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GLUTAMATE AS A PUTATIVE NEUROTRANSMITTER IN THE MOLLUSC, *LYMNAEA STAGNALIS*

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Abstract—Bath-applied glutamate (10–1000 μM) produced excitatory and inhibitory responses on numerous identified neurons of the mollusc *Lymnaea stagnalis*. Using both *in situ* and *in vitro* preparations, glutamate or glutamate agonists produced a depolarization in identified neurons right pedal dorsal 1 and right pedal dorsal 2 and 3. However, attempts to block glutamate-evoked responses with glutamate antagonists were unsuccessful. We examined a potential glutamatergic neuron, visceral dorsal 4. Exogenous application of the peptides (GDPFLRFamide and SDPFLRFamide) could mimic the inhibitory, but not the excitatory effects of visceral dorsal 4 on its postsynaptic cells, implying the presence of a second transmitter. We tested the possibility that glutamate is this second neurotransmitter by using excitatory synapses between visceral dorsal 4 and postsynaptic cells right pedal dorsal 2 and 3, right pedal dorsal 1, visceral F group and right parietal B group neurons. Of all the putative neurotransmitters tested, only glutamate had consistent excitatory effects on these postsynaptic cells. Also, the amplitude of the right pedal dorsal 2 and 3 excitatory postsynaptic potentials was reduced in the presence of *N*-methyl-D-aspartate and other glutamate agonists, suggesting desensitization of the endogenous transmitter receptor.

In conclusion, some identified *Lymnaea* neurons respond to glutamate via a receptor with novel pharmacological properties. Furthermore, a *Lymnaea* interneuron may employ glutamate as a transmitter at excitatory synapses. Copyright © 1996 IBRO. Published by Elsevier Science Ltd.

Key words: excitatory amino acid, synapse, CNS, invertebrate, receptor.

Glutamate is a prominent neurotransmitter in both the vertebrate and the invertebrate nervous system. Glutamate receptors are crucial for fast excitatory neurotransmission and their activation has been linked to the induction of certain forms of synaptic plasticity (long-term potentiation (LTP) and long-term depression (LTD)).[44] In addition, glutamate receptors mediate the excitotoxicity believed to underlie neuronal death associated with ischemia and some neurodegenerative diseases.[11,16,42] However, far less is known about the physiological role of glutamate in the invertebrate CNS. Glutamate mediates neuromuscular transmission in insects[62] and crayfish,[1] and *N*-methyl-D-aspartate (NMDA)-like receptors are involved in LTP of *Aplysia* sensory-to-motoneuron synapses.[36] It has been proposed that long-term facilitation of this sensorimotor synapse is the result of up-regulation of glutamate receptors on the motoneurons.[60] Furthermore, glutamate is involved in feeding behaviour in *Helisoma*,[26,49] and in the initiation of swimming activity in the leech.[7] In addition to its role in synaptic transmission, it has been reported that glutamate can promote neuronal regeneration in the *Helisoma* CNS as well as sprouting of both isolated and intact *Helisoma* neurons.[10]

The simplest classification of vertebrate glutamate receptors divides them into two general groups. The ligand-gated ion channels, or ionotropic receptors, include receptors activated by NMDA, kainate and (±)-alpha-amino-3-hydroxy-5-methylisoxasole-4-propionic acid hydrobromide (AMPA). The metabotropic or G-protein-linked receptors, however, are activated by ibotenate, (+)-quisqualic acid and *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (t-ACPD).[44] Vertebrate glutamate receptor pharmacology is reasonably well understood, but less is known about the pharmacology of glutamate responses in molluscan neurons. Glutamate-responsive neurons have been identified in the molluscan CNS,

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Abbreviations: ACh, acetylcholine; t-ACPD, *trans*-1-aminocyclopentane-1,3-dicarboxylic acid; AMPA, (±)-alpha-amino-3-hydroxy-5-methylisoxasole-4-propionic acid hydrobromide; AP-4, (±)-2-amino-4-phosphonobutyric acid; AP-5, DL-2-amino-5-phosphonovaleric acid; CNQX, 6-cyano-7-nitroquioxiline-2,3-dione; EPSC, excitatory postsynaptic current; EPSP, excitatory postsynaptic potential; GDP-β-S, guanine 5′-(β-thio)diphosphate; Gpp(NH)p, 5′-guanylylimidodiphosphate; GTPδS, guanosin 5′-(trihydrogen diphosphate); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; LTD, long-term depression; LTP, long-term potentiation; MK-801, dizocilpine maleate; NMDA, *N*-methyl-D-aspartate; PTMA, phenyltrimethylammonium chloride; RPeD1, neuron right pedal dorsal 1; RPeD2/3, neurons right pedal dorsal 2 or 3; VD4, neuron visceral dorsal 4; VE, visceral E group neurons.

most of them being non-NMDA-like, for example in *Planorbarius*,[6] *Helix*,[21,63] *Aplysia*[27,59] and *Helisoma*.[49] NMDA-like responses have been observed in the light yellow cells of *Lymnaea*.[45] Also, the glutamate receptor(s) on motoneurons of *Aplysia* share some pharmacological similarity with NMDA-like receptors.[13] The only metabotropic-like glutamate receptors identified in molluscs are the G-protein-dependent hyperpolarizing response in *Planorbarius*[6] and the Ca^{2+}/calmodulin-dependent glutamate-induced potassium current in neurons of the land snail *Euhadra*.[46]

Although genes for the known types of major vertebrate glutamate receptors have been cloned,[5] the only cloned and expressed invertebrate glutamate receptors are from *Drosophila* CNS and muscle.[55] Recently, a putative glutamate receptor subunit was cloned from the *Lymnaea* CNS.[15] This clone displays a moderate degree of homology with rat AMPA-selective glutamate receptor subunits.[14,15] From a phylogenetic point of view, it will be interesting to compare the sequence and pharmacological profile of *Lymnaea* glutamate receptors with other invertebrate and vertebrate glutamate receptors. For example, it has been proposed that glutamate-gated ion channels, which are believed to have evolved early in phylogeny, can be structurally (and evolutionary) linked to voltage-gated ion channels.[65]

The first objective of this study was to examine the effects of glutamate and various glutamate agonists on a number of identified neurons from the pond snail *Lymnaea stagnalis*. The second objective was to test whether glutamate is a neurotransmitter used by the identified cardiorespiratory interneuron, visceral dorsal 4 (VD4). Neuron VD4 makes excitatory, inhibitory and biphasic connections with many neurons in the *Lymnaea* CNS.[3,41,56,57,58] However, only its inhibitory connections can be mimicked by the application of the FMRFamide-like peptides, SDPFLRFamide and GDPFLRFamide, which are present in VD4.[56] Consequently, a second transmitter must be responsible for the excitatory effects of VD4.

EXPERIMENTAL PROCEDURES

Animals

The experiments employed a stock of the mollusc *Lymnaea stagnalis*, raised and maintained in aqua-culture at the University of Calgary.[51] Animals used for electrophysiology and cell culture had a shell length of 15–20 mm (age one to two months).

Dissections and salines

The standard dissection procedures used for the experiments are described in Magoski *et al.*[38] The composition of normal *Lymnaea* saline was (in mM): NaCl 51.3, KCl 1.7, $CaCl_2$ 4.1, $MgCl_2$ 1.5 and HEPES 5.0, pH 7.9. The composition of $6 \times Ca^{2+}/6 \times Mg^{2+}$ saline was (in mM): NaCl 51.7, KCl 1.7, $CaCl_2$ 24.6, $MgCl_2$ 1.5, $MgSO_4$ 7.5 and HEPES 5.0, pH 7.9. The salines were perfused through the chamber (0.5 ml) at a rate of about 2 ml/min.

Electrophysiology

Recordings were made using single-barrel borosilicate micropipettes. Saturated K_2SO_4 was used as the electrolyte, and the electrode resistance was about 20 MΩ. Current clamp data were collected with Getting microelectrode amplifiers. A Dagan 8100, discontinuous single-electrode voltage clamp was used for voltage-clamp experiments. The voltage and current were displayed on a Tektronix dual beam storage oscilloscope and recorded on a Gould 2 channel chart recorder.

Cell culture

The cell culture technique was described initially by Ridgway *et al.*[51] and in further detail by Magoski *et al.*[38] For voltage-clamp experiments, we used acutely isolated somata, without neurites, plated in defined medium for 3–6 h. Defined medium consisted of serum-free 50% Liebowitz-15 medium (Gibco, special order) with added inorganic salts (NaCl 40 mM, KCl 1.7 mM, $CaCl_2$ 4.1 mM, $MgCl_2$ 1.5 mM, HEPES 10 mM, pH 7.9) and 20 μM of gentamycin.

Drug application

Drugs were delivered to neurons by either bath or pressure application. For pressure application, glutamate was dissolved in saline containing 0.05% Fast Green. The solution was loaded into a fire-polished pipette that was connected to a General Valve Picospritzer II (pressure range: 20–40 psi).

