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Nucleotide-induced calcium oscillations

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Extracellular nucleotides elicit cytosolic free calcium oscillations in Arabidopsis

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ABSTRACT
Extracellular ATP induces a rise in the level of cytosolic free calcium ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) in plant cells. To expand our knowledge about the function of extracellular nucleotides in plants, the effects of several nucleotide analogs and pharmacological agents on [Ca\(^{2+}\)]\(_{\text{cyt}}\) changes were studied, using transgenic Arabidopsis expressing aequorin or the FRET-based Ca\(^{2+}\) sensor Yellow Cameleon 3.6. Exogenously applied CTP caused elevations in [Ca\(^{2+}\)]\(_{\text{cyt}}\) that displayed distinct time- and dose-dependent kinetics compared to the purine nucleotides, ATP or GTP. The inhibitory effects of antagonists of mammalian P2 receptors and calcium influx inhibitors on nucleotide-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations were distinct between CTP and purine nucleotides. These results suggest that distinct recognition systems may exist for the respective types of nucleotides. Interestingly, a mutant lacking the heterotrimeric G protein Gβ subunit exhibited a remarkably higher [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation in response to all tested nucleotides in comparison with wild type. The data suggest a role for Gβ in negatively regulating extracellular nucleotide signaling and points to an important role for heterotrimeric G proteins in modulating the cellular effects of extracellular nucleotides. The addition of extracellular nucleotides induced multiple temporal [Ca\(^{2+}\)]\(_{\text{cyt}}\) oscillations, which could be localized to specific root cells. The oscillations were attenuated by a vesicle trafficking inhibitor, indicating that the oscillations likely require ATP release via exocytotic secretion. The results reveal new molecular details concerning extracellular nucleotide signaling in plants and the importance of fine control of extracellular nucleotide levels to mediate specific plant cell responses.
INTRODUCTION

The calcium ion, Ca\(^{2+}\), is a ubiquitous second messenger that is used to regulate a wide range of cellular processes (Clapham, 2007). A number of plant environmental and developmental responses are encoded to distinct Ca\(^{2+}\) signal patterns with specific frequencies and amplitudes of cytosolic free Ca\(^{2+}\) concentration, [Ca\(^{2+}\)\(_{\text{cyt}}\)]. These signal patterns can take the form of pulsating [Ca\(^{2+}\)\(_{\text{cyt}}\) spiking/oscillations (Berridge et al., 2003). In plants, such [Ca\(^{2+}\)\(_{\text{cyt}}\) oscillations occur in various cell types (e.g., stomatal guard cells, pollen tubes, and legume root hairs) and play a critical role in responding to environmental signals (Evans et al., 2001; Oldroyd and Downie, 2008; McAinsh and Pittman, 2009).

ATP is a ubiquitous compound in all living cells; it not only provides the energy to drive many biochemical reactions, but also functions in signal transduction as a substrate for kinases, adenylate cyclases, etc. However, ATP was also shown to be an essential signaling agent outside of cells in animals, where it is referred to as extracellular ATP. Extracellular ATP is involved in numerous cellular processes, including neurotransmission, immune responses, cell growth, and cell death (Khakh and Burnstock, 2009). In mammalian cells, plasma membrane-localized receptors, purinoceptors of the P2X and P2Y classes, bind ATP, as well as other nucleotides, at the cell surface to activate intracellular signaling cascades via second messengers. Binding of extracellular ATP to P2X receptors gates calcium influx; whereas activation of P2Y receptors stimulates recruitment of heterotrimeric G proteins to trigger cytoplasmic signaling and gene expression. As a common phenomenon, the activated receptors induce the elevation of [Ca\(^{2+}\)\(_{\text{cyt}}\)], which in turn activates production of downstream messengers, such as nitric oxide (NO) and reactive oxygen species (ROS) (Shen et al., 2005; Fields and Burnstock, 2006).

A possible physiological role for extracellular ATP in plants was first reported in studies in which exogenously applied ATP was found to stimulate closure of the Venus flytrap (Jaffe, 1973), to induce the formation of nucleases in excised Avena leaves (Udvardy and Farkas, 1973), and to induce potassium ion
uptake into cells of maize leaf slices (Lüttge et al., 1974). Over the past several years, extracellular ATP was found to be an important signaling compound in plants that induces various plant responses, including root-hair growth (Lew and Dearnaley, 2000; Kim et al., 2006), stress responses (Thomas et al., 2000; Jeter et al., 2004; Song et al., 2006), gravitropism (Tang et al., 2003), cell viability (Chivasa et al., 2005), pathogen responses (Chivasa et al., 2009) and thigmotropism (Weerasinghe et al., 2009). The release of extracellular ATP from root cells was directly imaged by Kim et al. (2006) using a luciferase construct engineered to bind to plant cell wall cellulose. Recently, using this reporter, Weerasinghe et al. (2009) measured the release of ATP from root cells in response to touch. This documentation of the presence of extracellular ATP in plants at levels sufficient to induce cellular responses suggests that extracellular ATP likely plays an important role throughout plant growth and development. However, no P2 receptor homologs have been identified in plants, despite the fact that plants share a number of cellular responses to ATP with animal cells. For example, addition of exogenous ATP or ADP triggers an increase in \([\text{Ca}^{2+}]_{\text{cyt}}\) levels in whole seedlings, dissected root tissues, and root epidermal protoplasts of Arabidopsis (Demidchik et al., 2003; Jeter et al., 2004; Demidchik et al., 2009). The production of ROS in response to ATP addition was detected in various plant tissues (Kim et al., 2006; Song et al., 2006; Wu et al., 2008; Demidchik et al., 2009). More recently, the plasma membrane NADPH oxidase AtRBOHC was shown to be required for extracellular ATP-induced ROS production in Arabidopsis primary roots (Demidchik et al., 2009). Extracellular ATP also stimulates the production of NO in tomato culture cells and in *Salvia miltiorrhiza* hairy roots (Foresi et al., 2007; Wu and Wu, 2008). These reports suggest that extracellular ATP signals across the plasma membrane by triggering elevation in \([\text{Ca}^{2+}]_{\text{cyt}}\), which activates production of downstream messengers. Ultimately, these cell responses induce the expression of various genes, such as MAPKs, LOX, and ACS6 (Jeter et al., 2004; Song et al., 2006), and cause physiological responses, as described above.
In animal cells, extracellular ATP-evoked elevations in \([Ca^{2+}]_{cyt}\) are often observed in the form of oscillations that result from the transient opening of \(Ca^{2+}\) channels located either in the plasma membrane or in cytosolic \(Ca^{2+}\) stores. Intracellular calcium release is often mediated through phospholipase C (PLC)-mediated signaling coupled to heterotrimeric G proteins (Mahoney et al., 1992; Visegrady et al., 2000; Hanley et al., 2004). In plants, plasma membrane \(Ca^{2+}\) permeable channels are known to contribute to extracellular ATP-induced \([Ca^{2+}]_{cyt}\) elevation (Demidchik et al., 2009). However, neither the mechanisms underlying extracellular ATP-evoked \(Ca^{2+}\) signaling nor the possible involvement of heterotrimeric G proteins has been characterized in plants.

In order to explore their roles as possible ligands of putative nucleotide receptors, the plant \([Ca^{2+}]_{cyt}\) response to six different nucleotides (see Figure 1a) was measured using Arabidopsis seedlings expressing one of two \([Ca^{2+}]_{cyt}\) sensors, either aequorin or the FRET-based \(Ca^{2+}\) sensor Yellow Cameleon 3.6 (YC3.6). The pyrimidine nucleotide CTP, as well as the purine nucleotides ATP and GTP, induced a strong elevation of \([Ca^{2+}]_{cyt}\) in seedlings. Interestingly, the effects of all the nucleotides on \(Ca^{2+}\) signaling were negatively regulated by a heterotrimeric G protein \(\beta\) subunit, AGB1. The addition of ATP to aequorin expressing seedlings induced distinct \([Ca^{2+}]_{cyt}\) oscillations in the presence of the apyrase inhibitor NGXT191. However, in the absence of this inhibitor, such \([Ca^{2+}]_{cyt}\) oscillations could be localized to specific root cell layers using YC3.6 fluorescence. Given the importance of \([Ca^{2+}]_{cyt}\) oscillations in intracellular signaling, the data suggest an important, unexplored role of extracellular ATP in the plant signaling pathways.

