The influence of glutamic and aminoacetic acids on the excitability of the liverwort

Conocephalum conicum

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KEYWORDS
Action potential; Glutamate; Glycine; Liverwort; Voltage transient

Summary
Intracellular microelectrode measurements revealed that a resting potential (RP), an action potential (AP) and a calcium component of AP (named voltage transient, VT) can be influenced by glutamic acid (Glu) and aminoacetic acid (glycine, Gly) in the liverwort Conocephalum conicum. In the continuous presence of 5 mM Glu or 5 mM Gly, the RP hyperpolarized constantly and the plants became desensitized to the excitatory amino acids (Glu or Gly). Under such circumstances, the amplitudes of APs evoked by stimuli other than Glu or Gly grew, as did their calcium components (VTs). The sudden application of 1–15 mM Glu or Gly to a thallus not yet desensitized resulted in an excitation, i.e. a single AP or AP series. Aspartate (Asp) could not substitute for Glu in any way.

Simultaneous action of both amino acids acted synergically to trigger APs. The same phenomenon was observed when glycine solution was enriched with N-methyl-D-aspartic acid (NMDA). Gly-induced APs were totally hindered by 1 mM D-amino-5-phosphonopentanoic acid (AP5) – an inhibitor of ionotropic glutamate receptors of the NMDA kind. Glu-induced APs could be totally suppressed by 1 mM AP5 as well as by 1 mM 6,7-dinitroquinoxaline-2,3-dione (DNQX) – an inhibitor of AMPA/KA receptors. DNQX also completely blocked the calcium component of Glu-evoked APs. After DNQX treatment, the only response to Glu was a membrane potential hyperpolarization (like the Glu response in a desensitized plant). It was concluded that the Glu-induced depolarization and hyperpolarization are separate phenomena.

Abbreviations: A9C, anthracene-9-carboxylic acid; AP, action potential; AP5, D-amino-5-phosphonopentanoic acid; Asp, aspartic acid; DC, direct current; DNQX, 6, 7-dinitroquinoxaline-2,3-dione; iGluR, ionotropic glutamate receptor; GLR, iGluR-like receptor; Glu, glutamic acid; Gly, glycine (aminoacetic acid); NMDA, N-methyl-D-aspartic acid; RP, resting potential; t1/2, half-time of membrane potential changes; TEA, tetraethylammonium; VT, voltage transient

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Introduction

Membrane potential deviations can be perceived as a signal of environmental changes, as a number of external factors (first messengers), either directly or—more often—indirectly, interfere with membrane channels, pumps or carriers. In response to different damaging and non-damaging stimuli, the excitable liverwort *Conocephalum conicum* is able to generate action potentials (APs). (Paszewski et al., 1982; Dziubinska et al., 1983; Trebacz and Zawadzki, 1985; Favre et al., 1999). The most common mechanism of AP in plants includes transient activation of Ca$^{2+}$-, Cl$^{-}$- and K$^{+}$-channels in sequence (Williamson and Ashley, 1982; Lunevsky et al., 1983; Kourie, 1994). Treated with anthracene-9-carboxylic acid (A9C) (anion channel inhibitor) and tetraethylammonium (TEA) (potassium channel inhibitor), the liverwort loses excitability and the calcium component of AP (called VT – voltage transient after Trebacz et al., 1997) is detected only in a stimulated cell. VT amplitude depends on stimulus strength and it decreases, which makes the differentiation between APs and VTs clear (Krol and Trebacz, 1999; Krol et al., 2003). It appears that voltage-gated Cl$^{-}$- and K$^{+}$-channels are sufficient for the propagation of excitation, as voltage-dependent Ca$^{2+}$-currents themselves may not be able to pass from cell to cell even in excitable tissues (Hagiwara and Naka, 1964). Still, various Ca$^{2+}$-channels are necessary for the complete transduction of environmental signals. Thus, the calcium component of AP is very likely to play a messenger role in a stimulated cell, while self-propagating Cl$^{-}$- and K$^{+}$-fluxes are likely to “pass on” the calcium-coded message from one cell to another.

