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 phosphatidylinositol-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, 400 μmol l$^{-1}$). The mGluR-selective antagonist (RS)-α-methyl-4-carboxyphenylglycine [(RS)-MCPG, 1 mmol l$^{-1}$] significantly reduced glutamate-evoked increases in [Ca$^{2+}$]$_i$ by 20%. Incubation of the ganglia with the endoplasmic ATPase inhibitor cyclopiazonic acid (CPA, 10 μmol l$^{-1}$) caused a significant (53%) reduction of glutamate-induced [Ca$^{2+}$]$_i$ transients, while incubation with lithium ions (2 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 [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 phosphatidylinositol-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; Hémart et al. 1995) or long-term potentiation (Ito and Sugiyama, 1991; Hémart et al. 1995).
Bashir et al. (1993) of synaptic transmission. In addition, the activation of mGluRs has been shown to induce the production of Ca^2+ channels (Chavis et al. 1994) and voltage- or Ca^2+-activated K^+ channels (Charpak et al. 1990; Baskys, 1992) and can cause Ca^2+ release from intracellular stores, both in neurons (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 the pharmacological profile of mGluR-mediated presynaptic inhibition at the crayfish synapse (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 Ca^2+ release in invertebrate nerve or glial cells. A sequence homology of approximately 45% to vertebrate group I 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 Ca^2+ concentration ([Ca^{2+}]_i) and the membrane potential (E_{m}) of leech neurone glial cells. The results demonstrate that leech giant glial cells express ionotropic and metabotropic glutamate receptors, the latter mediating InsP3-dependent Ca^2+ release from intracellular stores. To our knowledge, this is the first evidence that intracellular Ca^2+ 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, 1995). 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/disparge (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, CaCl_2 2, MgCl_2 1, Heps 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 dimethyl sulphoxide (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-amino-cyclopentane-1,3-dicarboxylic acid (t-ACP), L(+)-2-amino-3-phosphonopropionic acid (L-AP3), L(+)-2-amino-4-phosphonobutyric acid (L-AP4), (RS)-α-methyl-4-carboxyphenylglycine ([RS]-MCPP), (RS)-1-amino-4-carboxylic acid ([RS]-AIDA), (S)-2-amino-2-methyl-4-phosphonobutyric acid (MAP4) and (S)-4-carboxyphenylglycine ([S]-4-CPP) 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 [Ca^{2+}] 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). [Ca^{2+}] was calculated using the equation described by Grynkiewicz et al. (1985):

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

where \( R \) is the fluorescence ratio measured experimentally, \( R_{min} \) is the fluorescence ratio for Ca^{2+}-free and \( R_{max} \) for Ca^{2+}-

...
saturated conditions, and $S_f/S_b$ 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 ± 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±13.7 nmol l$^{-1}$ ($N=64$) at a mean membrane potential of −66.5±11.7 mV ($N=60$). Fig. 1 shows the $[Ca^{2+}]_{i}$ and $E_m$ responses elicited by glutamate (500 μmol l$^{-1}$) in comparison with responses elicited by kainate (10 μmol 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 μmol 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±13.6 nmol l$^{-1}$ ($N=11$, Fig. 1), while 200 μmol l$^{-1}$ glutamate raised the $[Ca^{2+}]_{i}$ by 17.0±10.9 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 μmol l$^{-1}$ or 200 μmol 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 μmol l$^{-1}$ glutamate elicited a maximal depolarization of 6.3±11.1 mV ($N=6$) or biphasic responses with a depolarization of 6.2±11.8 mV followed by a hyperpolarization of −4.4±10.8 mV ($N=5$), while 200 μmol l$^{-1}$ glutamate elicited a maximal depolarization of 3.8±10.3 mV ($N=35$) or biphasic responses with a depolarization of 3.4±10.4 mV followed by a hyperpolarization of −3.2±10.5 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 μmol l$^{-1}$) produced $[Ca^{2+}]_{i}$ transients of 58.6±17.4 nmol l$^{-1}$ ($N=11$) and membrane depolarizations of 14.6±12.6 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±186.9 nmol l$^{-1}$ ($N=4$) and to membrane depolarizations of 38.0±11.8 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±12.1% ($N=4$) of the control value for kainate and to 1.6±10.7% ($N=4$, Fig. 1) for K$^+$, while the glutamate-induced $[Ca^{2+}]_{i}$ transients were only decreased to 68.5±19.9% ($N=6$). This suggests that

