

INTRACELLULAR Ca^{2+} RELEASE MEDIATED BY METABOTROPIC GLUTAMATE RECEPTOR ACTIVATION IN THE LEECH GIANT GLIAL CELL

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Summary

We have investigated the effects of glutamate and glutamate receptor ligands on the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and the membrane potential (E_m) of single, identified neuropile glial cells in the central nervous system of the leech *Hirudo medicinalis*. Exposed glial cells of isolated ganglia were filled iontophoretically with the Ca^{2+} indicator dye Fura-2. Application of glutamate ($200\text{--}500\ \mu\text{mol l}^{-1}$) caused biphasic membrane potential shifts and increases in $[\text{Ca}^{2+}]_i$, which were only partly reduced by either removing extracellular Ca^{2+} or blocking ionotropic glutamate receptors with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, $50\text{--}100\ \mu\text{mol l}^{-1}$). Metabotropic glutamate receptor (mGluR) ligands had the following rank of potency in inducing a rise in $[\text{Ca}^{2+}]_i$: quisqualate (QQ, $200\ \mu\text{mol l}^{-1}$) > glutamate ($200\ \mu\text{mol l}^{-1}$) > L(+)-2-amino-3-phosphonopropionic acid (L-AP3, $200\ \mu\text{mol l}^{-1}$) > *trans*-1-aminocyclopentane-1,3-dicarboxylic

acid (*t*-ACPD, $400\ \mu\text{mol l}^{-1}$). The mGluR-selective antagonist (RS)- α -methyl-4-carboxyphenylglycine [(RS)-MCPG, $1\ \text{mmol l}^{-1}$] significantly reduced glutamate-evoked increases in $[\text{Ca}^{2+}]_i$ by 20%. Incubation of the ganglia with the endoplasmic ATPase inhibitor cyclopiazonic acid (CPA, $10\ \mu\text{mol l}^{-1}$) caused a significant (53%) reduction of glutamate-induced $[\text{Ca}^{2+}]_i$ transients, while incubation with lithium ions ($2\ \text{mmol l}^{-1}$) resulted in a 46% reduction. The effects of depleting the Ca^{2+} stores with CPA and of CNQX were additive. We conclude that glutamate-induced $[\text{Ca}^{2+}]_i$ transients were mediated by activation of both Ca^{2+} -permeable ionotropic non-NMDA receptors and of metabotropic glutamate receptors leading to Ca^{2+} release from intracellular Ca^{2+} stores.

Key words: central nervous system, excitatory amino acid, Fura-2, *Hirudo medicinalis*, invertebrate, leech.

Introduction

Glutamate is one of the commonest excitatory neurotransmitters in the central nervous systems of vertebrates and invertebrates. Glutamate receptors are classified as ionotropic or metabotropic glutamate receptors. The ionotropic receptors are ligand-gated ion channels and are subdivided into the *N*-methyl-D-aspartate (NMDA) and the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors by reference to their artificial agonists (Nakanishi, 1992; Watkins *et al.* 1990). Metabotropic glutamate receptors (mGluRs) are coupled to GTP-binding proteins (G-proteins) with a broad spectrum of targets, e.g. phospholipases, adenylate cyclases or ion channels (for reviews, see Schoepp and Conn, 1993; Pin and Duvoisin, 1995). Recently, eight different mGluRs have been cloned (Duvoisin *et al.* 1995). These can be divided into three subgroups from their sequence homology and their functional and pharmacological properties (Nakanishi, 1992; Watkins and Collingridge, 1994): mGluR1 and mGluR5 (group I) are positively linked to phospholipase C, thus stimulating hydrolysis of phosphatidyl inositol-4,5-

bisphosphate into inositol-1,4,5-trisphosphate (InsP_3) and 1,2-diacylglycerol, and are strongly activated by quisqualate; mGluR2 and mGluR3 (group II) are negatively coupled to adenylate cyclase and are activated by *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD), whereas mGluR4, mGluR6, mGluR7 and mGluR8 (group III), also negatively coupled to adenylate cyclase, are sensitive to L(+)-2-amino-4-phosphonobutyrate (L-AP4).

In vertebrate central nervous systems, mGluRs have been shown to be expressed in many cell types, e.g. in hippocampal (Baskys, 1992) or thalamic (Salt and Eaton, 1996) neurones, in neurones of the retina and the olfactory bulb (Duvoisin *et al.* 1995; Nakanishi, 1995) and in glial cells (Prezeau *et al.* 1994; Petralia *et al.* 1996). They are believed to be involved in mechanisms of memory and learning (Kaba *et al.* 1994; Riedel, 1996) by modulating synaptic transmission (Gerber *et al.* 1993; Fitzsimonds and Dichter, 1996), and they are thought to mediate long-term depression (Hartell, 1994; Hémar *et al.* 1995) or long-term potentiation (Ito and Sugiyama, 1991;

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Bashir *et al.* 1993) of synaptic transmission. In addition, the activation of mGluRs has been shown to induce the inhibition of Ca²⁺ channels (Chavis *et al.* 1994) and voltage- or Ca²⁺-activated K⁺ channels (Charpak *et al.* 1990; Baskys, 1992) and can cause Ca²⁺ release from intracellular stores, both in neurones (Linden *et al.* 1994; Geiling and Schild, 1996) and in different types of glial cells in culture (Holzwarth *et al.* 1994; Kim *et al.* 1994; Brune and Deitmer, 1995) and *in situ* (Kriegler and Chiu, 1993; Porter and McCarthy, 1995, 1996).

Much less is known about mGluRs in invertebrates. Evans *et al.* (1992) reported a hyperpolarization evoked by *t*-ACPD in the Schwann cell surrounding the squid giant axon. A pertussis-toxin-sensitive, G-protein-coupled glutamate receptor, the glutamate_B receptor, has been reported to depress synaptic transmission at the lobster neuromuscular junction (Miwa *et al.* 1987, 1993), while a pertussis-toxin-insensitive mGluR mediated presynaptic inhibition at the crayfish neuromuscular junction (Shinozaki and Ishida, 1992). However, little is known about the pharmacological profile of these glutamate receptors, their possible homology with vertebrate mGluRs, and whether mGluR activation results in intracellular Ca²⁺ release in invertebrate nerve or glial cells. A sequence homology of approximately 45% to vertebrate group II mGluRs has recently been found for two mGluRs cloned from *Drosophila melanogaster* (Parmentier *et al.* 1996).

