Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 7163–7167, August 1993
Neurobiology

L-Glutamate may be the fast excitatory transmitter of *Aplysia* sensory neurons

(synapses/chemical transmitters/amino acid transmitters)

NICHOLAS DALE* AND ERIC R. KANDEL

Center for Neurobiology and Behavior and Howard Hughes Medical Institute, Columbia University, College of Physicians and Surgeons, 722 West 168th Street, New York, NY 10032

Contributed by Eric R. Kandel, March 15, 1993

ABSTRACT **Although modulation of synaptic transmission between *Aplysia* mechanosensory and motor neurons has been an important model for processes thought to underlie simple forms of learning and memory, the nature of the fast excitatory transmitter utilized by the sensory neurons has remained obscure. To identify the sensory neuron transmitter, we first examined the detailed properties of the synaptic response evoked in motor neurons cocultured with pleural sensory neurons. The excitatory postsynaptic current had a nonlinear current–voltage relation with a reversal potential between 0 and 10 mV and a plateau region between −40 and −70 mV. When the concentration of Mg^{2+} in the artificial sea water was lowered to 5 mM, the current–voltage relation of the excitatory postsynaptic current became linear, suggesting that Mg^{2+} blocks the postsynaptic receptor in a voltage-dependent manner. After screening a variety of small molecules, we found that L-glutamate could mimic the actions of the sensory neuron transmitter: responses to L-glutamate also had a reversal potential between 0 and 10 mV and a nonlinear current–voltage relation that could be made linear by lowering external Mg^{2+}. To demonstrate further similarity of action between L-glutamate and the endogenous transmitter, we utilized four antagonists (kynurenate, 6,7-dinitroquinoxaline-2,3-dione, D-aspartate, and D-glutamate) to block in a dose-dependent manner the actions of L-glutamate and the natural transmitter. We therefore suggest that the sensory neurons use a glutamate-like transmitter and favor L-glutamate itself, because no other naturally occurring amino acid that we have studied has had similar actions. As the postsynaptic receptor for the sensory neuron transmitter is weakly blocked in a voltage-dependent manner by Mg^{2+}, the excitatory receptors innervated by the *Aplysia* sensory neuron may represent a distant precursor of the vertebrate *N*-methyl-D-aspartate receptor.**

Several of the mechanisms that contribute to memory storage for different forms of learning in the gill-withdrawal reflex in *Aplysia* have been located to the synapses between the siphon sensory neurons and their follower interneurons and motor neurons (1). For example, short- and long-lasting changes in the efficacy of transmission at these synapses contribute to habituation and sensitization, elementary forms of nonassociative learning (2, 3). Although the cellular and molecular mechanisms for modulating synaptic function have been well studied, the fast transmitter released by sensory neurons has not been identified. Identification of the transmitter used by the sensory neurons would allow study of several previously inaccessible mechanisms that are potentially important for learning, such as alterations in the synthesis of transmitter and its postsynaptic receptors. After screening a number of small molecules, we focused on L-glutamate, an excitatory transmitter at many central synapses in the vertebrate nervous system (4, 5). Our evidence suggests that L-glutamate or a very similar molecule may be the fast transmitter for sensory neurons.

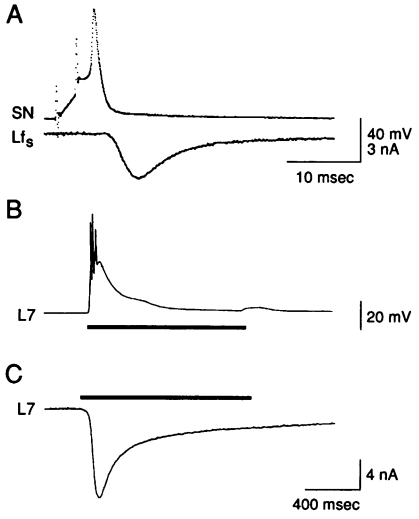

FIG. 1. Glutamate simulates the actions of the natural transmitter. (*A*) An excitatory postsynaptic current (EPSC) evoked by intracellular stimulation of a sensory neuron (SN) cocultured with an L_{f_s} motor neuron. (*B*) The response of a cultured L7 motor neuron to 10 mM L-glutamate under current clamp. (*C*) The current evoked by application of L-glutamate to a different L7 motor neuron in culture. Holding potential, −50 mV in *A* and *C*; resting potential, −60 mV in *B*.

