Comparison of Effects of Endogenous and Exogenous Excitatory Amino Acids on Retzius Nerve Cells of the Leech

Srdjan LOPICIC, Vladimir NEDELIJKOV, Dusan CEMERIKIC, Zoran DUDVARSKI, Dragan PAVLOVIC, and Nedjo CUTURA

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L-glutamate (L-glu) is the principal excitatory neurotransmitter in both vertebrates and invertebrates. The excitatory effect of L-glu on the nervous system was first demonstrated by HAYASHI (1954). Second in abundance and significance among excitatory amino acids (EAAs) is L-aspartate (L-asp). Also ubiquitous throughout the animal kingdom, it shares many of glutamate’s excitatory actions. Both take part in a vast variety of physiological processes in the nervous system and, through excitotoxicity, have a presumed pathophysiological role.

β-N-oxalylamino-L-alanine (L-BOAA) is an excitatory amino acid found in the grass pea (Lathyrus sativus). When chronically consumed through dietary intake, it causes a disease known as neurolathyrism in humans and animals (RAO 1978; COHN & STREIFLER 1981a,b; YAN et al. 2006).

All three EAAs have been extensively studied in numerous preparations, including Retzius nerve cells of the leech (RNCL). It has been shown that L-glu has a direct excitatory effect on RNCL, with increased permeability for sodium and potassium, the latter being passive and sodium dependent (JAMES & WALKER 1978; JAMES & WALKER 1979). It has also been shown that the L-glu-induced excitatory effect on RNCL can be diminished by the non-NMDA (AMPA/kainate) receptor antagonist DNQX (DIERKES et al. 1996).

The depolarizing effect of L-asp on RNCL accompanied by an increase of membrane permeability for sodium and potassium was demonstrated by JAMES & WALKER (1978, 1979), and JAMES et al. (1980b). They postulated that the effect of L-asp on membrane potential (E_m) is carried by these ions. This has subsequently been proven by CEMERIKIC et al. (1988) through usage of ion-selective microelectrodes, who concluded that, similarly to L-glu, the efflux of potassium is a passive and sodium dependent event.

The first electrophysiological investigations of the effect of L-BOAA on RNCL were performed by NEDELIJKOV et al. (1979), and NEDELIJKOV (1983), showing a depolarizing effect with increase in membrane conductance. This effect was attenuated in sodium-free Ringer solution, indicating a similar mechanism to that of L-asp and L-glu. The role of inward sodium and outward potassium fluxes was proven by CEMERIKIC et al. (2001).

Interestingly, although there is a multitude of data regarding the effect of L-glu, L-asp, and L-BOAA on E_m of RNCL, the data on potency and effectiveness of these substances on RNCL is either lacking or inconclusive.
Bearing in mind the importance of L-glu, L-aspartate, and L-BOAA in the physiology and pathophysiology of both invertebrates and vertebrates, we conducted a comparison of their electrophysiological effects on our model.

Methods

The experiments were performed at room temperature (22-25°C) on Retzius nerve cells in the isolated segmental ganglia of the ventral nerve cord of the leech *Haemopis sanquisuga*. All procedures related to use of the animals were conducted in compliance with ethical guidelines guaranteed by the institution’s ethical committee.

The animals were purchased from a local dealer and kept in aquaria in batches of up to 20 leeches per aquarium in dechlorinated tap water in a refrigerator at +4°C. Water was changed twice a week.

The leeches were first anaesthetized in 10% ethanol. Then, the ventral nerve cord with its enveloping blood sinus was removed from the animal in short segments of three to four ganglia via a ventral longitudinal incision. The removed segments were immediately transferred to 2.5 ml plastic chamber with leech Ringer and fixed by means of fine steel clips. The blood sinus was then dissected away.

The plastic chamber was then placed in a grounded Faraday’s cage. Identification and penetration of the cells was performed in the cage under a MBS stereomicroscope. The Retzius cells were identified by their position on the ventral surface of the ganglion, their size and by their bioelectrical properties.

Prior to the experiments the chamber was flushed with fresh Ringer solution. A microelectrode was dipped into the solution and allowed 20-30 min for equilibration.

To change the solution the chamber was continuously flushed with a volume of fluid at least 5 times that of the chamber volume. The perfusion rate was such that the impaled microelectrode remained inside the cell during and after the perfusion, and was usually completed in 10-15 s.

