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Excitotoxicity of *Lathyrus Sativus* Neurotoxin in Leech Retzius Neurons

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Summary

The effects of *Lathyrus sativus* neurotoxin were studied on the cell membrane potential and cellular cation composition in Retzius nerve cells of the leech *Haemopis sanguisuga*, with ion-selective microelectrodes using liquid ion-exchangers. Bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min depolarized the cell membrane potential and decreased the input resistance of directly polarized membrane in Retzius neurons. At the same time the cellular Na^+ activity increased and cellular K^+ activity decreased with slow but complete recovery, while the intracellular Ca^{2+} concentration was not changed. Na^+-free Ringer solutions inhibited the depolarizing effect of the neurotoxin on the cell membrane potential. Zero-Ca^{2+} Ringer solution or Ni^{2+}-Ringer solution had no influence on the depolarizing effect of the neurotoxin on the cell membrane potential. It is obvious that the increase in membrane conductance and depolarization of the cell membrane potential are due to an influx of Na^+ into the cell accompanied by an efflux of K^+ from the cell.

Key words

Input membrane resistance • Intracellular Ca^{2+} concentration • Intracellular Na^+ and K^+ activities • *Lathyrus sativus* excitotoxicity • Leech Retzius neurons

Introduction

Prolonged ingestion of the legume of the plant *Lathyrus sativus* in man leads to a clinical syndrome, known as neurolathyrism, characterized by muscle weakness as a result of the destruction of corticospinal tract neurons, which causes a serious health problem in India and Ethiopia because of the lack of alternate food sources during periods of drought (Spencer *et al.* 1986). In cultured neurons from the mouse spinal cord the application of *Lathyrus sativus* neurotoxin produced cell membrane potential depolarization and an increase in input conductance (MacDonald and Morris 1984). It is suggested that this potent neuronal excitant acts preferentially on glutamate receptors of the non-NMDA type, i.e. on quisqualate-kainate receptors (MacDonald and Morris 1984, Ross *et al.* 1989, Willis *et al.* 1993). Glutamate has been identified as one of the most common excitatory neurotransmitters in the central nervous system of vertebrates and invertebrates (Gardner and Walker 1982, Takeuchi 1987). Glutamate receptors are divided into ionotropic and metabotropic subtypes. The ionotropic receptors are ligand-gated ion channels and are subdivided, according to their selective agonists, into N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) / kainate

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receptors. Metabotropic receptor subtypes are coupled to a transducer protein, a G protein, and modulate the production of intracellular messengers (Collingridge and Lester 1989, Schoepp and Conn 1993).

Because of the capability of glutamate to produce both excitation and toxicity in neurons, the term "excitotoxicity" was proposed to characterize this particular form of neuronal degeneration (Whetsell and Shapira 1993). Considerable interest has been focused on the molecular mechanisms underlying the glutamate-induced neurodegeneration. Excessive activation of ionotropic and metabotropic glutamate receptors induces acute effects of important Na^+, H^+, Ca^{2+} fluxes causing "excitotoxic" neuronal swelling and long-term effects of Ca^{2+} influx (Hicks and Conti 1996). Glutamate induces cell death by an elevation of cytosolic free Ca^{2+} in neurons, thereby leading to the generation of free radicals and the activation of proteases, phospholipases and endonucleases, as well as the transcriptional activation of specific "cell death" programs (Llinas and Sugimori 1990).

Neurons of the mammalian central nervous system degenerate when exposed to high concentrations of L-glutamate and a number of its (endogenous and exogenous) chemically structural analogs (Whetsell and Shapira 1993). One of these exogenous analogs of L-glutamate is β-N-oxalyl-L-α-β-diaminopropionic acid from peas of the plant *Lathyrus sativus*. We studied its excitotoxic effects on the cell membrane potential, on the input resistance of directly polarized membrane and on the cellular ion activities of Na^+, K^+ and Ca^{2+} in Retzius neurons of ganglia of the leech *Haemopis sanguisuga*. Preliminary results were previously reported (Cemerikic and Nedeljkov 1998).

Methods

Experiments were performed at room temperature on the Retzius neurons of isolated abdominal segmental ganglia of the ventral nerve cord of the leech *Haemopis sanguisuga*. The method of dissection was described previously (Beleslin 1971). Free segmental ganglia were placed in a 2.5 ml plastic chamber with leech Ringer solution of the following composition (in mmol/l): NaCl 115, KCl 4, $CaCl_2$ 2, NaH_2PO_4 0.3, Na_2HPO_4 1.2 (pH = 7.2). To change the solution, the chamber was flushed continuously with a volume of fluid at least 10 times larger than that of the chamber volume. Neurotoxin β-N-oxalyl-L-α-β-diaminopropionic acid (obtained from Anesthesia Research Department, McGill University, Montreal, Canada) from peas of the plant *Lathyrus sativus* was used by bath perfusion dissolved in the Ringer solution in a concentration of 10^{-6}, 10^{-5}, $5x10^{-5}$ and 10^{-4} mol/l. In the Na^+-free Ringer solution, 115 mmol/l NaCl were completely replaced with an equal amount of Tris (hydroxymethyl) aminomethane-Cl (Tris-Ringer solution) and NaH_2PO_4 and Na_2HPO_4 were omitted. In the Ca^{2+}-free Ringer solution $CaCl_2$ was omitted and 0.5 mmol EGTA was added. The Ringer solution containing Ni^{2+} was prepared by adding 2 mmol $NiCl_2$ to the standard Ringer solution.