A gravity-driven Y-tube system[33] was used to establish the dose–response curve and rank order of efficacies of glutamate agonists. One line of the Y-tube was connected to several drug reservoirs and the other line to a suction pump. This system delivered a large volume of drug through the application pipette with a tip diameter of 5–15 μm. This enables fast, local application of known concentrations of different drugs through the same application pipette.

Guanine 5′-(β-thio)diphosphate (GDP-β-S) was applied intracellularly, according to the method described in Haydon *et al.*[22] and Magoski *et al.*[37] GDP-β-S (10 mM) was dissolved in intracellular saline (in mM): KCl 50, $CaCl_2$ 2.56, ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*,*N*′,*N*′-tetraacetic acid 5, and HEPES 10, adjusted to pH 7.9 with KOH, with 0.01% Fast Green. The cell was loaded with the pipette solution via a General Valve Picospritzer II, allowing simultaneous membrane potential monitoring and GDP-β-S injection. GDP-β-S was injected until the soma was the same colour as the electrode solution (pulses 50–100 ms, over 1–3 min).

Drugs

The following agents were employed: phenyltrimethylammonium chloride (PTMA) were from Aldrich Chemical Co.; FMRFamide from Bachem; kainic acid, (+)-quisqualic acid, AMPA, ketamine hydrochloride, (±)-ibotenic acid, dizocilpine maleate (MK-801), 6-cyano-7-nitroquioxiline-2,3-dione (CNQX), (±)-2-amino-4-phosphonobutyric acid (AP-4), DL-2-amino-5-phosphonovaleric acid (AP-5), and t-ACPD from Research Biochemicals International; and L-glutamate monosodium salt, D-glutamate monosodium salt, NMDA, L-homocysteic acid, L-aspartic acid monosodium salt, kynurenic acid, DL-alpha-aminopimelic acid, dopamine (3-hydroxytyramine), serotonin (5-hydroxytryptamine), DL-octopamine (1-(*p*-hydroxyphenyl)-2-aminoethanol hydrochloride), histamine (dihydrochloride), acetylcholine (ACh) chloride, hexamethonium bromide, GABA, glycine, FLRFamide, and GDP-β-S from Sigma.

GDPFLRFamide and SDPFLRFamide were gifts from J. P. Riehm of the University of West Florida. SKPYMRFamide was a gift from Dr J. Burke, University of Sussex.

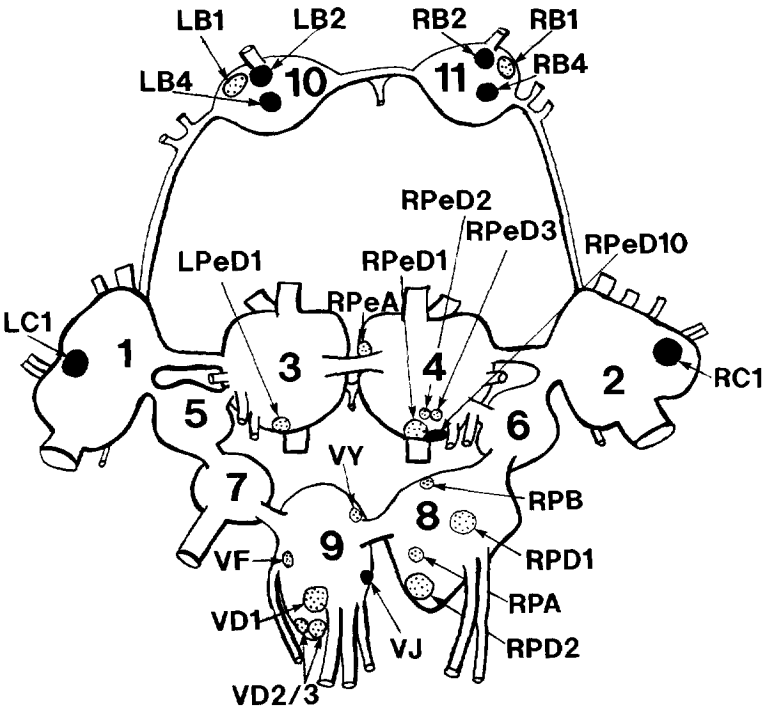


Fig. 1. Schematic diagram of the *Lymnaea* CNS with identified neurons responsive to glutamate. The ganglia are numbered as follows: 1 and 2 (left and right cerebral ganglia), 3 and 4 (left and right pedal ganglia), 5 and 6 (left and right pleural ganglia), 7 and 8 (left and right parietal ganglia), 9 (visceral ganglion), 10 and 11 (left and right buccal ganglia). Neurons are abbreviated as follows: RC1, right cerebral one; LC1, left cerebral one; RPeD1, right pedal dorsal one; RPeD2, right pedal dorsal two; RPeD3, right pedal dorsal three; RPeD10, right pedal dorsal ten; RPeA, right pedal A group; LPeD1, left pedal dorsal one; RPD1, right parietal dorsal one; RPD2, right parietal dorsal two; RPA, right parietal A group; RPB, right parietal B group; VD1, visceral dorsal one; VD2, visceral dorsal two; VD3, visceral dorsal three; VJ, visceral J; VY, visceral yellow group; VF, visceral F group; RB1, right buccal one; LB1, left buccal one; RB2, right buccal two; LB2, left buccal two; RB4, right buccal four; LB4, left buccal four. For neuronal groups (RPeA, RPA, RPB, VF and VY), we have depicted only one neuron indicating the approximate centre of the cluster. The neurons which respond to glutamate with depolarization are labelled with dots, the neurons which respond with hyperpolarization as black circles.

All peptides were made up fresh before each experiment from frozen aliquots stored at millimolar concentration at −20°C.

RESULTS

This study was carried out in two stages. First, we examined the effect of glutamate on the membrane potential of identified *Lymnaea* neurons. Second, the hypothesis that was tested that an identified interneuron (VD4) in the *Lymnaea* CNS is glutamatergic.

Effect of glutamate on identified Lymnaea *neurons*

L-Glutamate, from here on referred to as glutamate, was pressure applied (1 mM in the pipette) on various identified *Lymnaea* neurons (Figs 1 and 2). We chose visually identified neurons, typically ones with a known functional role, e.g., the interneurons right pedal dorsal 1 (RPeD1)[58] and C1,[40] motoneurons such as RPA,[58] RPeA[57] and VJ.[58] Of the responsive neurons, most of them were depolarized by glutamate, in a dose-dependent manner, at resting membrane potential (Fig. 2A); however, eight neurons, VJ, RPeD10,[35] left and right C1, left and right B2[4] and left and right B4,[4] were hyperpolarized by glutamate (Fig. 2B).

Dose–response curves for neurons right pedal dorsal 2 or 3 (RPeD2 or RPeD3)[35] (n = 4; Fig. 3) showed that the glutamate receptors bound glutamate with a relatively low affinity ($ED_{50} = 68 \pm 7$ μM, for four curves) in a positively co-operative manner (Hill coefficient = 3.3 ± 1.2; curves were fitted with the use of the PC program GRAPHPAD, Version 4.0).

To characterize the pharmacological profile of the glutamate response, we tested the effect of glutamate agonists and antagonists on neurons RPeD1, RPeD2 and RPeD3. These particular neurons where chosen because they receive input from the identified interneuron VD4,[57] which, as we subsequently propose, may be glutamatergic. All of the tested agonists (100 μM), NMDA, kainic acid, AMPA, (+)-quisqualic acid, ibotinic acid and alpha-homocysteic acid, depolarized these neurons with different efficacy (n = 16 preparations; Figs 4 and 5). However, D-glutamate and aspartic acid were ineffective (n = 4; data not shown).