Electrophysiological recordings

The membrane potential was recorded using standard single-barreled glass microelectrodes. Micropipettes were pulled from capillary tubings with internal filament (od 1.5, id 0.84, CGF150-4, WPI) on a vertical puller (Narishige, Japan) and then filled with 3 mol/l KCl shortly after being pulled. The tip diameter of the electrodes was less than 1 μm, tip potentials were less than 5 mV, and the microelectrode resistance was 15-25 MΩ in standard Ringer solution (for composition see solutions).

The potentials were amplified using a high input impedance amplifier (Winston Electronics, model 1090). Microelectrodes were connected to the amplifier via an Ag-AgCl wire. The ground electrode was an Ag-AgCl wire in a separate chamber filled with Ringer solution connected to the experimental chamber by a 3 mol/l KCl 3% agar bridge. The recordings were displayed on a two-channel oscilloscope (Hameg) and permanently recorded on a pen recorder (Linseis, Selb, Germany).

Solutions

The Ringer solution used in these experiments had the following composition (in mmol/l): NaCl 115.5, KCl 4, CaCl$_2$ 2, NaH$_2$PO$_4$ 0.3, Na$_3$HPO$_4$ 1.2 (pH=7.2). Excitatory amino acids L-glutamate, L-aspartate, and L-BOAA (all from Sigma), were kept in concentrated aqueous stock solutions. Adequate concentrations of the amino acids were prepared by pipetting appropriate amounts of the stock solution to the leech Ringer solution shortly before use.

Data analysis

All results are expressed as means ± S.E.M. with n indicating number of trials. Dose dependent curves were fitted using equation (1)

$$y = Y_{min} + \frac{Y_{max} - Y_{min}}{1 + 10^{(\log_{10} IC_{50} - \log_{10} IC_{eff})}}$$

Comparisons between mean values was made with a two-tailed paired Student’s t-test. P-values of less than 0.05 were considered significant.

Results

Effects of L-glutamate on membrane potential

In the first set of experiments we tested the effect of different concentrations of L-glutamate (L-glu) on the resting membrane potential of the Retzius nerve cells of the leech. As shown in Table 1, 10$^{-3}$ mol/l L-glu caused a rise in frequency of spontaneous action potential firing without significant depolarization (0.00 ± 0.00 mV, P>0.05, n=3). L-glu in concentration of 5x10$^{-3}$ mol/l caused a significant depolarization (4.50±0.35 mV, P<0.01, n=3; Fig. 1B), with a depolarizing block, followed by an afterhyperpolarization on washout, while a dose of 10$^{-2}$ mol/l L-glu produced a rapid and stable depolarization of 14.83±1.38 mV (P<0.01, n=6; Fig. 1C), also followed by a depolarizing block and a more.
Fig. 1. Effects of bath application of L-glutamate, L-aspartate, and L-BOAA on the resting membrane potential of the Retzius nerve cell.

Table 1

<table>
<thead>
<tr>
<th>Substance</th>
<th>Dose (mol/l)</th>
<th>RMP (mV)</th>
<th>MP on substance application (mV)</th>
<th>Depolarization (mV)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamate</td>
<td>1•10⁻³</td>
<td>-41.50±3.89</td>
<td>-41.50±3.89</td>
<td>0.00±0.00</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5•10⁻³</td>
<td>-41.00±1.52</td>
<td>-36.50±1.77</td>
<td>4.50±0.35</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1•10⁻²</td>
<td>-49.25±3.56</td>
<td>-33.25±2.53</td>
<td>14.83±1.38</td>
<td>6</td>
</tr>
<tr>
<td>L-aspartate</td>
<td>1•10⁻³</td>
<td>-46.67±5.44</td>
<td>-46.00±4.90</td>
<td>0.67±0.54</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>5•10⁻³</td>
<td>-42.00±1.63</td>
<td>-36.00±1.70</td>
<td>6.00±1.47</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>1•10⁻²</td>
<td>-51.27±1.71</td>
<td>-33.60±1.31</td>
<td>17.67±1.18</td>
<td>15</td>
</tr>
<tr>
<td>L-BOAA</td>
<td>1•10⁻⁶</td>
<td>-44.29±2.54</td>
<td>-43.71±2.46</td>
<td>0.57±0.28</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1•10⁻⁵</td>
<td>-45.20±2.16</td>
<td>-40.40±2.17</td>
<td>4.33±0.51</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>5•10⁻⁵</td>
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<td>-37.00±1.86</td>
<td>11.71±1.08</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>1•10⁻⁴</td>
<td>-46.67±0.90</td>
<td>-31.00±1.80</td>
<td>15.67±1.89</td>
<td>6</td>
</tr>
</tbody>
</table>