In the present study, we have investigated the effects of glutamate and several mGluR-selective agonists or antagonists on the intracellular free Ca²⁺ concentration ([Ca²⁺]_i) and the membrane potential (*E*_m) of leech neuropile glial cells. The results demonstrate that leech giant glial cells express ionotropic and metabotropic glutamate receptors, the latter mediating InsP₃-dependent Ca²⁺ release from intracellular stores. To our knowledge, this is the first evidence that intracellular Ca²⁺ release is mediated by mGluRs in an invertebrate nervous system. Some of the results have previously been communicated in abstract form (Lohr *et al.* 1996).

Materials and methods

Preparation

Experiments were performed on isolated segmental ganglia of the leech *Hirudo medicinalis* L. The preparation and dissection procedures have been described previously (Munsch and Deitmer, 1992). In brief, individual ganglia were removed from the ventral nerve cord and pinned, ventral side upwards, into a Sylgard-lined experimental chamber (volume ≤ 0.2 ml). The ventral ganglionic capsule was removed, and the ganglia were incubated in collagenase/dispase (2 mg ml⁻¹, Boehringer Mannheim, Germany) for 30 min. After enzyme treatment, the ventral neurones were removed mechanically, leaving the two giant glial cells at the surface of the neuropile.

Solutions

The normal superfusion saline had the following composition (in mmol l⁻¹): NaCl 85, KCl 4, CaCl₂ 2, MgCl₂ 1, Hepes 10, pH adjusted to 7.4 with NaOH. L-Glutamate (Sigma, Germany) was

kept in a stock solution of 100 mmol l⁻¹ at 4 °C. Cyclopiazonic acid (CPA, Sigma), thapsigargin (Sigma) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Cookson, UK) were dissolved in dimethylsulphoxide (DMSO) at concentrations of 50 mmol l⁻¹ and frozen at -20 °C; the compounds were added to the final solution from these stock solutions, so that the final concentration of DMSO did not exceed 0.2%. Caffeine (Sigma) was added directly to the perfusion saline. L-Quisqualate (QQ), DL-2-amino-5-phosphonopentanoic acid (DL-AP5), *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD), L-(+)-2-amino-3-phosphonopropionic acid (L-AP3), L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), (RS)-α-methyl-4-carboxyphenylglycine [(RS)-MCPG], (RS)-1-aminoindan-1,5-dicarboxylic acid [(RS)-AIDA], (S)-2-amino-2-methyl-4-phosphonobutyric acid (MAP4) and (S)-4-carboxyphenylglycine [(S)-4-CPG] were purchased from Tocris Cookson and were dissolved in 50 mmol l⁻¹ aqueous NaOH as stock solutions of 20 mmol l⁻¹. Drugs from these stock solutions were added to the perfusion saline immediately before an experiment, and the pH was readjusted to 7.4.

Measurement of intracellular [Ca²⁺] and membrane potential

Dye injection into leech glial cells and the measurement and calibration of Fura-2 fluorescence have been described previously (Munsch and Deitmer, 1995). Fura-2 pentapotassium salt (Molecular Probes, USA) was dissolved in 0.1 mol l⁻¹ KCl at a concentration of 12 mmol l⁻¹. The tip of one channel of a theta-type micropipette was filled with the dye solution, whereas the other channel was filled with 3 mol l⁻¹ KCl. Both channels were connected to bridge amplifiers (Intra 747, World Precision Instruments, USA, and Axoclamp 2B, Axon Instruments, USA) with chlorided silver wires. After inserting the micropipette into a glial cell, Fura-2 was injected into the cell by a constant negative current of approximately 1–5 nA until a 10- to 20-fold emission fluorescence value, compared with the background fluorescence before dye injection, was achieved. The dye was continuously injected throughout the experiment with a smaller current of approximately -1 nA to maintain Fura-2 fluorescence. Simultaneously, the membrane potential was recorded through the KCl-filled channel.

Fura-2 fluorescence was measured by a Deltascan dual-excitation spectrofluorimeter (PTI, Wedel, Germany) using excitation wavelengths of 350 nm and 380 nm (bandwidth 4 nm). Emission fluorescence from the cell soma was collected over a range of wavelengths from 510 nm to 530 nm by a photon-counting photomultiplier tube. The photomultiplier output signal and the bridge amplifier output signals were recorded on a personal computer using data-acquisition software (Felix, PTI, Germany). [Ca²⁺]_i was calculated using the equation described by Grynkiewicz *et al.* (1985):

$$[\text{Ca}^{2+}]_i = K_D \times S_f/S_b \times (R - R_{\min})/(R_{\max} - R),$$

where *R* is the fluorescence ratio measured experimentally, *R*_{min} is the fluorescence ratio for Ca²⁺-free and *R*_{max} for Ca²⁺-

saturated conditions, and Sf_2/Sb_2 is the fluorescence ratio for Ca^{2+} -free/ Ca^{2+} -bound dye at 380 nm. K_D is the apparent dissociation constant between Fura-2 and Ca^{2+} , and was determined to be 204 nmol l^{-1} for the leech giant glial cell.

Measurements are given as mean values \pm the standard error of the mean (S.E.M.) with N indicating the number of experiments. Statistical differences were checked using the Student's t -test for unpaired or, if possible, paired data ($P < 0.05$).

Results

Glutamate-induced membrane potential shifts and $[Ca^{2+}]_i$ transients

The steady-state $[Ca^{2+}]_i$ of the neuropile glial cells, as calculated from Fura-2 fluorescence, was $71.8 \pm 13.7 \text{ nmol l}^{-1}$ ($N=64$) at a mean membrane potential of $-66.5 \pm 11.7 \text{ mV}$ ($N=60$). Fig. 1 shows the $[Ca^{2+}]_i$ and E_m responses elicited by glutamate ($500 \mu\text{mol l}^{-1}$) in comparison with responses elicited by kainate ($10 \mu\text{mol l}^{-1}$) or by an elevation of the extracellular K^+ concentration to 20 mmol l^{-1} to activate voltage-gated Ca^{2+} channels. Glutamate-induced $[Ca^{2+}]_i$ increases were approximately half as large as kainate-induced increases at these concentrations (see also Deitmer and Munsch, 1994). $[Ca^{2+}]_i$ transients evoked by 20 mmol l^{-1} K^+ reached amplitudes of up to 600 nmol l^{-1} , due to the large depolarization which activates voltage-gated Ca^{2+} channels in these cells (Munsch and Deitmer, 1992, 1995). Removal of extracellular Ca^{2+} reduced the kainate- and the high- $[K^+]$ -induced $[Ca^{2+}]_i$ transients to less than 10%, while the glutamate-mediated transients were affected by less than 50%. After re-addition of extracellular Ca^{2+} , the $[Ca^{2+}]_i$ transients elicited by application of glutamate, kainate or elevation of $[K^+]$ recovered completely (Fig. 1).

