Arabidopsis DORN1 extracellular ATP receptor; activation of plasma membrane K+-and Ca2+-permeable conductances

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The first reports in the 1970s of extracellular ATP (eATP) effects on algal cytoplasmic streaming and Venus fly trap closure received little attention (Jaffe, 1973; Williamson, 1975). By the time interest in plant eATP revived, work on animals had revealed the existence of plasma membrane (PM) receptors for eATP that function in such processes as pain perception and vasodilation (Burnstock, 2016). Plant research is now catching up. eATP effects on roots (gravitropism, growth and development), hypocotyl (elongation), pollen (germination and tube growth), stomatal aperture, and cell viability are now documented (reviewed by Clark et al., 2014, Cho et al., 2017). Furthermore, eATP is implicated in immunity, abiotic stress responses and nodulation (reviewed by Clark et al., 2014, Cho et al., 2017). eATP increases in response to wounding, mechanical stimulation, abiotic stress, abscisic acid, glutamate and chitin (Dark et al., 2011; Cho et al., 2017). eATP can increase plant free Ca2+ (cytosolic, nuclear, mitochondrial), phosphatidic acid, nitric oxide, and reactive oxygen species (ROS) as potential second messengers in signalling (Demidchik et al., 2003, 2009; Loro et al., 2012; Cho et al., 2017). Until recently, perception of eATP was very much a “black box” mechanism as no equivalents of animal receptors were apparent in higher plant genomes. A breakthrough came with the discovery of Arabidopsis thaliana DORN1 (Does not Respond to Nucleotides 1) as a PM eATP receptor (Choi et al., 2014). Analysis of dorn1 mutants has shown that this receptor governs eATP-induced [Ca2+]cyt elevation in young seedlings and also a specific transcriptional response enriched in wound-responsive genes (Choi et al., 2014).
Delineation of the first higher plant eATP receptor will now spur on attempts to identify the immediate targets of its serine/threonine kinase activity and downstream components of the signalling pathway it commands. eATP affects plasma membrane Ca\(^{2+}\), K\(^+\) and Na\(^+\) fluxes (Demidchik et al., 2011; Zhao et al., 2016) but none have yet been shown to rely on DORN1. eATP-activated Ca\(^{2+}\) fluxes are mediated by channel proteins in Arabidopsis root epidermal and guard cell PM but their genetic identities are unknown (Demidchik et al., 2009; Wang et al., 2014; Zhu et al., 2017). eATP- and eADP-induced K\(^+\) fluxes have been detected at the Arabidopsis root epidermis using extracellular K\(^+\)-selective microelectrodes (Demidchik et al., 2011). eADP was found (by using patch clamp electrophysiology) to activate channel-mediated K\(^+\) efflux and Ca\(^{2+}\) influx conductances (Demidchik et al., 2011). eATP-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations than leaves (Tanaka et al., 2010) and robust DORN1 expression (Cho et al., 2017).