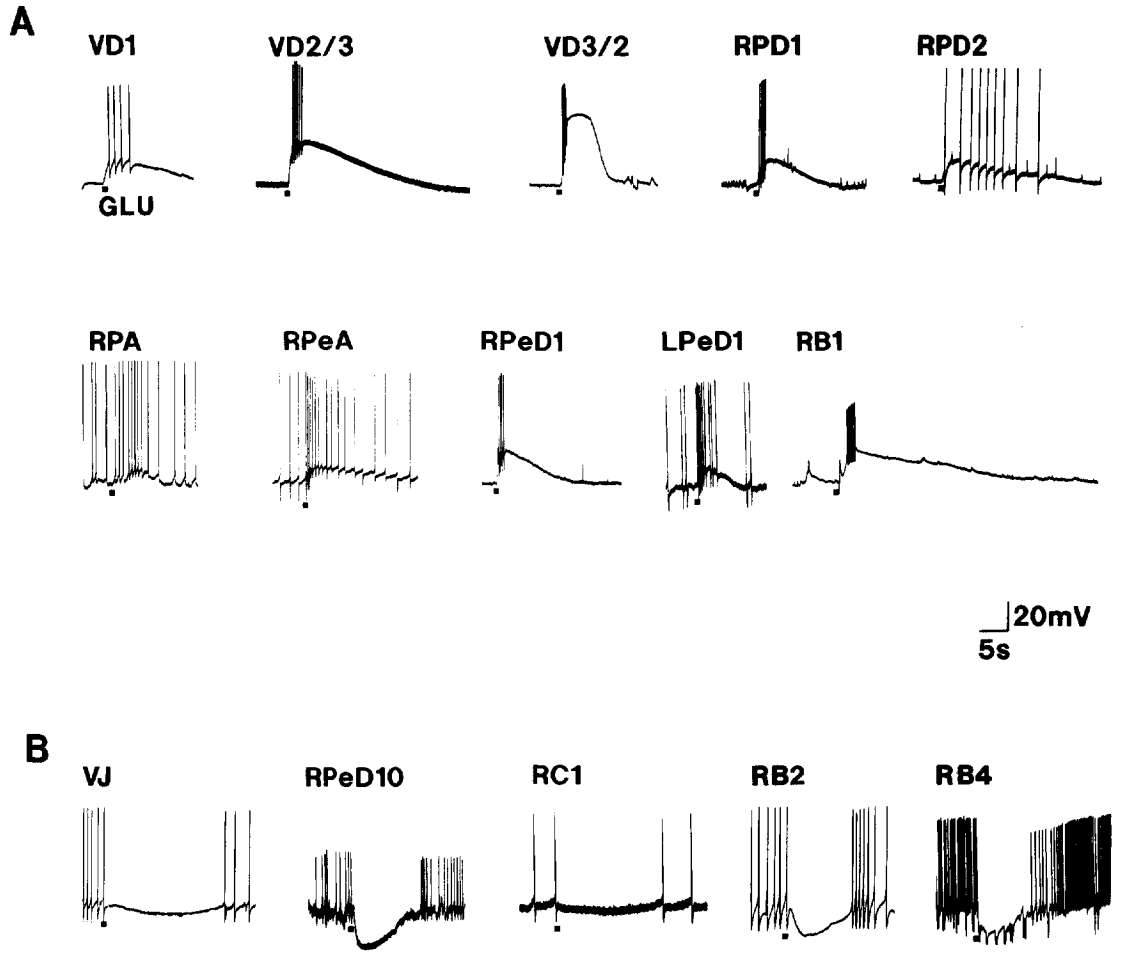


Fig. 2. Glutamate-induced excitation and inhibition. Glutamate was applied by pressure ejection for a period of 1 s (1 mM in the pipette, 1 s, $n = 4$–8 for each cell type). (A) Glutamate-induced excitation on different identified neurons. Recordings were made at the resting membrane potential: VD1 (−64mV), VD2/3 (−62mV), VD3/2 (−68mV) (neurons VD2 and VD3 can not be distinguished from each other), RPD1 (−62mV), RPD2 (−60mV), RPA (−56mV), RPeA (−54mV), RPeD1 (−60mV), LPeD1 (−50mV), RB1 (−68mV). (B) Glutamate-induced inhibition on five identified neurons: VJ (−52mV), RPeD10 (−44mV), RC1 (−60mV), RB2 (−56mV), RB4 (−60mV).

To rule out the possibility that the effect of the glutamate agonists was indirect, and due to the activation of other neurons in the brain, we tested the effect of agonists on acutely isolated RPeD2/3 somata plated in defined medium (Fig. 5). The agonists produced depolarizations in the isolated somata similar to those observed *in situ*. The rank order of efficacy of the glutamate agonists (100 μM) established *in vitro* ($n = 4$) and *in situ* (n = 12) for RPeD2/3 was: (+)-quisqualic acid>kainic acid>L-glutamate >homocysteic acid>AMPA>NMDA>ibotenic acid.

We tested several "classical" glutamate antagonists (Table 1) for their ability to block the glutamate-evoked response in RPeD2/3. None of the antagonists could block the glutamate response. A similar insensitivity to glutamate antagonists was reported for the glutamate response of *Helisoma* salivary gland cells.[2] Thus, the pharmacological profile of this *Lymnaea* glutamate receptor(s) does not correspond to any of the known vertebrate glutamate receptor subtypes.

A putative glutamatergic neuron

Recent evidence has suggested that molluscan neurons use glutamate as a neurotransmitter.[2,12,13,48,49,50,53] The hypothesis that the *Lymnaea* cardiorespiratory interneuron, VD4, uses glutamate as a neurotransmitter was tested. Previous work indicated that the inhibitory effects of VD4 could be attributed to the peptides GDPFLRFamide and SDPFLRFamide.[56] However, the transmitter responsible for the excitatory effects of VD4 is not known. Consequently, we tested the effect of a number of classical neurotransmitters (glutamate, GABA, glycine, ACh, dopamine, histamine, octopamine and serotonin) on some of the postsynaptic

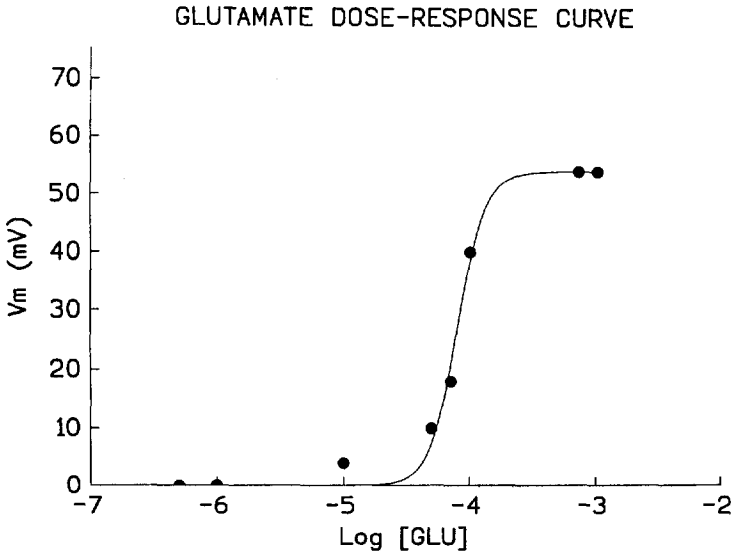


Fig. 3. Dose–response curve for bath application of glutamate. The dose–response curve was established by bath application of glutamate on RPeD3, a neuron which responds to glutamate with excitation (see Fig. 1B). The fitted curve has a Hill slope of 4 and ED_{50} of 80 μM. The average value of ED_{50} from four experiments was 68 ± 7 μM, and Hill slope of 3.3 ± 1.2. A Hill coefficient of 3.3 indicates that more than one molecule of glutamate must be bound to open the channel.

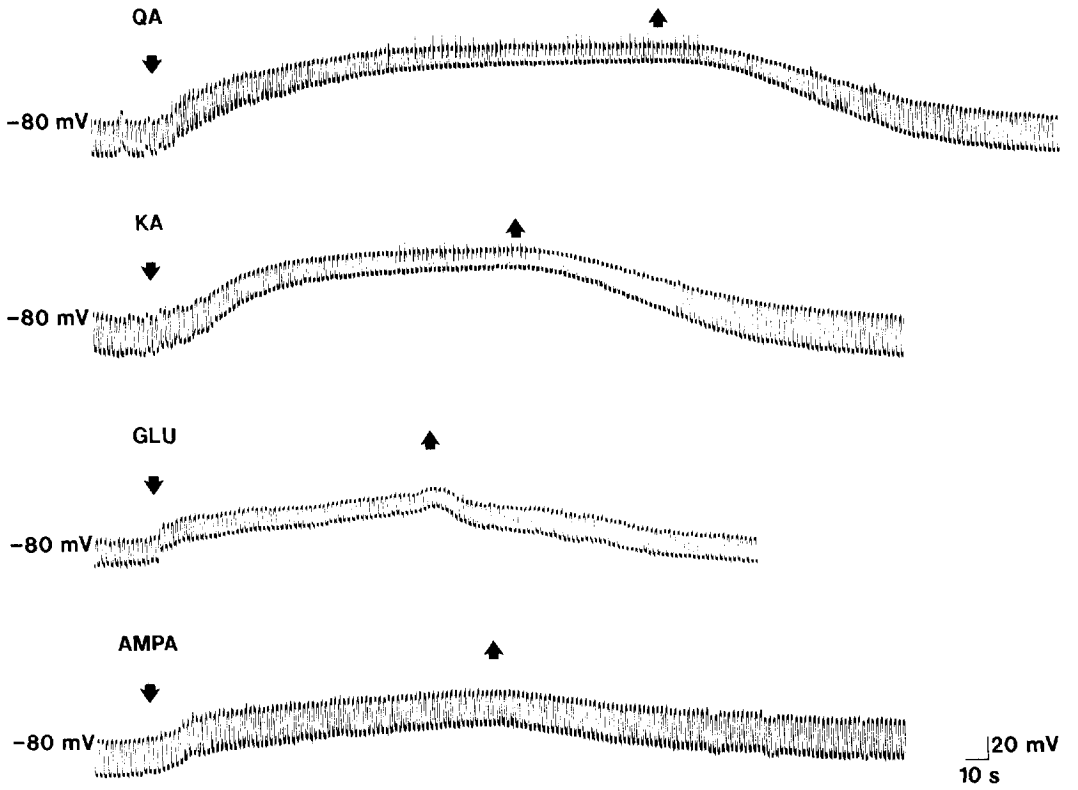


Fig. 4. Depolarizing effects of glutamate and glutamate agonists on neuron RPeD3. Glutamate and glutamate agonists (QA, KA, AMPA) were bath applied at concentrations of 100 μM, in $6 \times Ca^{2+}$/ $6 \times Mg^{2+}$ saline. A downward arrow indicates when the agonist entered the bath, and an upward arrow indicates wash. The change in membrane resistance was monitored by the application of hyperpolarizing pulses (0.5nA, 1s).