Electrical methods

The cell membrane potential was measured with conventional 3 mol/l KCl microelectrodes made from thick wall capillary tubings having an internal fiber (1.2 mm outside diameter, 0.6 mm inside diameter, Frederick Haer, Brunswick, ME). Microelectrodes were fabricated on a horizontal puller (PD-5, Narishige Instrument Lab., Tokyo, Japan). The tip diameter was <1 μm, tip potentials were <5 mV, and the input resistance was 10-20 MΩ in standard Ringer solution. For measurements of the input resistance of directly polarized membrane a high input impedance bridge amplifier (Winston Electronics, model 1090) was used to inject a current through the recording microelectrode. The amplitude of the recorded voltage produced by rectangular hyperpolarizing current pulses (0.3-0.6 nA, 500 ms duration, applied at 0.1-0.2 Hz) was used as a measure of the membrane input resistance.

The manufacture and siliconisation of double-barreled K^+ and Na^+ selective microelectrodes (with a tip diameter <1 μm), using Corning 477317 liquid K^+ exchanger resin or sodium ionophore I cocktail A (Fluka, Buchs, Switzerland), respectively, were the same as those described by Cemerikic *et al.* (1982).

The characteristics of the double-barreled K^+ selective microelectrodes were as follows: the slope for a 10-fold change in K^+ concentration was –58.8±0.69 mV (n=8), the selectivity coefficient ($K_{K\text{-}Na}$) was 0.08±0.004 (n=8), the input resistance in the Ringer solution was 6×10^9 Ω, and the response time was in the order of 1 s, as calibrated in single salt solutions of 0.1 and 0.01 mol/l KCl and 0.1 mol/l NaCl. Intracellular K^+ activity ($(K^+)_c$) was calculated directly from the calibration curves of each microelectrode in pure KCl solutions.

The characteristics of the double-barreled Na^+ selective microelectrodes were as follows. The slope for a 10-fold change in Na^+ concentration was –50±0.4 mV (n=5), the selectivity coefficient (K_{Na-K}) was 0.05±0.008 (n=5), the input resistance in the Ringer solution was 2-5 x 10^{10} Ω, and the response time was in the order of 1 s, as calibrated in single salt solutions of 0.1 and 0.01 mol/l NaCl and 0.1 mol/l KCl. Intracellular Na^+ activity ($(Na^+)_c$) was calculated directly from the calibration curves of each microelectrode in pure NaCl solutions.

Single-barreled Ca^{2+} selective microelectrodes were made from the same glass, filled with a neutral carrier Ca ligand ETH 129 (Fluka I cocktail A, Buchs, Switzerland) and backfilled with a 10^{-7} mol/l Ca buffer solution (pH 7.4) containing 125 mmol/l K^+ prepared according to Amman *et al.* (1987). Calibration solutions for Ca^{2+} selective microelectrodes with 10^{-3}, 10^{-4} and 10^{-5} mol/l Ca^{2+} concentrations (pCa 3, 4, 5) were made by a single dilution of a 10^{-2} mol/l $CaCl_2$ solution. 10^{-6}, 10^{-7}, 10^{-8} and 10^{-9} mol/l Ca buffer solutions (pCa 6, 7, 8, 9) containing an intracellular background of 125 mmol/l K^+ (pH 7.4) were prepared according to Amman *et al.* (1987). The characteristics of Ca^{2+} selective microelectrodes were as follows: the slopes of the Ca^{2+} selective microelectrodes (n=5) were 27.3±1.35 mV, 28.4±2.1 mV, 113±4.35 mV, 10.9±1.45 mV, 5±0 mV and 4.2±2.0 mV between pCa 3 and 4, pCa 4 and 5, pCa 5 and 6, pCa 6 and 7, pCa 7 and 8, and pCa 8 and 9, respectively. Ca^{2+} selective microelectrodes exhibit a super-Nernstian slope between pCa 5 and 6, and sub-Nernstian slopes below pCa 6. Breaking of the tip of microelectrodes did not eliminate the non-ideal behavior of the microelectrode response. Similar non-linearity in the slope of Ca^{2+} selective microelectrodes for calibration in Ca buffer solutions has been reported by Kubota *et al.* (1990). The time required for a 90 % response in test solutions pCa 6, 7, 8 and 9 was about 2 s. Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_c$) was calculated directly from the calibration curves of each microelectrode in Ca buffer solutions after making allowances for the cell membrane potential. Conventional and Ca^{2+} selective microelectrodes were inserted sequentially into a pair of Retzius cells in the same ganglion, as they are electrically well-coupled and have about the same cell membrane potential. The validity of this two-cell approach was tested previously (Cemerikic *et al.* 1988).

The microelectrodes were connected to a high impedance probe of a dual electrometer (Analog Devices Ad 515 L, Norwood, MA) *via* an Ag-AgCl wire, and the voltage signals were recorded by a two-channel recorder (Linseis, Selb, Germany) and digitally displayed on LED panels. The ground electrode was an Ag-AgCl wire connected to the experimental chamber by a 3 mol/l KCl 3 % agar bridge.