RESULTS

Extracellular nucleoside triphosphates increase \([Ca^{2+}]_{cyt}\) in Arabidopsis seedlings

In animal cells, different P2 receptor subtypes have distinct agonist specificities for extracellular nucleotides. In order to explore extracellular nucleotide
specificity in plants, we characterized responses to various nucleoside
triphosphates (NTPs). The effect of nucleotide addition was measured by
characterizing the \([\text{Ca}^{2+}]_{\text{cyt}}\) changes each elicited in Arabidopsis seedlings
expressing the calcium reporter protein aequorin (Knight et al., 1996). The six
NTPs, ATP, GTP, ITP, CTP, TTP, and UTP (their nitrogenous bases are shown in
Figure 1a) were applied to 5-day-old aequorin transgenic seedlings and the
bioluminescence signal detected with a photon-counting, charge-coupled device
(CCD) camera (Figure 1b). The purine-based nucleotides, ATP, GTP, and ITP
caused strong transient elevations in bioluminescence signals, which comprised
two distinguishable peaks; the first peak occurred at 30-40 sec and the second at
80-90 sec after nucleotide application. This result led us to further examine the
nature of this biphasic \([\text{Ca}^{2+}]_{\text{cyt}}\) response. The plant \([\text{Ca}^{2+}]_{\text{cyt}}\) response was
analyzed in the presence of various inhibitors, Gd\(^{3+}\), La\(^{3+}\) (Ca\(^{2+}\) channel inhibitors),
and U-73122 (a PLC inhibitor) with measurements pooled for 10-60 sec (the initial
peak) and 60-120 sec (the second elevation in \([\text{Ca}^{2+}]_{\text{cyt}}\)) after ATP addition. The
first peak of ATP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) elevation was significantly inhibited by 50-100
\(\mu\text{M Gd}^{3+}\); whereas the second peak was only inhibited at higher concentrations
(\(\geq 150 \mu\text{M}\)). The ATP effect on \([\text{Ca}^{2+}]_{\text{cyt}}\) was nullified at 500 \(\mu\text{M Gd}^{3+}\) (Figure 2a and
2b). A similar result was obtained with La\(^{3+}\) treatment (data not shown). In contrast,
in the presence of 5-10 \(\mu\text{M U-73122}\), a slight inhibition (~20%) was observed in the
second peak, but not in the first peak (Figure 2c and 2d). An inactive analog of U-
73122, U-73343, had no effect on the ATP-induced \([\text{Ca}^{2+}]_{\text{cyt}}\) response (data not
shown). Based on these results, we conclude that the first peak of ATP-induced
\([\text{Ca}^{2+}]_{\text{cyt}}\) elevation is likely due to external Ca\(^{2+}\) entry; whereas the second peak
likely corresponds to both the external Ca\(^{2+}\) entry and release of Ca\(^{2+}\) from interior
stores.

CTP (a pyrimidine-based nucleotide) also elicited a significant elevation in
\([\text{Ca}^{2+}]_{\text{cyt}}\); but neither TTP nor UTP caused a significant response at up to 100 \(\mu\text{M}\)
(Figure 1b). There was a notable difference in the kinetics of the \([\text{Ca}^{2+}]_{\text{cyt}}\) response
between CTP and purine-based NTPs. The kinetics of the bioluminescence signal
induced by CTP exhibited a broad peak at 100-150 sec after application; whereas, as noted above, the purine-based NTPs induced a sharp and transient increase in Ca\(^{2+}\) levels. Moreover, the CTP-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation was inhibited only by Gd\(^{3+}\) and La\(^{3+}\), but not by U-73122 (see Supplemental Figure S1), tentatively suggesting that this [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation is primarily generated by Ca\(^{2+}\) influx that is likely distinct from PLC-mediated Ca\(^{2+}\) mobilization.

To compare the magnitude of the effects of CTP and other NTPs, the response over 400 sec was integrated to calculate overall [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels. Figure 3a shows the [Ca\(^{2+}\)]\(_{\text{cyt}}\) levels induced by the NTPs were induced in the following descending order at 100 µM: ATP = GTP > ITP = CTP >> TTP, UTP. However, this order was changed at higher nucleotide concentrations (500 µM) as follows: ATP = CTP ≥ GTP > ITP, TTP, UTP (Figure 3b). Closer examination revealed that the calcium response with 100 µM ATP was confined primarily to root tissues, while at the higher concentration (500 µM) leaf tissues also responded (data not shown). This may account for the relative differences seen between these treatments.

To better characterize the effects of NTPs on the [Ca\(^{2+}\)]\(_{\text{cyt}}\) response, we applied NTPs over a range of concentrations from 0.1-1000 µM (Figure 3c). The effect of ATP on [Ca\(^{2+}\)]\(_{\text{cyt}}\) was stronger than any of the other nucleotides, and was easily detectable at 10 µM (P < 0.05) and saturated at 250 µM. The GTP effect was comparable to the ATP effect at 100 µM, and reached a plateau level of 50% of that seen with ATP after 250 µM; whereas ITP exhibited a similar dose-dependent curve as GTP. The effect of CTP was comparable to that of ATP at higher concentration (>250 µM), although moderate effects were found at lower concentrations (e.g., 60% of the ATP effect at 100 µM). TTP and UTP were only effective at concentrations greater than 250 µM. Because all of the nucleotides were applied as sodium salts, we assessed the effect of NaCl on the [Ca\(^{2+}\)]\(_{\text{cyt}}\) response. NaCl up to 5 mM caused no significant [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation (P>0.4 versus water treatment control; data not shown). Thus, the effects of NTPs on [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation are specific, even at high concentrations (up to 1000 µM). Taken together, these results suggest that plants indeed exhibit cellular responses with specificities
that depend on the nitrogenous base structures of the nucleotides (Figure 1a), which may reflect the specificity of individual receptor mechanisms.

**Mammalian P2 receptor antagonists inhibit extracellular NTP-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) responses in Arabidopsis seedlings**

A variety of P2 receptor antagonists have been characterized based on their ability to inhibit specific mammalian P2 receptor subtypes (Ralevic and Burnstock, 1998). While these compounds have been used in plant studies, due to the lack of well-characterized plant P2 receptors, their activity against plant extracellular ATP receptors is assumed but not proven. For example, the non-selective P2 receptor antagonists, suramin and PPADS, inhibited extracellular ATP-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) response in excised Arabidopsis roots (Demidchik et al., 2003). In theory, it may be possible to distinguish distinct plant NTP recognition mechanisms by their differing sensitivity to these various antagonists. These methods are well established for animal studies (Ralevic and Burnstock, 1998; González et al., 2005). Hence, we expanded upon earlier studies by including additional antagonists; i.e., reactive blue-2, iso-PPADS, Evans blue, and TNP-ATP, in addition to suramin and PPADS.