The activation of cation currents by glutamic acid (Glu) is a key event underlying the excitation phenomenon in animal nerve tissues. The possibility that Glu also activates plant channels arose along with the discovery of ionotropic glutamate receptor (iGluR)-like genes in *Arabidopsis thaliana* (GLR – Lam et al., 1998; Lacombe et al., 2001). It was postulated then that amino acid-based cell-to-cell signalling evolved before the divergence of animals and plants (Chiu et al., 1999, 2002). The report of Dennison and Spalding (2000) showed evidence that Glu causes a large transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ and an accompanying membrane depolarization in *Arabidopsis*. Following this, Dubos et al. (2003) showed that Glu-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increases can be hindered successfully by a specific inhibitor of iGluR. Glutamate-activated Na$^{+}$ and Ca$^{2+}$ fluxes in patch-clamped protoplasts have been reported recently by Demidchik et al. (2004). These Glu-induced currents were voltage insensitive and likely mediated by non-selective channels of yet unknown function. The molecular approach of Kim et al. (2001) attributed GLR to calcium uptake during ionic stress. In fact, 20 apparent homologues of iGluRs in the *Arabidopsis* genome suggest that they may have a number of different physiological roles, including light signal transduction (Lam et al., 1998), photomorphogenesis (Brenner et al., 2000), Ca$^{2+}$ signalling (Dennison and Spalding, 2000), protection against ionic stresses (Kim et al., 2001; Sivaguru et al., 2003), nutrient uptake (Kim et al., 2001), nitrogen assimilation/reassimilation (Lam et al., 1995), amino acid-regulated signalling and metabolism (Davenport, 2002). All the 20 members of the AtGLR gene family are expressed genes (Chiu et al., 2002). However, very few data ascribe a channel function to a specific AtGLR gene (AtGLR2.1 – Kim et al., 2001; AtGLR1.1 – Kang and Turano, 2003, Kang et al., 2004; AtGLR3.4 – Meyerhoff et al., 2004, 2005).

The recent work of Meyerhoff et al. (2004, 2005) has focused on a touch- and cold-sensitive member of the GLR family, again in *Arabidopsis*. Upon Glu treatment, the plasma membrane of mesophyll cells depolarized and this depolarization was Ca$^{2+}$ dependent, transient in nature and subject to desensitization. In this way, the response resembles the calcium component of APs. Since all the above-mentioned papers consider one plant – *Arabidopsis* – the present study was meant to obtain more information about Glu action in other plants and its influence on plant excitability. *Conocephalum conicum* was chosen as it easily excites, its calcium component of APs is feasible to be revealed, and its electrophysiology is well documented by our research team. The liverwort is a lower plant that belongs to the phylogenetically oldest terrestrial plants (Qiu et al., 1998). Our data are in good agreement with the common origin of the amino acid-based cell-to-cell signalling (Chiu et al., 1999, 2002). They also seem to hint at the existence of GLR (an ion channel) in lower plants.
Materials and methods

Conocephalum conicum L. was collected together with soil in a forest near Zwierzyniec (Poland) and then grown in a greenhouse under natural light, highly moist conditions and in a constant temperature of 25 °C. The 3–4 cm thalli of Conocephalum were uprooted, thoroughly rinsed with tap water and mounted in an experimental chamber.

