Glutamate-Gated Calcium Fluxes in Arabidopsis[1]

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It is well accepted that endogenous and environmental signals can influence cellular activities by changing $[Ca^{2+}]_{cyt}$ (Malhó et al., 1998; Sanders et al., 1999). Despite the importance of this mechanism for coupling stimuli to responses (Malhó et al., 1998), the molecules responsible for generating increases in $[Ca^{2+}]_{cyt}$ during cell signaling in plants are not known at the genetic level. The results presented here raise the possibility that ligand-gated ion channels in plants such as those predicted by the discovery of ionotropic glutamate receptor (iGluR)-like genes in Arabidopsis (Lam et al., 1998) are key components of a Ca^{2+} influx mechanism important to signal transduction.

In animal brains iGluR channels mediate fast chemical transmission across synapses by increasing the permeability of the post-synaptic cell membrane to K^+, Na^+, and Ca^{2+} after binding Glu released by the presynaptic cell (Hille, 1992; Hollmann and Heinemann, 1994; Dingledine et al., 1999). The resulting Ca^{2+} entry in particular has been associated with long-term potentiation of the synapse, a physiological adaptation important to the learning process (Baudry and Lynch, 1993; Bliss and Collinridge, 1993). The Glu receptor homologs recently identified in plants (Lam et al., 1998) are too divergent from animal iGluRs to know with any certainty what ligand(s) gate them, what ions are conducted in the open state, and in which membrane(s) of the cell they function (Chiu et al., 1999). Thus the identification of iGluR sequences in the Arabidopsis genome raises intriguing questions about the physiological functions of neurotransmitter-gated channels in plant cells.

The possibility that Glu gates Ca^{2+}-permeable channels at the plasma membrane of plant cells was explored by measuring $[Ca^{2+}]_{cyt}$ in transgenic seedlings expressing aequorin, a Ca^{2+}-sensitive luminescent protein (Knight et al., 1991). As shown in Figure 1A, Glu application immediately triggered a very large, transient spike in $[Ca^{2+}]_{cyt}$. In separate experiments the effect of Glu on membrane potential (V_m) was measured with intracellular microelectrodes inserted into root apices of intact seedlings. Figure 1A also shows that switching the bathing medium from 1 mM KCl to 1 mM K-Glu induced a large and rapid depolarization of the membrane, as would be expected if the abrupt increase in $[Ca^{2+}]_{cyt}$ was due to Glu opening Ca^{2+}-permeable channels at the plasma membrane. The average peak change in V_m induced by 1 mM Glu was 55 ± 7 mV ($n = 6$ seedlings). This positive shift in V_m, though consistent with Glu gating an inward electrogenic Ca^{2+} current across the plasma membrane, may also be due to secondary effects of the increased $[Ca^{2+}]_{cyt}$ on other ion transporters. Another scenario to consider is that Glu directly gates channels permeable to ions such as Cl^- (Cully et al., 1994) in addition to Ca^{2+}-permeable channels to cause the depolarization. And last, an electrogenic Glu-uptake mechanism (Boorer et al., 1996) may also contribute to the electrical response. Because these and perhaps other scenarios are not mutually exclusive, more electrophysiological studies of the connection between the large Glu-gated changes in $[Ca^{2+}]_{cyt}$ and the effect on V_m are warranted.

Figure 1B shows that the magnitude and time course of the rapid increase in $[Ca^{2+}]_{cyt}$ was similar to the well-studied response to cold shock, i.e. in the micromolar concentration range and completed within several seconds (Knight et al., 1991, 1996; Lewis et al., 1997). Treatment with 1 mM Glu induced a response that was typically hundreds of fold higher than the control injection of equimolar KCl, which produced a touch response that may reflect Ca^{2+} entering the cytoplasm from internal stores (Haley et al., 1995; Legué et al., 1997). The post-peak shoulder apparent in the selected response to Glu was often, but not always observed. Activation of iGluRs by Glu causes very similar Ca^{2+} changes in cells of the mammalian nervous system (Kirischuk et al., 1999; Obrietan and van den Pol, 1999).

