from inflorescences (inf) and embryos (emb). Growth regulators were used at 10 mg/dm^3. Values represent averages from 5 replicates ± SE.

Growth regulator treatment	Efflux of ions [μg/l]							
	[K^+]				[Ca^{2+}]			
	Tissue cultures							
	NE inf	E inf	NE emb	E emb	NE inf	E inf	NE emb	E emb
0	3.5 ± 0.2	4.5 ± 0.3	4.0 ± 0.2	4.8 ± 0.4	3.1 ± 0.2	4.0 ± 0.3	2.5 ± 0.2	3.3 ± 0.2
2,4-D	3.7 ± 0.1	3.0 ± 0.1	4.8 ± 0.3	4.5 ± 0.2	3.3 ± 0.3	3.0 ± 0.3	4.0 ± 0.3	3.1 ± 0.3
IAA	3.9 ± 0.3	3.0 ± 0.1	5.0 ± 0.3	3.5 ± 0.3	3.5 ± 0.2	3.5 ± 0.3	5.0 ± 0.4	2.8 ± 0.1
Kinetin	4.9 ± 0.4	3.6 ± 0.1	4.9 ± 0.2	4.3 ± 0.3	3.5 ± 0.1	3.9 ± 0.2	5.0 ± 0.4	3.0 ± 0.1
Zeatin	4.1 ± 0.3	2.8 ± 0.2	4.7 ± 0.2	4.3 ± 0.2	3.8 ± 0.3	2.8 ± 0.1	5.0 ± 0.3	3.0 ± 0.2

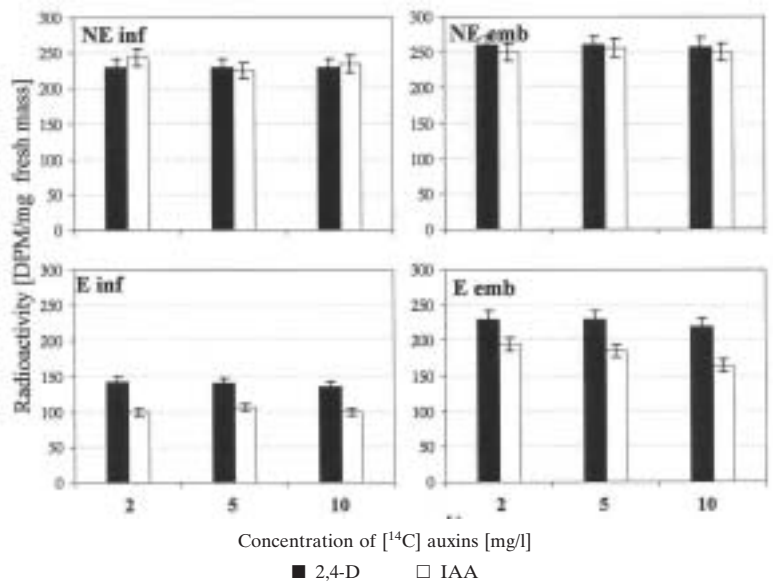

Fig. 2. Radioactivity of non-embryogenic (NE) and embryogenic (E) winter wheat tissue cultures initiated from inflorescences (inf) and embryos (emb) after incubation with [^{14}C]-IAA (320 MBq/mmol) or [^{14}C]-2,4-D (380 MBq/mmol). Auxins were used at 2, 5 and 10 mg/l. For details see Materials and Methods. Values represent averages from 5 replicates ± SE.

auxins (IAA, 2,4-D) > zearalenone > cytokinins (kinetin, zeatin).

Changes in the efflux of K^+ and Ca^{2+} ions from winter wheat cells were similar to those of the total ion leakage (Table II).

Incubation in 2, 5 and 10 mg dm^{-3} of [^{14}C]-IAA or [^{14}C]-2,4-D resulted in almost the same level of auxin accumulation (DPM mg^{-1} fresh mass) in the NE cells (Fig. 2). In E cells, auxin accumulation was lower than in NE cells and in a higher degree dependent on chemical structure of the auxin used: accumulation of 2,4-D was slightly higher than that of IAA.

The endogenous IAA level was in both emb and inf cultures higher in E cells than in NE cells and slightly higher in calli derived from the inflorescences (Table III).