cells which are known to be depolarized by VD4; namely, VF, RPB[3] and RPeD2/3 (Fig. 6). We also tested RPeD1, which often receives a biphasic input from VD4[3] (see Fig. 11A). Only glutamate had a depolarizing effect on all of these neurons (Table 2), which suggests that glutamate may be the excitatory

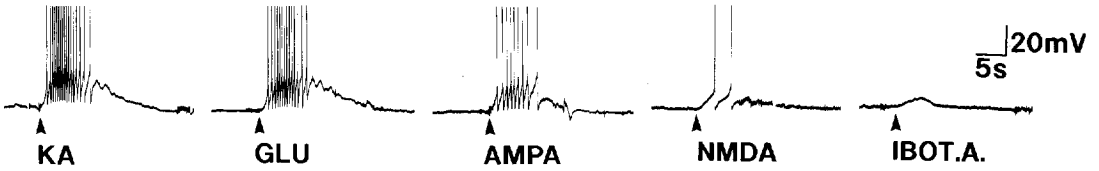


Fig. 5. Depolarizing effects of glutamate agonists on an isolated RPeD2 neuron. The agonists were applied by Y-tube at a concentration of 100 μM. The neuron was plated in defined medium for 5 h.

Table 1. Glutamate antagonists tested

Antagonist	Concentrations
Alpha-aminopimelic acid ($n = 4$)	0.001 mM; 0.01 mM
AP-4, AP-5 ($n = 8$)	0.1 mM; 0.2 mM
CNQX ($n = 7$)	0.01 mM; 0.1 mM
Ketamine ($n = 4$)	0.1 mM; 1 mM
Kynurenic acid ($n = 3$)	0.1 mM
MK-801 ($n = 8$)	0.01 mM; 0.1 mM; 1 mM

The number of neurons (*in situ*) tested for each antagonist is indicated in brackets below the response sign.

transmitter in VD4. Recently, Kellett *et al.*[30] suggested the possibility that VD4 expresses another peptide, SKPYMRFamide. They also showed that when SKPYMRFamide was applied to a visceral E group (VE) neuron, a postsynaptic cell excited by VD4, it produced excitation. In the present study, when SKPYMRFamide was applied (0.1 mM and 1 mM, bath and pressure application) to all of the VD4 postsynaptic cells listed in Table 2, as well as the two electrically connected VE cells[9] ($n = 3$), it did not elicit responses (Table 2).

Because VD4 produces a very consistent and robust excitatory postsynaptic potential (EPSP) in RPeD2 and RPeD3, we focused on identification of the transmitter at these synapses. Dopamine, ACh and glutamate each had excitatory effects on RPeD2/3 (Table 2). In two previous studies, it was shown that VD4 was not immunoreactive for dopamine,[18,64] hence dopamine was unlikely to be a co-transmitter in VD4. ACh in some cases hyperpolarized neurons RPeD2/3, although VD4 in the same preparations excited these cells (Table 2). In the cases when ACh depolarized RPeD2/3 neurons, we attempted to block the EPSP using the ACh receptor antagonists hexamethonium (100 μM) and PTMA (100 μM), which have been shown to block excitatory cholinergic transmission in *Lymnaea*.[66] Applied together, PTMA and hexamethonium could not block the RPeD2/3 EPSPs, although they blocked the ACh response (data not shown). Consequently, the only remaining candidate neurotransmitter was glutamate.

To test the extent to which exogenous glutamate mimicked the endogenous transmitter of VD4, we next examined the *I–V* relationships of the glutamate response and the synaptic current in RPeD2/3 (Fig. 7A). The left panel of Fig. 7B shows *I–V* curves for the glutamate-induced response (open circles) and the excitatory postsynaptic current (EPSC), obtained in the same *in situ* preparation. The right panel of Fig. 7B shows the *I–V* curve of the glutamate-induced current obtained from an acutely isolated RPeD3 plated in defined medium. The EPSC (*in situ*) and the glutamate-induced inward current (*in situ* and *in vitro*) shared a similar increase in membrane conductance and voltage dependence. In both cases the reversal potential was estimated to be between +20 and +30 mV ($n = 4$).

We attempted to block the synapse from VD4 to RPeD2/3 using several glutamate receptor antagonists (Table 1). None of these antagonists was effective (data not shown). We therefore performed a cross-desensitization experiment using the VD4 to RPeD2/3 synapse. Specifically, we tested the possibility that glutamate, or a glutamate agonist, applied in the bath, would cross-desensitize the receptor for the endogenous transmitter. The amplitude of the response to pressure-applied glutamate decreased when the CNS was perfused with 0.1 mM NMDA, indicating desensitization of the glutamate response (Fig. 8A). Similarly, the EPSP was decreased in the presence of 0.1 mM NMDA (Fig. 8B; $n = 3$). The same effect was observed with bath-applied glutamate ($n = 5$; Fig. 9B), (+)-quisqualic acid ($n = 3$), kainic acid ($n = 3$) and AMPA ($n = 2$) (data not shown), but not with t-ACPD ($n = 2$; Fig. 9A).

Considering that the EPSP is slow (latency is 200–400 ms), and that postsynaptic neurons respond to (+)-quisqualic acid and ibotenic acid (Fig. 4 and Fig. 5), which are the agonists for vertebrate metabotropic receptors, we investigated the possibility that the EPSP/glutamate-induced depolarization resulted from the activation of a metabotropic glutamate receptor. To this end, we tested the effect of both the selective metabotropic receptor agonist, t-ACPD, and a non-hydrolysable GDP analog, GDP-β-S.

Bath application of t-ACPD (0.1 mM) did not affect the membrane potential of RPeD3, nor did it change the amplitude of the EPSP (Fig. 9A; $n = 2$). We also looked at the action of intracellularly injected GDP-β-S (10 mM),[29] a procedure commonly used to block G-protein-mediated responses, including dopamine responses in *Lymnaea* neurons.[37]