Application of $500 \mu\text{mol l}^{-1}$ glutamate (for 1 min) induced elevations in $[Ca^{2+}]_i$ that ranged from 17.8 to 50.7 nmol l^{-1} and averaged $30.2 \pm 13.6 \text{ nmol l}^{-1}$ ($N=11$, Fig. 1), while $200 \mu\text{mol l}^{-1}$ glutamate raised the $[Ca^{2+}]_i$ by $17.0 \pm 10.9 \text{ nmol l}^{-1}$ with a range of 9.7 – 33.8 nmol l^{-1} ($N=53$, Fig. 2). There appeared to be no correlation between the basal $[Ca^{2+}]_i$ and the amplitudes of the glutamate-induced $[Ca^{2+}]_i$ increases. $[Ca^{2+}]_i$ transients induced by $500 \mu\text{mol l}^{-1}$ or $200 \mu\text{mol l}^{-1}$ glutamate were accompanied either by depolarizations, consisting of two components (Fig. 1, see Fig. 4), or by biphasic membrane potential shifts, i.e. a depolarization followed by a hyperpolarization (Fig. 2). $500 \mu\text{mol l}^{-1}$ glutamate elicited a maximal depolarization of $6.3 \pm 11.1 \text{ mV}$ ($N=6$) or biphasic responses with a depolarization of $6.2 \pm 11.8 \text{ mV}$ followed by a hyperpolarization of $-4.4 \pm 10.8 \text{ mV}$ ($N=5$), while $200 \mu\text{mol l}^{-1}$ glutamate elicited a maximal depolarization of $3.8 \pm 10.3 \text{ mV}$ ($N=35$) or biphasic responses with a depolarization of $3.4 \pm 10.4 \text{ mV}$ followed by a hyperpolarization of $-3.2 \pm 10.5 \text{ mV}$ ($N=20$). The mechanism of the glutamate-evoked hyperpolarization, previously described in several invertebrate preparations including the leech (Mat Jais *et al.* 1983; Evans *et al.* 1992; Miwa *et al.* 1987; Osborne, 1996), was not further examined in the present study.

Kainate ($10 \mu\text{mol l}^{-1}$) produced $[Ca^{2+}]_i$ transients of $58.6 \pm 17.4 \text{ nmol l}^{-1}$ ($N=11$) and membrane depolarizations of $14.6 \pm 12.6 \text{ mV}$ ($N=7$). Elevation of the extracellular K^+ concentration from 4 mmol l^{-1} to 20 mmol l^{-1} led to rapid and large $[Ca^{2+}]_i$ increases of $397.4 \pm 186.9 \text{ nmol l}^{-1}$ ($N=4$) and to membrane depolarizations of $38.0 \pm 11.8 \text{ mV}$ ($N=4$). In nominally Ca^{2+} -free solution, the $[Ca^{2+}]_i$ increases evoked by kainate or by the elevation of $[K^+]$ were reduced to $6.9 \pm 12.1 \%$ ($N=4$) of the control value for kainate and to $1.6 \pm 10.7 \%$ ($N=4$, Fig. 1) for K^+ , while the glutamate-induced $[Ca^{2+}]_i$ transients were only decreased to $68.5 \pm 19.9 \%$ ($N=6$). This suggests that

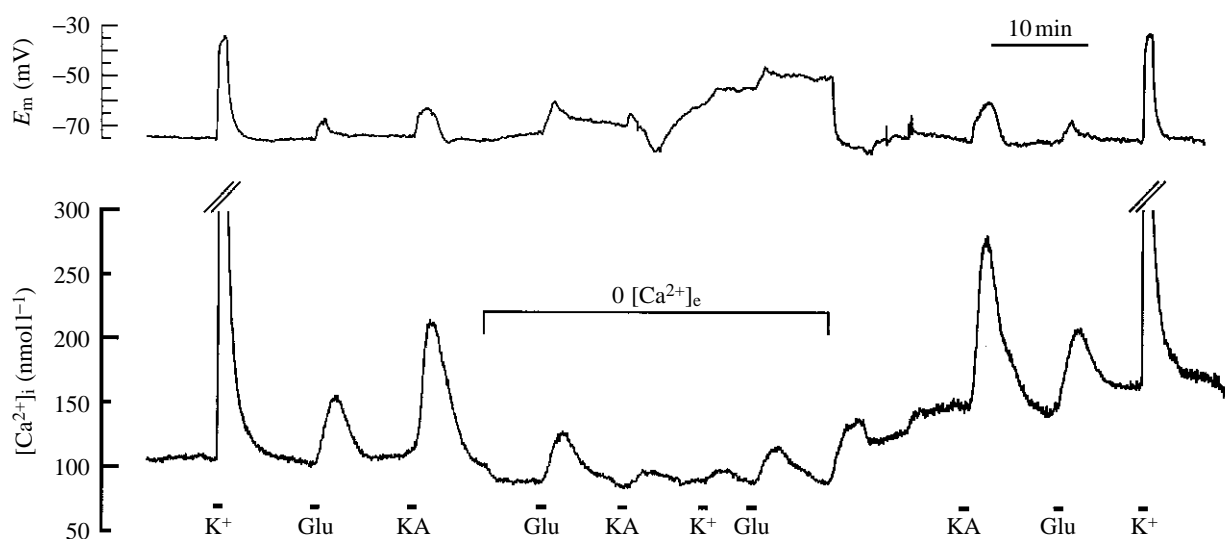


Fig. 1. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$, lower trace) and membrane potential responses (E_m , upper trace) of an individual neuropile glial cell evoked by glutamate (Glu, $500 \mu\text{mol l}^{-1}$), by kainate (KA, $10 \mu\text{mol l}^{-1}$) and by an elevation of the extracellular K^+ concentration (K^+ , 20 mmol l^{-1}) before, during and after removal of external Ca^{2+} ($0 [Ca^{2+}]_e$). The $[Ca^{2+}]_i$ transients shown here are the largest transients observed throughout the experiments. Note that the K^+ -induced $[Ca^{2+}]_i$ transient (to approximately 600 nmol l^{-1}) was clipped.