If the increase in $[Ca^{2+}]_{cyt}$ triggered by Glu resulted at least in part from flux across the plasma membrane from the apoplasm, impermeant channel blockers and external chelators of Ca^{2+} should reduce the response. The results in Figure 2A demonstrate that pretreatment with La^{3+}, a frequently used blocker of plasma membrane Ca^{2+} channels, inhibited the Ca^{2+} spike to the low level induced by the control treatment. Chelating extracellular Ca^{2+} by pre-treating seedlings with EGTA was similarly inhibitory (Fig. 2B). The combined evidence support our suggestion that Glu triggers an influx of Ca^{2+} across the plasma membrane and this leads to a dramatic change in $[Ca^{2+}]_{cyt}$. Calcium-induced Ca^{2+}-release from inter-

[1] This work was supported by the National Science Foundation (career award no. IBN–9734478 to E.P.S.).

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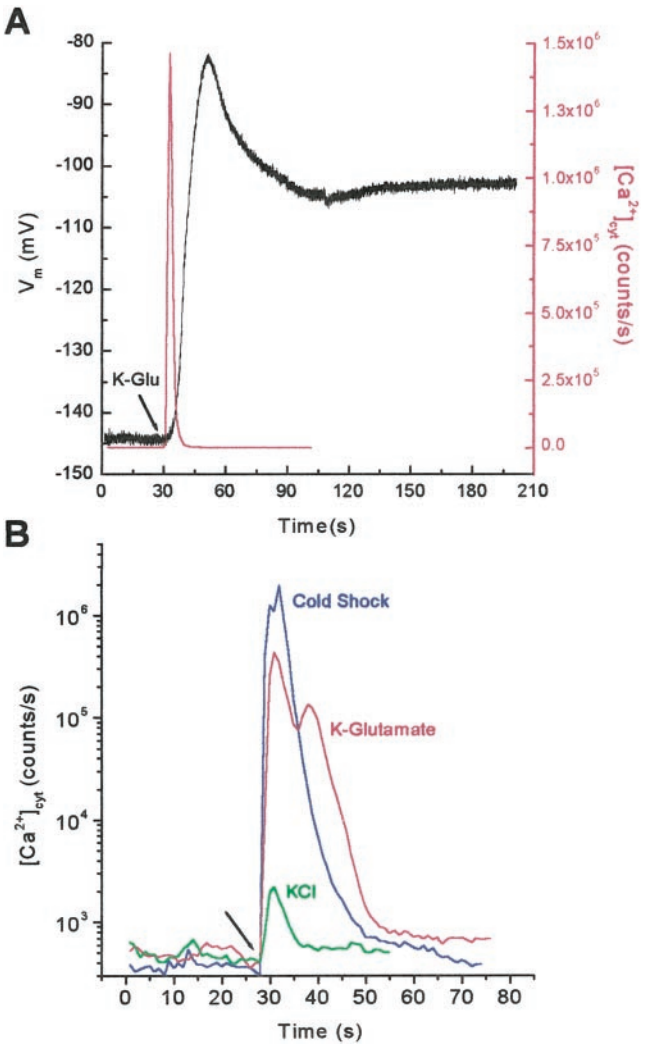

Figure 1. Glu triggers a large transient increase in $[Ca^{2+}]_{cyt}$ and an accompanying membrane depolarization. A, The red trace shows Ca^{2+}-dependent luminescence from whole aequorin-expressing Arabidopsis seedlings (5- to 8-d-old) measured with a luminometer as described previously (Lewis et al., 1997). The black trace shows the response of V_m measured by impaling a cell near the root apex with an intracellular microelectrode as previously described (Spalding et al., 1999). Intact seedlings between 7- and 14-d-old were used for the V_m measurements. Glu at a final concentration of 1 mM was delivered as the K^+ salt. The pH was buffered at 5.7 with 2.3 mM MES [2-(*N*-morpholino)-ethanesulfonic acid]. B, The kinetics and magnitude of the change in $[Ca^{2+}]_{cyt}$ induced by Glu resembles the response to cold shock, but is much larger than the touch response induced by the control treatment. Cold shock was achieved by injecting 0°C 1 mM KCl into the luminometer cuvette, whereas the control treatment was room temperature 1 mM KCl. Glu was delivered as 1 mM K-Glu and all solutions were buffered at pH 5.7. Arrow indicates the time of treatment.

nal stores such as the vacuole may also contribute (Allen et al., 1995).