Electrophysiological experiments were performed as previously described (Trebacz et al., 1997). The transmembrane potential was measured with 3 M KCl-filled glass microelectrodes Ag/AgCl (Hilgenberg, Malsfeld, Germany) connected to a VF-4 buffer amplifier (World Precision Instruments, Sarasota, FL, USA). The output signals were digitized by an AD converter and registered on a hard drive. For AP recording, the experiments started after at least 1 h-long incubation of the plant material under dim light (2 μmol m⁻² s⁻¹) in a standard solution containing 1 mM KCl, 1 mM CaCl₂, and 50 mM sorbitol (pH 7). For VT occurring, the thalli of Conocephalum conicum were immersed for 2–3 h in the standard solution supplemented with 2 mM A9C, and 10 mM TEA (pH 7). By this time, the VT amplitude grew to its maximal value (Krol and Trebacz, 1999). Electrical stimuli were carried out by two silver electrodes impaled into a thallus. A 4.5 V battery connected to a voltage divider was used to elicit APs. Light stimulation (80–180 μmol m⁻² s⁻¹) was executed after 10 min of darkness. To evoke rapid membrane potential changes by the other stimuli, the thalli were transiently and locally washed with 0.5 mL of

(a) respective amino acid solutions (1–20 mM glutamate or 1–20 mM glycine) for excitatory amino acid effects;
(b) standard solution cooled to 1 °C for cold stimulation. The local drop of temperature was by 8–10 °C as measured by a thermocouple located near the microelectrode (Krol et al., 2004).

In experiments considering synergic action of amino acids (Fig. 4), the single thallus was cut into 5 × 5 mm² squares, which were thereafter treated as clones. Inserted in the experimental chamber, a single fragment was simulated only once by a respective amino acid solution (from 0.01 up to 10 mM). From the total stimulation of all fragments originating from one thallus, the threshold concentration for the thallus was set.

Because of the variability of amplitudes among different thalli, the data were standardized (%) for statistical analysis (Table 1). The APs and VTs during the first 1–2 h of recording were taken as a control (100%). After a quick exchange of the experimental solution for the one supplemented with 50 mM LaCl₃, or 1 mM 6,7-dinitroquinoxaline-2,3-dione (DNQX), or 1 mM D-amino-5-phosphonopentanoic acid (AP5), or 5 mM glycine, or 5 mM glutamate, or 5 mM aspartate (Asp), all the changes in AP or VT amplitudes (A) and durations (t₁/₂) were assessed as a percent of the control (APs or VTs during the respective first hours). Each experiment was repeated at least 3 times. STATISTICA was used for estimation of significance. The values in the tables are given as a mean ± SE. The representative traces are shown in the figures.

Results

Different effects of glutamate and glycine – lay in variable responsiveness of thalli

In response to different stimuli such as light, a direct current (DC), a temperature drop and glutamate, glycine or N-methyl-D-aspartate (NMDA) application, Conocephalum conicum was able to generate APs (noted as APlight, APel, APcold, APGlut, APGly, and APNMDA, respectively). Depending on the kind of a stimulus, the amplitude of APs differed slightly within a plant. However, it varied distinctly between separate thalli and the significant correlation between AP amplitude and the resting potential (RP) values was visible (Table 1). The differences between thalli in VT records were even clearer and less correlated with RP (Table 1). The high dependence of VT on the stimulus strength (Krol and Trebacz, 1999; Krol et al., 2003) may help to explain lower correlation between the RP value and VT amplitudes. Here, we report primarily on the APs and respective VTs evoked by excitatory amino acids, as APs induced by the other stimuli have already been characterized in Conocephalum conicum (and so they could serve as a positive control in the course of the experiments presented) and published (Paszewski et al., 1982; Dziubinska et al., 1983; Trebacz and Zawadzki, 1985; Trebacz et al., 1994, 1997; Favre et al., 1999; Krol and Trebacz, 1999; Krol et al., 2003).