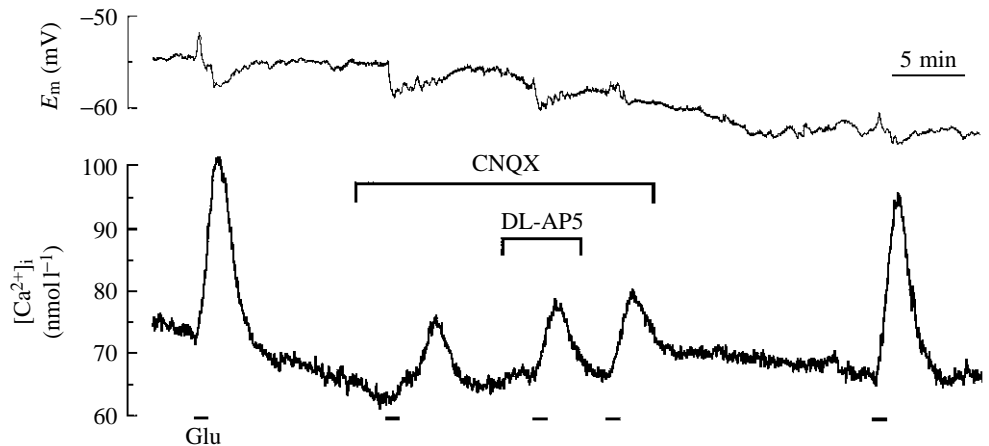


Fig. 2. Effects of the ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, $100\ \mu\text{mol l}^{-1}$) and DL-2-amino-5-phosphonopentanoic acid (DL-AP5, $100\ \mu\text{mol l}^{-1}$) on glutamate-induced ($200\ \mu\text{mol l}^{-1}$) $[\text{Ca}^{2+}]_i$ and membrane potential responses of an individual glial cell.

both Ca^{2+} influx and release of Ca^{2+} from intracellular stores contributed to the total $[\text{Ca}^{2+}]_i$ response elicited by glutamate, in contrast to the $[\text{Ca}^{2+}]_i$ transients evoked by kainate and high $[\text{K}^+]$, which appeared to be primarily due to Ca^{2+} influx. In addition, in Ca^{2+} -free saline, the membrane slowly depolarized, presumably because of a decrease in K^+ permeability (W. Nett and J. W. Deitmer, unpublished observation), and kainate or high $[\text{K}^+]$ elicited smaller depolarizations, which may also have reduced the Ca^{2+} influx (Fig. 1; see also Munsch *et al.* 1994).

Pharmacological characterization of the glutamate-induced responses

In the presence of $50\ \mu\text{mol l}^{-1}$ 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an inhibitor of ionotropic, non-NMDA glutamate receptors, the amplitude of the $[\text{Ca}^{2+}]_i$ increase induced by $200\ \mu\text{mol l}^{-1}$ glutamate was reduced to $53.6 \pm 15.6\%$ of the control level ($N=13$, not shown). A further reduction to $49.2 \pm 15.1\%$ of the control value ($N=6$, Fig. 2) occurred when $100\ \mu\text{mol l}^{-1}$ CNQX was used. No significant difference between $50\ \mu\text{mol l}^{-1}$ and $100\ \mu\text{mol l}^{-1}$ CNQX was found, indicating that $50\ \mu\text{mol l}^{-1}$ CNQX was sufficient to block the majority of the non-NMDA receptors. Furthermore, CNQX reduced the early depolarization evoked by glutamate, but left the subsequent depolarization or hyperpolarization unchanged. Additional application of the NMDA-receptor antagonist DL-2-amino-5-phosphonopentanoic acid (DL-AP5, $100\ \mu\text{mol l}^{-1}$) had no effect on the glutamate-induced $[\text{Ca}^{2+}]_i$ transients or the membrane potential shifts ($N=5$, Fig. 2). Thus, the glutamate-mediated responses appeared to consist of a CNQX-sensitive component, presumably mediated by non-NMDA receptor activation, and a CNQX- and DL-AP5-resistant component, presumably mediated by metabotropic activation.

The effects of various compounds known to exhibit agonistic or antagonistic effects on vertebrate mGluRs were examined on the glutamate-evoked responses in the leech glial cell. Among these, quisqualate (QQ, $200\ \mu\text{mol l}^{-1}$), *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD, $400\ \mu\text{mol l}^{-1}$) and L(+)-2-amino-3-phosphonopropionic acid (L-AP3, $200\ \mu\text{mol l}^{-1}$) evoked $[\text{Ca}^{2+}]_i$ increases and/or

membrane potential responses (Fig. 3), whereas no responses were induced by L(+)-2-amino-4-phosphonobutyric acid (L-AP4, $200\ \mu\text{mol l}^{-1}$) (not shown here).

Incubation with $200\ \mu\text{mol l}^{-1}$ QQ induced $[\text{Ca}^{2+}]_i$ increases of $13.8 \pm 11.4\ \text{nmol l}^{-1}$ ($N=10$, Fig. 3A), accompanied by either a depolarization or a hyperpolarization of 4–13 mV. Since QQ can also activate ionotropic, non-NMDA receptors (Mayer and Miller, 1990; Watkins *et al.* 1990), the QQ-induced responses were examined in the presence of CNQX. In five out of a series of six experiments, $100\ \mu\text{mol l}^{-1}$ CNQX failed to inhibit the QQ-mediated transients, but in one experiment, CNQX reduced the $[\text{Ca}^{2+}]_i$ transient by 26% and the depolarization of 8 mV by 50%.