**Plasma membrane cation-permeable conductances under control conditions**

DORN1 expression is high at the root apex and is maintained there as the root ages; expression declines with age in distal epidermis (Cho et al., 2017). Therefore epidermal protoplasts were isolated from excised root apices (<3 mm) of 9-16 days old Col-0 (parental wild type) and two dorN1 mutants. The dorN1-1 mutant has a point mutation in its cytosolic serine-threonine kinase domain while the dorN1-3 mutant has a T-DNA insertion in the extracellular legume-type lectin domain; both lesions result in failure in eATP-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation (Choi et al., 2014). Both mutants were confirmed as homozygous. Mean (± SE) protoplast diameter was 16 ± 0.5 µm (n = 40), smaller than those in a previous study from the mature epidermis (20 µm) isolated using the same method (Demidchik et al., 2011). The “whole cell” mode of patch clamping was applied, permitting measurement of net current as ions are conducted through PM channels at different trans-membrane voltages, and reported as the overall current/voltage (I/V) relationship. Under control conditions, one type of overall non-linear conductance was most commonly observed in Col-0 (Figure 1a, left panel and Supplemental Figure S1a) and both dorN1 mutants (Supplemental Figure S1b, c). The time constant of half-activation of Col-0 outward currents at 23 mV was 88.4 ± SE 8.5 ms (n = 12). This is reminiscent of the depolarization-activated non-selective cation channels (NSCC) in different plant species (Wegner and de Boer, 1997; Zhang et al., 2002; Shabala et al., 2006). In all genotypes, both
inward currents (evoked by hyperpolarised voltages) and outward currents (evoked by
depolarised voltages) were greatly inhibited by the cation channel blocker, Gd$^{3+}$ (Supplemental
Figure S1a-c). The Gd$^{3+}$-insensitive conductance was not investigated further. Tail-current
analysis of the Col-0 outward conductance revealed a reversal potential ($E_{rev}$) of -90.2 ± 8.9
mV ($n = 8$). Thus, permeability ratios $P_K/P_{Cl}$ and $P_{Ca}/P_K$ of 13.7 ± 5.4 and 0.04 ± 0.01,
respectively, were estimated using the Goldman equation. A high selectivity for $K^+$ was also
found in the NSCC studies and also for PM $K^+$ channels in root cells of *Arabidopsis* (Maathuis
and Sanders, 1995). Voltage ramping minimised activation of the Col-0 outward conductance
to delineate the inward conductance (Supplemental Figure 2) and yielded an $E_{rev}$ of -8.2 ± 4.7
mV ($n = 6$) and $P_{Ca}/P_K$ of 1.9 ± 0.7. This indicates $Ca^{2+}$ influx contributing to the inward
conductance. Single channel studies are now required to delineate channel contribution to the
Gd$^{3+}$-sensitive $K^+$- and $Ca^{2+}$-permeable conductances.

eATP activates $K^+$- and $Ca^{2+}$-permeable conductances in wild type root plasma membrane

Before addition of ATP, at least two recordings were performed to confirm the stability of
currents (Figure 1b, -3 and 0 mins). ATP was added as its disodium salt and in controls with
an equivalent Na$^+$ concentration applied as NaCl, there was no current activation
(Supplemental Figure 3), confirming that any responses would be caused by ATP. Addition of
300 µM ATP increased the outward currents and initiated small ‘spiky’ inward currents at
hyperpolarized voltage after 3 minutes (Figure 1a, b). The kinetics of the inward conductance
resembled the hyperpolarization-activated $Ca^{2+}$ conductance (HACC) found in previous studies
on epidermal PM (*e.g.*, Demidchik *et al.*, 2009). Current activation was transient (Figure 1c),
as was previously observed with eATP- and eADP-activated PM conductances in protoplasts
from mature epidermis (Demidchick *et al.*, 2009, 2011). Eight minutes after ATP application,
maximum current values (at +43 and -217 mV) were significantly greater than those prior to
treatment ($p < 0.01$, Student's $t$-test; Figure 1c, d) and inward rectification of the
hyperpolarization-activated currents became apparent. Analysis of the eATP-activated currents
(control $I/V$ subtracted from eATP $I/V$ at 8 minutes; Figure 1d, insert) revealed a positive shift
of $E_{rev}$ to -64.5 ± 16.8 mV, indicating a greater $Ca^{2+}$ permeability in eATP-induced inward
currents ($E_{Ca}$ at + 161 mV). It is therefore likely that eATP transiently increased $Ca^{2+}$
conductivity to deliver $Ca^{2+}$ to the cytosol, although increased $Cl^-$ permeation is also possible.
However, neither outward nor inward currents in Col-0 were stimulated by the same concentration of eADP (300 µM; Supplemental Figure 4).