For clarification of our data, we split all plants into two categories: “excitable” by amino acids and “unexcitable”, when the administration of glutamate or glycine solutions of up to 20 mM
concentration did not result in APs. For a highly responsive thallus, addition of 0.5 mL of 1 mM glutamic acid to a bath chamber of 4 mL capacity was sufficient to trigger AP. Moreover, such a specimen generated a series of APs in response to 5 mM Glu application (Fig. 1). In the long run, the series waned and only single AP responses were recorded. The same phenomenon corresponded with glycine (Gly) action. Though the series of AP were much more spectacular after 5 mM Gly administration (Fig. 2), they also decreased in number with time and with successive stimulations. Occasionally, instead of AP series, there was only a single AP recorded for 5–10 min. Two series could be triggered one after another if we applied both amino acids in alternating order, but not when we used the same excitatory amino acid twice (the response to the 2nd stimulation was a single AP or none). Therefore, we stimulated the thalli with Glu and Gly alternately and the stimulation by Glu or Gly was with the frequency of one per hour. It was consistent with Meyerhoffs’ et al. (2004, 2005) data, which showed that the Glu-induced membrane potential changes require a 1-h-long refractory period.

In the case of “Glu-, Gly-unexcitable” thalli, the response to amino acid addition was only a transient RP hyperpolarization, sometimes followed by a transient depolarization, but never by APs (Fig. 3). It seems that the main difference between “excitable” and “unexcitable” individuals lies in various excitation thresholds. Because of the high variability in responsiveness among thalli, the attempt to set a mean threshold

Table 1. Mean values of RP, AP amplitudes and VT amplitudes obtained from the separate thalli of Conocephalum conicum under control conditions (standard solution for AP and standard with A9C and TEA for VT)

<table>
<thead>
<tr>
<th>No. of experiment</th>
<th>AP&lt;sub&gt;Glu&lt;/sub&gt; (mV)</th>
<th>AP&lt;sub&gt;Gly&lt;/sub&gt; (mV)</th>
<th>AP&lt;sub&gt;el&lt;/sub&gt; (mV)</th>
<th>AP&lt;sub&gt;light&lt;/sub&gt; (mV)</th>
<th>AP&lt;sub&gt;cold&lt;/sub&gt; (mV)</th>
<th>AP&lt;sub&gt;NMDA&lt;/sub&gt; (mV)</th>
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<td>96.5</td>
<td>144.1</td>
<td>144.1</td>
<td>163.4</td>
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</table>

Mean ± SD (all experiments) 74 ± 7 71 ± 6 80 ± 7 89 ± 8 86 ± 7 88 ± 7 138 ± 3

Correlation with RP -0.677* -0.775* -0.849* -0.790* -0.716* -0.970* 1

Table 2. Mean values of VT amplitudes obtained from the separate thalli of Conocephalum conicum under control conditions (standard solution for AP and standard with A9C and TEA for VT)

<table>
<thead>
<tr>
<th>No. of experiment</th>
<th>VT&lt;sub&gt;Glu&lt;/sub&gt; (mV)</th>
<th>VT&lt;sub&gt;Gly&lt;/sub&gt; (mV)</th>
<th>VT&lt;sub&gt;light&lt;/sub&gt; (mV)</th>
<th>VT&lt;sub&gt;cold&lt;/sub&gt; (mV)</th>
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<td>78.0</td>
<td>91.5</td>
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<td>92.4</td>
</tr>
</tbody>
</table>

Mean ± SD (all experiments) 64 ± 8 43 ± 10 142 ± 11 98 ± 5 141 ± 5

Correlation with RP -0.482 -0.981* -0.441 -0.658 1

*Statistically significant correlation (p<0.05).
concentration of excitatory amino acids was immaterial. Instead, when no AP appeared in response to 20 mM Glu or Gly, we did examine long-term amino acid effects on APel, APlight and APcold as well as on VTlight and VTcold, continuously submerging the thallus into 5 mM amino acid bath solution. From that point, we started to name the thallus as desensitized.

We never observed VT after a stimulation of 5 mM Glu in any of the thalli examined. However, with the 15 mM glutamate solution, VT appeared (Fig. 1). The same holds true for Gly – no less than 15 mM was sufficient to evoke VTgly (Fig. 2).