![Fig. 1](image-url) 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 μmol l$^{-1}$), by kainate (KA, 10 μmol 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.
both Ca$^{2+}$ influx and release of Ca$^{2+}$ from intracellular stores contributed to the total [Ca$^{2+}$]; response elicited by glutamate, in contrast to the [Ca$^{2+}$]; transients evoked by kainate and high [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 [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 μmol l$^{-1}$ 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), an inhibitor of ionotropic, non-NMDA glutamate receptors, the amplitude of the [Ca$^{2+}$]; increase induced by 200 μmol l$^{-1}$ glutamate was reduced to 53.6±15.6 % of the control level (N=13, not shown). A further reduction to 49.2±15.1 % of the control value (N=6, Fig. 2) occurred when 100 μmol l$^{-1}$ CNQX was used. No significant difference between 50 μmol l$^{-1}$ and 100 μmol l$^{-1}$ CNQX was found, indicating that 50 μmol 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 μmol l$^{-1}$) had no effect on the glutamate-induced [Ca$^{2+}$]; 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 mGlurRs were examined on the glutamate-evoked responses in the leech glial cell. Among these, quisqualate (QQ, 200 μmol l$^{-1}$), trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD, 400 μmol l$^{-1}$) and L(+)-2-amino-3-phosphonopropionic acid (L-AP3, 200 μmol l$^{-1}$) evoked [Ca$^{2+}$]; increases and/or membrane potential responses (Fig. 3), whereas no responses were induced by L(+)-2-amino-4-phosphonobutyric acid (L-AP4, 200 μmol l$^{-1}$) (not shown here).

Incubation with 200 μmol l$^{-1}$ QQ induced [Ca$^{2+}$]; increases of 13.8±11.4 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 μmol l$^{-1}$ CNQX failed to inhibit the QQ-mediated transients, but in one experiment, CNQX reduced the [Ca$^{2+}$]; transient by 26 % and the depolarization of 8 mV by 50 %.

Incubation with t-ACPD (400 μmol 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 [Ca$^{2+}$]; of 3–5 nmol l$^{-1}$ were obtained in four out of seven experiments, while in three experiments no changes in [Ca$^{2+}$]; were found.

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

![Fig. 2. Effects of the ionotropic glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 100 μmol l$^{-1}$) and DL-2-amino-5-phosphonopentanoic acid (DL-AP5, 100 μmol l$^{-1}$) on glutamate-induced (200 μmol l$^{-1}$) [Ca$^{2+}$]; and membrane potential responses of an individual glial cell.](image1)

![Fig. 3. Changes in [Ca$^{2+}$]; and membrane potential responses evoked by the putative metabotropic glutamate receptor agonists quisqualate (QQ, 200 μmol l$^{-1}$) (A), trans-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD, 400 μmol l$^{-1}$) (B) and L(+)-2-amino-3-phosphonopropionic acid (L-AP3, 200 μmol l$^{-1}$) (C).](image2)
Effect, L-AP3 has also been shown to stimulate phosphatidyl inositol hydrolysis via mGluR activation (Mistry et al. 1996). In leech neurone glial cells, the application of 200 μmol l⁻¹ L-AP3 induced [Ca²⁺]ᵢ increases of 4.7±10.6 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 mmol l⁻¹], the glutamate-mediated [Ca²⁺]ᵢ transients were significantly reduced, on average to 80.5±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 [Ca²⁺]ᵢ responses. Together with glutamate, QQ was the most potent agonist, even when non-NMDA receptors were blocked by CNQX. The increases in [Ca²⁺]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 [Ca²⁺]ᵢ transients (Fig. 5B). (RS)-1-aminoindan-1,5-dicarboxylic acid [(RS)-AIDA, 500 μmol l⁻¹], reported to inhibit selectively and potently group I mGluRs (Pellicciani et al. 1995), and (S)-2-amino-2-methyl-4-phosphonobutyric acid (MAP4, 400 μmol 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 μmol l⁻¹], known to antagonize group I mGluRs, but with agonistic potency on group II mGluRs (Sekiyama et al. 1996), elicited small [Ca²⁺]ᵢ increases of 5–10 nmol l⁻¹ (N=4) and biphasic membrane potential shifts (not shown).

**Origin of intracellular Ca²⁺ release**

The activation of mGluRs can lead to phosphatidyl inositol hydrolysis-mediated Ca²⁺ release from intracellular stores such as the endoplasmic reticulum (Pearce et al. 1986; Sladeczek et al. 1988). To investigate the involvement of glutamate-mediated Ca²⁺ release from intracellular stores, we incubated ganglia in 10 μmol l⁻¹ cyclopiazonic acid (CPA), an inhibitor of the endoplasmic reticulum Ca²⁺-ATPase (Golovina et al. 1996; Mason et al. 1991). The application of CPA itself evoked a moderate rise in [Ca²⁺]ᵢ of 28.1±13.5 nmol l⁻¹ (N=9).