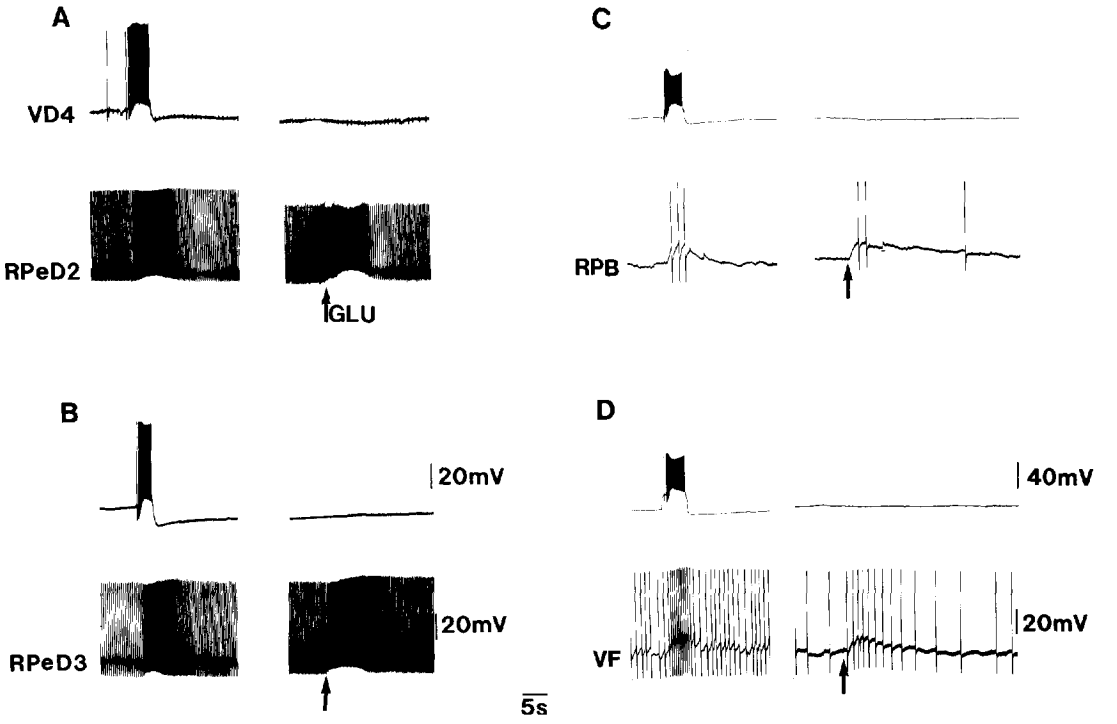


Fig. 6. Identified interneuron VD4 makes excitatory synapses with postsynaptic cells RPeD2, RPeD3, RPB and VF. Neuron VD4 makes a presumed monosynaptic connection to RPeD2, RPeD3, RPB and VF neurons (left panel of A, B, C and D). A burst of action potentials evoked by current injection into neuron VD4 (upper trace) resulted in the excitation of the postsynaptic neurons (lower trace). VD4 did not have consistent excitatory effects on every cell in the VF and RPB neuronal clusters, in some neurons VD4 evoked an IPSP and in some a biphasic PSP. In those same postsynaptic neurons where EPSPs were evoked, pressure-applied glutamate (1 mM, 1 s, application indicated by arrow) produced an excitatory effect (right panel of A, B, C and D). The EPSPs and the effect of glutamate were induced at resting membrane potential: RPeD2 (−56mV), RPeD3 (−54mV), RPB (−50mV) and VF (−46mV).

Loading the cell with GDP-β-S did not affect the EPSP or glutamate response of RPeD2/3 ($n = 4$ and 4; Fig. 10). Given this result, and the ineffectiveness of t-ACPD, the extension of the experiments to include pertussis toxin treatment, another commonly used procedure to test for the involvement of G-protein, was not justified. Furthermore, not all G-protein-mediated responses are sensitive to pertussis toxin.[8,23,39]

To examine further the glutamatergic nature of VD4, we investigated the effect of glutamate on another postsynaptic cell of VD4, neuron RPeD1. Neuron VD4 often makes a biphasic (i.e. depolarization followed by longer lasting inhibition) connection with RPeD1 (Fig. 11A). The hyperpolarizing phase in RPeD1 can be mimicked by exogenous application of either GDPFLRFamide or SDPFLRFamide.[56] We tested the effect of both GPDFLRFamide and glutamate on RPeD1. GDPFLRFamide (1 mM in the pipette), as expected, produced a long-lasting hyperpolarization, while glutamate (1 mM in pipette) elicited a depolarization (Fig. 11B). When applied together, glutamate and GDPFLRFamide (mixed in the pipette at the same concentrations as before) evoked a biphasic response (depolarization preceding a longer lasting hyperpolarization). This biphasic response, which could be repeatedly evoked in four preparations, closely resembled the effect of VD4 stimulation.

DISCUSSION

Effect of glutamate on identified Lymnaea *neurons*

Unlike vertebrate neurons, molluscan neurons respond to the exogenous application of glutamate with both excitation and inhibition involving different ionic mechanisms.[6,27,49,53] Accordingly, we observed both excitatory and inhibitory glutamate responses. The only previous report regarding glutamate in *Lymnaea* described a depolarizing response on the light yellow cells;[45] however, in that study other neurons were not investigated. In the present study, almost all of the identified neurons tested in *Lymnaea* responded to glutamate, and most of these responses were depolarizing (Figs 1 and 2). Similarly, a large number of glutamate-responsive neurons has been found in the CNS of the pulmonate molluscs *Helix*[63] and *Planorbarius*.[6] Considering the diverse physiological role of these glutamate responsive neurons, it would suggest that glutamate has a role in several types of behavior.

Table 2. The effect of "classical neurotransmitters" on the postsynaptic cells of VD4: RPeD1, RPeD2, RPeD3, RPB and VF

	RPeD2	RPeD3	RPB	VFR	RPeD1
Glutamate	+ (48)	+ (61)	+ (10)	+ (12)	+ (8)
GABA	− (6)	+ or − (3) (3)	+/− (5)	0 or − (3) (5)	+ (3)
Glycine	0 (4)	0 (4)	0 (3)	0 (3)	0 (3)
Acetylcholine	+ or − (6) (5)	+ or − (4) (4)	− (6)	− (6)	+ (5)
Dopamine	+ (9)	+ (10)	− (5)	− (6)	− (4)
Histamine	− (7)	− (7)	+ (5)	+ (8)	+ (3)
Octopamine	− (4)	+ (3)	− (5)	− (6)	not tested
Serotonin	− (8)	+ (10)	− (6)	− (6)	+ (3)
FMRFamide	− (6)	− (4)	− (2)	− (2)	− (3)
GDPFLRFamide	− (3)	− (2)	not tested	not tested	− (5)
SDPFLRFamide	− (3)	− (2)	not tested	not tested	− (3)
SKPYMRFamide	0 (3)	0 (2)	0 (2)	0 (2)	0 (2)

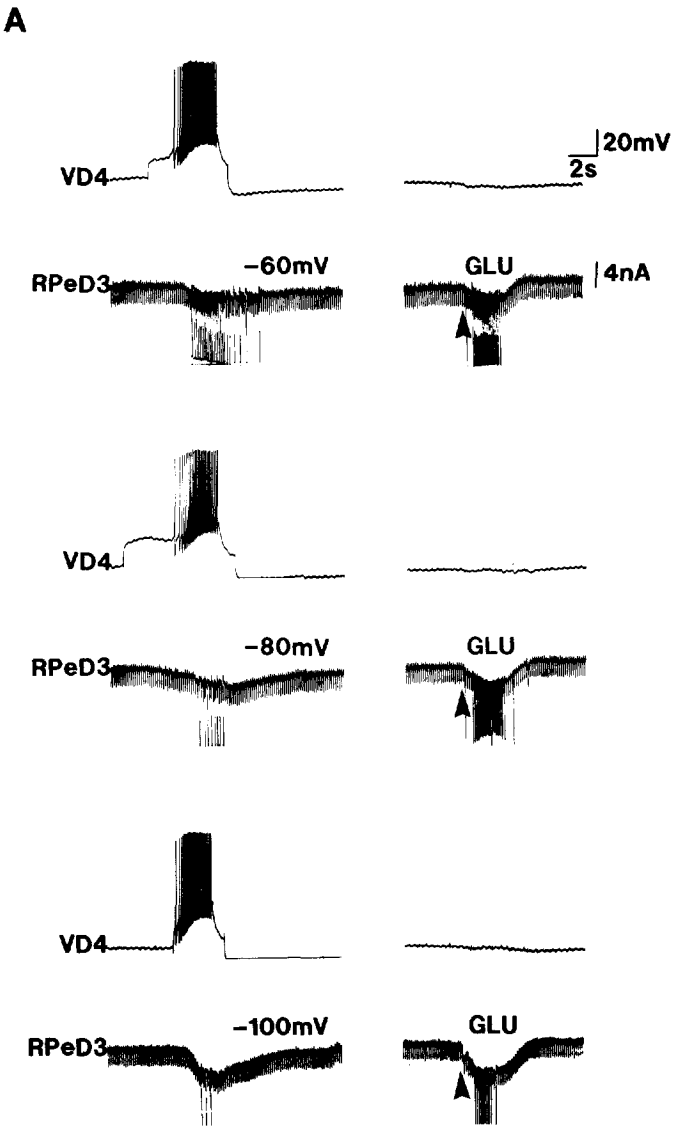
Each neurotransmitter was bath applied at 100 μM in $6 \times Ca^{2+}/6 \times Mg^{2+}$ saline.
(+) indicates depolarization,
(−) indicates hyperpolarization,
(+/−) indicates biphasic effect (depolarization followed by hyperpolarization),
(0) indicates no effect.
The number of neurons tested for each neurotransmitter is indicated in brackets below the response sign.