As shown in Figure 4, PPADS inhibited the ATP-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation, which is consistent with previous reports (Demidchik et al., 2003). PPADS also inhibited the [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevations induced by the other NTPs. In contrast to earlier studies in which dissected roots or root protoplasts were used (Demidchik et al., 2003; Demidchik et al., 2009), suramin showed no significant effect on purine nucleotide-induced responses in our experiments (Figure 4a to 4c). These contrasting results might be attributed to the tissue-specific sensitivity to suramin or differences of tissue conditions between intact seedlings and protoplasts or dissected roots. However, suramin caused significant inhibition of CTP-induced [Ca\(^{2+}\)]\(_{\text{cyt}}\) elevation (Figure 4d). This result suggests that CTP recognition by plant cells may be distinct from recognition of purine-based NTPs. Interestingly, only the effects of purine NTPs were significantly inhibited (by more than 60%) by Evans blue and TNP-ATP (Figure 4a to 4c). This result again suggests distinct recognition
systems for purine nucleotides versus CTP. We also verified the inhibitory effects of the antagonists on the biphasic kinetics of the purine nucleotide-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ responses. Our results showed that the antagonists reduced both peaks equally. However, we did notice that only the treatment with TNP-ATP appeared to more strongly inhibit the first Ca$^{2+}$ peak, relative to that of the second. Given that the first peak is most likely due to influx of external Ca$^{2+}$ and the second peak is composed of Ca$^{2+}$ both from exterior and interior stores, these results suggest that the antagonists, except for TNT-ATP, may inhibit the putative receptors involved in regulation of Ca$^{2+}$ flux both from exterior and interior store.

To examine the specificity of the antagonists to NTP-induced responses, we applied another stimulus, cold treatment, known to cause elevation in Ca$^{2+}$ levels but not thought to act through ATP signaling (Figure 4e). The $[\text{Ca}^{2+}]_{\text{cyt}}$ elevation induced by the addition of ice-cold water was not inhibited by most of the antagonists used, except iso-PPADS and Evans blue. Based on these results, iso-PPADS and Evans blue might be considered to have nonspecific effects on NTP-induced responses.

However, all antagonists used in this study, with the exception of suramin, exhibited some side effects at higher concentration (>300 µM) that reduced the bioluminescence signal even with respect to discharging of all of the remaining aequorin in the tissues (data not shown), implying that these antagonists might inhibit the enzymatic activity of aequorin and/or the luminescence signal itself. Therefore, we were unable to pursue experiments with higher concentrations.

The extracellular NTP-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ response is enhanced in heterotrimeric G protein mutants

Because P2Y receptor-dependent $[\text{Ca}^{2+}]_{\text{cyt}}$ responses in mammalian cells are exclusively mediated through the functions of heterotrimeric G proteins, we wondered whether plant heterotrimeric G proteins are involved in extracellular NTP-induced Ca$^{2+}$ signaling. Since the G$\alpha$ (GPA1) and G$\beta$ (AGB1) subunits are encoded by single-copy genes in the Arabidopsis genome, a genetic approach was
used to study their involvement in the NTP-induced Ca$^{2+}$ response. The single G protein mutants, *gpa1*-4 and *agb1*-2, and the double mutant, *gpa1*-4;*agb1*-2, were generated in the aequorin transgenic background by cross-pollination (see Materials and Methods). The individual mutants and the double mutant were then subjected to assays for the ATP-induced [Ca$^{2+}$]$_{cyt}$ response.

Upon addition of ATP to the mutants, *gpa1*-4 and *agb1*-2, the latter mutant exhibited at least a 3.5-fold stronger [Ca$^{2+}$]$_{cyt}$ response; whereas the response of the *gpa1*-4 mutant was similar to wild type (Figure 5). These data suggest that extracellular ATP-induced Ca$^{2+}$ signaling is negatively regulated by the G$\beta$ subunit AGB1 or the G$\beta$γ complex. The [Ca$^{2+}$]$_{cyt}$ response in the *gpa1*-4;*agb1*-2 double mutant was similar to that seen in *agb1*-2, but not that of *gpa1*-4. These results indicate that G$\beta$(γ), not the G$\alpha$ subunit GPA1, is involved in nucleotide-induced Ca$^{2+}$ signaling, rather than AGB1 acting through epistatic effects on GPA1. Similar results to those observed upon ATP addition were obtained with addition of GTP, ITP or CTP (Figure 5). These data imply that these nucleotides, after being recognized on the plasma membrane, may share a common intracellular signaling pathway to the triggering of Ca$^{2+}$ increase that is negatively regulated by AGB1.

We also observed similar hypersensitivity of *agb1* and *gpa1;agb1* mutants to the poorly hydrolyzable analogs of ATP and ADP, ATP$\gamma$S and ADP$\beta$S (data not shown). Again, this result suggests that AGB1 is regulating nucleotide signaling following receptor recognition, and not via regulation of apoplastic ATP (e.g., by changing ectoapyrase activity).

Poorly-hydrolyzable nucleotide analogs induce [Ca$^{2+}$]$_{cyt}$ oscillations: inhibition of ectoapyrase increases the number of [Ca$^{2+}$]$_{cyt}$ oscillations induced by extracellular nucleotides

In order to insure that the responses seen where to the nucleotides and not the result of hydrolysis, we examined the [Ca$^{2+}$]$_{cyt}$ response to poorly hydrolyzable (thiol containing) analogs of adenine-based nucleotides (i.e., ATP$\gamma$S and ADP$\beta$S), which are relatively less susceptible to hydrolysis (Figure 6). ATP, ADP and their
poorly-hydrolyzable analogs induced significant increases in \([Ca^{2+}]_{cyt}\); whereas AMP and its poorly-hydrolyzable analog had no significant effect. We also confirmed that adenosine, adenine, and adenine derivatives (e.g., zeatin, kinetin, benzyl adenine), as well as inorganic phosphate, had no significant effects compared to mock treated controls. These data suggest that the increased \([Ca^{2+}]_{cyt}\) is a specific response to nucleoside tri- and diphosphate. Intriguingly, the effect of ADP\(\beta\)S (poorly-hydrolyzable ADP) was significantly stronger than all adenine nucleotides tested; the response was 1.5- to 2-fold higher than that of ATP, ATP\(\gamma\)S, or ADP (\(P<0.04, P<0.03,\) and \(P<0.01\) versus ADP\(\beta\)S, respectively). The fact that poorly-hydrolyzable analogs of both ATP and ADP have activities with different magnitude could indicate the existence of multiple and distinct receptor mechanisms for specific nucleotides.

A number of reports suggest that plant extracellular ATP levels could be modulated through the action of ectoapyrases (Steinebrunner et al., 2003; Wolf et al., 2007; Wu et al., 2007; Govindarajulu et al., 2009), i.e., membrane associated nucleoside tri- and diphosphohydrolases with the predicted catalytic domain in the apoplast. Hence, it is possible that the differential response to ADP and ADP\(\beta\)S was due to the hydrolysis of nucleotides by ectoapyrase. Previously, Windsor et al. (2003) identified a plant apyrase inhibitor, NGXT191. Application of NGXT191 alone had no effect on \([Ca^{2+}]_{cyt}\) (data not shown). However, consistent with hydrolysis of ADP by ectoapyrase, in the presence of NGXT191, seedlings responded to ADP addition to a level equivalent to that seen with addition of ADP\(\beta\)S alone (Figure 7a). Most notably, in the presence of NGXT191, ADP induced temporal \([Ca^{2+}]_{cyt}\) oscillations that lasted for 150 sec with ~20 sec periodicity for four to six peaks (Figure 7b and 7c; Supplemental Movie S1). Interestingly, the \([Ca^{2+}]_{cyt}\) oscillations were also induced by other nucleotides, such as ADP\(\beta\)S and ATP\(\gamma\)S, in the presence of NGXT191 (Supplemental Table S1). Similar \([Ca^{2+}]_{cyt}\) oscillation responses were also observed in the G protein mutants, although the number of the oscillations was slightly reduced in the \textit{agb1-2} and
gpa1-4; agb1-2 mutants (Figure 7d; Supplemental Table S2). Note that the initial peak before the start of the ADP-induced oscillations appears to be reduced by NGXT191 treatment in Figure 7b. However, this observation was not significant since it did not occur in all the experiments conducted.