Data analysis

All results were expressed as means ± S.E.M. with n indicating the number of neurons. Comparison between mean values were made with a paired Student´s t-test. $P<0.05$ values were considered significant.

Table 1. Effects of bath application of *Lathyrus sativus* neurotoxin on the cell membrane potential (V_m), cellular sodium activity $(Na^+)_c$, cellular potassium activity $(K^+)_c$, and cellular calcium ion concentration $[Ca^{2+}]_c$ in the Retzius nerve cells of the leech.

	V_m (mV)	**$(Na^+)_c$ (mmol/l)**	**$(K^+)_c$ (mmol/l)**	**$[Ca^{2+}]_c$ (nmol/l)**
Control	–40.8±1.51	13.3±0.80	84.0±3.40	33.6±9.15
Lathyrus sativus	–30.7±1.34	33.5±2.82	67.5±2.72	33.6±9.15
(10^{-4} mol/l)	P<0.001	P<0.001	P<0.005	NS
Δ	10.1±0.52	20.1±2.95	16.4±2.47	0
n	15	5	7	3

Data are means ± S.E.M., n = the number of neurons, NS = not significant.

Results

In pivotal experiments, the effects of bath application of 10^{-6}, 10^{-5}, 5 x 10^{-5} and 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min were studied on the cell membrane potential of Retzius neurons. The concentration of 10^{-6} mol/l had no influence on the cell membrane potential of –43±3.34 mV (n=6), the concentration of 10^{-5} mol/l showed insignificant depolarization of the cell membrane potential of´ –46.8±4.63 mV (n=5) by 2.5±0.44 mV (n=5, P>0.05), while the concentration of $5x10^{-5}$ mol/l induced rapid and stable depolarization of the cell membrane potential of –47.6±2.13 mV (n=5) by 8.6±1.93 mV (n=5, P<0.05). The bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min evoked rapid and stable depolarization of the mean cell membrane potential of –40.8±1.51 mV (Table 1) by 10.1±0.52 mV (n=15). The effect of *Lathyrus sativus* neurotoxin on the cell membrane potential was dose-dependent and the concentration of 10^{-4} mol/l was chosen as an experimental final concentration (Fig. 1A). In addition, this depolarization was always followed by a transient hyperpolarization of the cell membrane potential, during recovery or washout with control Ringer solution, by –5.43±0.93 mV (n=15). This transient hyperpolarization of the cell membrane potential during recovery from *Lathyrus sativus* neurotoxin application was inhibited completely when the Na-K pump was inhibited during exposure to zero-K-Ringer solution, indicating that it was due to the activation of the electrogenic Na-K pump. As illustrated in Figure 1B, after 25 min of exposure to zero-K-Ringer solution the application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 4 min depolarized the cell membrane potential from –44 mV to –19 mV, which was followed by further depolarization to –16.5 mV after washout with zero-K-Ringer solution. Only during the recovery with control Ringer solution, under conditions of Na-K pump reactivation, the cell membrane potential hyperpolarized to the control value of –45 mV within 10 min.

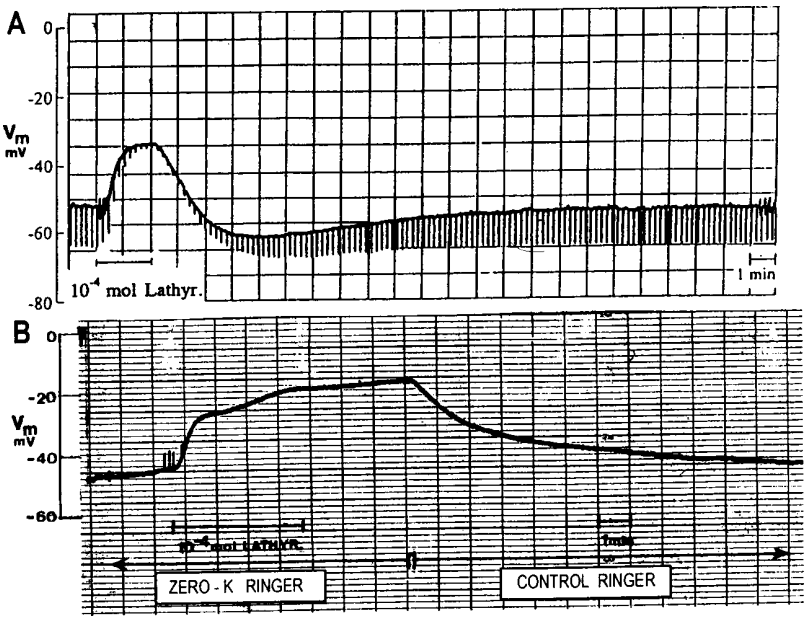

Fig. 1. *(A) Bath application of 10^{-4} mol/l Lathyrus sativus neurotoxin depolarized the cell membrane potential of –53 mV by 18 mV and after 3 min of recovery the cell membrane potential became transiently hyperpolarized to –62 mV and caused a decrease in the input resistance of directly polarized membrane from 25 MΩ to 4 MΩ.. (B) Bath application of 10^{-4} mol/l Lathyrus sativus neurotoxin after 25 min of exposure to zero-K-Ringer solution depolarized the cell membrane potential of –44 mV by 25 mV and washout with zero-K Ringer solution further depolarized the cell membrane potential to –16.5 mV.*

Bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min caused a significant decrease (P<0.05) in the input resistance of directly polarized membranes by 69 % from 25±0 MΩ (n=2) to 7.95±3.95 MΩ (n=2). Figure 1A illustrates that bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 2 min caused depolarization of the cell membrane potential of –53 mV by 18 mV which was accompanied by a decrease in the input resistance of directly polarized membrane from 25 MΩ to 4 MΩ. How could we explain this depolarization of the cell membrane potential and the decrease in the input resistance of directly polarized membrane after the exposure to *Lathyrus sativus* neurotoxin?