**Excitatory amino acid effects on sensitive thalli**

As mentioned above, 5 mM Glu or 5 mM Gly was sufficient to trigger at least a single AP in *Conocephalum conicum*. To ascertain that we were dealing with APs, the places of stimulation and recording were separated. We also stimulated the thallus with the increasing amino acid concentrations (up to 20 mM) and the amplitudes of Glu- and Gly-induced APs did not depend on the stimulus strength (amino acid concentration).

Since Dubos et al. (2003) showed that Gly and Glu act synergically to modulate \([\text{Ca}^{2+}]_{\text{cyt}}\), we also were able to show in *Conocephalum conicum* the analogical synchronization of both amino acid actions. Simultaneous application of 0.1 mM Glu and Gly triggered APs of \(75 \pm 13\) mV amplitude \((n = 4)\), while the administration of either amino acid alone of 0.1 mM concentration had no effect.
Only 5 mM Glu evoked AP in the thallus presented (Fig. 4a). As Gly plays a key role in stimulating iGluR of NMDA kind, we used NMDA to evoke APs (Table 1). Moreover, we were able to show that Gly and NMDA also act synergically (Fig. 4b), which is consistent with the activation of iGluR of NMDA types. Prolonged incubation with 1 mM NMDA cancelled APGlu, AP Gly, AP Pnmda.

The Glu- and Gly-induced APs could be totally blocked by lanthanum, as were all other APs (Fig. 5a). A concentration of 50 mM LaCl3 began to exert its inhibitory effects after an hour when we were dealing with the intact thalli. Total inhibition was never observed before the end of the 2nd hour of La3+-treatment. The data presented in Table 2 are the averages of 3-h-long La3+-action on the whole plants. In the case of thallus fragments (see Materials and methods), 5 mM LaCl3 was sufficient to inhibit APGlu completely within half an hour (Fig. 5b). As La3+ is a very non-specific inhibitor of excitability, we used 1 mM DNQX – an inhibitor of iGluRs of AMPA/KA kind – to keep Glu-induced stimulation separate. This very specific inhibitor of the channel in question caused a total block of Glu-induced APs in all the thalli examined (n = 4), simultaneously having no effects on the other APs (Fig. 6a). DNQX action was evident within half an hour, causing a successive amplitude inhibition until a complete blockade. In one of the 4 thalli investigated, DNQX also blocked Gly-induced excitation. This scheme was enhanced with VTs. All the thalli (n = 3) did not respond to Glu after 2-hour-long DNQX treatment but as many as 2 of them were not reacting to Gly either (Table 3). Fig. 6b shows the record of the thalli displaying slightly hindered VTGly and vanished VTGlu after 2 h of 1 mM DNQX incubation. For a splendid VTlight record, it is obvious that light-induced calcium fluxes are insensitive to DNQX (Fig. 6b).

AP5 is the most potent inhibitor of the ionotropic glutamate receptors of the NMDA kind, which necessitate Gly (apart from Glu) for their

![Figure 4](image_url)

**Figure 4.** The synergic action of (A) Glu and Gly (AP could not be evoked by Glu below 5 mM unless there was the simultaneous application of Glu and Gly); (B) Gly and NMDA (AP could not be evoked by Gly below 1 mM nor NMDA below 10 mM unless Gly and NMDA were simultaneously applied). For further explanation, see Materials and methods.

![Figure 5](image_url)

**Figure 5.** The inhibition of excitability by LaCl3: (A) 50 mM LaCl3 exerted its influence for 3 h on an intact thallus (APGlu, AP Gly, APcold, APlight and APel were triggered by 15 mM amino acid concentration, 1°C standard, light of 180 μmolm−2s−1 and DC of 4 V, respectively); (B) 5 mM blocked APGlu irreversibly within half an hour if a small fragment of a thallus was examined (see Materials and methods).
activation. The thallus treated with 1 mM AP5 overnight could not be stimulated by Glu, Gly or NMDA, whereas its responses to light and cold were unchanged (Fig. 7, Table 2).