To characterize the [Ca^{2+}]_{cyt} oscillations, we assessed the effects of the Ca^{2+} channel blockers (Gd^{3+} and Verapamil) and a PLC inhibitor (U-73122) on nucleotide responses in the presence of NGXT191. All of these chemicals completely abolished the [Ca^{2+}]_{cyt} oscillations induced by ATP and ADP in the presence of NGXT191 (Table 1), indicating that the [Ca^{2+}]_{cyt} oscillations are likely orchestrated by Ca^{2+} influxes from exterior and interior stores.

We initially presumed from the observed [Ca^{2+}]_{cyt} oscillations that exogenously applied nucleotides undergo immediate degradation by ectoapyrases, and also that a continuous nucleotide stimulus causes the [Ca^{2+}]_{cyt} oscillation response only in the absence of ectoapyrase activity. In order to investigate the possibility that the relative instability of ATP and ADP affected the [Ca^{2+}]_{cyt} response kinetics, we tested the effect of the poorly hydrolyzable nucleotides ATP\gamma S and ADP\beta S (Figure 6). One would presume that these compounds would effectively result in a more continuous and stable stimulus of nucleotides at the cell surface. The kinetics of the [Ca^{2+}]_{cyt} response seen with ATP\gamma S or ADP\beta S was very similar to that found with the native nucleotides (data not shown); that is, oscillations were only seen in the presence of NGXT191 (Figure 7b).

Thus, it is difficult to reconcile how ectoapyrase inhibition increases the number of nucleotide-induced [Ca^{2+}]_{cyt} oscillations. An alternative hypothesis is that the initial addition of ATP or ADP triggers serial release of endogenous ATP that under normal conditions is rapidly degraded by ectoapyrases before generating the serial [Ca^{2+}]_{cyt} oscillations (see Discussion). This would explain why the ectoapyrase inhibitor NGXT191 was required for [Ca^{2+}]_{cyt} oscillations even when poorly-hydrolyzable nucleotides were added. It was reported that extracellular ATP is released from plant cells by plasma membrane ABC transporters and exocytosis (Thomas et al., 2000; Kim et al., 2006). Therefore, to determine which secretion
system is responsible for the phenomenon, we treated plants with brefeldin A (BFA), which is a vesicle trafficking inhibitor, and 1-naphthylphthalamic acid (NPA), which is an inhibitor of the ABC transporters belonging to the multidrug resistance-like p-glycoprotein subclass (MDR/PGPs), especially PGP1 and PGP19 (Murphy et al., 2002; Geisler et al., 2003). The number of oscillations and frequency of occurrence of the $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations were remarkably diminished in presence of BFA, but not NPA (Figure 7e; Table 1). These results imply that the $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations are likely to be induced by released ATP (or some other compound(s)) secreted by vesicle trafficking.

**NTP-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ responses show cell type specific kinetics**

The specificity of the kinetics of $[\text{Ca}^{2+}]_{\text{cyt}}$ response to each nucleotide led us to ask whether these changes reflected different NTP responses being exhibited by different cell types and tissues, or whether individual cells could respond to all NTP types but with different parameters. The experiments using aequorin transgenic seedlings indicated that the majority of the $[\text{Ca}^{2+}]_{\text{cyt}}$ response to nucleotides ($\leq 100 \mu\text{M}$) was confined to the root tissues. Hence, the response of roots to NTP addition was examined utilizing plants expressing the GFP-based cameleon sensor. This sensor was originally developed for use in mammalian cells (Miyawaki et al., 1997) and was first applied to plants in guard cells (Allen et al., 1999). We applied a version with enhanced fluorescence resonance energy transfer signal, YC3.6 (Nagai et al., 2004) and confocal ratio imaging of plant roots (Monshausen et al., 2008) to follow the dynamics of the NTP-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ response with cellular resolution.

Supplemental Figure S2 shows that when the response was averaged over the root tip, ATP caused rapid changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ consistent with the kinetics monitored by aequorin. Moreover, the cellular analysis possible with this confocal approach revealed complex cell-type specificity in the details of these kinetics (Figure 8); lateral root cap cells responded more slowly than the subtending cell layer to exogenous ATP, even though these cells would have experienced...
extracellular ATP before those in the underlying cell layers. Interestingly, the subtending layer was capable of exhibiting strong oscillations in [Ca\textsuperscript{2+}]\textsubscript{cyl} not seen in the overlying cell layer (Figures 8a and 8b; Supplemental Figure S3), although the occurrence and magnitude of these oscillations was variable (oscillations seen in 57% of cells, n=14). These changes suggest that there are cell type differences in response to ATP, possibly reflecting a variety of perception and/or response machinery in each cell type. Such differences did not reflect generic differences in the [Ca\textsuperscript{2+}]\textsubscript{cyl} responsiveness of different cell layers; for example, cold shock treatment also increased [Ca\textsuperscript{2+}]\textsubscript{cyl} but led to a very different pattern of cell type-selective response and recovery of [Ca\textsuperscript{2+}]\textsubscript{cyl} levels (Supplemental Figure S3; Supplemental Movies S2 and S3).

Aequorin measurements in the presence of BFA suggested that secretion plays a role in nucleotide release. Therefore, the effect of BFA was tested while monitoring [Ca\textsuperscript{2+}]\textsubscript{cyl} with YC3.6 in root cells. Similar to the effects seen using the aequorin expressing plants, the observed [Ca\textsuperscript{2+}]\textsubscript{cyl} changes at the single cell level following ATP treatment were diminished following a preincubation in 10 µM BFA (data not shown). Consistent with the fact that NGXT191 treatment was not essential to see calcium oscillations in root cells using YC3.6 fluorescence, preincubation of root for 30 min with 10 mg mL\textsuperscript{-1} NGXT191 did not affect the calcium response seen upon the addition of ATP (data not shown).

Differences in the kinetics of [Ca\textsuperscript{2+}]\textsubscript{cyl} response of individual cells were also observed in response to other nucleotides such as GTP and CTP (Figure 8c and Supplemental Movies S4 and S5). Analysis of the [Ca\textsuperscript{2+}]\textsubscript{cyl} increases in response to this range of NTPs in a single cell type (Figure 8c), suggests that a single cell is capable of perceiving all of the NTPs tested and translating these into a cellular Ca\textsuperscript{2+} signal. However, the differences in the kinetics of the Ca\textsuperscript{2+} change could carry information as to the NTP triggering the response. Thus, the different Ca\textsuperscript{2+} dynamics seen in response to different NTPs monitored using aequorin are unlikely to simply reflect different cell types each responding to a specific NTP but suggest one cell can respond to different NTPs, possibly through multiple receptor systems.
DISCUSSION

Intracellular calcium is a ubiquitous signaling component in plant cells that is increased in response to many physiological stimuli, such as light, touch, pathogenic elicitors, plant hormones and abiotic stresses (Rudd and Franklin-Tong, 2001). Extracellular ATP also alters $[\text{Ca}^{2+}]_{\text{cyt}}$ levels presumably mediated through receptors on the plasma membrane (Demidchik et al., 2009). Our new data begin to reveal additional complexity of the plant cell response to extracellular ATP.