Figure 2 shows the effect of bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min on

$(Na^+)_c$. Concomitantly with the depolarization of the cell membrane potential of –41 mV by 11 mV, $(Na^+)_c$ rapidly increased from 11.6 mmol/l to 42.3 mmol/l. After 14 min of recovery the cell membrane potential returned to –41 mV and $(Na^+)_c$ to 18.4 mmol/l. In this instance, a differential output of the double-barreled Na^+ selective microelectrode is not shown. The average increase of $(Na^+)_c$ during bath application of *Lathyrus sativus* neurotoxin for 3 min was 20.1±2.95 mmol/l (Table 1), indicating a rapid influx of Na^+ into the cell.

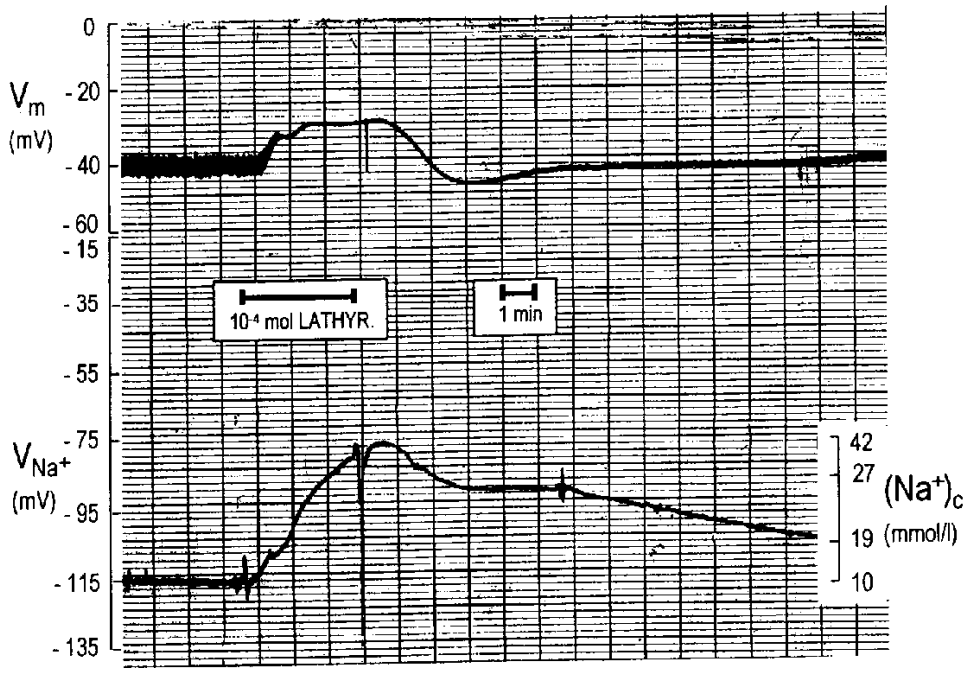

Fig. 2. *The effect of bath application of Lathyrus sativus neurotoxin on $(Na^+)_c$. During 3 min bath application of 10^{-4} mol/l Lathyrus sativus neurotoxin, the cell membrane potential of –41 mV (V_m, upper trace) became depolarized by 11 mV, while $(Na^+)_c$ of 11.6 mmol/l (V_{Na}, lower trace, electrochemical potential of Na^+ selective barrel) increased to 42.3 mmol/l, with slow recovery of $(Na^+)_c$ to 18.4 mmol/l after 14 min. $(Na^+)_c$ is derived from the difference $V_{Na} – V_m$, as a differential output of double-barreled Na^+ selective microelectrode is not shown.*

Figure 3 shows the effect of bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 6 min on $(K^+)_c$. While the cell membrane became depolarized from –42 mV to –32 mV, $(K^+)_c$ decreased from 84 mmol/l to 70 mmol/l, as shown in the differential recordings from a double-barreled K^+ selective microelectrode. After 10 min of recovery, the cell membrane potential and $(K^+)_c$ slowly and completely recovered to control values. The average decrease of $(K^+)_c$ during bath application of *Lathyrus sativus* neurotoxin for 3 min was 16.4±2.47 mmol/l (Table 1), indicating a rapid efflux of K^+ from the cell.