Effects of excitatory amino acids in desensitized thalli

The RP fluctuated minimally unless there was any alternation in the experimental environment (e.g. in light, temperature or bath composition). With a shift of RP ($-138 \pm 3$ mV, $n = 16$) toward more negative values due to the presence of either 5 mM Glu or 5 mM Gly ($-152 \pm 3$ mV; $n = 16$ or $-157 \pm 2$; $n = 15$, respectively), AP and VT amplitudes grew markedly (Fig. 8). The most remarkable feature of Gly action was the outstanding extension of AP/VT duration (Fig. 8). The significant increases in a half time of AP/VT were also recorded after Glu application. None was seen after 5 mM Asp addition. In the course of 6-h-long experiments, the administration of 5 mM aspartate to the bath caused no significant deviation in RP ($140 \pm 1$ mV, $n = 6$). The long-lasting effects of both excitatory amino acids and of Asp are compiled in Table 3.

Discussion

Excitable cells of plants appear to have much more in common with excitable animal cells than previously thought. The series of AP in response to Glu and Gly presented here, and never reported for any plant so far explored, seems a considerable analogy to the animal kingdom. Our inquiry into glutamate-induced APs in an excitable liverwort supports the probability of the genuine iGlur existence in plants. Such a hypothesis has come along with the identification of GLR genes in a plant genome (Lam et al., 1998). Since Lam’s et al. (1998) discovery, much attention has been focused on the understanding of GLR protein structure, function and location. GLR genes are predicted to encode proteins resembling an ancestral glutamate receptor from which both metabotropic (mGlur) and ionotropic (iGlur) receptors have been derived (Turano et al., 2001). With high sequence and structural homology to animal iGlurRs, plant GLRs seem to form a non-selective channel facilitating cation passage under glutamate binding (Davenport, 2002). These channels are inhibited by DNQX (Lam et al., 1998; Brenner et al., 2000; Dubos et al., 2003) or AP-5 (Sivaguru et al., 2003) and blocked by Mg$^{2+}$ (Nagata et al., 2004) or La$^{3+}$ (Dennison and Spalding, 2000). These channel receptors are also generally assumed to be placed in the plasma membrane (Davenport, 2002; Meyerhoff et al., 2005). The high expression of GLR in the roots (Chiu et al., 2002) appears to be important for regulating ion uptake from soil (Kim et al., 2001; Davenport, 2002). This fits well with the numerous observations that Glu activates cation fluxes in the intact roots of Arabidopsis thaliana (Dennison and Spalding, 2000; Sivaguru et al., 2003; Demidchik et al., 2004). In addition, the comparatively strong vascular expression of AtGLRs (Chiu et al., 2002) points to a plausible GLR involvement either in regulating the transport of metabolites in the phloem (Lam et al., 1995) or in cation utilization from the xylem (Kim et al., 2001). The expansive vascular localization of GLRs might also allude to their involvement in long-distance signalling (with the use of AP?). One could assume that, in an analogous fashion to animal iGlurRs, plant GLRs also might be responsible for AP initiation. Thus, it seems reasonable to document the impact of glutamate on “our” excitable plant.

We stimulated Conocephalum conicum with glutamate solutions of increasing concentrations from 0.01 to 20 mM. We recorded AP$_{glu}$ responses that were inhibited by DNQX or AP5 and blocked by...
Figure 6. The records after 2 h of 1 mM DNQX action in Conocephalum conicum: (A) its inhibitory effects on AP\textsubscript{Glu} with simultaneously uninfluenced AP\textsubscript{Gly}, AP\textsubscript{cold}, AP\textsubscript{light} and AP\textsubscript{el} (cold, light and DC stimulus strength as in Fig. 5); (B) its effects on VT\textsubscript{Glu}, VT\textsubscript{Gly} and VT\textsubscript{light}. 
La²⁺. Having treated *Conocephalum conicum* with A9C and TEA, we were able to reveal a calcium component of AP, namely VT. VTglu (the calcium-dependent Glu-induced membrane potential changes) was totally inhibited by DNQX. Such results are consistent with the elaborated scheme of an AP ionic mechanism for plants, in which Ca²⁺ ions are indispensable for the initial membrane depolarization (Beilby, 1984; Tsutsui et al., 1987; Trebacz et al., 1994; Biskup et al., 1999; Tazawa and Kikuyama, 2003; Krol et al., 2006). They also fit with the hypothesis that plant GLR proteins assemble into calcium-permeable ion channels.