It is worth discussing whether pyrimidine and purine nucleotides, other than ATP, can function as extracellular ligands in plants. Although animal P2 receptors were originally identified as receptors for purine nucleotides, they have diverse ligand specificities. For example, there are pyrimidine-recognizing or -preferring P2Y receptors, such as P2Y$_2$, P2Y$_4$, and P2Y$_6$, (Ralevic and Burnstock, 1998; Burnstock, 2007), suggesting that different P2 receptors display specificity for particular nucleotides. In plants, little information exists on the activity of various nucleotides and associated plant cell responses. For instance, early studies demonstrated that ATP and CTP, but not GTP or UTP, are involved in maintaining the water permeability of potato storage tissue (Stuart, 1973). UTP in addition to purine NTPs causes potassium ion uptake into the cells of maize leaf slices (Lüttge et al., 1974). Recently, GTP, CTP, and UTP (as well as ATP) were found to induce superoxide production in Arabidopsis leaves (Song et al., 2006). These NTPs prevented cell death induced by the toxin fumonisin B1 in Arabidopsis (Chivasa et al., 2005), whereas GTP and CTP—but not UTP—had similar attenuating effects as ATP on systemic acquired resistance induced by tobacco mosaic virus (Chivasa et al., 2009). Some authors suggested that the effects of non-ATP nucleotides are probably the result of an artifact caused by protection of endogenous extracellular ATP from degradation (Ostrom et al., 2000; Lazarowski et al., 2003; Chivasa et al., 2005). An alternative view would be that these differences might reflect differing nucleotide affinities of a single receptor protein. However, our results raise the
possibility that individual plant cells are in fact competent to recognize nucleotides in distinctly different manners. In particular, CTP caused a $[\text{Ca}^{2+}]_{\text{cyt}}$ response that was quite distinct from that of the other nucleotides whether monitored at the whole plant (i.e., with aequorin, Figure 1) or single cell (i.e., with YC3.6, Figure 8). For example, the time- and dose-dependent kinetics of $[\text{Ca}^{2+}]_{\text{cyt}}$ response upon CTP addition were distinct in shape from those resulting from purine nucleotides (Figures 1, 2, and 8). The CTP-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ changes were composed of $\text{Ca}^{2+}$ influx from the extracellular space; whereas ATP-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increases were dependent on $\text{Ca}^{2+}$ influx and release of calcium from intracellular stores (as judged by our pharmacological studies; see Figures 2 and Supplemental Figure S1). Additionally, the profiles of the inhibitory effects of mammalian P2 receptor antagonists were distinct for the purine and CTP-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ responses (Figure 4). Finally, our imaging data indicate that a single cell type is capable of responding to a range of nucleotides (Figure 8c), suggesting that these differences in characteristics do not simply reflect differential response kinetics of different tissue types each responding to only a single NTP. Thus, our observations, together with previous studies, suggest that multiple receptors or signaling mechanisms likely exist in plant cells to mediate responses activated by extracellular nucleotides.

Two classes of P2 receptors in mammals mediate extracellular ATP signaling; the ionotropic receptor is responsible for fast cell response and the metabotropic receptor is involved in the response that has a longer latency after stimuli in comparison to ionotropic signaling (Lechner and Boehm, 2004). In mammals, these two activities show tissue specificity (Burnstock, 2007); occasionally, either or both ionotropic and metabotropic receptors exist together even in the same cell type, e.g., in rat spiral ganglion neurons (Ito and Dulon, 2002). We observed biphasic kinetics of $[\text{Ca}^{2+}]_{\text{cyt}}$ induced by ATP using whole seedlings of Arabidopsis (Figure 2), suggesting that plants do possess two types of signal transduction in response to extracellular ATP, each of which may be activated by ionotropic or metabotropic extracellular ATP receptors. Demidchik et
al. (2009) reported that Ca\textsuperscript{2+} mobilization via metabotropic extracellular ATP signaling activates a plasma membrane ionic conductance in protoplasts isolated from the mature root epidermis. The current study and that of Demidchik et al. (2009) use different plant material and, therefore, may not be directly comparable. However, we are tempted to speculate that either or both classes of ATP receptors may mediate extracellular ATP signaling depending on the plant cell type. Such questions cannot be adequately addressed until the putative plant ATP receptors are identified and their cell specific activities can be studied.

In metabotropic P2 receptor signaling, heterotrimeric G proteins are typically required to stimulate PLC-mediated Ca\textsuperscript{2+} release from the ER (Ralevic and Burnstock, 1998; Burnstock, 2007). The PLC pathway is also tentatively implicated in plant cell ATP signaling as evidenced by the fact that U-73122 (a PLC inhibitor) partially inhibited ATP-induced [Ca\textsuperscript{2+}]\textsubscript{c} responses (Figure 2c and 2d). However, the involvement of G proteins in the extracellular ATP-induced calcium signaling in plant cells is unlikely to be identical to that seen in animals. Most notably, the elevation of [Ca\textsuperscript{2+}]\textsubscript{c} upon ATP addition in the agb1-2 mutant was significantly stronger than that seen in the wild type or gpa1-4 mutant, suggesting that the G\beta subunit AGB1 negatively regulates extracellular nucleotide signaling. Furthermore, we detected no significant differences in the ratio of the first peak of elevated [Ca\textsuperscript{2+}]\textsubscript{c} to the second peak between wild type and G protein mutants (data not shown). As the PLC inhibitor U-73122 preferentially inhibited the second peak (figure 2c and 2d), this failure to alter the relative magnitudes of either peak in the [Ca\textsuperscript{2+}]\textsubscript{c} response indicates that plant G proteins are unlikely to be involved in the PLC-mediated Ca\textsuperscript{2+} signaling pathway. The nature of the mechanism underlying the regulation of nucleotide-induced [Ca\textsuperscript{2+}]\textsubscript{c} responses by AGB1 is still unknown. However, it is possible that the observed hyper-responsiveness to nucleotides in the agb1 mutant is attributable to a general lack of cellular control in desensitizing extracellular nucleotide signaling, as is the case for touch-induced desensitization of ATP release (Weerasinghe et al., 2009).
Interestingly, similar to \textit{agb1-2} mutant plants, the double mutant \textit{gpa1-4;agb1-2} showed significantly enhanced [Ca$^{2+}$]$_{c_{yt}}$ responses to ATP addition. If this process were controlled by canonical heterotrimeric G protein signaling, G\(\beta(\gamma)\) mediated signaling should also be affected upon disruption of G\(\alpha\) and vice versa, because G\(\beta\gamma\) and G\(\alpha\) are mutually dependent for appropriate membrane targeting and for interaction with receptors (Marrari et al., 2007). Hence, we might have concluded that AGB1 is epistatic to GPA1. However, the \textit{gpa1} mutant displayed no detectable differences compared with wild type in its nucleotide-included [Ca$^{2+}$]$_{c_{yt}}$ elevation. Moreover, the behavior of the double mutant was indistinguishable from the single mutant in these assays. Therefore, we conclude that AGB1 is involved in nucleotide-induced Ca$^{2+}$ signaling independently of G\(\alpha\) subunit, rather than that AGB1 acts downstream of GPA1 in this signaling pathway. This conclusion is similar to that made in published reports describing the phenotypes altered only in \textit{agb1} mutants on silique shape (Lease et al., 2001), cell death associated with the unfolded protein response (Wang et al., 2007), and root waving and skewing responses (Pandey et al., 2008). In agreement with the conclusion of Pandey et al. (2008), we propose that the AGB1 subunit functions in an NTP-induced [Ca$^{2+}$]$_{c_{yt}}$ response without forming a G\(\alpha\)\(\beta\gamma\) heterotrimeric complex.

In experiments using Arabidopsis seedlings expressing aequorin, we found that the addition of single doses of ATP, ADP, or poorly-hydrolyzable analogs in the presence of a plant apyrase inhibitor, NGXT191, induced multiple temporal [Ca$^{2+}$]$_{c_{yt}}$ oscillations, in contrast to the transient [Ca$^{2+}$]$_{c_{yt}}$ response in the absence of ectoapyrase inhibition (Figure 7; Supplemental Table S1). Our initial conclusion from these experiments was that nucleotide hydrolysis by ectoapyrases resulted in a rapid loss of extracellular nucleotides, and that a sustained [Ca$^{2+}$]$_{c_{yt}}$ response was seen only when these enzymes were inhibited. However, this conclusion is inconsistent with the data in which a single application of poorly hydrolyzable analogs, ATP\(\gamma\)S or ADP\(\beta\)S, did not cause oscillations (data not shown), but in the presence of the apyrase inhibitor oscillations occurred (Supplemental Table S1).
ATPγS and ADPβS are known to be relatively resistant to hydrolysis by ectonucleotidases (Picher et al., 1996; Gendaszewska-Darmach et al., 2003). Therefore, the long-term hydrolysis rate is likely not the critical component causing the [Ca²⁺]ₖᵢᵣ oscillations observed (Figure 7). Therefore, one possibility is that initial ATP addition triggers the serial release of small amounts of endogenous ATP, which is essential for the subsequent calcium oscillations that are only apparent with ectoapyrase inhibition. This model would explain why poorly-hydrolyzable nucleotides were also able to cause the [Ca²⁺]ₖᵢᵣ oscillations.