Incubation with *t*-ACPD ($400\ \mu\text{mol l}^{-1}$), a widely used mGluR-selective agonist with preference for group II receptors (Nakanishi, 1992), elicited only small depolarizations of 1–2 mV (Fig. 3B). Small increases in $[\text{Ca}^{2+}]_i$ of 3–5 nmol l^{-1} were obtained in four out of seven experiments, while in three experiments no changes in $[\text{Ca}^{2+}]_i$ were found.

L-AP3 was one of the first antagonists specific for mGluRs to be described (Schoepp *et al.* 1990). Besides its antagonistic

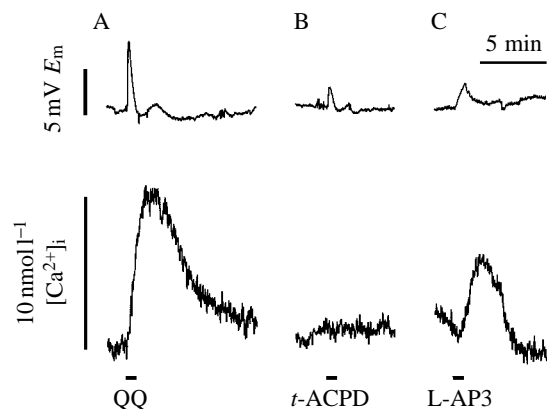


Fig. 3. Changes in $[\text{Ca}^{2+}]_i$ and membrane potential responses evoked by the putative metabotropic glutamate receptor agonists quisqualate (QQ, $200\ \mu\text{mol l}^{-1}$) (A), *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD, $400\ \mu\text{mol l}^{-1}$) (B) and L(+)-2-amino-3-phosphonopropionic acid (L-AP3, $200\ \mu\text{mol l}^{-1}$) (C).

effect, L-AP3 has also been shown to stimulate phosphatidylinositol hydrolysis *via* mGluR activation (Mistry *et al.* 1996). In leech neuropile glial cells, the application of $200\ \mu\text{mol l}^{-1}$ L-AP3 induced $[\text{Ca}^{2+}]_i$ increases of $4.7 \pm 10.6\ \text{nmol l}^{-1}$ ($N=8$) and led to a membrane depolarization or hyperpolarization of 1–3 mV (Fig. 3C).

In the presence of the mGluR antagonist (RS)- α -methyl-4-carboxyphenylglycine [(RS)-MCPG, $1\ \text{mmol l}^{-1}$], the glutamate-mediated $[\text{Ca}^{2+}]_i$ transients were significantly reduced, on average to $80.5 \pm 15.4\%$ of control values ($N=8$, $P<0.05$). The second phase of the membrane potential responses was inhibited in four out of eight experiments (Fig. 4), while in four other experiments (RS)-MCPG had no effect on the glutamate-induced membrane potential responses.

Fig. 5 summarizes the quantitative evaluation of the pharmacological profile of the glutamate-mediated $[\text{Ca}^{2+}]_i$ responses. Together with glutamate, QQ was the most potent agonist, even when non-NMDA receptors were blocked by CNQX. The increases in $[\text{Ca}^{2+}]_i$ induced by *t*-ACPD or L-AP3 were significantly ($P<0.05$) smaller than those elicited by glutamate or QQ (Fig. 5A).

Among the putative antagonists, only (RS)-MCPG significantly reduced the glutamate-mediated $[\text{Ca}^{2+}]_i$ transients (Fig. 5B). (RS)-1-aminoindan-1,5-dicarboxylic acid [(RS)-AIDA, $500\ \mu\text{mol l}^{-1}$], reported to inhibit selectively and potently group I mGluRs (Pellicciari *et al.* 1995), and (S)-2-amino-2-methyl-4-phosphonobutyric acid (MAP4, $400\ \mu\text{mol l}^{-1}$), a group III mGluR antagonist (Jane *et al.* 1994), did not significantly alter glutamate-induced responses. (S)-4-carboxyphenylglycine [(S)-4-CPG, 200 – $500\ \mu\text{mol l}^{-1}$], known to antagonize group I mGluRs, but with agonistic potency on group II mGluRs (Sekiyama *et al.* 1996), elicited small $[\text{Ca}^{2+}]_i$ increases of 5 – $10\ \text{nmol l}^{-1}$ ($N=4$) and biphasic membrane potential shifts (not shown).

Origin of intracellular Ca^{2+} release

The activation of mGluRs can lead to phosphatidylinositol hydrolysis-mediated Ca^{2+} release from intracellular stores such

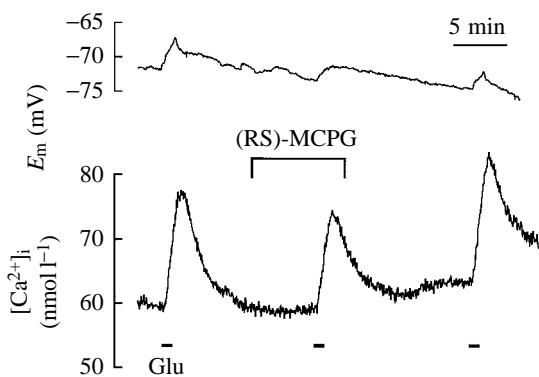


Fig. 4. Effect of the metabotropic glutamate receptor antagonist (RS)- α -methyl-4-carboxyphenylglycine [(RS)-MCPG, $1\ \text{mmol l}^{-1}$] on the glutamate-induced ($200\ \mu\text{mol l}^{-1}$) $[\text{Ca}^{2+}]_i$ and membrane potential responses.

as the endoplasmic reticulum (Pearce *et al.* 1986; Sladeczek *et al.* 1988). To investigate the involvement of glutamate-mediated Ca^{2+} release from intracellular stores, we incubated ganglia in $10\ \mu\text{mol l}^{-1}$ cyclopiazonic acid (CPA), an inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase (Golovina *et al.* 1996; Mason *et al.* 1991). The application of CPA itself evoked a moderate rise in $[\text{Ca}^{2+}]_i$ of $28.1 \pm 13.5\ \text{nmol l}^{-1}$ ($N=9$).

