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Extracellular nucleotide effects on the electrical properties of growing *Arabidopsis thaliana* root hairs

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Abstract

Extracellular nucleotides depolarize the membrane potential of growing root hairs. ATP and ADP (at 1 mM) caused depolarizations of about 100 mV. The relative effectiveness of other nucleotides was ATP = ADP = GTP > AMP > TTP (= adenosine) > CTP. Phosphate had no effect, indicating that the membrane potential changes were not a consequence of nucleotide hydrolysis and phosphate uptake. The ATP and ADP effects were characterized in more detail: half-maximal depolarization occurred at 0.4 mM for ATP, and at 10 μ M for ADP; membrane conductance was unchanged after treatment with either nucleotide. After wash-out, the potential usually did not completely recover, and conductance declined. Additions of ADP at a concentration resulting in depolarization did not affect cytosolic Ca²⁺ levels as monitored directly with dextran-conjugated calcium green or indirectly by cytoplasmic streaming (which was unaffected). Growth increased slightly (22–38%) after ADP perfusion. Since purines were more potent, the cause of the depolarization could be a plant homolog of an animal purinergic receptor. The surprisingly high specificity of the ADP effect on the membrane potential suggests ADP may function as an extracellular message, but its potential function is unknown. It may serve as a signal during cellular wounding, or as a sensor of bacterial/fungal activity near the root surface. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Multiple effects of extracellular ATP are well known in animal systems [1]. It is a neurotransmitter, but also functions as an extracellular messenger for a variety of non-neuronal cells as well. It generally acts by binding to purinoceptors which may be either ion channels [2,3] or G-protein coupled receptors [4]. Generally, effective concentrations are considerably lower than 1 mM. Purinergic effects have also been observed in the ciliated protist *Paramecium*: extracellular GTP in-

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duces oscillating membrane depolarizations and a change in swimming behaviour, an aversion response [5].

In plants, it is clear that the adenine nucleotides play key regulatory roles in intracellular metabolism in plants, and in fact can regulate ion channel activity [6], but to our knowledge no one has ever examined the possibility that nucleotides play an extracellular regulatory role. Indeed, recent research indicates that extracellular nucleotides would be effectively scavenged for their phosphate due to extracellular apyrase activity [7] which may also play a role in initial responses in Rhizobia binding during nodulation [8]. To determine if extracellular nucleotides could play a role in extracellular signal transmission, their effects on the electrical properties of growing root hairs [9], compared to phosphate, were examined.

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2. Materials and methods

2.1. Seedling preparation

Bleach-sterilized seeds of Arabidopsis thaliana L. (Columbia wildtype) were sown in tissue culture dishes containing 0.95-1.0% gellan gum (ICN Biochemicals, Cleveland, OH 44128) in APW 5 (0.1 mM KCl, CaCl₂ and MgCl₂, 1.0 mM Mes (2-[N-Morpholino]ethanesulfonic acid). The seedlings were grown under fluorescent lights (350-400 lux) at 25°C. After 7-14 days, APW 7 (0.1 mM KCl, CaCl₂ and MgCl₂, 1.0 mM Mops (3-[N-Morpholino]propanesulfonic acid) and 0.5 mM NaCl, pH adjusted to 7.0 with NaOH) was added to the culture dishes. Roots that grew partially embedded in the surface of the gellan gum were chosen for impalements, so that growing root hairs could be impaled without handling the roots, thus avoiding wounding responses. When growing root hairs (growth rates were about 1 μ m min⁻¹) had reached a length of 30-100 µm, they were impaled with double-barrel micropipettes. Previous experiments demonstrated that growth usually continued after impalement with the micropipette [9].

2.2. Micropipette fabrication

Double-barreled micropipettes were constructed by placing two borosilicate (KG-33) capillaries with internal filaments (Garner Glass Company, Claremont, CA 91711) within a nichromium heating filament, heating, then twisting 360°. The capillaries were then pulled on a vertical pipette puller (Sutter Model P-30, San Rafeal, CA 94912). The micropipettes were filled with 3 M KCl.

2.3. Electrophysiology

The micropipette barrels were connected by AgCl electrodes to IE-251 electrometers (input impedance 10^{11} ohms, Warner Instruments, Hamden, CT 06514). After placement in the APW 7, the electrodes were tested for crosstalk by injecting 1 nA current through one electrode and checking for significant voltage deflections in the other electrode; crosstalk was nil to minimal (< 5%).

Grounding was via a 2 mm capillary containing 3 M KCl (to match the micropipette KCl concentration) in 2% agar connected to a AgCl electrode. A water immersion objective (see Section 2.6) was used, and caused a secondary shunt to ground. This resulted in minimal additional noise (<2.5 mV peak to peak) and did not affect measurements of electrode resistance.