In mammals, ATP release can be induced by ATP or also other agonists, e.g., ADP and UTP (Yang et al., 1994; Matsuo et al., 1997; Cotrina et al., 1998; Anderson et al., 2004). Moreover, several reports described an interaction between ATP release and [Ca²⁺]ₖᵢᵣ oscillations. For example, spontaneous [Ca²⁺]ₖᵢᵣ oscillations required nucleotide release in canine renal epithelia (Geyti et al., 2008). ATP is also rhythmically released to control the intercellular synchronization of [Ca²⁺]ₖᵢᵣ oscillations in rat cardiac myocytes in a phenomenon dependent on purinoceptor signaling (Kawahara and Nakayama, 2007; Nakayama et al., 2007). In general, the release of ATP to the extracellular matrix can be mediated by exocytosis, anion channels, and ABC transporters in mammals (Lazarowski et al., 2003). In plants, ATP release was reported to occur mediated by AtPGP1 and vesicle trafficking (Thomas et al., 2000; Kim et al., 2006). We found that BFA, not NPA, specifically impaired the nucleotide-induced [Ca²⁺]ₖᵢᵣ oscillations (Figure 7e and Table 1). This result strengthens our hypothesis that the nucleotide-induced [Ca²⁺]ₖᵢᵣ oscillations requires ATP release mediated by vesicle trafficking, such as exocytosis. However, we cannot rule out the possibility that MDR/PGPs, other than AtPGP1, are required, since verapamil, which inhibited nucleotide-induced [Ca²⁺]ₖᵢᵣ responses (Table 1), also acts as an inhibitor of mammalian MDR/PGPs (Horio et al., 1989), as well as Ca²⁺ channels.

When monitored at a single cell level, applied ATP induced [Ca²⁺]ₖᵢᵣ oscillations (Figures 8b and Supplemental Figure S3a; Supplemental Movie S2) without addition of the ectoapyrase inhibitor NGXT191. However, such oscillations
were limited to specific cell types and were variable in occurrence. Considering that the aequorin-based $[\text{Ca}^{2+}]_{\text{cyt}}$ measurement results from integrated data of $[\text{Ca}^{2+}]_{\text{cyt}}$ elevations/oscillations in single cells (Dodd et al., 2006), our observations suggest that ectoapyrase activity may vary between cell types, possibly in an environmentally responsive manner, and is likely low around those root cells exhibiting $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations visualized with YC3.6. Addition of NGXT191 may act to lower ectoapyrase activity around all cell types allowing aequorin to monitor oscillations that develop across the entire plant. Inclusion of the inhibitor with the ATP did not elicit clear oscillations in the root tip cells analyzed suggesting that the oscillatory changes revealed by aequorin arise from outside this region.

It is impossible to exclude the possibility that some fraction of NGXT191 might cross the plasma membrane and inhibit intracellular apyrase activity. Although the cellular functions of plant intracellular apyrases have not been studied, there is a well-developed model for apyrase function in the Golgi and ER in yeast and Caenorhabditis elegans related to control of protein glycosylation (Hirschberg et al., 1998; Uccelletti et al., 2008). It was reported that glycosylation of Ca$^{2+}$ sensors in the ER regulates store-operated Ca$^{2+}$ influx (Taylor, 2006; Czyz et al., 2009). It is interesting to speculate that disruption of the regulation system of intracellular Ca$^{2+}$ flux by NGXT191 might contribute to ATP-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations. Evidence of intracellular apyrase activity correlated to protein glycosylation rate in ER would be needed to support this model. It is also important to note that, although NGXT191 has been used to inhibit apyrases in plants, it is known to have low, but measurable, inhibitor activity against alkaline phosphatase (Windsor et al., 2003). Results obtained with any pharmacological reagent should be interpreted with caution and it is for precisely this reason that we sought independent confirmation of the ATP-induced calcium oscillations utilizing transgenic plants expressing the YC3.6 chameleon calcium reporter (see discussion below).

What is the function of nucleotide-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations? In plants, specific $[\text{Ca}^{2+}]_{\text{cyt}}$ oscillations are known to be crucial to the control of stomatal
function, pollen tube and root hair growth, and the response of legume root hairs to rhizobial-produced Nod factor signals (Evans et al., 2001; Monshausen et al., 2008; Oldroyd and Downie, 2008; McAinsh and Pittman, 2009). It is tempting to propose that extracellular ATP functions as a component of these processes to elicit the $[Ca^{2+}]_{c}_{yt}$ oscillations. For example, previous reports demonstrated a critical role for ectoapyrases during the early steps of legume root hair infection by rhizobia (Etzler et al., 1999; McAlvin and Stacey, 2005; Govindarajulu et al., 2009). Presumably, these ectoapyrases are controlling the level of extracellular ATP at the root hair surface. Indeed, the addition of exogenous ADP to soybean root hairs was shown to significantly increase nodulation (Govindarajulu et al., 2009). Utilizing a reporter system that directly imaged extracellular ATP levels, Kim et al. (2006) demonstrated the release of extracellular ATP from the tips of growing legume root hairs. Kim et al. (2006) and Wu et al. (2008) also implicated extracellular ATP in the plant cell response to specific pathogen associated elicitors (e.g., chitin). Consistent with these findings, chitin addition to aequorin-expressing Arabidopsis seedlings in the presence of NGXT191 induced $[Ca^{2+}]_{c}_{yt}$ oscillations similar to those shown in Figure 7c (K. Tanaka and G. Stacey, unpublished data). Hence, the data are suggestive of a critical and largely unexplored role for extracellular ATP release in mediating $[Ca^{2+}]_{c}_{yt}$ signaling essential for a variety of plant cellular responses.

When monitored at a cellular level using the YC3.6 $Ca^{2+}$ sensor, we observed that NTP addition elicited changes in $[Ca^{2+}]_{c}_{yt}$ that did not occur synchronously across the root. Thus, ATP and GTP caused elevations in $[Ca^{2+}]_{c}_{yt}$ that were broadly similar in magnitude and progression through the root tip but that were markedly different in magnitude and spatial and temporal characteristics from CTP (Figure 8 and Supplemental Figure S2; Supplemental movies S2, S4, and S5). These NTP-specific $[Ca^{2+}]_{c}_{yt}$ signatures may well provide information to the root as to the NTP driving the $Ca^{2+}$ signaling and have the potential to elicit stimulus-specific downstream responses (McAinsh and Pittman, 2009). Further investigation of this new and emerging role for extracellular nucleotides as a critical extracellular
signal in plants is likely to provide novel insight into the basic mechanisms of plant
cell growth and environmental response.

MATERIALS AND METHODS
Reagents
All chemicals and enzymes, except those mentioned elsewhere, were
purchased from Sigma-Aldrich. Stock solution of nucleotides, antagonists of
mammalian P2 receptors (Tocris Bioscience) and calcium cannel blockers were
prepared in water at 100 mM and stored at -20ºC until use. NGXT191 (a kind gift of
Dr. Stanley J. Roux, University of Texas, USA), NPA (Frinton Laboratories), U-
73122, and BFA were dissolved in dimethylsulfoxide. The final concentration of the
solvent under assay conditions did not exceed 0.1% (v/v).