All data presented as mean ± SEM; RMP – resting membrane potential; MP – membrane potential; n – number of trials.
pronounced afterhyperpolarization. In both cases cells fully recovered and the membrane potential returned to its resting values. Representative diagrams from these experiments are shown in Figure 1.

Effects of L-aspartate on membrane potential

Table 1 also shows the effects of increasing concentrations of L-aspartate (L-asp) on resting membrane potential of RNCL. The smallest applied dose of $10^{-3}$ mol/l of L-asp did not lead to a significant depolarization ($0.67\pm0.54$ mV, $P>0.05$, $n=3$, Fig. 2A). On the other hand, doses of $5 \times 10^{-3}$ mol/l and $10^{-2}$ mol/l both produced significant depolarization of $6.00\pm0.47$ mV ($n=3$) and $17.67\pm1.18$ mV ($n=15$), respectively (traces in Figs 2B & 2C). In both cases depolarization is accompanied by a block in spontaneous firing of action potentials, as well as a steep afterhyperpolarization, followed by full recovery.

Effects of β-N-oxalylamino-L-alanine on membrane potential

β-N-oxalylamino-L-alanine (L-BOAA) also exerts excitatory action on Retzius nerve cells, similarly to the previously described substances, but in much smaller concentrations. It did not give rise to a significant depolarization when applied in the lowest of the chosen concentrations, i.e. $10^{-6}$ mol/l ($0.57\pm0.28$ mV, $P>0.05$, $n=7$; Fig. 3A). The dose of $10^{-5}$ mol/l L-BOAA produces a mild depolarization ($4.33\pm0.51$, $P<0.01$, $n=6$), which is not followed by an hyperpolarization, as shown in Figure 3B.

Figures 3C, and 3D depict the consequences of administration of $5 \times 10^{-5}$ mol/l and $10^{-4}$ mol/l L-BOAA. A dose of $5 \times 10^{-5}$ mol/l L-BOAA elicits a significant depolarization of $11.71\pm1.08$ mV ($P<0.01$, $n=7$). The highest applied concentration ($10^{-4}$ mol/l) produces the strongest effect ($15.67\pm1.89$ mV, $P<0.01$, $n=6$) with a full depolar-
izing block and a deep afterhyperpolarization. Effects of all four concentrations were followed by full recovery of the cell’s resting membrane potential. Table 1 provides a more detailed view of the numerical data.

Analysis of dose dependency

Since effects of all three substances on the membrane potential of Retzius nerve cells increase with the rising dose, indicating dose dependency, we performed a formal analysis of dose dependency using formula (1) (results presented in Table 2), and a graphical analysis using the plots of this function, shown in Figure 4.

Both analytical (value of EC_{50}) and graphical (position of the respective curve on the graph) results suggest the following order of potencies: L-BOAA >> L-asp ≈ L-glu, or, given numerically with L-glu taken as a reference, 233:1.08:1.00.
Discussion

Since excitatory amino acids (EAAs) play a significant role in both physiology and pathophysiology of the nervous system from invertebrates to man, we have examined and compared the electrophysiological effects of two endogenous amino acids ubiquitous across species, L-aspartate (L-asp), and L-glutamate (L-glu), and an exogenous amino acid β-N-oxalylamino-L-alanine (L-BOAA). Although each of these amino acids has previously been examined individually in our laboratory and by other authors, a comparison between them is either lacking or inconclusive.

Virtually equal potencies of L-asp and L-glu in our experiments are not in concert with the results of James and Walker (1978) and James et al. (1980b), which indicate that L-glu is approximately 11 times more potent than L-asp on Retzius nerve cells of the leech (RNCL). This discrepancy may be explained by differences in experimental techniques. In the above-mentioned papers, the substances were applied by means of direct administration in 20 ml of leech Ringer solution. We, on the other hand, applied the substances by completely exchanging the volume of the experimental chamber (for details see methods). It is also noteworthy that James and Walker (1978) and James et al. (1980b) did not perform a dose-dependency analysis, and did not calculate potencies based on multiple doses of the same substance before comparison, but rather compared the effects of a single dose of both substances.