2.4. Voltage-clamping

Current-voltage measurements using voltageclamp [10] have been described previously [9,11]. Voltage clamping for current-voltage measurements was computer-driven through a data acquisition board and operational amplifier configured for voltage clamping. A voltage clamp duration of 50 ms was used. A bi-polar staircase (alternating positive and negative clamps) of voltage clamps followed by a 50 ms voltage clamp at the resting potential minimized possible hysteresis effects. Data sampling (voltage and current) were performed during the last 10 ms of the voltage clamp.

2.5. Growth

Root hair lengths were measured every 2 min for 20 min before and after ADP addition. Growth rates were estimated from linear regression fits of length versus time.

2.6. Microscopy

A Nikon microscope was used with a 40X water immersion objective (NA 0.75) under bright field conditions. During experiments, the image was observed on a CCD video camera (Model KP-M1, Hitachi Denshi Ltd.) and displayed on a monitor. It was often possible to observe cytoplasmic streaming in the cells; however this depended on focus, which could not be adjusted after the experiment began because it would cause the micropipette to shift.

2.7. Calcium-imaging

Dextran-conjugated calcium green-1 dye (10000 molecular weight, Molecular Probes Inc., Eugene, OR) was pressure injected into the root hairs. The tip of the single-barrel micropipette was filled with 1 mM dye, then backfilled with 100 mM KCl. An air bubble between the dye solution and the backfilling solution prevented dye dilution due to diffusion into the backfilling solution. The mi-

cropipette was placed in a holder with both a micropipette port and a pressure port. Pressure was controlled by a water-filled 50 ml syringe attached to the pressure port with thick-wall tygon tubing. Before impalement, pressure was applied to the micropipette. After impalement, additional pressure introduced the dye into the cell. Dye fluorescence was monitored on a confocal microscope (Bio-Rad) fitted with a krypton-argon laser and a BHS filter block (488 nm, DF10 excitation; 510 nm LP dichroic; 515 nm LP emission filters). A $40 \times$ water immersion objective (NA 0.75) was used with a 2.5 or 3 zoom factor to obtain confo-



Fig. 1. The effect of nucleotides on the membrane potential (upper panel) and current-voltage relations (lower panel) of growing *Arabidopsis thaliana* root hairs. The example is for a 0.5 mM treatment with ATP, given as marked. The impalement and the removal of the micropipette are shown at the beginning and end of the voltage trace. Vertical bars overlaying the voltage trace represent times when current–voltage measurements were performed. The lower panel shows these measurements. All three replicates before (circles), after ATP treatment (triangles) and after wash-out (squares) are shown together. For all experiments, initial conductance was 165 ± 56 nS (12); 0.5 mM ATP had a negligible effect (150 ± 50 nS (12)), however, the conductance declined after wash-out (123 ± 24 nS (10)). Similar results were obtained using GTP and ADP.

2.8. Nucleotide treatment

The nucleotides were purchased from either Sigma (Sigma Chemical Company, St. Louis, MO, 63178 USA) or ICN (ICN Pharmaceuticals Inc., Costa Mesa, CA, 92626 USA) as highest purity sodium salts. They were dissolved in APW 7 at the final concentration, and pH adjusted with NaOH. The solutions were usually made fresh daily as needed. For treatment, perfusion was performed to change solutions; for confocal microscopy, a stock solution was added directly to the dish.

Where statistics are shown, means \pm standard deviations (sample size) are used.

3. Results and discussion

An example of electrophysiological measurements is shown in Fig. 1. Upon impalement, the measured potential was normally about -180 to -190 mV. ATP treatment (0.5 mM) caused a large depolarization. For GTP, ADP and ATP, a biphasic decline in the potential was sometimes, though not always, observed, and incomplete recovery upon wash-out was commonly observed. The conductance of the membrane, measured by voltage clamp tended to remain the same before and after treatment with these purine nucleotides, but declined after wash-out.

The extent of depolarization caused by a variety of nucleotides, all at concentrations of 1 mM, is shown in Table 1. The purine nucleotides, ATP, GTP and ADP had the greatest effect. The results using AMP were quite variable, ranging from no effect to large depolarizations. The cause of this variability is not known, but was not due to time of the experiment, or the age of the AMP solution. Apparently, individual root hairs exhibit differing sensitivity to AMP. TTP and CTP had minimal to nil effect, as did phosphate at 0.5 mM. Phosphate transport into the root hairs presumably occurs via a $nH^+/H_2PO_4^-$ symport [12]. However, the nucleotide effects are not due to apyrase activity followed by phosphate uptake [7], since phosphate addition is without any significant effect on the

Table 1 Effect of nucleotides, adenosine and phosphate on the membrane potential of growing *Arabidopsis thaliana* root hairs^a

Compound	Initial potential	Depolarization	Sample size	
GTP	-194 ± 23	91 ± 34	9	
ATP	-182 ± 21	103 ± 33	6	
ADP	-191 ± 9	94 ± 29	7	
AMP	-192 ± 10	32 ± 47	11	
TTP	-201 ± 23	34 ± 18	6	
CTP	-195 ± 23	9 <u>+</u> 6	5	
Adenosine	-190 ± 18	29 <u>+</u> 31	12	
Phosphate	-189 ± 9	3 ± 9	4	

^a In each case, the concentration of the compound was 1 mM in APW7 (except Na_2PO_4 , 0.5 mM). AMP either caused a strong depolarization or had no effect, resulting in a large standard deviation. The cause of this inconsistent result is not known. The purines ADP, ATP, and GTP had the strongest effects on the membrane potential. TTP had some effect, CTP and phosphate had virtually no effect.