At the same time, during 3 min of bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin the mean $[Ca^{2+}]_c$ value of 33.6±9.15 nmol/l (Table 1) was not changed, indicating that Ca^{2+} did not enter the cell for this average 10.1±0.52 mV depolarization of the cell membrane potential by *Lathyrus sativus* neurotoxin (Table 1). Figure 4 illustrates the effect of bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin on $[Ca^{2+}]_c$. Concomitantly with the depolarization of the cell membrane potential of –40 mV by 10 mV, $[Ca^{2+}]_c$ value of 30 nmol/l was not changed. During 20 min application of Na^+-free Tris-Ringer solution, the depolarizing effect of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min on the cell membrane potential was significantly reduced ($P<0.001$) to 3.7±0.8 mV (n=5). As Na^+-free Ringer solution inhibits the depolarizing effect of *Lathyrus sativus* neurotoxin on the cell membrane potential, it is obvious that the increase in membrane conductance and the depolarization of the cell membrane were due to the influx of Na^+ into the cell accompanied by an efflux of K^+ from the cell.

To confirm this finding, we used Ca^{2+}-free Ringer solution (containing 0.5 mmol/l EGTA) to suppress any major Ca^{2+} influx and Ringer solution with 2 mmol/l Ni^{2+} to block voltage-dependent Ca^{2+} channels in the Retzius neurons (Munsch and Deitmer 1995).

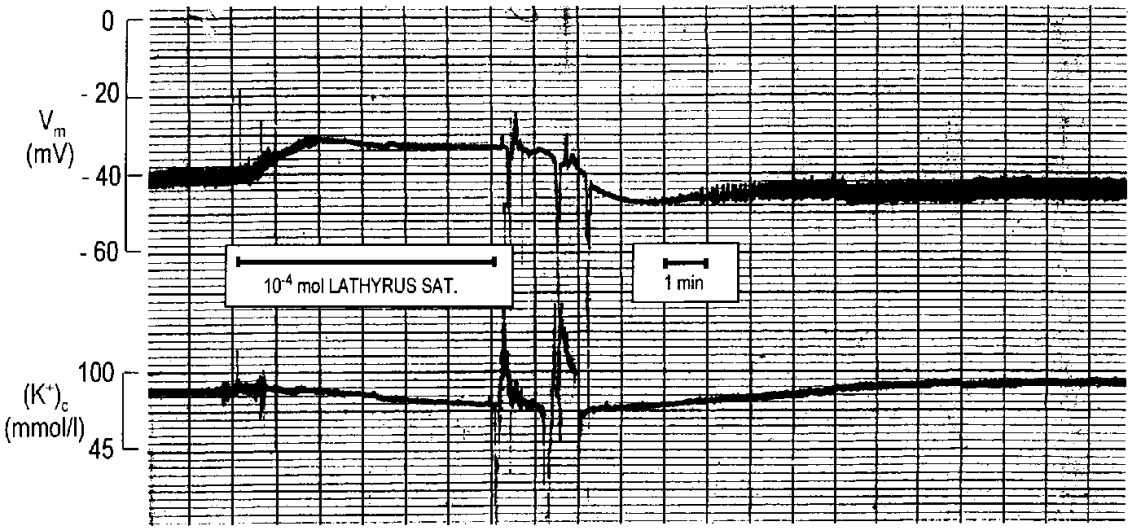

Fig. 3. *The effect of bath application of 10^{-4} mol/l Lathyrus sativus neurotoxin on $(K^+)_c$. The cell membrane potential of –42 mV (V_m, upper trace) became depolarized by 10 mV, while $(K^+)_c$ of 84 mmol/l (lower trace) decreased by 14 mmol/l, with complete recovery, as recorded with a double-barreled K^+ selective microelectrode.*

