

Effects of chemical inhibitors and apyrase enzyme further document a role for apyrases and extracellular ATP in the opening and closing of stomates in *Arabidopsis*

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Abbreviations: ABA, abscisic acid; eATP, extracellular ATP; ROS, reactive oxygen species; NO, nitric oxide; RBOHD/F, NADHP oxidase homolog sub-units D/F; DTT, dithiothreitol; DPI, diphenyleneiodonium; GCA2, growth controlled by ABA 2; ABI2, Abscisic Acid-Insensitive2; PA, phosphatidic acid; gpa, α -subunit of the *Arabidopsis* heterotrimeric G Protein

In *Arabidopsis* leaves there is a bi-phasic dose-response to applied nucleotides; i.e., lower concentrations induce stomatal opening, while higher concentrations induce closure. Two mammalian purinoceptor antagonists, PPADS and RB2, block both nucleotide-induced stomatal opening and closing. These antagonists also partially block ABA-induced stomatal closure and light-induced stomatal opening. There are two closely related *Arabidopsis* apyrases, AtAPY1 and AtAPY2, which are both expressed in guard cells. Here we report that low levels of apyrase chemical inhibitors can induce stomatal opening in the dark, while apyrase enzyme blocks ABA-induced stomatal closure. We also demonstrate that high concentrations of ATP induce stomatal closure in the light. Application of ATP γ S and chemical apyrase inhibitors at concentrations that have no effect on stomatal closure can lower the threshold for ABA-induced closure. The closure induced by ATP γ S was not observed in gpa1–3 loss-of-function mutants. These results further confirm the role of extracellular ATP in regulating stomatal apertures.

Extracellular ATP (eATP), which has now become recognized as a signaling agent in plants, can regulate growth in a variety of plant cell and tissue types.¹ The application of micromolar concentrations of ATP can induce $[Ca^{2+}]_{cyt}$ fluctuations and promote growth-altering accumulation of ROS and NO in diverse tissues of diverse plants. Nitric oxide (NO) and reactive oxygen species (ROS) are important in guard cell responses. In our original study of this signaling response we found that ATP γ S-induced stomatal closing was dependent on the production of ROS and NO.² Importantly we reported that both ABA and light-induced changes in stomatal aperture were preceded by an increase in the eATP levels of guard cells. A subsequent report by Hao et al. (2012)³ found that applied ATP promoted stomatal opening, but, in contrast to the results we obtained using ATP γ S, they did not observe stomatal closing in *Arabidopsis* or *Vicia faba* leaves in response to applied ATP. In this study we carry out additional tests that address questions raised by the findings of Hao et al.

(2012),³ and provide new data consistent with a proposed model for eATP regulation of stomatal aperture.

Application of 5 μ M or 15 μ M ATP γ S in the dark induces stomatal opening,² while application of 25 μ M ATP γ S or more does not have an effect in the dark. Hao et al. (2012) confirmed a role for eATP in stomatal opening, showing that applied ATP at concentrations as high as 1 mM induce stomatal opening.³ Regarding eATP-induced stomatal opening, we hypothesized that moderate inhibition of ectoapyrase activity by application of low concentrations of chemical apyrase inhibitors would cause naturally occurring levels of eATP to increase resulting in stomatal opening. We found that, similar to treatment with 15 μ M ATP γ S, treatment of leaves with two different apyrase inhibitors at a concentration of 1.5 μ g/mL also induces stomatal opening (Fig. 1A).

We reported that application of 150 μ M ATP γ S or more in the light induces stomatal closure,² but Hao et al. (2012)³ did