L-BOAA produced a dose-dependent depolarization of RNCL. The excitatory action of L-BOAA has been previously described in the mammalian nervous system, e.g. on cat spinal interneurons and Betz cells (Watkins et al. 1966), and cat spinal cord and cuneate nucleus (Krniević et al. 1977). As for our model, a similar effect in potency of L-BOAA was reported by Nedeljkov et al. (1979), Cemerić and Nedeljkov (1998), and Cemerić et al. (2001).

Our results show that L-BOAA is over 233 times more potent than L-glu. James et al. (1980a) report that kainate is about 100 times more potent than L-glu on RNCL. Dierkes et al. (1996) applied Kainate on RNCL in concentration of 10^{-4} mol/l which produced depolarization comparable to that of 10^{-3} mol/l L-BOAA in our experiments. Dörner et al. (1994) constructed dose response curves for Kainate and L-glu on RNCL that are almost identically positioned as our dose response curves for L-BOAA and L-glu. Furthermore, investigations of the ionic mechanism of action of Kainate (Dörner et al. 1990; Löhrke & Deitmer 1996; Muller et al. 2003) are in agreement with the ionic mechanism of action of L-BOAA reported by Cemerić et al. (2001).

Taken together, the conclusion may be drawn that L-BOAA is similar in potency, and possibly mechanism, to kainate, and significantly more potent than L-asp and L-glu.

This has been reported on other models also. The ability of L-BOAA, kainate, and L-glu to produce acute excitotoxicity in embryonic chick retina (Zeevlak & Nicklas 1989), inhibition of [3H]-AMPA binding to receptors in membranes from post-mortem human brain (Sawutz et al. 1995), and electrophysiological studies on frog spinal cord (Pearson & Nunn 1981) and on fetal spinal cord neurons of mice in culture (MacDonald & Morris 1984), all show analogous order of potency for L-BOAA and other excitatory amino acids, as well as its similarity to kainate.

This may indicate that at least these EAAs have similar effects in leeches and some vertebrates, and that results obtained in leeches could be used for broader generalizations, a conclusion similar to

<table>
<thead>
<tr>
<th>Substance</th>
<th>( V_{max} ) (mV)</th>
<th>( EC_{50} ) (mol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-glutamate</td>
<td>16.56</td>
<td>0.0065</td>
</tr>
<tr>
<td>L-aspartate</td>
<td>22.62</td>
<td>0.0007</td>
</tr>
<tr>
<td>L-BOAA</td>
<td>15.80</td>
<td>0.00003</td>
</tr>
</tbody>
</table>

\( V_{max} \) – maximal calculated effect (maximal depolarization); \( EC_{50} \) – half maximal effective concentration.

Fig. 4. Dose dependency curves for L-glutamate (Glu), L-aspartate (Asp), and L-BOAA. Data points and error bars omitted for clarity.
that of GARDNER and WALKER (1982), who compared the actions of 4-methyl-homoibotenate, 4-bromo-homoibotenate and β-amino-5-hydroxy-3-methyl-4-isoxazole propionic acid (AMPA) on mammalian neurons and RNCLs.

The high potency of L-BOAA shown in this paper, and its similarity to kainate, a well established neuroexcitatory agent capable of inducing neurotoxicity and seizures, strengthen the case for the neurotoxic role of this excitatory amino acid from Lathyrus seeds.

Furthermore, L-BOAA is not only a potent excitatory agent, but it also disrupts mitochondrial processes in specific areas of the nervous system causing oxidative stress (RAVINDRANATH 2002; DIWAKAR & RAVINDRANATH 2007; SHINOMOL & MURALIDHARA 2007), and sensitizes neurons to depolarization by other excitatory amino acids (CHASE et al. 2007).

Therefore, although the quantity of L-BOAA present in Lathyrus sativus beans is relatively small, the high potency of this amino acid, along with other properties discussed above, could render it a powerful and effective neurotoxin.

We conclude that L-glutamate, L-aspartate and β-N-oxalylamino-L-alanine all have excitatory properties on Retzius nerve cells of the leech Hae-mopis sanguisuga, and that L-BOAA is by far the most potent of the three.

Acknowledgements

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References

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