In the presence of CPA, [Ca²⁺]ᵢ transients elicited by glutamate or QQ (200 μmol l⁻¹) were significantly (P<0.05) smaller than those elicited by the agonists alone (Glu or QQ, 200 μmol l⁻¹). The increases in [Ca²⁺]ᵢ induced by glutamate were inhibited in four out of eight experiments (Fig. 5A), while in four other experiments (RS)-MCPG had no effect on the glutamate-induced membrane potential responses.

**Fig. 5.** (A) Mean increases in [Ca²⁺]ᵢ evoked by glutamate (Glu, 200 μmol l⁻¹), quisqualate (QQ, 200 μmol l⁻¹), trans-1-aminoindan-1,3-dicarboxylic acid (t-ACPD, 400 μmol l⁻¹) and L(+)-2-amino-3-phosphonopropionic acid (L-AP3, 200 μmol l⁻¹). Experiments with glutamate and quisqualate were performed in the presence of 50 or 100 μmol l⁻¹ CNQX. (B) The relative amplitude of glutamate-induced (200 μmol l⁻¹) [Ca²⁺]ᵢ transients during the application of the putative mGluR antagonists (RS)-α-methyl-4-carboxyphenylglycine [(RS)-MCPG, 1 mmol l⁻¹], (RS)-1-aminoindan-1,5-dicarboxylic acid [(RS)-AIDA, 500 μmol l⁻¹] or (S)-2-amino-2-methyl-4-phosphonobutyric acid (MAP4, 400 μmol 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).
application of 200 μmol l⁻¹ glutamate were significantly decreased, on average to 47.0±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 [Ca²⁺] transient increases were depressed to 13.4±15.5 % of control levels (N=5), indicating that the effects of depleting the intracellular Ca²⁺ stores and of blocking the ionotropic receptors on the glutamate-induced transients are additive.

Another inhibitor of the endoplasmic reticulum Ca²⁺-ATPase, thapsigargin (1 μmol l⁻¹, N=5), and the ryanodine receptor ligand caffeine (10 μmol l⁻¹, N=3) had no effect on resting [Ca²⁺] or glutamate-mediated responses (results not shown).

We have also tested the effect of 2 mmol l⁻¹ Li⁺, known to interrupt inositol recycling by inhibiting the enzyme inositol monophosphatase (Halleher and Sherman, 1980), on the glutamate-induced [Ca²⁺] signals. After application of Li⁺ for 15–20 min, the [Ca²⁺] increases evoked by glutamate were reduced to 53.8±13.0 % of the control values (N=6, Fig. 6B), suggesting that the [Ca²⁺] transients are partly mediated by phosphatidyl inositol hydrolysis. After a 40 min wash-out of Li⁺, the [Ca²⁺] responses to glutamate recovered to control values.

The effects of inhibiting Ca²⁺ influx through non-NMDA receptors and inhibiting Ca²⁺ release from intracellular stores on the [Ca²⁺] transients evoked by glutamate are shown in Fig. 7. Inhibition of glutamate-induced Ca²⁺ influx by CNQX caused a reduction of the glutamate-induced [Ca²⁺] transients to 50–60 % of the control level, and similar values were observed after withdrawal of extracellular Ca²⁺. A similar reduction in the size of the [Ca²⁺] transients was also obtained when intracellular Ca²⁺ stores were depleted by CPA or when phosphatidyl inositol hydrolysis-mediated Ca²⁺ release was suppressed by Li⁺. Thus, the glutamate-induced responses appeared to be due to Ca²⁺ influx through ionotropic receptors and to Ca²⁺ release from intracellular stores mediated by metabotropic receptors. This view was further supported by the additive effects of depleting intracellular Ca²⁺ stores with CPA and blocking non-NMDA receptors with CNQX.

Discussion

The present study provides the first evidence for mGluR-mediated intracellular Ca²⁺ 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 Ca²⁺ transient as a result of (1) Ca²⁺ influx through Ca²⁺-permeable ionotropic receptors, and (2) Ca²⁺ release from intracellular stores mediated by metabotropic receptors, presumably via the InsP₃-mediated pathway.