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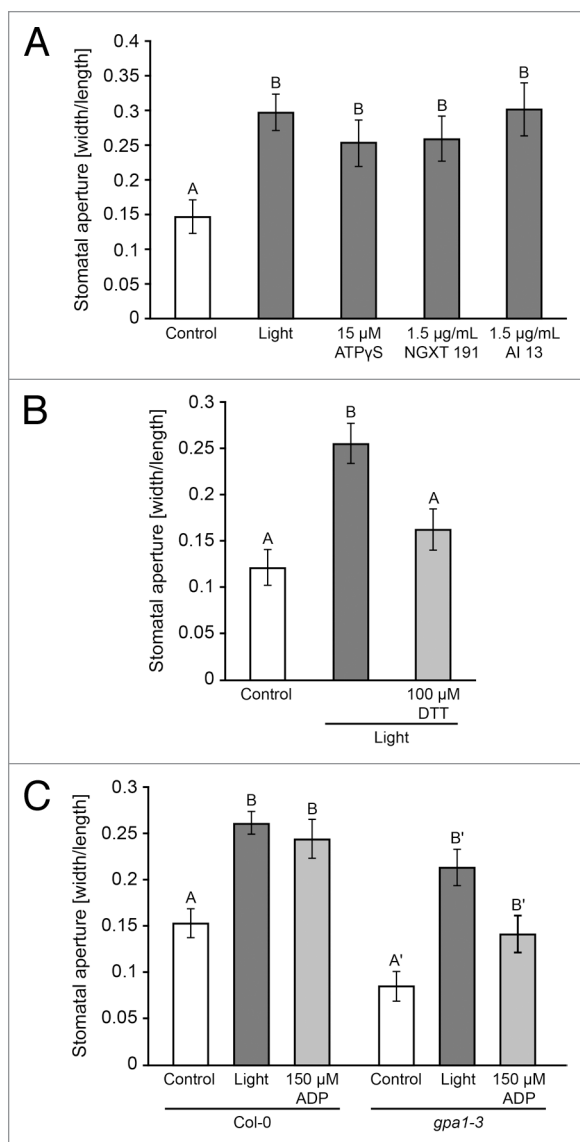


Figure 1. (A) Treatment with light induces stomatal opening. Treatment with 15 μM ATP γS or 1.5 $\mu\text{g}/\text{mL}$ apyrase inhibitor NGXT191 or 1.5 $\mu\text{g}/\text{mL}$ apyrase inhibitor #13 induces stomatal opening in the dark. (B) Treatment with 100 μM DTT blocks light-induced opening. (C) Treatment with light and treatment with 150 μM ADP in the dark induces stomatal opening in Col-0 and the *gpa1-3* mutant. Apertures measured in epidermal peels as width/length after 2 h treatment of whole leaves. Different letters above bars = mean values that are significantly different from one another as determined by Student *t*-test ($p < 0.05$; $n > 50$).

not observe stomatal closure when treating leaves with ATP. In our previous experiments we found that application of ATP γS can induce changes in plant growth at 10-fold lower concentrations than ATP, presumably because applied ATP is hydrolyzed by ectoapyrases or other phosphatases. Thus our expectation was that stomatal closure induced by applied $\geq 150 \mu\text{M}$ ATP γS would also be induced by ATP but at 10-fold higher concentrations ($\geq 1.5 \text{ mM}$), so we performed closing experiments using ATP and found that $\geq 1.5 \text{ mM}$ ATP did indeed induce stomatal closure (Fig. 2A). Interestingly, just as application of soluble potato

apyrase blocked stomatal opening in the light,³ we found that it could block ABA induced-closure (Fig. 2B).

The observation that purinoceptor antagonists can partially block ABA-induced closure and that ABA treatment of leaves induces a rapid increase in eATP levels suggests that eATP is part of the ABA signal transduction pathway. In order to further test this hypothesis we examined whether eATP and ABA could act synergistically to induce stomatal closure. We found that 0.1 μM ABA alone was not enough to induce stomatal closure in our system so we combined this concentration of ABA with concentrations of apyrase inhibitor and ATP γS that also were too low alone to have an effect on stomatal closure. However, we found that combining low levels of ABA with either low levels of two different apyrase inhibitors or low levels of ATP γS resulted in stomatal closure (Fig. 2C and D).

ATP γS -induced stomatal closure and ATP-induced stomatal opening require production of H_2O_2 by NADPH oxidase (RBOHD/F).^{2,3} Application of H_2O_2 induces stomatal closure in *Arabidopsis*,⁴ while application of CuCl_2 and ascorbic acid to introduce hydroxyl radicals induces opening at 0.1 mM, but not above 0.5 mM.³ These observations suggest that the biphasic response to ATP γS may be a biphasic response to ROS, with lower amounts of ATP γS causing production of lower amounts of ROS.