Table 3. The average percentage change in the amplitudes (A) and durations (t₁/₂) of *AP*<sub>light</sub>, *AP*<sub>cold</sub>, *AP*<sub>el</sub>, *VT*<sub>light</sub> and *VT*<sub>cold</sub> in the liverwort *Conocephalum conicum* under the respective amino acid treatments

<table>
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<th>Treatment (mM)</th>
<th><em>AP</em>&lt;sub&gt;light&lt;/sub&gt; (%)</th>
<th><em>AP</em>&lt;sub&gt;cold&lt;/sub&gt; (%)</th>
<th><em>AP</em>&lt;sub&gt;el&lt;/sub&gt; (%)</th>
<th><em>VT</em>&lt;sub&gt;light&lt;/sub&gt; (%)</th>
<th><em>VT</em>&lt;sub&gt;cold&lt;/sub&gt; (%)</th>
<th>RP standard (mV)</th>
<th>RP A9C, TEA (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 Glu A</td>
<td>154 ± 11* 119 ± 3*</td>
<td>139 ± 18* 188 ± 12*</td>
<td>136 ± 14* -152 ± 3*</td>
<td>-160 ± 3*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t₁/₂</td>
<td>124 ± 11* 131 ± 13*</td>
<td>97 ± 10 120 ± 7*</td>
<td>124 ± 6*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Gly A</td>
<td>120 ± 7* 130 ± 8*</td>
<td>162 ± 19* 114 ± 6*</td>
<td>110 ± 4* -157 ± 2*</td>
<td>-151 ± 2*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t₁/₂</td>
<td>320 ± 40* 245 ± 26*</td>
<td>252 ± 45* 590 ± 118*</td>
<td>156 ± 15*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Asp A</td>
<td>88 ± 3 93 ± 2</td>
<td>85 ± 7 -140 ± 1</td>
<td>-150 ± 6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>t₁/₂</td>
<td>92 ± 4 89 ± 7</td>
<td>97 ± 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From each experiment, the percentage was calculated in relation to the first control hour(s) of the experiment. The means ±SE come from at least 3 different repetitions. The RP values represent means ± SE from all experiments under the respective treatment, and for the significance assessment, they are compared to the control experiments from Table 1.

*Statistically significant difference (p < 0.05).

Figure 7. The inhibition of *AP*<sub>Glu</sub>, *AP*<sub>Gly</sub> and *AP*<sub>NMDA</sub> after an overnight incubation in 1 mM AP5. AP responses to light and cold stayed unaffected.

Figure 8. The traces of respective APs and VTs recorded after *Conocephalum conicum* was awash for an hour in (A) 5 mM glycine; (B) 5 mM glutamate. The arrows are the moments of respective stimulations. The records with stars go for the stimulation under respective amino acid conditions.

The use of another acidic amino acid, aspartate (Asp), was to depict Glu specific effects. Asp, though very similar to Glu in the structure, could
not mimic Glu effects. The inability of Asp to substitute Glu has been shown recently (Brenner et al., 2000; Dubos et al., 2003). On the other hand, NMDA and Gly were very similar to Glu in their actions. Many earlier reports and some very recent ones suggested that Gly is a signalling molecule in plants. The recent results of Dubos et al. (2003) provided evidence that Glu and Gly can act synergically, sharing the predicted binding site within a receptor protein. Also, Meyerhoff et al. (2004) reported that, upon Glu as well as Gly treatment, there was a plasma membrane depolarization in Arabidopsis. In animal systems, the Glu receptors of NMDA type require glycine as a co-agonist, and Gly is the more potent co-agonist for them (Dingledine et al., 1999).