In the presence of CPA, $[\text{Ca}^{2+}]_i$ transients elicited by

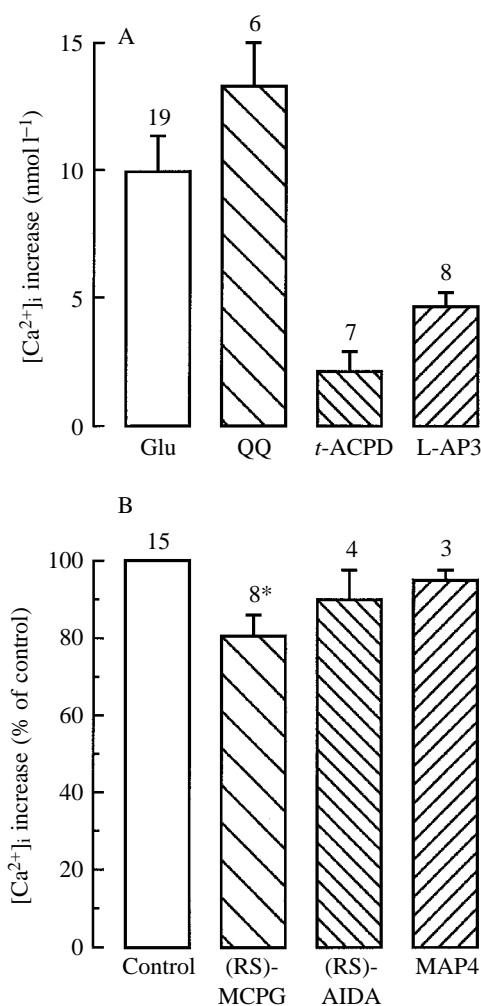


Fig. 5. (A) Mean increases in $[\text{Ca}^{2+}]_i$ evoked by glutamate (Glu, $200\ \mu\text{mol l}^{-1}$), quisqualate (QQ, $200\ \mu\text{mol l}^{-1}$), *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD, $400\ \mu\text{mol l}^{-1}$) and L-(+)-2-amino-3-phosphonopropionic acid (L-AP3, $200\ \mu\text{mol l}^{-1}$). Experiments with glutamate and quisqualate were performed in the presence of 50 or $100\ \mu\text{mol l}^{-1}$ CNQX. (B) The relative amplitude of glutamate-induced ($200\ \mu\text{mol l}^{-1}$) $[\text{Ca}^{2+}]_i$ transients during the application of the putative mGluR antagonists (RS)- α -methyl-4-carboxyphenylglycine [(RS)-MCPG, $1\ \text{mmol l}^{-1}$], (RS)-1-aminoindan-1,5-dicarboxylic acid [(RS)-AIDA, $500\ \mu\text{mol l}^{-1}$] or (S)-2-amino-2-methyl-4-phosphonobutyric acid (MAP4, $400\ \mu\text{mol l}^{-1}$) plotted with respect to the glutamate-induced response in the absence of antagonists (Control). The number of experiments is given above each treatment. Error bars represent + S.E.M.; the asterisk indicates a significant difference from the control value ($P<0.05$).

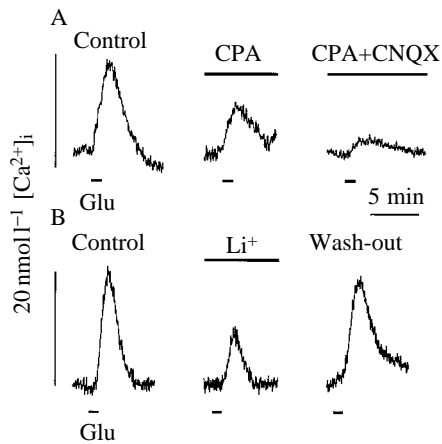


Fig. 6. (A) Effects of cyclopiazonic acid (CPA, $10\mu\text{mol l}^{-1}$) alone, and of both CPA ($10\mu\text{mol l}^{-1}$) and CNQX ($50\mu\text{mol l}^{-1}$), on the $[\text{Ca}^{2+}]_i$ transient evoked by $200\mu\text{mol l}^{-1}$ glutamate (Glu). (B) Effect of Li^+ (2mmol l^{-1}), pre-exposed for 20 min, on the glutamate-induced $[\text{Ca}^{2+}]_i$ transient. After wash-out of Li^+ for 40 min, the $[\text{Ca}^{2+}]_i$ response had recovered to almost its control value.

application of $200\mu\text{mol l}^{-1}$ glutamate were significantly decreased, on average to $47.0\pm 14.7\%$ of the control levels ($N=9$, Fig. 6A). The effect of CPA was irreversible. During co-application of CPA and CNQX, glutamate-induced $[\text{Ca}^{2+}]_i$ increases were depressed to $13.4\pm 15.5\%$ of control levels ($N=5$), indicating that the effects of depleting the intracellular Ca^{2+} stores and of blocking the ionotropic receptors on the glutamate-induced transients are additive.

Another inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase, thapsigargin ($1\mu\text{mol l}^{-1}$, $N=5$), and the ryanodine receptor ligand caffeine (10mmol l^{-1} , $N=3$) had no effect on resting $[\text{Ca}^{2+}]_i$ or glutamate-mediated responses (results not shown).

We have also tested the effect of 2mmol l^{-1} Li^+ , known to interrupt inositol recycling by inhibiting the enzyme inositol monophosphatase (Hallcher and Sherman, 1980), on the glutamate-induced $[\text{Ca}^{2+}]_i$ signals. After application of Li^+ for 15–20 min, the $[\text{Ca}^{2+}]_i$ increases evoked by glutamate were reduced to $53.8\pm 13.0\%$ of the control values ($N=6$, Fig. 6B), suggesting that the $[\text{Ca}^{2+}]_i$ transients are partly mediated by phosphatidylinositol hydrolysis. After a 40 min wash-out of Li^+ , the $[\text{Ca}^{2+}]_i$ responses to glutamate recovered to control values.