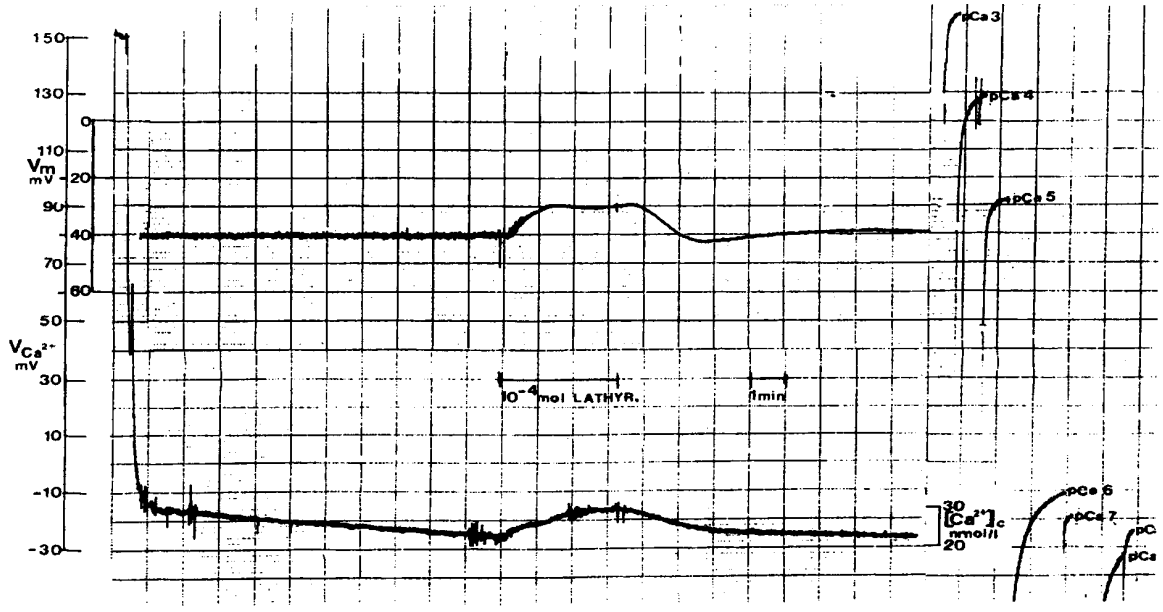

Fig. 4. *The effect of bath application of 10^{-4} mol/l Lathyrus sativus neurotoxin on $[Ca^{2+}]_c$. The cell membrane potential of –40 mV (V_m, upper trace) was depolarized by 10 mV, while $[Ca^{2+}]_c$ of 30 nmol/l was not changed (V_{Ca}, lower trace, electrochemical potential of Ca^{2+} selective single-barreled microelectrode). $[Ca^{2+}]_c$ is derived from the difference $V_{Ca} - V_m$. Both microelectrodes were inserted into a pair of Retzius cells as given in the Methods. The calibration procedure for Ca^{2+} selective microelectrode is also indicated.*

Exposure to Ca^{2+}-free Ringer solution for 20 min had no significant influence (P>0.05) on the depolarization of a cell membrane potential (–36.6±3.08 mV, n=3) by 8.7±1.2 mV (n=3), induced with a bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min, compared to the depolarization of the cell membrane potential by

10.1±0.52 mV (Table 1) in control Ringer solution. Exposure to Ringer solution with 2 mmol/l Ni^{2+} for 20 min also had no significant influence (P>0.05) on the depolarization of the cell membrane potential (–45.6±3.34 mV, n=3) by 9.83±2.04 mV (n=3), induced by the bath application of 10^{-4} mol/l *Lathyrus sativus* neurotoxin for 3 min, compared to the depolarization of the cell membrane by 10.1±0.52 mV (Table 1) in control Ringer solution.

Discussion

The present results have shown that excitation of Retzius neurons with *Lathyrus sativus* neurotoxin causes dose-dependent cell membrane potential depolarization with a concomitant increase in cell membrane input conductance, as well as an increase in cellular Na^+ activity and a decrease in cellular K^+ activity without changes in intracellular Ca^{2+} concentration. As was shown previously, raising the extracellular Mg^{2+} concentration, which has been shown to inhibit synaptic transmission in the leech nervous system (Stuart 1970), did not influence the excitation effect of *Lathyrus sativus* neurotoxin on Retzius neurons. This indicates that the effect of this neurotoxin is due to the direct action on Retzius neurons (Nedeljkov *et al.* 1979). Our results exclude the possibility that the cellular Na^+ activity increases during action of *Lathyrus sativus* neurotoxin, is due to the synaptic input. It is also suggested that this potent neuronal excitant acts preferentially on glutamate receptors of the non-NMDA type, i.e. AMPA/kainate receptors (MacDonald and Morris 1984, Ross *et al.* 1989, Willis *et al.* 1993). It was recently shown in Retzius neurons that ion channels coupled to the ionotropic glutamate receptors of the AMPA/kainate type are not permeant to Ca^{2+} (Dierkes *et al.* 1996, Lohrke and Deitmer 1996). It is most probable that the excitotoxic effects on Retzius neurons observed in our study are mediated by the effects of *Lathyrus sativus* neurotoxin on this ionotropic AMPA/kainate glutamate receptor.

It was observed that the dose-dependent cell membrane potential depolarization, the increase in cell membrane input conductance and the enhanced cellular Na^+ activity caused by *Lathyrus sativus* neurotoxin were due to a Na^+ influx from the external medium, because the *Lathyrus sativus* neurotoxin only caused small, insignificant depolarization of the cell membrane potential in the absence of external Na^+. Furthermore, the lack of hyperpolarization of the cell membrane potential during recovery in zero-K-Ringer solution indicates that this is due to the activation of electrogenic Na-K exchange pump stimulated by the increased cellular Na^+ activity during bath application of *Lathyrus sativus* neurotoxin. The Na-K pump, which is most likely electrogenic as in most other cells, hyperpolarized the cell membrane potential and reversed the high levels of accumulated Na^+ to control values during 15 min of recovery with control Ringer solution. Consequently, the *Lathyrus sativus* neurotoxin is probably acting on the AMPA/kainate glutamate receptor in Retzius neurons (Dierkes *et al.* 1996, Lohrke and Deitmer 1996) to increase Na^+ permeability (and thus the input conductance) and to depolarize the cell membrane potential towards the Na^+ equilibrium potential. This process is arrested at a stable level of cell membrane potential depolarization during maximal *Lathyrus sativus* neurotoxin response, probably by K^+ efflux from the cell balancing a part of the inward Na^+ current. The resulting increase of extracellular K^+ might be responsible for some portion of the initial depolarization of cell membrane potential induced by *Lathyrus sativus* neurotoxin. A large influx of Na^+ resulting in the cell membrane potential depolarization presumably leads to a secondary influx of Cl^- and water and “excitotoxic” cell swelling.