**eATP-induced current activation does not occur in dorn1 mutants**

Under control conditions, there was no significant difference between the overall Col-0 conductance and that of *dorn1-1* (Figure 2a,b: Tail current analysis of outward current, $E_{rev}$ -88.8 ± 9.3 mV; $P_K/P_{Cl}$ 9.4 ± 3.0; n=5). Consistent with Col-0, the epidermal PM currents recorded from both *dorn1* mutants were blocked by Gd$^{3+}$ (Supplemental Figure S1b, c). In contrast to Col-0, *dorn1-1* did not respond to 300 µM ATP (Figure 2a,b,c), neither did *dorn1-3* (Figure 2d,e). As DORN1 binds ATP with a $K_d$ of 45.7 nM (Choi *et al.*, 2014), the lack of response to this high concentration of ATP supports the currents’ lying downstream of this receptor in Col-0.

**DORN1 is involved in eATP activation of Arabidopsis root epidermal PM currents**

DORN1 governs eATP’s transient activation of a Ca$^{2+}$-permeable inward conductance at hyperpolarized voltages (Figure 1c, d; Figure 2). With resting PM voltage in epidermal cells from *Arabidopsis* roots varying between -153 and -129 mV (Maathuis and Sanders, 1993), eATP-induced HACC-like currents could initiate [Ca$^{2+}$]$_{cyt}$ elevation for wound signalling (Choi *et al.*, 2014) and promote the eATP-induced depolarization of *Arabidopsis* PM (Lew and Dearnaley, 2000). DORN1 also governs eATP’s transient activation of a K$^+$-permeable outward conductance (probably NSCC) at depolarized voltages (Figure 1c, d; Figure 2). In whole *Arabidopsis* roots, the same concentration of eATP (300 µM) was shown by Demidchik *et al.* (2011) to induce K$^+$ efflux at the root epidermis. The K$^+$ efflux conductance found in the present study would be a strong candidate for the root eATP-induced K$^+$ efflux pathway. This may have significance for eATP and DORN1 function in pathogen- or stress-induced K$^+$ loss in cell death (Demidchik, 2014).

DORN1 commands [Ca$^{2+}$]$_{cyt}$ elevation in response to both eATP and eADP (Choi *et al.*, 2014). The eADP concentration used here (300 µM) was found previously to activate PM K$^+$ efflux and Ca$^{2+}$ influx conductances in Col-0 protoplasts from mature root epidermis (Demidchik *et al.*, 2011) but in the present study it had no effect on those from the root apex (Supplemental...
Figure 4). A possible explanation is a cell-specific secondary effect of ADP that negates DORN1’s activation of channels. Nevertheless, the relationship between DORN1 and PM conductances found here for eATP may well prove valuable in the search for the molecular identities of the contributory channels. Root PM conductances activated by eATP independently of DORN1 may also yet be discovered.

References


Figure 1. Exogenous ATP activates $K^+$ and $Ca^{2+}$ currents in Col-0 root epidermal plasma membrane.

a. Representative current traces from whole cell recordings of Arabidopsis thaliana Col-0 before (left panel) and after 3 minutes’ ATP treatment (300 µM; right panel). Baseline membrane voltage was held at -137 mV prior to a step-wise voltage protocol of 20 mV increments. b. Effect of 300 µM eATP on $I/V$ relationships. Data are means ± SE ($n = 6$), recorded 3 minutes before ATP addition, immediately before addition (0 minutes) and 3 minutes after. Inward current below the $V$ axis is mainly $Ca^{2+}$ influx. Outward current above the $V$ axis is mainly $K^+$ efflux from cytosol. Equilibrium potentials for $K^+$ ($E_K$) and $Cl^-$ ($E_{Cl^-}$) are annotated. $E_{Ca} = + 161$ mV. eATP transiently increased inward and outward currents. Data are means ± SE of current recorded at -217 and +43 mV respectively ($n = 3$ to 6). ** marks significant difference from control ($p < 0.01$, Student’s $t$-test). d. $I/V$ relationships 8 minutes after ATP addition and (insert) difference $I/V$ to reveal the eATP-activated currents ($n = 4$). Bath solution comprised 20 mM CaCl$_2$, 0.1mM KCl, 20 µM NaCl, 5 mM MES-Tris, pH 5.6. Pipette solution comprised 40 mM K-gluconate, 10 mM KCl, 0.4 mM CaCl$_2$, 1mM BAPTA, 2mM MES-Tris, pH 7.2 (Demidchik et al., 2011).