Thus, we stimulated Conocephalum conicum with 1–20 mM Gly and made the records of APgly and VTgly. Moreover, we were able to trigger AP with simultaneous application of Glu and Gly (Fig. 4a) at sub-threshold concentrations (when applied separately, i.e. 0.01–0.1 mM). The synergic action of Gly and NMDA was also undeniable in Conocephalum conicum (Fig. 4b). Once the thallus was incubated with 1 mM NMDA for several hours, no APGlu, APGly or APNMDA could be stimulated any longer. To further delineate APgly and APgly, we used AP5 – an iGluR antagonist for NMDA receptors. APgly, APgly and APNMDA vanished completely after the treatment with AP5.

Though DNQX did not necessarily exert its inhibitory effects on APgly and VTgly (in contrast to Dubos et al.’s (2003) results), our data still may indicate that sensitivities to Glu and Gly rely on different affinities of a single receptor channel, resulting from the assembly of different receptor subunits, and especially that Gly seems to be a more potent GLR agonist (Fig. 2, Dubos et al., 2003). Obviously, Glu and Gly competed with each other for a common binding site. Therefore, plants might truly switch their “susceptibility” between Gly and Glu by changing the expression of different GLR subunits, as suggested by Dubos et al. (2003).

Apart from triggering APs, Glu and Gly caused an immediate RP hyperpolarization. As shown in Fig. 1, both responses (hyperpolarization and depolarization) appeared simultaneously. The impaled micro-electrode recorded the results of both responses. Such a “sum” might explain the small amplitudes of APgly, APgly, VTgly and VTgly (in comparison to the AP triggered by the other stimuli – Table 1). Glu-induced hyperpolarization persisting under DNQX (Fig. 6a) or AP5 (Fig. 7) treatment additionally proves that the depolarization and the hyperpolarization are separate phenomena governed by distinct mechanisms. APs might be the consequences of iGluR activation, while the long-term membrane hyperpolarization under Glu or Gly treatment fits with the scheme in which Glu and Gly are transported into a cell by H+-coupled forces. The evidence available to date supports the notion that a high number of H+-ATPases is expressed in the plasma membrane (Axelsen and Palmgren, 2001). Therefore, in the long run the enhancement of APlight, APcold and APet amplitudes after Glu/Gly addition could be an outcome of a subsequent membrane hyperpolarization (a growth of AP amplitudes is positively correlated with the magnitude of RP hyperpolarization). Our data show RP hyperpolarization under Glu or Gly treatment even after A9C and TEA addition (Table 3). Thus, the Glu/Gly-provoked hyperpolarization elicits the enhancement of VTlight and VTcold amplitudes under persistent excitatory amino acid administration. Such results may be the consequences of either “an energized status” of plasma membrane allowing Ca2+ ions beyond their equilibrium to enter a cell more rapidly, or the restricted activation of Kout channels (a restrained cation efflux allows AP to grow – Roelfsema and Prins, 1997), or both. The outstanding broadening of AP/VT records in the presence of Gly and Glu (to a lesser extent) is obscure. Nevertheless, as long as the extension of AP duration is accompanied by VT extension, long-lasting APs can be accounted for by the decelerated Ca2+-removal.

It seems that being the main signalling components of metabolism and of N–C status, as well as of stress conditions, Glu and Gly are well sensed by cell membranes (receptors). Our final conclusion is that plants may keep genuine iGluR previously attributed to animals only. Moreover, many different Glu receptors (ion channels and transporters) might coexist, having multifarious functions in the plasma-lemma of the liverwort Conocephalum conicum.

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