Furthermore, it was shown by Dierkes *et al.* (1996) that a kainate-induced intracellular Ca^{2+} concentration increase was detectable when the cell membrane potential in Retzius neurons was depolarized to about –30 mV. It closely corresponds to the threshold potential at which the intracellular Ca^{2+} concentration increase, caused by raising the extracellular K^+ concentration, had occurred. This indicates that Ca^{2+} enters the cell through voltage-dependent Ca^{2+} channels activated by cell membrane potential depolarization below the threshold value. It is concluded that voltage-gated Ca^{2+} channels are activated at this threshold potential thus leading to a Ca^{2+} influx into the cell (Dierkes *et al.* 1996). As the cellular Ca^{2+} transient was reversibly blocked by Ni^{2+} added to the Ringer solution, it is suggested that Retzius neurons do indeed possess voltage-gated Ca^{2+} channels mediating the rise of intracellular Ca^{2+} concentration (Munsch and Deitmer 1995). As the depolarization of the cell membrane potential evoked by *Lathyrus sativus* neurotoxin in our experiments reaches a value of about –30 mV, which is

the threshold value of the cell membrane potential for influx of Ca^{2+} *via* voltage-dependent Ca^{2+} channels in Retzius neurons (Dierkes *et al.* 1996), the intracellular Ca^{2+} concentration was not changed in this way during the exposure to *Lathyrus sativus* neurotoxin in our experiments. This is also confirmed by experiments with zero-Ca-Ringer solution and Ni^{2+}- Ringer solution neither of which had any influence on the magnitude of depolarization of the cell membrane potential by *Lathyrus sativus* neurotoxin. The Ca^{2+}-dependent K^+ channels which may play a role in generating the cell membrane potential of leech Retzius neurons were shown to exist (Frey *et al.* 1993). In Ni^{2+}-Ringer solution, both the cell membrane potential depolarization and cellular Ca^{2+} transients evoked by kainate were completely but reversibly suppressed in leech glial cells (Munsch *et al.* 1994).

A complete absence of the cellular Ca^{2+} response is surprising. At a typical intracellular K^+ concentration background corresponding Ca^{2+} selective microelectrodes showed Ca^{2+} microelectrode response curves with a detection limit at pCa 9. Ca^{2+} selective microelectrodes rapidly respond within a few seconds after cellular impalements, but a slope below pCa 6 may not be sufficient to record small and/or fast calcium changes. But a kainate-induced intracellular Ca^{2+} concentration increase in Retzius neurons, detectable when the cell membrane potential is depolarized beyond –30 mV, is in the micromolar range and remains elevated for up to tens of minutes (Dierkes *et al.* 1996). The cellular Ca^{2+} transients in these ranges should be detected with the Ca^{2+} selective microelectrodes used. Thus an absence of the cellular calcium response in our experiments (at a the depolarization of the cell membrane potential to the value of about –30 mV) suggests that the AMPA/kainate glutamate receptor in Retzius neurons (demonstrated by Dierkes *et al.* (1996) and Lohrke and Deitmer 1996) is not Ca^{2+} permeable.

Furthermore, the applicability of an invertebrate model to excitotoxicity in mammals is limited because the properties of ionotropic glutamate receptor channels in invertebrates are different from any of the ionotropic glutamate receptor channels found in the mammalian brain. In the mammalian neurons expressing certain AMPA/kainate receptor subunits glutamate may trigger calcium-dependent intracellular events by activating non-NMDA receptors (Hollmann *et al.* 1991). Much less is known about the properties of glutamate receptor channels in invertebrates. A complementary DNA for the *Drosophila* glutamate receptor channel subunit has been cloned which is homologous to only 30 % of the types to be found in the mammalian central nervous system (Schuster *et al.* 1991). The pharmacological profile of the ionotropic glutamate receptor of the AMPA/ kainate type, the ion channel of which coupled to the glutamate receptor is not permeant to Ca^{2+} has been described in Retzius neurons (Dierkes *et al.* 1996, Lohrke and Deitmer 1996). Nevertheless, molecular data checking the homology of leech and mammalian ionotropic glutamate receptors are still lacking.

Unchanged intracellular Ca^{2+} concentration after the application of *Lathyrus sativus* neurotoxin in our study indicates that Ca^{2+} influx into the cell did not occur *via* the ion channel coupled to the AMPA/kainate glutamate receptor in Retzius neurons, as had been shown by Dierkes *et al.* (1996) and Lohrke and Deitmer (1996). Since the accumulation of intracellular Ca^{2+} after exposure to L-glutamate has been reported to mediate its excitotoxic effect resulting in neurodegeneration (Pellegrini-Giampietro *et al.* 1997, Dirnagl *et al.* 1999), it is probable that the *Lathyrus sativus* neurotoxin causes persistent depolarization of the cell membrane potential and hence damages neurons by the toxic accumulation of intracellular Ca^{2+} through voltage-dependent Ca^{2+} channels.

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References

AMMAN D, BUHRER T, SCHEFER U, MULLER M, SIMON W: Intracellular neutral carrier based Ca^{2+} microelectrode with subnanomolar detection limit. *Pflügers Arch* **409**: 223-228, 1987.

BELESLIN BB: Effects of different external media on the leech ganglion cells interaction. *Period Biol* **73**: 63-67, 1971.