The effects of inhibiting Ca^{2+} influx through non-NMDA receptors and inhibiting Ca^{2+} release from intracellular stores on the $[\text{Ca}^{2+}]_i$ transients evoked by glutamate are shown in Fig. 7. Inhibition of glutamate-induced Ca^{2+} influx by CNQX caused a reduction of the glutamate-induced $[\text{Ca}^{2+}]_i$ transients to 50–60% of the control level, and similar values were observed after withdrawal of extracellular Ca^{2+} . A similar reduction in the size of the $[\text{Ca}^{2+}]_i$ transients was also obtained when intracellular Ca^{2+} stores were depleted by CPA or when phosphatidylinositol hydrolysis-mediated Ca^{2+} release was suppressed by Li^+ . Thus, the glutamate-induced responses appeared to be due to Ca^{2+} influx through ionotropic receptors and to Ca^{2+} release from intracellular stores mediated by metabotropic receptors. This view was further supported by the

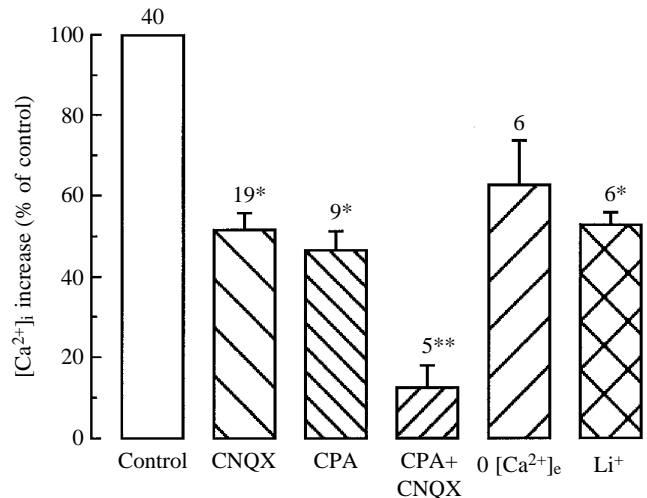


Fig. 7. Mean relative amplitudes of the $[\text{Ca}^{2+}]_i$ increases induced by $200\mu\text{mol l}^{-1}$ glutamate in the presence of CNQX ($50\text{--}100\mu\text{mol l}^{-1}$), cyclopiazonic acid (CPA, $10\mu\text{mol l}^{-1}$), both CPA ($10\mu\text{mol l}^{-1}$) and CNQX ($50\mu\text{mol l}^{-1}$), in nominally Ca^{2+} -free saline ($0[\text{Ca}^{2+}]_e$) and in the presence of Li^+ (2mmol l^{-1}), were plotted with respect to the $[\text{Ca}^{2+}]_i$ response as induced by $200\mu\text{mol l}^{-1}$ glutamate alone (Control). The number at the top of each column indicates the number of experiments. Error bars indicate + S.E.M. and asterisks indicate significant differences from control values (* $P < 0.05$; ** $P < 0.01$).

additive effects of depleting intracellular Ca^{2+} stores with CPA and blocking non-NMDA receptors with CNQX.

Discussion

The present study provides the first evidence for mGluR-mediated intracellular Ca^{2+} release in an invertebrate nervous system. Our results show that mGluRs are expressed by leech giant glial cells in the ganglionic neuropile; these cells also express ionotropic AMPA/kainate receptors but not NMDA receptors (Deitmer and Munsch, 1992, 1994; Munsch *et al.* 1994). Activation of both types of glutamate receptors present in these cells induces an intracellular Ca^{2+} transient as a result of (1) Ca^{2+} influx through Ca^{2+} -permeable ionotropic receptors, and (2) Ca^{2+} release from intracellular stores mediated by metabotropic receptors, presumably *via* the InsP_3 -mediated pathway.

Glutamate-mediated E_m and $[\text{Ca}^{2+}]_i$ transients

The E_m responses evoked by glutamate consist of two phases which can be separated by the use of antagonists of either ionotropic or metabotropic glutamate receptors. While the early CNQX-sensitive response is always a depolarization, the second (RS)-MCPG-sensitive response can include both a depolarization and a hyperpolarization. This second phase is presumably mediated by mGluRs by an unknown mechanism that is currently under investigation. Preliminary results suggest that a glutamate-activated chloride permeability might be involved in this component. In addition, the mGluR agonists QQ and L-AP3 can also induce depolarizations and hyperpolarizations. Interestingly,

glutamate-evoked biphasic membrane potential responses were observed in leech Retzius neurones (Mat Jais *et al.* 1983). However, these responses consist of an early hyperpolarization, depending on the concentration of extracellular chloride, followed by a later depolarization.

Glutamate at the concentrations used here (200 and 500 $\mu\text{mol l}^{-1}$) depolarizes the glial membrane by up to 8 mV, which is much less than the 15–20 mV required to activate voltage-gated Ca^{2+} channels (approximately at -50 mV and beyond; Munsch and Deitmer, 1992, 1995). In contrast, the depolarization of the glial membrane induced by kainate (10 $\mu\text{mol l}^{-1}$) is 2–4 times larger than the depolarization evoked by 500 $\mu\text{mol l}^{-1}$ glutamate and can activate voltage-dependent Ca^{2+} influx. $[\text{Ca}^{2+}]_i$ transients mediated by non-NMDA receptor activation in leech glial cells have been examined extensively in previous studies, and kainate has also been shown to produce a rise in $[\text{Ca}^{2+}]_i$ in voltage-clamped cells or when voltage-gated Ca^{2+} channels are blocked, indicating that kainate also induced Ca^{2+} influx through ionotropic receptor channels (Munsch *et al.* 1994; Munsch and Deitmer, 1997). Non-NMDA receptors with high Ca^{2+} permeability are also found in mammalian glial cells (Müller *et al.* 1992; Burnashev *et al.* 1992), but molecular data to check the homology of leech and vertebrate glutamate receptors are still lacking.

Since glutamate does not activate voltage-gated Ca^{2+} channels, and the glutamate-induced rise in $[\text{Ca}^{2+}]_i$ is only partly inhibited by the ionotropic receptor blocker CNQX, we conclude that the glutamate response is due to activation of both ionotropic and metabotropic receptors. These different pathways could be separated by the removal of external Ca^{2+} and by blocking the ionotropic receptors with CNQX. As expected, the glutamate response was reduced less in nominally Ca^{2+} -free saline, where some Ca^{2+} may still remain in the extracellular space, than by the ionotropic receptor blocker CNQX. As confirmed by the small response to kainate in the nominal absence of external Ca^{2+} , some Ca^{2+} could still have leaked into the cell *via* the ionotropic receptor channels (Fig. 1).