[Ca^{2+}]_{cyt} measurement by aequorin luminometry and confocal microscopy
Aequorin transgenic seedlings in Col-0 background (kindly provided by Dr.
Marc R. Knight, University of Oxford, UK) were sterilized for 5 min in 5% sodium
hypochlorite solution and then rinsed twice and resuspended in sterile water. The
seeds were then sown along a straight line on half-strength MS medium containing
1% (w/v) sucrose, 1% (w/v) agar, 0.05% (w/v) MES (pH 5.7), and Gamborg’s B5
vitamins. After incubation at 4ºC for 3 d to synchronize seed germination, Petri
dishes with the seeds were placed vertically in a plant growth chamber set at 22ºC
under continuous light conditions. Five-day-grown seedlings were transferred
individually to individual wells of a 96-well microplate, and incubated overnight at
room temperature in 50 µL reconstitution buffer containing 2mM MES (pH 5.7), 10
mM CaCl₂, and 10 µM coelenterazine (Nanolight Technology). After overnight
incubation, chemicals were applied at indicated final concentrations. Photon
emissions were detected using an intensified CCD camera, Photek 216 (Photek
Ltd.). At the end of each experiment, remaining aequorin was discharged by the
addition of an equal volume of solution containing 2 M CaCl₂ and 20% (v/v) ethanol.
Luminescence values were calibrated as calcium concentrations, according to Knight et al. (1996).

For cytoplasmic calcium imaging using confocal microscopy, Arabidopsis seedlings expressing the FRET-based Ca\(^{2+}\) sensor cameleon YC3.6 (Miyawaki et al., 1997; Allen et al., 1999; Nagai et al., 2004) were mounted in a thin layer of Phytagel media on 22 x 40 mm coverglass as described in Blancaflor et al. (Blancaflor et al., 1998). The root tips of 5 day old seedlings were exposed by cutting a window in the gel to which a 1x solution of 10 µM ATP, CTP, GTP, or media without NTPs were added. Roots were ratio imaged with the Zeiss LSM 510 microscope (Carl Zeiss Inc.) at intervals of 5 sec or 3 sec using the 40X objective with the 458-nm line of the argon laser exciting the YC3.6. The CFP (473–505 nm) and FRET-dependent (536–546 nm) emission were collected by using a 458-nm primary dichroic mirror and the Meta detector of the microscope. Images were pseudocolored (overlays of the CFP channel in green and the FRET channel in red) using the Zeiss LSM software. Ratios were calculated using iVision software (BioVision Technologies), statistical analysis was done using Excel (Microsoft Co.), and graphs were generated using KaleidaGraph (Synergy Software).

**Identification of heterotrimeric G protein mutants in aequorin transgenic background**

Heterotrimeric G protein mutants in the aequorin transgenic background were screened from F2 generation pools after cross-pollination between \textit{gpa1-4;agb1-2} double mutant (kindly provided by Alan M. Jones, University of North Carolina, USA) and aequorin transgenic Arabidopsis. Homozygous T-DNA insertions were selected by PCR using gene-specific primers (Jones et al., 2003; Ullah et al., 2003) and a T-DNA left-border-specific primer, LBb1 (5'-GCGTGGACCGCTTGCTGCAACT-3'). The presence of the aequorin transgene was confirmed in the F2 generation by detection of bioluminescence in its dissected cotyledon using reconstitution buffer and discharging solution (see
above); homozygosity was then determined in the F3 generation by the same method.

**Statistical Analysis**

All experiments were repeated at least three times and the data obtained were analyzed using ANOVA followed by Student’s $t$ test or Turkey-Kramer multiple comparison test. A difference with $P < 0.05$ was considered significant.

**SUPPLEMENTAL DATA**

The following materials are available in the online version of this article.

**Figure S1.** Effects of a PLC inhibitor and a Ca$^{2+}$ channel inhibitor on CTP-induced [Ca$^{2+}$]$_{cyt}$ response in Arabidopsis seedlings.

**Figure S2.** Effect of ATP, GTP or CTP on [Ca$^{2+}$]$_{cyt}$ response in the root tip of Arabidopsis seedlings monitored by YC3.6.

**Figure S3.** Effect of ATP and cold shock on [Ca$^{2+}$]$_{cyt}$ response in Arabidopsis root tips.

**Table S1.** Nucleotide-induced [Ca$^{2+}$]$_{cyt}$ oscillations in Arabidopsis.

**Table S2.** Nucleotide-induced [Ca$^{2+}$]$_{cyt}$ oscillations in heterotrimeric G protein mutants.

**Movie S1.** NGXT191 elicited ADP-induced [Ca$^{2+}$]$_{cyt}$ oscillations.

**Movie S2.** Effect of ATP on [Ca$^{2+}$]$_{cyt}$ response in the root tip of Arabidopsis seedlings.
Movie S3. Effect of cold shock on $[\text{Ca}^{2+}]_{\text{cyt}}$ response in the root tip of Arabidopsis seedlings.

Movie S4. Effect of GTP on $[\text{Ca}^{2+}]_{\text{cyt}}$ response in the root tip of Arabidopsis seedlings.

Movie S5. Effect of CTP on $[\text{Ca}^{2+}]_{\text{cyt}}$ response in the root tip of Arabidopsis seedlings.

ACKNOWLEDGEMENTS

We are grateful to Alan M. Jones for heterotrimeric G protein mutant Arabidopsis, Dr. Marc R. Knight for aequorin transgenic Arabidopsis, and Dr. Stanley J. Roux for providing NGXT191. Special thanks to Drs. Gary A. Weisman and Seth D. Findley (University of Missouri, USA) for critical comments on the manuscript.
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FIGURE LEGENDS

Figure 1. NTPs increase bioluminescence in aequorin-expressing transgenic Arabidopsis seedlings.

(A) Chemical structures of purine and pyrimidine derivatives.

(B) Individual 5-day-old aequorin seedlings were transferred to individual wells of a 96-well microplate, and incubated overnight in reconstitution buffer containing coelenterazine. Each NTP was then applied at a final concentration of 100 µM. The line graph shows time-dependent changes in photon counts from representative wells of each treatment (bin size = 50 frames, 1 sec, 20 bin smoothing). The inset picture is a pseudocolored photon-counting image integrated over 400 sec after nucleotide treatment calibrated to the inset scale.

Figure 2. Effects of a PLC inhibitor and a Ca²⁺ channel inhibitor on ATP-induced [Ca²⁺]ₖₒₜ response in Arabidopsis seedlings.

Arabidopsis seedlings harboring reconstituted aequorin were pre-incubated for 30 min with Gd³⁺ (a Ca²⁺ channel inhibitor) (a and b) or U-73122 (a PLC inhibitor) (c and d) at the indicated concentrations; 100 µM ATP was then applied. The remaining aequorin in the tissues was then discharged for [Ca²⁺]ₖₒₜ calculation (see Materials and Methods).

(a) and (b) Kinetic differences of ATP-induced bioluminescence in the presence or absence of Gd³⁺ (a) or U-73122 (b).

(c) and (d) Data were calculated as integrated [Ca²⁺]ₖₒₜ values over 10-60 sec or 60-120 sec (refer to the upper graphs) and then converted into a relative value to mock treatment. Asterisks indicate statistically significant differences compared with mock treatment control; *P<0.05, **0.001<P<0.01, ***P<0.001.

Figure 3. Effects of NTPs on [Ca²⁺]ₖₒₜ response in Arabidopsis seedlings.

Each integrated [Ca²⁺]ₖₒₜ was obtained from luminescence data recorded over 400 sec after treatment and discharging of the remaining aequorin signal (see Materials and Methods).
(a) and (b) Histograms show means with SE (n = 8) as integrated [Ca^{2+}]_{cyt} values that were recorded after treatment with 100 µM (a) or 500 µM NTPs (b). Different letters indicate statistically significant differences at P<0.05.
(c) Dose-dependent curves of NTP-induced [Ca^{2+}]_{cyt} responses.