CEMERIKIC D, NEDELJKOV V: Lathyrus neuroexcitotoxicity. *Yugoslav Physiol Pharmacol Acta* **34**: 53-63, 1998.

CEMERIKIC D, WILCOX CS, GIEBISCH G: Intracellular potential and K^+ activity in rat kidney proximal tubular cells in acidosis and K^+ depletion. *J Membr Biol* **69**: 159-165, 1982.

CEMERIKIC D, NEDELJKOV V, BELESLIN B: Effects of L-aspartate on cellular Na^+, K^+ and Cl^- activities in Retzius nerve cells of the leech. *Comp Biochem Physiol A* **89:** 67-74, 1988.

COLLINGRIDGE GL, LESTER RAJ: Excitatory amino acids receptors in the vertebrate central nervous system. *Pharmacol Rev* **40**: 143-210, 1989.

DIERKES PW, HOCHSTRATE P, SCHLUE WR: Distribution and functional properties of glutamate receptors in the leech central nervous system. *J Neurophysiol* **75**: 2312-2321, 1996.

DIRNAGL U, IADECOLA C, MOSKOWITZ MA: Pathobiology of ischaemic stroke: an integrated view. *Trends Neurosci* **22**: 391-397, 1999.

FREY G, HANKE W, SCHLUE WR: ATP-inhibited and Ca^{2+}-dependent K^+ channels in the soma membrane of cultured leech Retzius neurons. *J Membr Biol* **134**: 131-142, 1993.

GARDNER CR, WALKER RJ: The role of putative neurotransmitters and neuromodulators in annelids and related invertebrates. *Progr Neurobiol* **18**: 81-120, 1982.

HICKS TP, CONTI F: Amino acids as the source of considerable excitation in cerebral cortex. *Can J Physiol Pharmacol* **74**: 341-361, 1996.

HOLLMANN M, HARTLEY M, HEINEMANN S: Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* **252**: 851-853, 1991.

KUBOTA T, HAGIWARA N, FUJIMOTO M: Intracellular calcium measurements with PVC resin Ca selective microelectrodes in frog proximal tubules and sartorius muscle fibers. *Jpn J Physiol* **40**: 79-95, 1990.

LLINAS R, SUGIMORI M: Intracellular calcium concentration changes during cell death. In: *Neurotoxicity of Excitatory Amino Acids*, A GUIDOTTI (ed), Raven Press, New York, 1990, pp 1-10.

LOHRKE S, DEITMER J: Kainate responses of leech Retzius neurons in situ and in vitro. *J Neurobiol* **31**: 345-358, 1996.

MACDONALD JF, MORRIS ME: Lathyrus excitotoxin: mechanism of neuronal excitation by L-2-oxalylamino-3-amino- and L-3-oxalylamino-2-amino-propionic acid. *Exp Brain Res* **57**: 158-166, 1984.

MUNSCH T, DEITMER JW: Maintenance of Fura-2 fluorescence in glial cells and neurons of the leech central nervous system. *J Neurosci Methods* **57:** 195-204, 1995.

MUNSCH T, NETT W, DEITMER JW: Fura-2 signals evoked by kainate in leech glial cells in the presence of different divalent cations. *Glia* **11**: 345-353, 1994.

NEDELJKOV V, LEKIC D, BELESLIN B: Does Lathyrus sativus neurotoxin have a direct effect on Retzius nerve cells of the leech? *Period Biol* **81**: 658, 1979.

PELLEGRINI-GIAMPIETRO DE, GORTER JA, BENNETT MVL, ZUKIN RS: The GluR2 (GluR-B) hypothesis: Ca^{2+} -permeable AMPA receptors in neurological disorders. *Trends Neurosci* **20**: 464-470, 1997.

ROSS SM, ROY DN, SPENCER PS: Beta-N-oxalylamino-L-alanine action on glutamate receptors. *J Neurochem* **53**: 710-715, 1989.

SCHOEPP DD, CONN J: Metabotropic glutamate receptors in brain function and pathology. *Trends Pharmacol Sci* **14**: 13-20, 1993.

SCHUSTER CM, ULTSCH A, SCHLOSS P, COX JA, SCHMITT B, BETZ H: Molecular cloning of an invertebrate glutamate receptor subunit expressed in Drosophila muscle. *Science* **254**: 112-114, 1991.

SPENCER PS, ROY DN, LUDOLPH A, HUGON J, DWIVEDI MP, SCHAUMBURG HH: Lathyrism: evidence for the role of the neuroexcitatory amino acid BOAA. *Lancet* **2**: 1066-1067, 1986.

STUART AE: Physiological and morphological properties of motoneurones in the central nervous system of the leech. *J Physiol (Lond)* **209**: 627-646, 1970.

TAKEUCHI A: The transmitter role of glutamate in nervous systems. *Jpn J Physiol* **37**: 559-572, 1987.

WHETSELL WO, SHAPIRA NA: Neuroexcitation, excitotoxicity and human neurological disease. *Lab Invest* **68**: 372-387, 1993.

WILLIS CL, MELDRUM BS, NUNN PB, ANDERTON BH, LEIGH PN: Neuronal damage induced by beta-N-oxalylamino-L-alanine, in the rat hippocampus, can be prevented by a non-NMDA antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(F)quinoxaline. *Brain Res* **627**: 55-62, 1993.

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