MATERIALS AND METHODS

L_{f_s} and L7 motor and pleural sensory neurons were isolated and grown in culture by established methods (6, 7). Whole cell patch recording techniques, together with an Axoclamp 2A amplifier in the discontinuous single electrode voltage clamp mode, were used to record from the motor cells. The pipette solution contained 400 mM cesium methylsulfonate, 10 mM EGTA, and 50 mM Hepes and was adjusted to pH 7.4. The sensory neurons were penetrated with microelectrodes containing 2.5 M KCl. The artificial sea water contained 450 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 55 mM $MgCl_2$, 2.5 mM $NaHCO_3$, and 20 mM Hepes and was adjusted to pH 7.4. The low Mg^{2+} sea water contained 540 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 2.5 mM $NaHCO_3$, and 20 mM Hepes and was adjusted to pH 7.4.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EPSC, excitatory postsynaptic current; NMDA, *N*-methyl-D-aspartate; DNQX, 6,7-dinitroquinoxaline-2,3-dione; *I–V*, current–voltage.

*Present address: Department of Zoology, University of Bristol, Bristol BS8 1UG, United Kingdom.

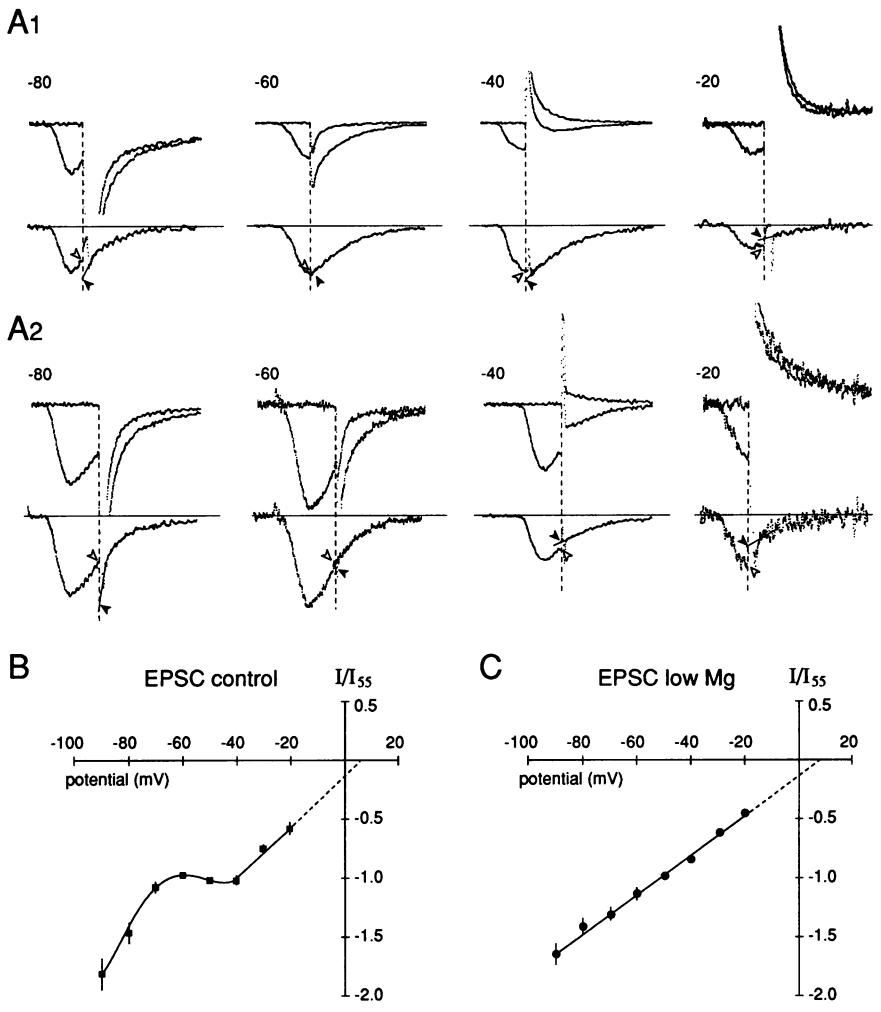

FIG. 2. The *I–V* relation of the synaptic current is nonlinear and can be altered by lowering extracellular Mg^{2+}. Sample traces (from different cells) of the EPSC recorded in L_{f_s} motor neurons clamped at −55 mV during a series of voltage steps superimposed on current during the voltage steps alone (top) together with the subtracted current records (bottom) for control sea water (*A1*) and low Mg^{2+} sea water (*A2*). Since the voltage step took about 1 msec to achieve the desired value, the instantaneous current was approximated by linear extrapolation back to time zero (the time of the voltage step). The current measured immediately before the voltage step is shown by open arrowheads, whereas that measured immediately after is shown by filled arrowheads. (*B* and *C*) Summary graph of the *I–V* relation for the EPSC taken from experiments on 10 different synapses in control sea water (*B*) and from 10 different synapses in sea water containing 5 mM Mg^{2+} (*C*).

RESULTS

Stimulation of siphon sensory neurons evoked fast inward synaptic currents in their target motor neurons (Fig. 1*A*). Rapid application of L-glutamate caused a large depolarization sufficient to make motor neurons fire spikes (Fig. 1*B*) that was evident under voltage clamp as a rapidly desensitizing inward current (Fig. 1*C*). To test in detail whether L-glutamate accurately mimicked the actions of the endogenous transmitter, we first characterized the current–voltage (*I–V*) relationship of the synaptic current.