In addition to ROS, ATP γS -induced closure requires production of NO by nitrate reductase, with production of ROS temporally preceding production of NO.² This is consistent with the fact that ABA-induced NO production is dependent on H_2O_2 production in *Arabidopsis*.⁵ Furthermore, ATP-induced NO production requires phosphatidic acid (PA) production in tomato,⁶ and PA inhibits ABI1 phosphatase activity to allow ROS production by RBOHD/F in ABA-induced closure in *Arabidopsis*.⁷ This potentially places PA production upstream of ROS production in ATP-induced closure.

ATP elicits two calcium spikes in *Arabidopsis* root hairs, the first occurring 30–40 s after application and the second 80–90 s after application, with the first spike involving Ca^{2+} influx and the second release of Ca^{2+} from internal stores.⁸ ATP-induced PA accumulation in tomato is not sensitive to EGTA, while ATP-induced NO production is,⁶ placing the first Ca^{2+} spike downstream of PA production but upstream of NO production. During ABA- and H_2O_2 -induced stomatal closure, H_2O_2 activates plasma membrane Ca^{2+} channels in a manner dependent on GCA2 and ABI2.^{4,9} This places the first Ca^{2+} spike downstream of H_2O_2 . Finally, NO-induced stomatal closure involves Ca^{2+} spikes in *Arabidopsis*,¹⁰ placing the second Ca^{2+} spike downstream of NO production and upstream of closure.

These results for ABA-induced and eATP-induced closure inspire several questions. Because these results predominantly concern stomatal closure, an important question is the degree to which ATP-induced opening differs from ATP-induced closure. H_2O_2 production is required for both ATP-induced opening and ATP γS -induced closure.^{2,3} If the mechanisms differ, then, they may differ downstream of H_2O_2 , although they may also differ due to amount of H_2O_2 produced as described above. Interestingly, cytokinin- and auxin-induced stomatal opening

Figure 2. (A) Treatment with 10 μM ABA induces stomatal closure in the light, as did 150 μM ATP γS and 1.5 mM ATP. (B) Treatment with 10 μM ABA induces stomatal closure in the light, but in combination with 8 units of potato apyrase closure is blocked. Treatment with either 0.1 μM ABA or 75 μM ATP γS alone does not change stomatal apertures, but in combination induce stomatal closure in the light. (C) Treatment with either 0.1 μM ABA or 1.5 $\mu\text{g}/\text{mL}$ apyrase inhibitor #13 alone does not change stomatal apertures, but in combination induce stomatal closure in the light. (D) Combining 10 μM ABA with 8 units of boiled apyrase has no effect on ABA-induced closure. (E) Treatment with 10 μM ABA induces stomatal closure in both Col-0 and the *gpa1-3* mutant in the light, however treatment with 250 μM ATP γS in the light only induces stomatal closure in Col-0. Apertures measured in epidermal peels as width/length after 2 h treatment of whole leaves. Different letters above bars = mean values that are significantly different from one another as determined by Student *t*-test ($p < 0.05$; $n > 50$).

involves a decrease in hydrogen peroxide levels,¹¹ as does inhibition of stomatal closure by ethylene in *Vicia faba*.¹² This is in contrast to the requirement for ROS in ATP-induced opening. It should be noted, however, that pretreatment with DTT, a ROS scavenger, prevents light-induced opening (Fig. 1B), although the effects of DPI, an NADPH oxidase inhibitor, on light-induced opening have not been tested.