Glutamate-mediated E₉₀ and [Ca²⁺]; transients

The E₉₀ 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 μmol 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 Ca²⁺ channels (approximately at −50 mV and beyond; Munsch and Deitmer, 1992, 1995). In contrast, the depolarization of the glial membrane induced by kainate (10 μmol l⁻¹) is 2–4 times larger than the depolarization evoked by 500 μmol l⁻¹ glutamate and can activate voltage-dependent Ca²⁺ influx. [Ca²⁺], 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 [Ca²⁺], in voltage-clamped cells or when voltage-gated Ca²⁺ channels are blocked, indicating that kainate also induced Ca²⁺ influx through ionotropic receptor channels (Munsch et al. 1994; Munsch and Deitmer, 1997). Non-NMDA receptors with high Ca²⁺ 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 Ca²⁺ channels, and the glutamate-induced rise in [Ca²⁺], 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 Ca²⁺ and by blocking the ionotropic receptors with CNQX. As expected, the glutamate response was reduced less in nominally Ca²⁺-free saline, where some Ca²⁺ 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 Ca²⁺, some Ca²⁺ could still have leaked into the cell via the ionotropic receptor channels (Fig. 1).

**Intracellular Ca²⁺ release**

The glutamate-induced [Ca²⁺], transients were reduced by approximately 50% after incubation of the cells with CPA, an inhibitor of the Ca²⁺-ATPase of intracellular Ca²⁺ stores (Golovina et al. 1996; Mason et al. 1991), suggesting that there is glutamate-mediated intracellular Ca²⁺ release. The CPA-resistant part of the [Ca²⁺], response was largely inhibited by CNQX, suggesting that intracellular Ca²⁺ release contributes to approximately half of the glutamate-induced [Ca²⁺], transients. Interestingly, thapsigargin, assumed to interact at the same endoplasmic Ca²⁺-ATPase as CPA in vertebrate preparations (Mason et al. 1991), had no effect on the intracellular Ca²⁺ stores of the leech neuropile glial cell. The present results also show that CPA blocks the Ca²⁺ pump irreversibly in leech glial cells, in contrast to vertebrate cells (Golovina et al. 1996). Since caffeine could not alter the basal [Ca²⁺], in these glial cells, it is unlikely that Ca²⁺-induced Ca²⁺ release contributed to the glutamate-evoked response. This suggests that leech glial cells have no ryanodine-sensitive intracellular Ca²⁺ stores.

The metabotropically evoked [Ca²⁺], transient induced by glutamate was much smaller than the rise in [Ca²⁺], elicited ionotropically by 10 μmol l⁻¹ kainate in experiments on voltage-clamped glial cells, where kainate did not activate voltage-dependent Ca²⁺ influx (Munsch et al. 1994; Munsch and Deitmer, 1997). Indeed, in comparison with metabotropically mediated [Ca²⁺], transients in vertebrate glial cells (Cornell-Bell and Finkbeiner, 1991; de Barry et al. 1991; Brune and Deitmer, 1995), the [Ca²⁺], responses in leech glial cells appear rather small. This is also the case for the serotonin-evoked rise in [Ca²⁺], 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 Ca²⁺ and/or to the presence of relatively few Ca²⁺ stores in leech glial cells. In addition, the application of CPA only induced a rather small rise in basal [Ca²⁺], (30 nmol l⁻¹).

Further evidence for metabotropically mediated Ca²⁺ release from intracellular stores being one component of the glutamate-evoked [Ca²⁺], response came from experiments using Li⁺, which is known to interfere with inositol recycling (Haller and Sherman, 1980; Nahorski et al. 1991). At a concentration of 2 mmol l⁻¹, Li⁺ reduced a similar fraction of the glutamate-evoked [Ca²⁺], transient as did CPA, which supports the suggestion that this component of the [Ca²⁺], 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, 1994; Schoep 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 Ca²⁺ 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 [Ca²⁺], transient. Although QQ may also activate ionotropic, non-NMDA receptors (Sladecek et al. 1988; Watkins et al. 1990), the QQ-induced [Ca²⁺], 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 (Schoep et al. 1990; Nakashiba, 1992), exerted weak agonistic effects in leech glial cells. Third, the frequently used mGluR antagonist (RS)-MCPP partly reduced the glutamate-induced [Ca²⁺], transient. However, the pharmacological profile of the glutamate-mediated [Ca²⁺], responses observed in leech glial cells differs from that observed for InsP₃-mediated intracellular Ca²⁺ release in vertebrate cells. In Purkinje cells, t-ACPD induced large [Ca²⁺], transients, which were strongly blocked by (RS)-MCPP (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 Ca²⁺ 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 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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