Figure 2. dorn 1-1 and dorn 1-3 failed to respond to exogenous ATP.

a. Representative current traces from Arabidopsis thaliana dorn 1-1 before (left panel) and after 3 minutes’ ATP treatment (300 µM; right panel). b. There was no effect of eATP on $I/V$ relationships of dorn 1-1 3 minutes after eATP application and (c) no effect over an extended time period. d. There was no effect of eATP on $I/V$ relationships of dorn 1-3 3 minutes after eATP application and (e) no effect over an extended time period. Data are presented as means ± SE ($n = 5$ in b and c; $n = 3$ in d and e). Bath solution comprised 20 mM CaCl$_2$, 0.1mM KCl, 20 µM NaCl, 5 mM MES-Tris, pH 5.6. Pipette solution comprised 40 mM K-gluconate, 10 mM KCl, 0.4 mM CaCl$_2$, 1mM BAPTA, 2mM MES-Tris, pH 7.2 (Demidchik et al., 2011).

Fig. S1. Gd$^{3+}$ inhibits whole-cell currents from epidermal plasma membrane of Col-0 and dorn1 mutants.

Fig. S2. Voltage ramping to determine $Ca^{2+}$ permeability of the Col-0 inward conductance.

Fig. S3. Exogenous NaCl has no effect on Col-0 currents.

Fig. S4. Exogenous ADP does not induce currents in Col-0 apical root epidermal plasma.
membrane.

**Key words** *Arabidopsis*, ATP, calcium, channel, DORN1, extracellular, potassium

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**Author contributions** The study was conceived by LW and JMD. LW and KAW generated and analysed the data. LW and JMD wrote the paper.
Figure 3. Exogenous ATP activates K⁺ and Ca²⁺ currents in Col-0 root epidermal plasma membrane.

a. Representative current traces from whole cell recordings of Arabidopsis thaliana Col-0 before (left panel) and after 1 minute's ATP treatment (300 μM; right panel). Baseline membrane voltage was held at -177 mV prior to a step-wise voltage protocol of 20 mV increments. b. Effect of 300 μM eATP on U'/V' relationships. Data are means ± SE (n = 6), recorded 3 minutes before ATP addition, immediately before addition (0 minutes) and 3 minutes after inward current below the Y axis is mainly Ca²⁺ influx. Outward current above the Y axis is mainly K⁺ efflux from cytoplasm. Equilibrium potentials for K⁺ (E钾) and Ca²⁺ (E钙) are indicated. E钾 = E钙 = +161 mV. eATP transiently increased inward and outward currents. Data are means ± SE of current recorded at -217 and +43 mV respectively (n = 6). ** denotes significant difference from control (p < 0.01, Student's t-test). c. U'/V' relationships 8 minutes after ATP addition and (insert) difference U'/V' to reveal the eATP-activated currents (n = 4). Bath solution comprised 20 mM CaCl₂, 0.1 mM KCl, 20 mM NaCl, 5 mM MES–Tris, pH 5.6. Pipette solution comprised 40 mM K-glutamate, 10 mM KCl, 0.4 mM CaCl₂, 1 mM 3-APTA, 2 mM MES–Tris, pH 7.2 (Demidchik et al., 2011).
Figure 2. *dros* l-1 and *dros* l-3 failed to respond to exogenous ATP.

a. Representative current traces from *dros* l-1 before (left panel) and after 3 minutes' ATP treatment (500 μM; right panel). b. There was no effect of cATP on Vr relationships of *dros* l-1 3 minutes after cATP application and (c) no effect over an extended time period.

d. There was no effect of cATP on Vr relationships of *dros* l-3 5 minutes after cATP application and (e) no effect over an extended time period. Data are presented as means ± SE (n = 5 in b and c; n = 3 in d and e). Recording conditions and protocols were identical to those in Figure 1.