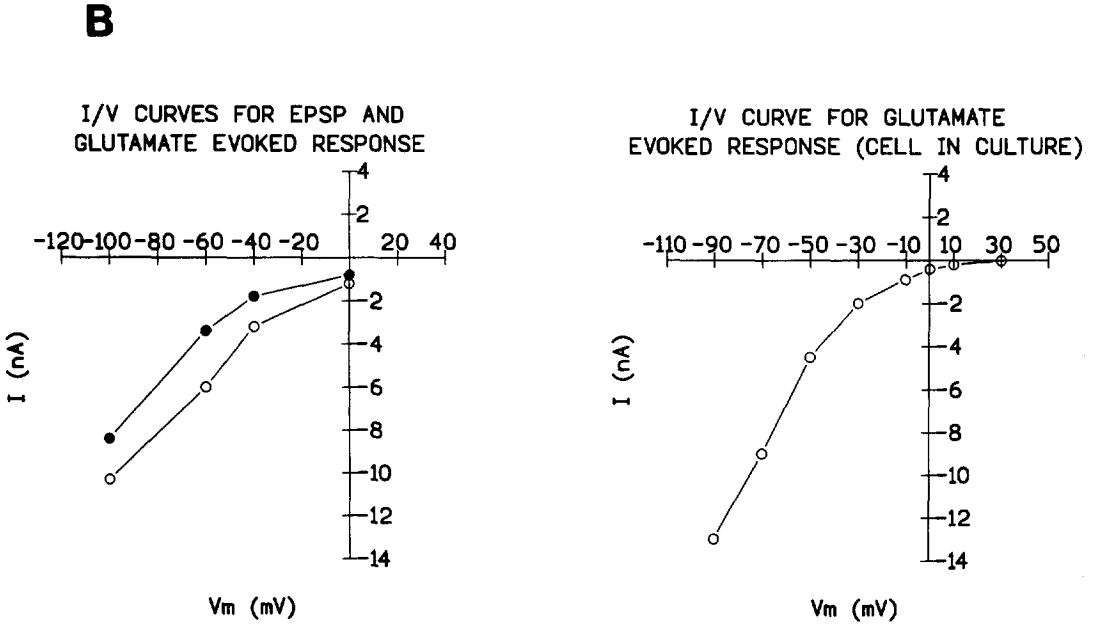
Pharmacology of the glutamate response

To the best of our knowledge, this study reports the first example of a glutamate receptor(s) on single cells (RPeD2/3 and RPeD1) that is activated by all classical glutamate agonists (NMDA, kainic acid, (+)-quisqualic acid, AMPA and ibotenate; Figs 4 and 5). The rank order of agonist efficacies implies that the glutamate receptors on RPeD1 and RPeD2/3 have the highest affinity for (+)-quisqualic acid and kainic acid. This in turn suggests that the receptor more closely resembles a vertebrate kainic acid/(+)-quisqualic acid type ionotropic receptor. However, the fact that the response cannot be blocked by CNQX, a competitive antagonist for kainic acid/(+)-quisqualic acid and AMPA/(+)-quisqualic acid receptors,[44] does not support this assumption. Parenthetically, CNQX could block kainic acid responses[59] and 6,7,dinitroquinoxaline-2,3-dione (DNQX) could block glutamate responses[13] of neurons in *Aplysia* and *Helisoma*,[49] although the glutamate response of *Helisoma* salivary gland cells was insensitive to CNQX.[2] RPeD1 and RPeD2/3 responded to NMDA, so we tested competitive (AP-4, AP-5, kynurenic acid) and non-competitive (MK-801) NMDA receptor antagonists,[44] which were ineffective. The same ineffectiveness of NMDA antagonists was reported for NMDA-induced depolarization in light yellow cells of *Lymnaea*.[45] The vertebrate NMDA receptor has the specific properties of voltage-dependent Mg^{2+} block and potentiation by glycine. We tested the effect of a zero Mg^{2+} solution with 10 μM glycine on the NMDA response, but no effect was detected ($n = 4$; data not shown).

The finding that (+)-quisqualic acid and ibotenate were effective agonists suggested that this *Lymnaea* receptor might belong to a family of metabotropic glutamate receptors. However, the selective metabotropic glutamate receptor agonist t-ACPD had no effect on RPeD2/3 (Fig. 9A). Also, intracellular

Fig. 7. *I–V* relationships for the EPSC and the glutamate evoked resonse in RPeD3. (A) Using single electrode voltage clamp, the membrane potential of RPeD3 was held at different voltages. The recordings presented here show the amplitudes of the EPSCs (left column) and inward currents induced by pressure applied glutamate (right column), at three different membrane potentials: −60, −80 and −100mV. Upper trace: intracellular recordings of VD4 membrane potential. Membrane conductance was monitored by the application of 10mV, 1s hyperpolarizing pulses. (B) *I–V* curves for the EPSP (●) and the glutamate-evoked response (○), both obtained from RPeD2 *in situ*. An *I–V* curve for the glutamate response was also established using RPeD3, acutely isolated and plated in defined medium. The experiments were performed within 3–5 h after plating.


A
VD4
20mV
2s
RPeD3
-60mV
GLU
4nA
VD4
RPeD3
-80mV
GLU
VD4
RPeD3
-100mV
GLU


B
I/V CURVES FOR EPSP AND GLUTAMATE EVOKED RESPONSE
-120 -100 -80 -60 -40 -20 20 40
4 2 -2 -4 -6 -8 -10 -12 -14
I (nA)
Vm (mV)
I/V CURVE FOR GLUTAMATE EVOKED RESPONSE (CELL IN CULTURE)
-110 -90 -70 -50 -30 -10 10 30 50
4 2 -2 -4 -6 -8 -10 -12 -14
I (nA)
Vm (mV)

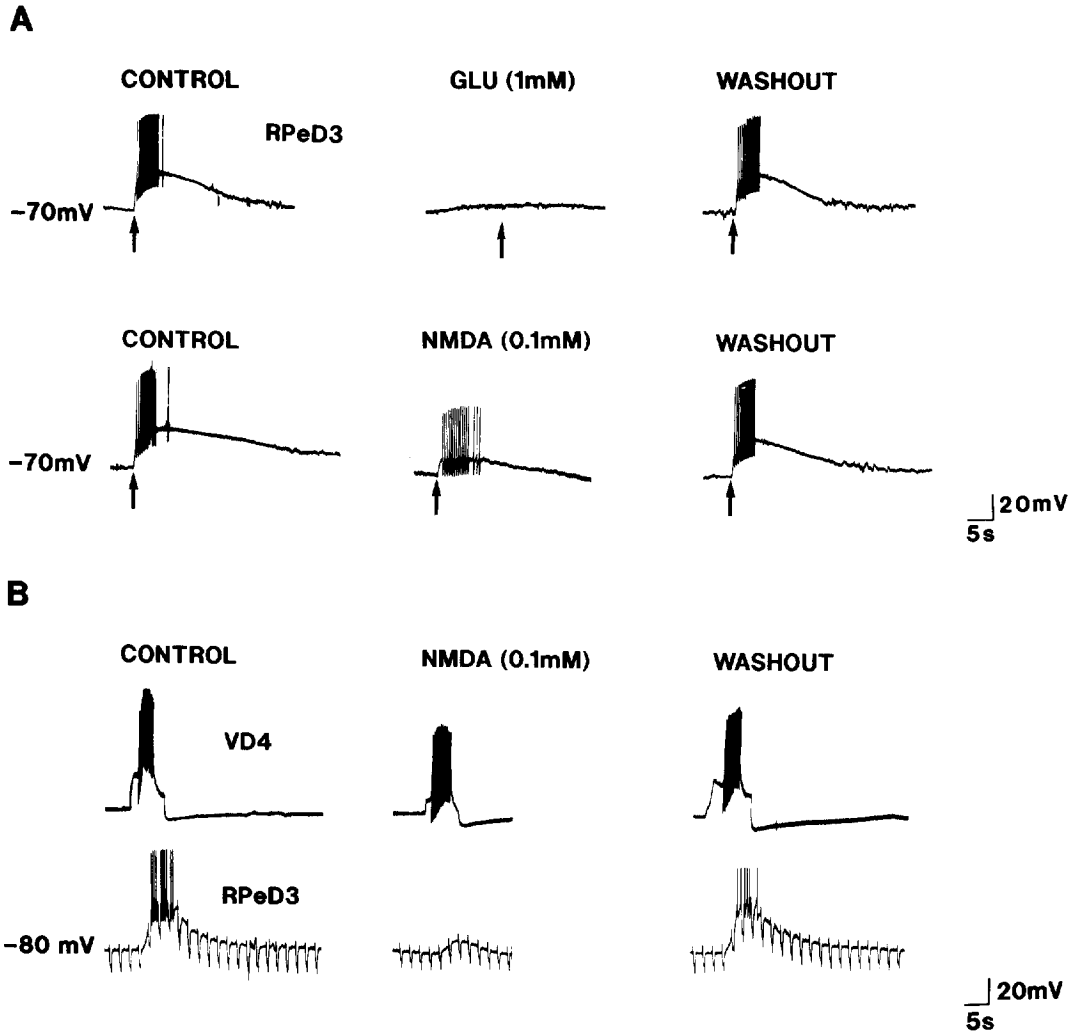


Fig. 8. Cross-desensitization between NMDA and the EPSP. (A) The effect of pressure applied glutamate (1 mM in the pipette, 20 ms) on RPeD3, in the presence of bath-perfused glutamate (1 mM) or NMDA (0.1 mM). Bath application of 1 mM of glutamate desensitized the glutamate receptor completely, so pressure applied glutamate did not induce any response. However, 0.1mM NMDA desensitized the glutamate receptors partially. (B) Recording of the excitatory response from VD4 to RPeD3 in the absence and presence of NMDA (0.1 mM). During the exposure to NMDA the EPSP was reduced in amplitude. The membrane potential of RPeD3 was held at −80mV. The input resistance of RPeD3 was measured by the injection of 0.5 nA, 0.5 s hyperpolarizing pulses.