Figure 4. Effects of antagonists of mammalian P2 receptors on nucleotide-induced [Ca^{2+}]_{cyt} response in Arabidopsis seedlings.
Arabidopsis seedlings harboring reconstituted aequorin were pre-incubated for 30 min with antagonists for mammalian P2 receptors at the indicated concentrations (10-300 µM). The indicated nucleotide was then applied: ATP (a), GTP (b), ITP (c), and CTP (d). Ice-cold water was applied as a negative control (e). The integrated [Ca^{2+}]_{cyt} values were calculated from normalized data of luminescence signal recorded over 400 sec after treatment and discharging of the remaining aequorin signal (see Materials and Methods). Histograms show the mean with SE as relative values to a single treatment of nucleotide. Non-selective P2 receptor antagonists are suramin, reactive blue-2 (RB2), and PPADS. Selective P2X antagonists are iso-PPADS, Evans blue (EB), and TNP-ATP. Mean [Ca^{2+}]_{cyt} values of single nucleotide treatment controls were as follows: ATP, 1.9 ± 0.19 µM; GTP, 1.8 ± 0.15 µM; ITP, 1.3 ± 0.06 µM; CTP, 1.2 ± 0.04 µM; Cold treatment, 2.6 ± 0.55 µM. Asterisks indicate statistically significant differences compared with single application of nucleotides; *P<0.05, **0.001<P<0.01, ***P<0.001.

Figure 5. Effects of nucleotides on [Ca^{2+}]_{cyt} response in heterotrimeric G protein mutant backgrounds.
NTPs (100 µM) were applied to three independent aequorin lines in gpa1-4, agb1-2, and gpa1-4;agb1-2 mutant backgrounds. Histograms show means with SE as relative values (Log2 ratio) to wild type background control. Note that aequorin seedlings in agb1 and gpa1-4;agb1-2 backgrounds exhibited hyper-responsiveness to each of the nucleotides used. Mean [Ca^{2+}]_{cyt} values of wild type controls were as follows: ATP, 2.1 ± 0.36 µM; GTP, 2.0 ± 0.22 µM; ITP, 1.3 ± 0.06
µM; CTP, 1.2 ± 0.06 µM. Asterisks indicate statistically significant differences compared with wild type aequorin control; *P<0.05, **0.001<P<0.01, ***P<0.001.

**Figure 6.** Effects of hydrolyzable and poorly-hydrolyzable nucleotide analogues on [Ca^{2+}]_{cyt} response in Arabidopsis seedlings.
Native nucleotides or poorly-hydrolyzable analogs were applied at a concentration of 100 µM. Experimental and analytical procedures were identical to those used in experiments of Figure 3. Different letters denote statistically significant difference (P<0.05).

**Figure 7.** NGXT191 elicited nucleotide-induced [Ca^{2+}]_{cyt} oscillations.
Arabidopsis seedlings harboring reconstituted aequorin were treated with 100 µM nucleotides after 30 min pre-incubation with NGXT191.
(a) Histogram represents each integrated [Ca^{2+}]_{cyt} response obtained from data recorded over 400 sec upon nucleotide treatments. Arrows indicate the NGXT191 concentration that evoked nucleotide-induced [Ca^{2+}]_{cyt} oscillations.
(b) ADP-induced [Ca^{2+}]_{cyt} oscillations in wild type. Arrows indicate amplitude peaks in the oscillations.
(c) Time-lapse movie of wild type aequorin transgenic seedlings treated with ADP after pre-incubation with NGXT191 (see Supplemental Movie S1). Frames from the recording at the indicated times (sec) are shown in a pseudo-colored photon-counting image.
(d) ADP-induced [Ca^{2+}]_{cyt} oscillations in agb1-2 mutant background.
(e) Effect of a vesicle trafficking inhibitor BFA on ADP-induced [Ca^{2+}]_{cyt} oscillations. Ten micromolar BFA was applied together with NGXT191 for pre-incubation.

**Figure 8.** Effects of NTPs on [Ca^{2+}]_{cyt} response in Arabidopsis seedlings monitored using yellow cameleon YC3.6.
Arabidopsis seedlings expressing YC3.6 were treated with either 10 µM ATP, CTP or GTP as indicated. Changes in [Ca^{2+}]_{cyt} monitored at 5 sec (a, b) or 3 sec (c)
intervals using confocal ratio imaging as described in Materials and Methods. 
$[\text{Ca}^{2+}]_{\text{cyt}}$ has been pseudocolor coded according to the inset scale. Representative of $n \geq 5$ roots per treatment.

(a) ATP-induced changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ imaged using YC3.6. Panels show time points in seconds following ATP treatment. Scale bar = 20 µm.

(b) ATP-induced changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ monitored in individual cells shown in (a).

(c) NTP-induced changes in $[\text{Ca}^{2+}]_{\text{cyt}}$ monitored in individual cell type a from panel (a).
Table 1. Effects of inhibitors on ADP- or ATP-induced [Ca$^{2+}$]$_{cyt}$ oscillations in Arabidopsis

Aequorin seedlings were treated with 100 µM nucleotides after simultaneous preincubation with 10 µg mL$^{-1}$ NGXT191 and indicated inhibitors.

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Pharmaceutical</th>
<th>No. of oscillations$^a$</th>
<th>Frequency of [Ca$^{2+}$]$_{cyt}$ oscillation occurrence$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Mock (H$_2$O)</td>
<td>4.6 ± 0.2</td>
<td>5/6</td>
</tr>
<tr>
<td></td>
<td>Mock (DMSO)</td>
<td>4.3 ± 0.3</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Gd$^{3+}$ (50 µM)</td>
<td>—</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Verapamil (100 µM)</td>
<td>—</td>
<td>0/9</td>
</tr>
<tr>
<td></td>
<td>U-73122 (1 µM)</td>
<td>—</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>NPA (50 µM)</td>
<td>3.9 ± 0.4</td>
<td>8/9</td>
</tr>
<tr>
<td></td>
<td>BFA (10 µM)</td>
<td>2.8 ± 0.4</td>
<td>2/12</td>
</tr>
<tr>
<td>ATP</td>
<td>Mock (H$_2$O)</td>
<td>4.4 ± 0.6</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Mock (DMSO)</td>
<td>4.0 ± 0.6</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Gd$^{3+}$ (50 µM)</td>
<td>—</td>
<td>0/6</td>
</tr>
<tr>
<td></td>
<td>Verapamil (100 µM)</td>
<td>—</td>
<td>0/9</td>
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<tr>
<td></td>
<td>U-73122 (1 µM)</td>
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</tr>
<tr>
<td></td>
<td>NPA (50 µM)</td>
<td>4.0 ± 0.4</td>
<td>8/9</td>
</tr>
<tr>
<td></td>
<td>BFA (10 µM)</td>
<td>3.0 ± 2.0</td>
<td>3/12</td>
</tr>
</tbody>
</table>

$^a$Samples that did not exhibit oscillations were omitted from the calculations.

$^b$Frequency of [Ca$^{2+}$]$_{cyt}$ oscillation occurrence is presented as the ratio: no. of occurrences/total no. of samples tested.
(a) Purine derivatives

- Adenine
- Guanine
- Hypoxanthine (for Inosine)

(b) Pyrimidine derivatives

- Cytosine
- Thymine
- Uracil

(b) Graph showing kinetics of hydrolysate formation over time and pseudocolor images.
(a) $\Delta [Ca^{2+}]_{cyt}$ (uM) in 400 sec at 100 uM NTPs

(b) $\Delta [Ca^{2+}]_{cyt}$ (uM) in 400 sec at 500 uM NTPs

(c) $\Delta [Ca^{2+}]_{cyt}$ (uM) in 400 sec as a function of Nucleotide concentration (uM)

Legend:
- ATP
- GTP
- ITP
- CTP
- TTP
- UTP

Significance levels:
- a
- b
- c
- d
- cd

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