La^{3+} also blocked the depolarization triggered by Glu without affecting the resting V_m (Fig. 2C), indicating that the depolarization is a consequence of the inward Ca^{2+} movement. However, La^{3+} is not a specific Ca^{2+}-channel blocker (Lewis and Spalding, 1998) and it may prevent the depolarization by blocking a separate Glu-gated conductance in addition to the Ca^{2+} pathway. Thus despite the fact that La^{3+} blocks the Ca^{2+} flux and the membrane depolarization, the exact relationship between the two Glu-

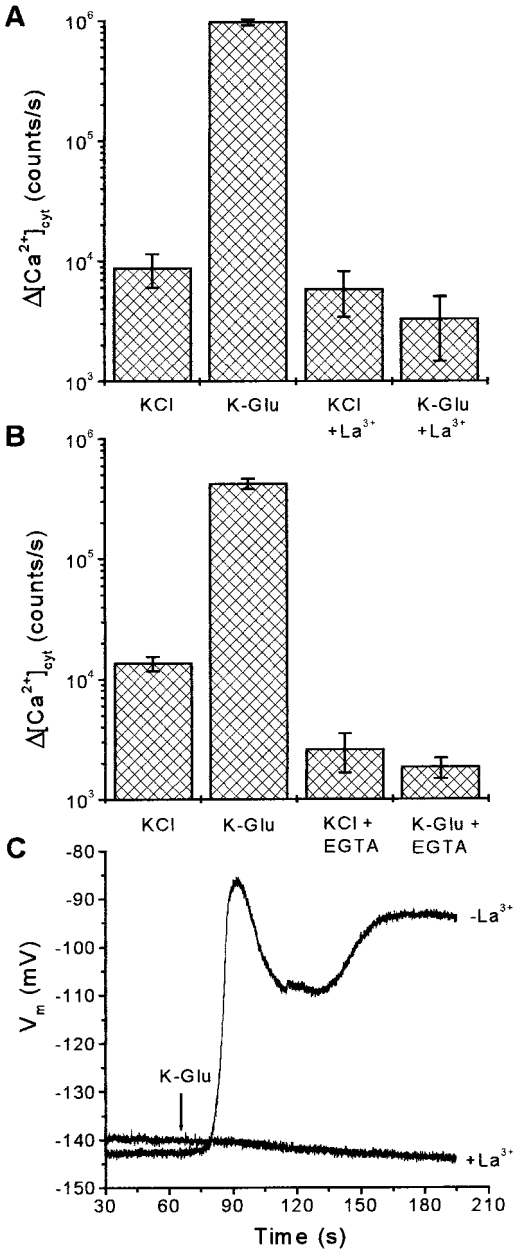

Figure 2. Inhibitory effects of La^{3+} and EGTA. A, Pretreatment of seedlings with 5 mM La^{3+}, an extracellular Ca^{2+}-channel blocker, inhibited the change in $[Ca^{2+}]_{cyt}$ induced by Glu, but had a much lesser effect on the 100-fold smaller response to the control treatment. B, Chelating extracellular Ca^{2+} by pretreatment with EGTA inhibited the Glu-induced Ca^{2+} response. The data plotted are means (±SEM) from three or four independent trials. C, Treatment of roots with La^{3+} blocked the Glu-induced depolarization without affecting the resting V_m. Roots were treated with 5 mM $LaCl_3$ before and during exposure to 1 mM K-Glu.

gated phenomena should be considered an open question. An alternative test would be to determine if EGTA treatment also inhibits the depolarization triggered by Glu, but stable recordings of V_m are difficult to obtain when extracellular Ca^{2+} is depleted to an extent that significantly affects its availability for inward fluxes. Patch-clamp studies of the ionic currents activated by Glu would be the preferred means of obtaining a biophysical description of the depolarization mechanism.