loading with GDP-β-S (Fig. 10B) did not change the glutamate response. Therefore, this glutamate receptor does not seem to have properties typical for the vertebrate metabotropic receptors. The only published G-protein-coupled glutamate response demonstrated in molluscan neurons is the hyperpolarizing glutamate response of *Planorbarius* neurons.[6] It is interesting to note that all the metabotropic-like glutamate receptors identified in the CNS of invertebrates are connected to the opening of potassium channels and membrane hyperpolarization.[6,19,43,46] To add to the pharmacological peculiarity of invertebrate glutamate receptors, there is a finding that, although (+)-quisqualic acid and t-ACPD produce slow hyperpolarizations in *Aplysia* buccal neurons, the response to metabotropic receptor agonists is independent of G-protein.[29]

The sequence of the cloned *Lymnaea* glutamate receptor suggests that some neurons in the *Lymnaea* CNS possess AMPA-like glutamate receptors.[14,15] However, the pharmacological analysis presented in this study indicates that RPeD1 and RPeD2/3 neurons possess a different type of glutamate receptor(s). Another possibility is that *Lymnaea* neurons express a mixture of various types of glutamate receptors, or glutamate receptors with a non-specific (maybe ancestral) pharmacological profile. This is supported by the finding that although the *Aplysia* motoneuron glutamate receptor shares some similarities with vertebrate NMDA receptors, such as

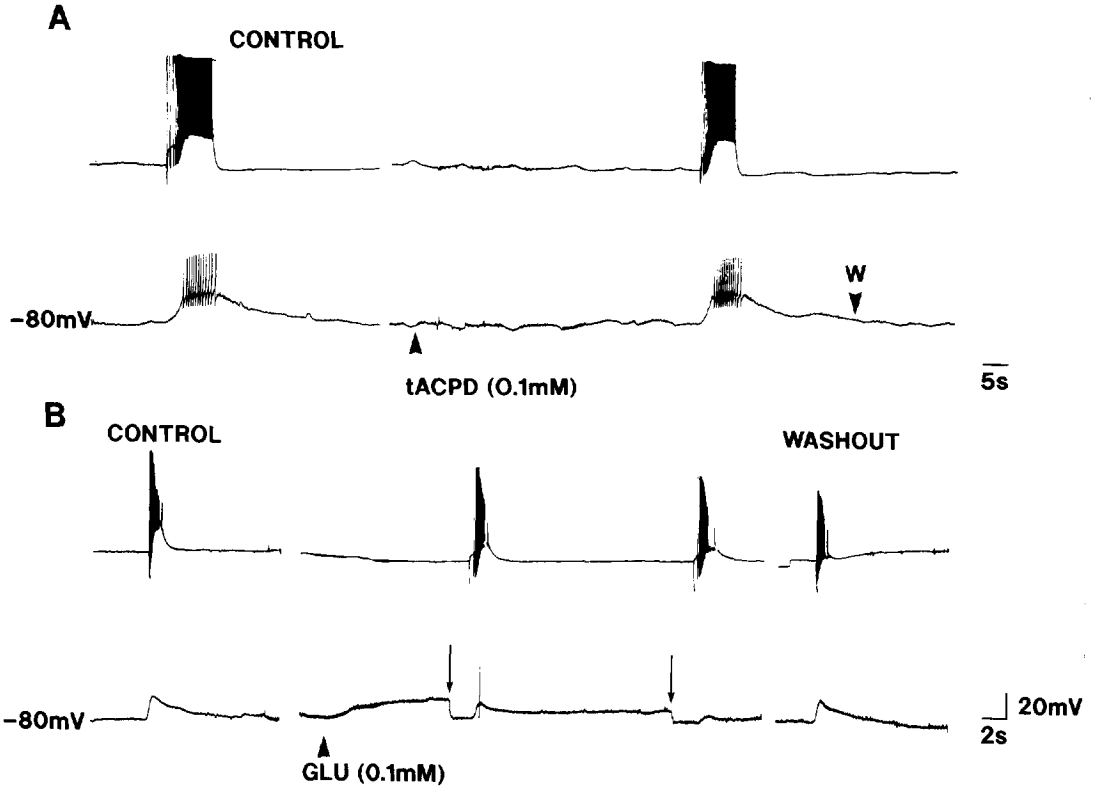


Fig. 9. Effect of t-ACPD and glutamate on both the membrane potential of neuron RPeD3 and the VD4 to RPeD3 synapse. (A) Simultaneous recordings from VD4 and RPeD3. A depolarizing stimulus injected into VD4, caused an EPSP in RPeD3. Bath application of 0.1 mM tACPD did not change the membrane potential of RPeD3, nor did it change the amplitude of the EPSP, indicating a lack of cross-desensitization. (B) In the same experiment, bath application of glutamate (0.1 mM) produced depolarization of RPeD3, and its continued presence decreased the amplitude of the EPSP, indicating cross-desensitization. The membrane potential of RPeD3 was held at −80 mV throughout the experiment. When glutamate depolarized RPeD3, additional hyperpolarizing current was injected into the cell to keep membrane potential at −80mV (indicated by the two thin arrows).

voltage-dependent Mg^{2+} block, the glutamate response is blocked by the kainic acid/(+)-quisqualic acid receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX).[13]

The relatively high threshold and maximal response obtained with 1mM glutamate resulted in a very steep glutamate dose–response curve, which is reflected in the high Hill coefficient (3.3 ± 1.2; Fig. 3). A comparable Hill coefficient was found for glutamate binding to crab neuromuscular junction.[32] Similarly, positive co-operative binding of glutamate was found for other snail neurons.[24,47,54]

The concentration of glutamate in *Lymnaea* haemolymph, as measured by high-performance liquid chromatography,[26] was found to be 6 μM (data not shown), which is an order of magnitude below the threshold for the tested glutamate response (Fig. 3). As expected, the blood concentration of glutamate did not change the membrane potential of the glutamate-responsive neurons. The same insensitivity of receptors to circulating glutamate was found in *Helisoma*, where the haemolymph glutamate concentration (40 μM) did not affect glutamate-responsive buccal neurons.[26] Also, the concentration of glutamate in *Aplysia* blood is an order of magnitude lower then the threshold for the glutamate response (0.8 vs 10 μM).[53] Thus, circulating glutamate is unlikely to affect neuronal receptors that participate in synaptic transmission.

A putative glutamatergic neuron

Recent evidence has suggested that glutamate is a neurotransmitter in the molluscan CNS. Glutamate is the proposed neurotransmitter at the giant synapse of the squid,[12,28] at the synapse between sensory neurons and motoneurons in *Aplysia*,[13] at buccal interneuron–motoneuron and neuroglandular synapses in *Helisoma*,[2,48,49] and at buccal neuromuscular synapses in *Aplysia*.[20] A possible glutamatergic neuron in the *Lymnaea* CNS is the cardiorespiratory interneuron, VD4. Because of its important physiological roles, this interneuron has been subjected to detailed analysis; for example, it is a part of the *Lymnaea* respiratory central pattern generator and it participates in cardioregulation.[9,25,57,58] To address the question of which neurotransmitter(s) is (are) released at VD4's excitatory and biphasic synapses,