Table III. Endogenous IAA concentration of non-embryogenic (NE) and embryogenic (E) winter wheat calli initiated from inflorescences (inf) and embryos (emb). Values represent averages from 3 replicates ± SE.

Object	IAA concentration [pmol·g^{-1} DW]
NE inf	74.3 ± 13.7
E inf	114.3 ± 23.4
NE emb	40.6 ± 4.6
E emb	93.7 ± 9.1

Discussion

Growth regulators are substances essential for *in vitro* cultures of plants, usually used in the concentration of 2–5 mg/l in the culture medium. Modifications in the composition and concentration of these substances influence the cell compe-

tence to embryogenesis and regeneration and are specific for different kinds of plant explants. In the presented experiments we used also higher growth regulator concentrations (to 30 mg/l) according to our earlier study where we observed the influence of these substances on the electric properties of wheat cells (Filek *et al.*, 2002).

Independently of the kind of explants (inf or emb), 2,4-D and zearalenone almost did not influence the NE relative influx, especially at lower concentrations. On the contrary, IAA, kinetin and zeatin markedly increased the NE membrane permeability. The similarity in 2,4-D and zearalenone action on the NE membranes, in spite of differences in their chemical structure and acidity, suggests that these substances specifically influenced membrane properties. Moreover, IAA induced the NE inf and NE emb cell leakage in the same way as kinetin or zeatin, again in spite of differences in their structure and charge (IAA bears a negative charge and kinetin and zeatin a positive one). In the case of winter wheat culture, 2,4-D could be replaced by zearalenone (unpubl. results). Thus, similarity in the interaction of phytohormones with membranes could be proposed to explain the similarity in their action.

Differences in the action of growth regulators on ion flux from NE and E cells can be explained in the context of their membrane composition. Blanckaert *et al.* (2000) demonstrated that important modifications occur in the lipid metabolism related to somatic embryogenesis in *Cichorium* leaves: the percentage of 18:3 acids decreased while that of 18:2 increased. Also Reidiboym-Talleux and Grenien-De March (1999) have concluded that somatic embryogenesis in *Prunus avium* is associated with an increase in phosphatidylcholine (PC) and triacylglycerols (TAG) which are enriched in 18:2 acids. An increase in the level of unsaturated fatty acids and a corresponding increase in membrane permeability are observed due to low temperature (Senser, 1982; Filek *et al.*, 1992). In the membranes of winter wheat calli induced from inf, a high level of non-saturated fatty acids was observed (Laggner *et al.*, 2003). However, when cholesterol is present in large amounts in the membranes, it acts as a permeability barrier for the membrane by introducing conformational changes of the lipid chains (Bloom *et al.*, 1991). It seems possible that the variation in cholesterol content provides for an effective control of the membrane stiffness and morphology, thereby controlling the vesicle-budding processes as well as the intermembrane distances (Raffy and Teissié, 1999). The embryogenic competence of winter wheat cells was connected with an increase in sterol concentration in the plasmalemma (Laggner *et al.*, 2003). In addition, the influence of phytohormones on membrane phospholipid charge suggests the strong interaction of phytohormones with these membrane constituents (Filek *et al.*, 2002). Stillwell *et al.* (1985) demonstrated that cytokinins increased the permeability of phosphatidylcholine bilayers in the liquid crystalline state. Thus, reducing the phospholipid/sterol ratio in E cells could decrease the number of interacting sites for phytohormones on the E membranes and in consequence also functioning of ion channels and pumps. This can result in decreasing ion efflux from E cells in the medium containing hormones. Besides that, Laggner *et al.* (2003) showed considerable changes in lipid structure of E membranes after kinetin and IAA treatment. In addition, the measurement of uptake of radioactively labelled auxins (IAA, 2,4-D) demonstrated higher capacity of the NE tissues to accumulate these auxins.

The importance of the endogenous auxin levels in the development of somatic embryos has been discussed elsewhere (Jiménez and Bangerth, 2001; Michalczuk *et al.*, 1992). Higher concentrations of endogenous IAA were found in embryogenic callus cultures (Table III), the cells of which had higher permeability. Treatment of NE cells with auxin led to an increase in membrane permeability. All these results suggest that auxin is an important factor in determining cell membrane permeability.

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