Glu is the primary natural ligand of iGluRs in the central nervous system although other non-native ligands are effective and have been used to classify receptor subtypes. The effectiveness of different ligands was tested using the aequorin reporter plants. Figure 3 demonstrates that Glu was clearly the most effective agonist tested (note the logarithmic y-scale). α-amino-3-hydroxy-5-methylisoxazole-4-propionate and N-methyl-D-aspartate, potent agonists of animal iGluRs, did not induce a response above the control treatment (approximately 2% of the L-Glu response). These non-native agonists of animal iGluRs also did not activate the *Synechocystis* GLU0 (Chen et al., 1999). It may be that affinity for α-amino-3-hydroxy-5-methylisoxazole-4-propionate and N-methyl-D-aspartate evolved in Glu receptors after the divergence of plants and animals. An alternative possibility is that Glu-gated Ca^{2+} entry in Arabidopsis does not involve iGluR-like molecules, but instead some unrelated Ca^{2+}-permeable pathway lacking affinity for typical iGluR agonists is responsible for the phenomenon.

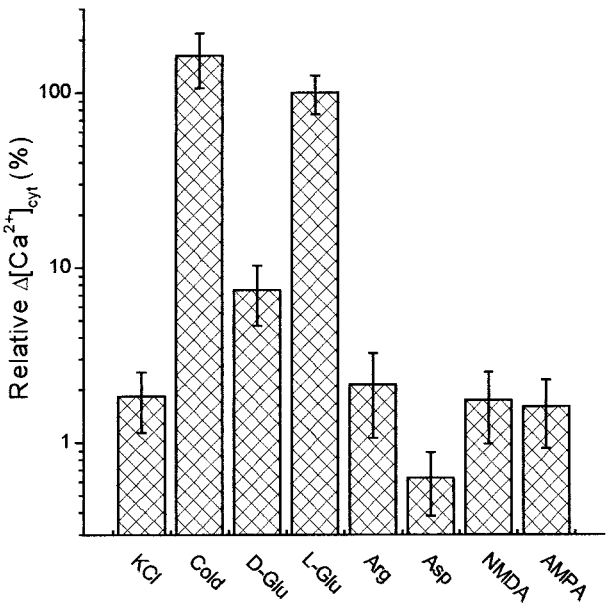

Figure 3. Relative effectiveness of related compounds. L-Glu was much more effective than other potential agonists, including D-Glu and the animal iGluR agonists, NMDA and AMPA. Note the logarithmic scale of the *y* axis, and that the response magnitudes are shown relative to the response induced by L-Glu. All compounds were administered at a final concentration of 1 mM. The plotted values are the means (±SE) of six independent trials.

The fact that the response to D-Glu was less than 10% of the L-Glu response indicates high stereochemical specificity of the binding site(s) on whatever molecules are responsible. Although Glu is clearly an effective ligand in a plausible concentration range, other ligands may be more physiologically important. The Arabidopsis genome contains several iGluR-like genes (Chiu et al., 1999) and that diversity may be matched by a similar diversity of agonists.

Information on the effective concentration range of Glu would help to establish a physiological context for this ligand-gated response in plants. The change in $[Ca^{2+}]_{cyt}$ induced by Glu increased between 0.3 and 3 mM, with the concentration for half-maximal response (EC_{50}) being approximately 1 mM (data not shown). This value is approximately 10-fold greater than the typical value for prokaryotic and animal iGluRs, but very similar to the EC_{50} of Cl^--permeable iGluRs from nematodes (Cully et al., 1994).

If Glu or some other related small organic acid is the primary endogenous ligand, then it is important to consider how and when the external ligand-binding site would experience 0.3 to 3 mM concentrations. Anion channels at the plasma membrane of plant cells are known to function in the transduction of several signals important to plant growth and development (Ward et al., 1995). These channels are relatively non-selective among anions and may conduct significant efflux of dicarboxylic anions such as malate (Hedrich, 1994; Schmidt and Schroeder, 1994), and therefore perhaps Glu, as well. When environmental or endogenous signals activate such anion channels, apoplastic Glu concentration may rise into the effective range, causing a transient change in $[Ca^{2+}]_{cyt}$ that serves to couple a stimulus to downstream responses. This hypothetical scenario may be most plausible in roots, where anion-channel mediated release of dicarboxylic acids has been proposed as a mechanism for combating Al^{3+} stress (Delhaize and Ryan, 1995; Ryan et al., 1997). Perhaps it is no coincidence that dissection experiments revealed most of the Ca^{2+} signal recorded from intact Arabidopsis seedlings was contributed by the root; leaves and cotyledons of young plants displayed smaller Glu responses (data not shown).