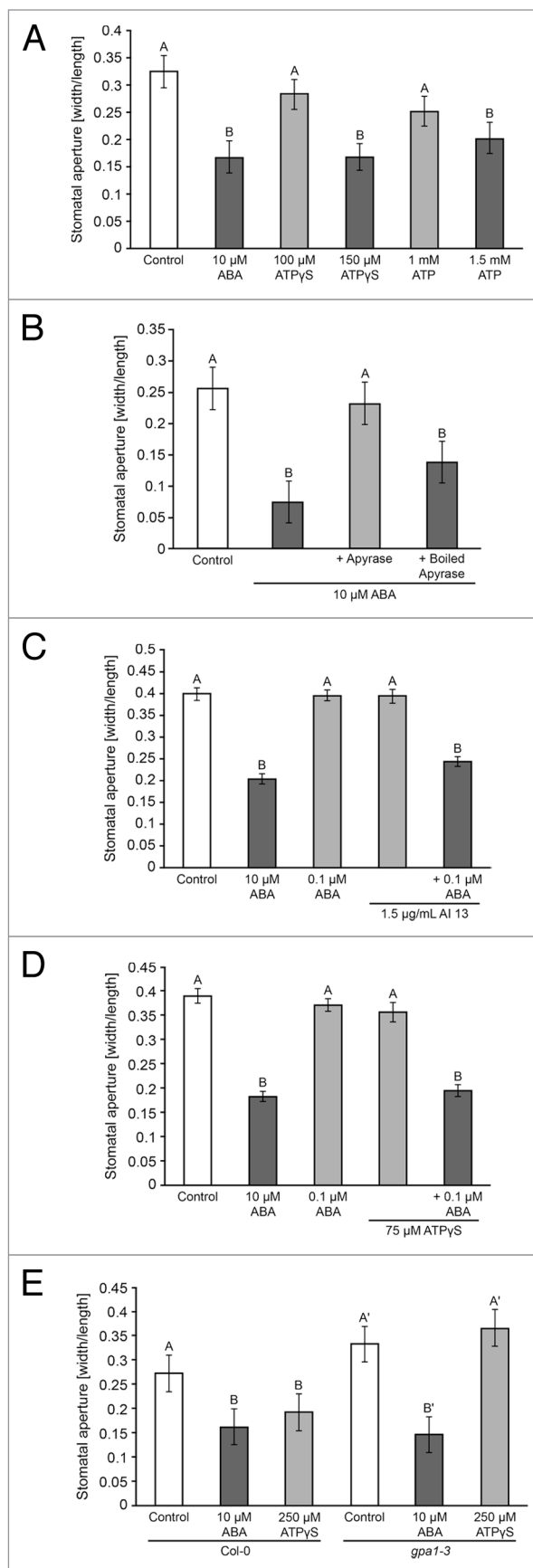
Another important question in plant extracellular nucleotide signaling that needs to be further addressed is possible differences between ADP and ATP signaling. Some results from physiological experiments using ADP βS and ATP γS suggest that eADP and eATP signaling occur through the same receptor, as they have similar effects in similar tissues and are antagonized by the same compounds.^{2,13–15} However, while eATP induces ROS accumulation in *Arabidopsis* root cells, eADP does not.¹⁶ More significantly, application of ADP induces inward Ca^{2+} currents within 1 s of application,¹⁶ while the first Ca^{2+} spike does not occur until 30 – 40 s after application of ATP.⁸ Together, these results suggest that eADP and eATP may act through different receptors. Specifically, the rapid Ca^{2+} influx caused by ADP in comparison to ATP suggests that ADP may act through an ion channel, while ATP may act through a G protein-linked receptor.

Hao et al. (2012) found that ATP does not induce stomatal opening in *gpa1-1* mutants.³ We found that ATP γS does not induce stomatal closing in the *gpa1-3* mutants (Fig. 2E) which indicates that an early step in the signaling pathway for both eATP-induced stomatal opening and closing involves heterotrimeric G proteins. We also found that, in contrast to ATP, ADP is able to induce opening in *gpa1-3* mutants (Fig. 1C), providing direct evidence that ATP and ADP act through different mechanisms in their regulation of stomatal aperture.

The observation that application of ATP γS and chemical apyrase inhibitors can act synergistically with ABA to induce closure provides additional evidence for cross-talk between extracellular nucleotide and ABA signal pathways during stomatal closure. Taken together these data suggest that the swelling and shrinking of guard cells induced by various stimuli may result in the release of ATP that helps regulate stomatal apertures.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.



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