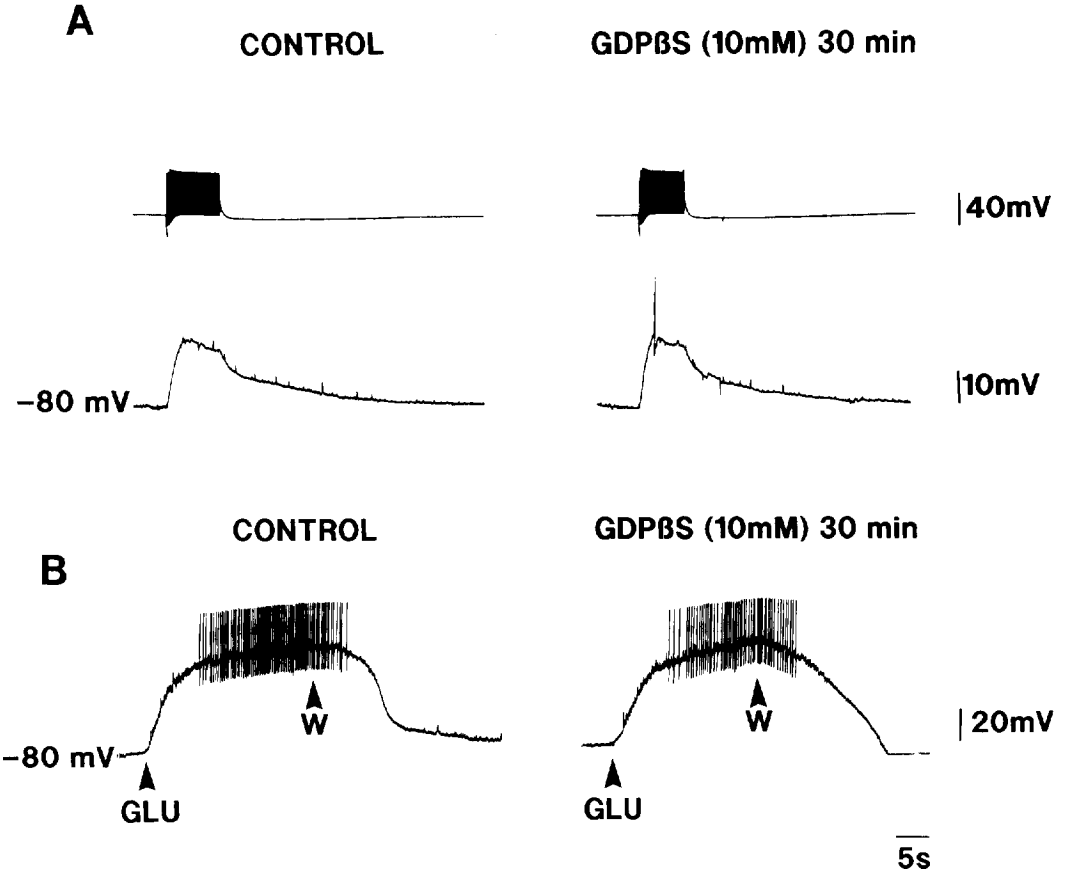


Fig. 10. The effect of GDPβS on the VD4 to RPeD3 EPSP and the RPeD3 glutamate response. (A) RPeD3 was intracellularly loaded with GDPβS (10 mM in pipette)[29] by pressure injection. The EPSP amplitude was neither changed after 30 min (shown in the figure), nor after 1 h following injection ($n = 4$). (B) The glutamate response of the same postsynaptic neuron (in A), was measured after loading the cell with GDPβS. The amplitude of the glutamate induced polarization was neither changed after 30 min (shown in the figure), nor after 1 h following injection ($n = 4$).

we tested the effects of a number of classical neurotransmitters on postsynaptic cells excited by VD4 (Table 2). Although all of the tested neurotransmitters depolarized some of the postsynaptic cells of VD4, the only neurotransmitter which consistently depolarized all of the postsynaptic cells was glutamate (Table 2). Hence we propose that glutamate is a plausible candidate for an excitatory neurotransmitter in VD4. Furthermore, histochemical and immunocytochemical data suggest that VD4 does not contain dopamine,[18,64] histamine,[61] octopamine[17] or serotonin.[31] There are, however, no published maps showing cholinergic, GABAergic or glutamatergic neurons in the *Lymnaea* CNS.

It is possible that VD4 uses different co-transmitters (SKPYMRFamide, glutamate, GABA, dopamine or ACh) at excitatory synapses with various postsynaptic cells; however, at the synapse with RPeD2/3, the only plausible candidate is glutamate (Table 2). Consistent with this hypothesis is the observation that the glutamate response induced on RPeD2/3 and the VD4-evoked EPSC both show a membrane conductance increase and a similar voltage dependence of the activated conductance (Fig. 7). Although vertebrate glutamate receptor antagonists can block some glutamate responses and synapses in molluscs,[13,49,59,60] every attempt to block both the glutamate response (Table 1) on neurons RPeD2/3 and the synaptic input from VD4 was unsuccessful. A similar insensitivity to glutamate receptor antagonists was reported for the neuroglandular synapse in *Helisoma*.[2] Because none of the glutamate antagonists was effective in the study involving RPeD2/3, we employed a cross-desensitization procedure with glutamate and glutamate agonists. The glutamate receptor(s) desensitizes when exposed to suprathreshold doses of glutamate or glutamate agonists (Fig. 8A). We showed that in the presence of NMDA and glutamate the EPSP also decreases (Figs 8B and 9B). Similar cross-desensitization experiments were performed to support a transmitter role for glutamate in the excitatory junction potential of *Aplysia* anterior aorta,[53] at the squid giant synapse,[12] at an *Aplysia* neuromuscular junction,[20] and at a *Helisoma* neuroglandular synapse.[2] The cross-desensitization experiment provides

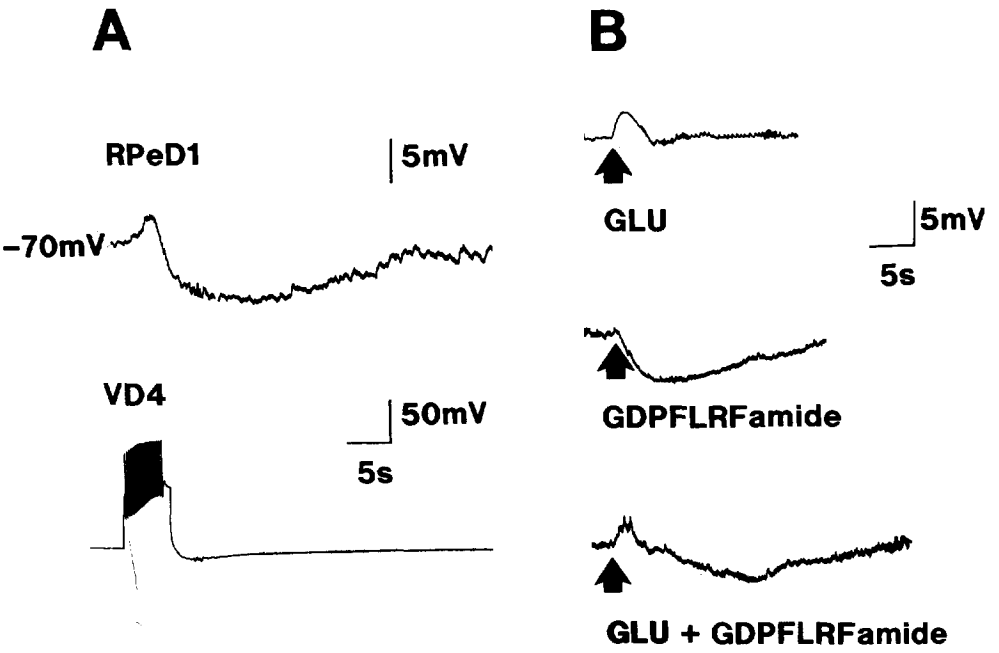


Fig. 11. Glutamate together with GDPFLRFamide mimics the biphasic connection from VD4 to RPeD1. (A) VD4 elicits a biphasic postsynaptic potential in RPeD1. The membrane potential of RPeD1 (upper trace) was held at −70mV in order to see both phases of the synaptic potential and prevent the neuron from spiking. VD4 (lower trace) was stimulated by applying a constant current pulse. The experiment was performed in $6 \times Ca^{2+}/6 \times Mg^{2+}$ saline. (B) Membrane potential recordings of RPeD1. Pressure application of glutamate (1 mM, 1 s, at arrow) produced a transient depolarization (upper trace). GDPFLRFamide (0.1 mM, 1 s) evoked a longer-lasting hyperpolarization (middle trace). When glutamate and GPFLRFamide were mixed in the same pipette, and applied (1 mM glutamate, 0.1 mM GDPFLRFamide, 1 s), they produced a biphasic effect, similar to the biphasic synaptic potential.

further support for a neurotransmitter function of glutamate in VD4, although demonstration of its presence and release from presynaptic terminals would be required to confirm such a role.

The synaptic connection between neurons VD4 and RPeD1 may be an example of co-transmission[34] of a classical neurotransmitter (glutamate) and a neuropeptide (GDPFLRFamide and/or SDPFLRFamide). Simultaneous application of glutamate and GDPFLRFamide can mimic the biphasic postsynaptic potential evoked by VD4 (Fig. 11). Hence, it is possible that VD4 uses glutamate as an excitatory transmitter at this synapse. Co-localization of neuropeptide and glutamate has been found in *Aplysia* buccal motoneurons (with small cardioactive peptides and FMRFamide)[20] and in mouse olfactory neurons (with carnosine).[52]

CONCLUSIONS

We have shown that many identified *Lymnaea* neurons respond to glutamate via a receptor with novel pharmacological properties, i.e. one sensitive to a majority of glutamate agonists and insensitive to classical glutamate antagonists. Furthermore, our results indicate the possibility that an identified cardiorespiratory interneuron (VD4) uses glutamate as a transmitter at excitatory synapses.

Our data add to the diversity of the repertoire of putative transmitters in gastropod molluscs. The complexity of gastropod neuropharmacology reflects the complex behaviour of these animals, and it may indicate that many transmitters emerged early in evolution.

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