Intracellular Ca^{2+} release

The glutamate-induced $[\text{Ca}^{2+}]_i$ transients were reduced by approximately 50% after incubation of the cells with CPA, an inhibitor of the Ca^{2+} -ATPase of intracellular Ca^{2+} stores (Golovina *et al.* 1996; Mason *et al.* 1991), suggesting that there is glutamate-mediated intracellular Ca^{2+} release. The CPA-resistant part of the $[\text{Ca}^{2+}]_i$ response was largely inhibited by CNQX, suggesting that intracellular Ca^{2+} release contributes to approximately half of the glutamate-induced $[\text{Ca}^{2+}]_i$ transients. Interestingly, thapsigargin, assumed to interact at the same endoplasmic Ca^{2+} -ATPase as CPA in vertebrate preparations (Mason *et al.* 1991), had no effect on the intracellular Ca^{2+} stores of the leech neuropile glial cell. The present results also show that CPA blocks the Ca^{2+} pump irreversibly in leech glial cells, in contrast to vertebrate cells (Golovina *et al.* 1996). Since caffeine could not alter the basal $[\text{Ca}^{2+}]_i$ in these glial cells, it is unlikely that Ca^{2+} -induced Ca^{2+} release contributed to the glutamate-evoked response. This

suggests that leech glial cells have no ryanodine-sensitive intracellular Ca^{2+} stores.

The metabotropically evoked $[\text{Ca}^{2+}]_i$ transient induced by glutamate was much smaller than the rise in $[\text{Ca}^{2+}]_i$ elicited ionotropically by 10 $\mu\text{mol l}^{-1}$ kainate in experiments on voltage-clamped glial cells, where kainate did not activate voltage-dependent Ca^{2+} influx (Munsch *et al.* 1994; Munsch and Deitmer, 1997). Indeed, in comparison with metabotropically mediated $[\text{Ca}^{2+}]_i$ transients in vertebrate glial cells (Cornell-Bell and Finkbeiner, 1991; de Barry *et al.* 1991; Brune and Deitmer, 1995), the $[\text{Ca}^{2+}]_i$ responses in leech glial cells appear rather small. This is also the case for the serotonin-evoked rise in $[\text{Ca}^{2+}]_i$ in these cells (Munsch and Deitmer, 1992), which is also likely to be mediated metabotropically. These small responses may be due to a poor capacity for storage of intracellular Ca^{2+} and/or to the presence of relatively few Ca^{2+} stores in leech glial cells. In addition, the application of CPA only induced a rather small rise in basal $[\text{Ca}^{2+}]_i$ (30 nmol l^{-1}).

Further evidence for metabotropically mediated Ca^{2+} release from intracellular stores being one component of the glutamate-evoked $[\text{Ca}^{2+}]_i$ response came from experiments using Li^+ , which is known to interfere with inositol recycling (Hallcher and Sherman, 1980; Nahorski *et al.* 1991). At a concentration of 2 mmol l^{-1} , Li^+ reduced a similar fraction of the glutamate-evoked $[\text{Ca}^{2+}]_i$ transient as did CPA, which supports the suggestion that this component of the $[\text{Ca}^{2+}]_i$ transient results from the same intracellular release process.

Phosphatidyl inositol hydrolysis following mGluR activation has been reported for a number of vertebrate cells (reviewed by Pin and Duvoisin, 1995; Schoepp and Conn, 1993), including mammalian glial cells (cf. Finkbeiner, 1995). It is suggested that this hydrolysis also occurs in leech glial cells in response to glutamate, leading to intracellular Ca^{2+} release.

Pharmacology of the metabotropic receptor response

The pharmacological evidence supports the conclusion that metabotropic receptors are activated by glutamate. First, QQ was the most potent agonist evoking a $[\text{Ca}^{2+}]_i$ transient. Although QQ may also activate ionotropic, non-NMDA receptors (Sladeczek *et al.* 1988; Watkins *et al.* 1990), the QQ-induced $[\text{Ca}^{2+}]_i$ response was only weakly affected by CNQX, indicating that it was mainly due to activation of mGluRs. Second, *t*-ACPD and L-AP3, which are known metabotropic receptor ligands (Schoepp *et al.* 1990; Nakanishi, 1992), exerted weak agonistic effects in leech glial cells. Third, the frequently used mGluR antagonist (RS)-MCPG partly reduced the glutamate-induced $[\text{Ca}^{2+}]_i$ transient. However, the pharmacological profile of the glutamate-mediated $[\text{Ca}^{2+}]_i$ responses observed in leech glial cells differs from that observed for InsP_3 -mediated intracellular Ca^{2+} release in vertebrate cells. In Purkinje cells, *t*-ACPD induced large $[\text{Ca}^{2+}]_i$ transients, which were strongly blocked by (RS)-MCPG (Hartell, 1994) or L-AP3 (Linden *et al.* 1994). In addition, the effects of other drugs, such as (S)-4-CPG and (RS)-AIDA, on mGluR-mediated intracellular Ca^{2+} release in

leech glial cells also deviate from their reported effects in mammals. Thus, the mGluRs in the leech neuropile glial cell show clear differences from vertebrate mGluRs with respect to their pharmacology, although they seem to share the same signal transduction pathway.

Other invertebrates also show both similarities and differences in their response to the activation of mGluRs. In the squid Schwann cell, *t*-ACPD was potent in eliciting a hyperpolarization, while L-AP3 blocked a membrane hyperpolarization evoked by glutamate (Evans *et al.* 1992). At the lobster neuromuscular junction, activation of the presynaptic glutamate_B receptors by glutamate induced a depression of synaptic transmission because of an increase in the K⁺ conductance (Miwa *et al.* 1987, 1993). This effect, mediated by a pertussis-toxin-sensitive G-protein, could be mimicked by application of QQ, but not of *t*-ACPD or L-AP4. Two mGluRs recently cloned from *Drosophila melanogaster*, DmGluRA and DmGluRB, showed sequence homologies of approximately 45% with mammalian mGluR2 and mGluR3 (Parmentier *et al.* 1996). In addition, DmGluRA and DmGluRB showed similarities in pharmacology and transduction mechanism to mGluR2 and mGluR3. Thus, these metabotropic receptors were clearly different from those described here for the leech glial cell, suggesting that the diversity of mGluRs of invertebrates might be similar to that of vertebrates.

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