The results presented here form the basis of our proposition that a key element of a mechanism for altering $[Ca^{2+}]_{cyt}$ in plant cells during signaling is similar to that responsible for neurotransmitter action in the central nervous system of animals. The evidence would be bolstered considerably if mutational studies revealed a link between specific iGluR-like genes and Glu-gated Ca^{2+} fluxes. Plant biologists interested in Ca^{2+} signaling are presently particularly well equipped to test this connection because there is a wealth of published details about iGluR-mediated Ca^{2+} signaling in neurons, the Arabidopsis genome is essentially sequenced and searchable, so-

phisticated reverse-genetic strategies are very practical, and electrophysiological techniques can measure function with high resolution. The stage for exciting developments in Ca^{2+} signaling is set.

ACKNOWLEDGMENT

We thank Jamie Verheyden for technical assistance.

Received September 11, 2000; accepted September 25, 2000.

LITERATURE CITED

Allen GJ, Muir SR, Sanders D (1995) Science **268:** 735–737

Baudry M, Lynch G (1993) *In* Baudry M, Thompson RF, Davis JL, eds, Synaptic Plasticity. MIT Press, Cambridge, MA, pp 87–115

Bliss TVP, Collinridge GL (1993) Nature **361:** 31–39

Boorer KJ, Frommer WB, Bush DR, Kreman M, Loo DDF, Wright EM (1996) J Biol Chem **271:** 2213–2220

Chen G-Q, Cui C, Mayer ML, Gouaux E (1999) Nature **402:** 817–821

Chiu J, DeSalle R, Lam H-M, Meisel L, Coruzzi G (1999) Mol Biol Evol **16:** 826–838

Cully DF, Vassilatis DK, Liu KK, Paress PS, Van der Ploeg LHT, Schaeffer JM, Arena JP (1994) Nature **371:** 707–711

Delhaize E, Ryan PR (1995) Plant Physiol **107:** 315–321

Dingledine R, Borges K, Bowie D, Traynelis SF (1999) Pharmacol Rev **51:** 7–61

Haley A, Russell AJ, Wood N, Allan AC, Knight M, Campbell AK, Trewavas AJ (1995) Proc Natl Acad Sci USA **92:** 4124–4128

Hedrich R (1994) Curr Top Membr **42:** 1–33

Hille B (1992) Ionic Channels of Excitable Membranes. Sinauer, Sunderland, MA, pp 140–169

Hollmann M, Heinemann S (1994) Ann Rev Neurosci **17:** 31–108

Kirischuk S, Kirchoff F, Matyash V, Kettenmann H, Verkhratsky A (1999) Neuroscience **92:** 1051–1059

Knight H, Trewavas AJ, Knight MR (1996) Plant Cell **8:** 489–503

Knight MR, Campbell AK, Smith SM, Trewavas AJ (1991) Nature **352:** 524–526

Lam H-M, Chiu J, Hsieh M-H, Meisel L, Oliveira IC, Shin M, Coruzzi G (1998) Nature **396:** 125–126

Legué V, Blancaflour E, Wymer C, Perbal G, Fantin D, Gilroy S (1997) Plant Physiol **114:** 789–800

Lewis BD, Karlin-Neumann G, Davis RW, Spalding EP (1997) Plant Physiol **114:** 1327–1334

Lewis BD, Spalding EP (1998) J Membr Biol **162:** 81–90

Malhó R, Moutinho A, van der Luit A, Trewavas AJ (1998) Phil Trans R Soc Lond B **353:** 1463–1473

Obrietan K, van den Pol A (1999) J Neurophysiol **82:** 94–102

Ryan PR, Skerrett M, Findlay GP, Delhaize E, Tyerman SD (1997) Proc Natl Acad Sci USA **94:** 6547–6552

Sanders D, Brownlee C, Harper JF (1999) Plant Cell **11:** 691–706

Schmidt C, Schroeder JI (1994) Plant Physiol **106:** 383–391

Spalding EP, Hirsch RE, Lewis DR, Qi Z, Sussman MR, Lewis BD (1999) J Gen Physiol **113:** 909–918

Ward JM, Pei Z-M, Schroeder JI (1995) Plant Cell **7:** 833–844