Fig. 2. Concentration dependence of ATP effects on depolarization of the membrane potential, and conductance. Halfmaximal depolarization was observed at about 0.4 mM (upper panel). Conductance tended to remain unchanged.

membrane potential. In addition, the absence of complete recovery after removal of the nucleotide, and the decline in the membrane conductance both indicate changes in the plasma membrane electrical properties more complex than expected for a $nH^+/H_2PO_4^-$ symport (Fig. 1).

Beause the purine nucleotides ATP and ADP

would be present at highest concentration in the cell compared to the other nucleotides, the concentration dependence of the depolarization was examined for these two compounds. Fig. 2 shows the concentration dependence for ATP. A $K_{1/2}$ (halfmaximal effect) of 0.44 mM was calculated from a Michaelis-Menten non-linear fit of the data There was no trend in conductance changes (Fig. 2), indicating that total ion flux was unchanged during the marked depolarization of the potential. In contrast to the relatively high $K_{1/2}$ for ATP, the concentration dependence for ADP-induced depolarization revealed a much lower concentration required for half-maximal depolarization, 10 µM (Fig. 3). For ADP as well, there was no trend in conductance changes (Fig. 3).

If the adenine nucleotide effects are due to the presence of a purinoceptor on the plasma membrane, it is possible that they mediate their effect by elevation of intracellular calcium concentrations, often observed in animal cells [1]. To assess this, dextran-conjugated calcium green was pressure injected into root hairs, 0.2 mM ADP in APW7 (final concentration) added to the extracellular medium and its effect on intracellular calcium observed. Over time, the fluorescence intensity in the absence of any addition tended to



Fig. 3. Concentration dependence of ADP effects on depolarization of the membrane potential, and conductance. Halfmaximal depolarization was observed at about 10 uM (upper panel). Changes in [ADP] caused no clear change in conductance.

decrease, possibly due to degradation of the dye in the cytoplasm. The addition of ADP did not the cause fluorescence to increase (data not shown). When cytoplasmic streaming could be observed during electrophysiology experiments, the addition of the nucleotides did not affect streaming. Since streaming is sensitive to intracellular calcium levels [13–15], both fluorescent dye and cytological observation suggest calcium levels do not increase during the ADP-induced depolarization.

Growth as well was relatively unaffected by ADP perfusion at various concentrations. The initial growth rate was $1.05 \pm 0.35 \ \mu m \ min^{-1}$ (29). There was a slight increase in growth rate (15–38%) after ADP addition, independent of [ADP] (from 0.1 to 0.8 mM).

The presence of this extracellular nucleotide effect on the membrane potential of the root hairs was not expected. Initially, relatively high concentrations were used in a general screen of a variety of nucleotides, but it was clear that the range of nucleotide concentrations that a root might be exposed to was considerably lower (cf [7]). The low $K_{1/2}$ for ADP (10 μ M) raises the possibility that the ADP effects in particular might have physiological significance. Indeed, production of ADP due to ATP hydrolysis could also contribute to an overall effect of the adenine nucleotides.

There are two sources of ADP at these concentrations. One is damaged cells. Concentrations of ATP and ADP are normally in the range of about 1-2 mM in the cell. Release of these compounds into the extracellular solution would result in significant dilution, but it is not unexpected that nearby cells could be exposed to ADP concentrations > 10 μ M. The second source of nucleotides is bacteria and fungi surrounding the growing root. In the latter case, there is a recent report that a lectin that binds to Nod factors and appears to function in the nodulation response also exhibits apyrase activity [8]. However, ADP is not the final product of apyrase activity.

It is more likely that ADP is a component of an extracellular transmission pathway for wound signalling. This function of a purinergic receptor has been proposed in *Paramecium* [5,16]. In this case, the evidence to support this interpretation is stronger, since the modifications in swimming behaviour are consistent with an avoidance reaction on the part of this free-swimming protist: to leave an area where there are wounded cells. In *Parame*-

cium, there is a well defined link between membrane electrical properties and swimming behaviour [17].

For the root hairs, the electrical responses are very clear-cut, but direct support for a wound response is lacking. Calcium transients are reported to occur upon wounding [18], but extracellular ADP affects neither calcium levels nor cytoplasmic streaming. The membrane electrical changes that do occur, however, indicate a longterm modification of ion transport properties. The persistent depolarized potential and conductance decline after wash-out suggest a decrease in ion transport activity at the plasma membrane. Given that the H⁺ ATPase is known to consume a high percentage of ATP turnover, it is possible that the membrane changes reflect conservation of ATP.

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