The Synaptic Response Has a Nonlinear *I–V* Relation. The synaptic potentials evoked by *Aplysia* sensory neurons decrease rapidly with repeated stimulation. To avoid the complicating effects of this homosynaptic depression, we voltage clamped motor cells at −55 mV and measured the instantaneous change in the synaptic current during a series of voltage steps from −90 to −20 mV (taking one step for each elicited EPSC). To obtain a leak-subtracted trace of the synaptic current during the voltage step, the current trace for the voltage step given alone was subtracted from the trace of the EPSC plus voltage step (Fig. 2 *A1* and *A2*). The instantaneous current following each voltage step was then expressed as a fraction of the synaptic current measured immediately before the step, to give a normalized measure of the synaptic current at each test voltage.

The instantaneous *I–V* relation of the EPSC in L_{f_s} motor neurons was nonlinear, having a plateau region between −40 and −70 mV (Fig. 2*A1*) and an extrapolated reversal potential between 0 and 10 mV (Fig. 2*B*). This *I–V* relation was reminiscent of that of the vertebrate NMDA receptor, which has a region of negative slope conductance that is caused by voltage-dependent blockade of the receptor channel by Mg^{2+} (8, 9). To test whether the nonlinear *I–V* relationship of the EPSC might also result from a blocking action of Mg^{2+}, we lowered Mg^{2+} in sea water from 55 mM to 5 mM. This caused the *I–V* relation of the EPSC to become linear (Figs. 2 *A* and *C*), suggesting that Mg^{2+} may partially block the ion channel underlying the EPSC at voltages ranging from −40 to around −70 mV and permeate the channel at more negative potentials.

To mimic the endogenous transmitter, the actions of any putative transmitter must have the following properties: (*i*) a reversal potential between 0 and 10 mV, (*ii*) a nonlinear *I–V* relation with a plateau region between −40 and −70 mV, and (*iii*) a weak voltage-dependent blocking action of extracellular Mg^{2+}.

L-Glutamate Evokes an Inward Current in L_{f_s} and L7 Motor Neurons. To test the extent to which exogenous L-glutamate

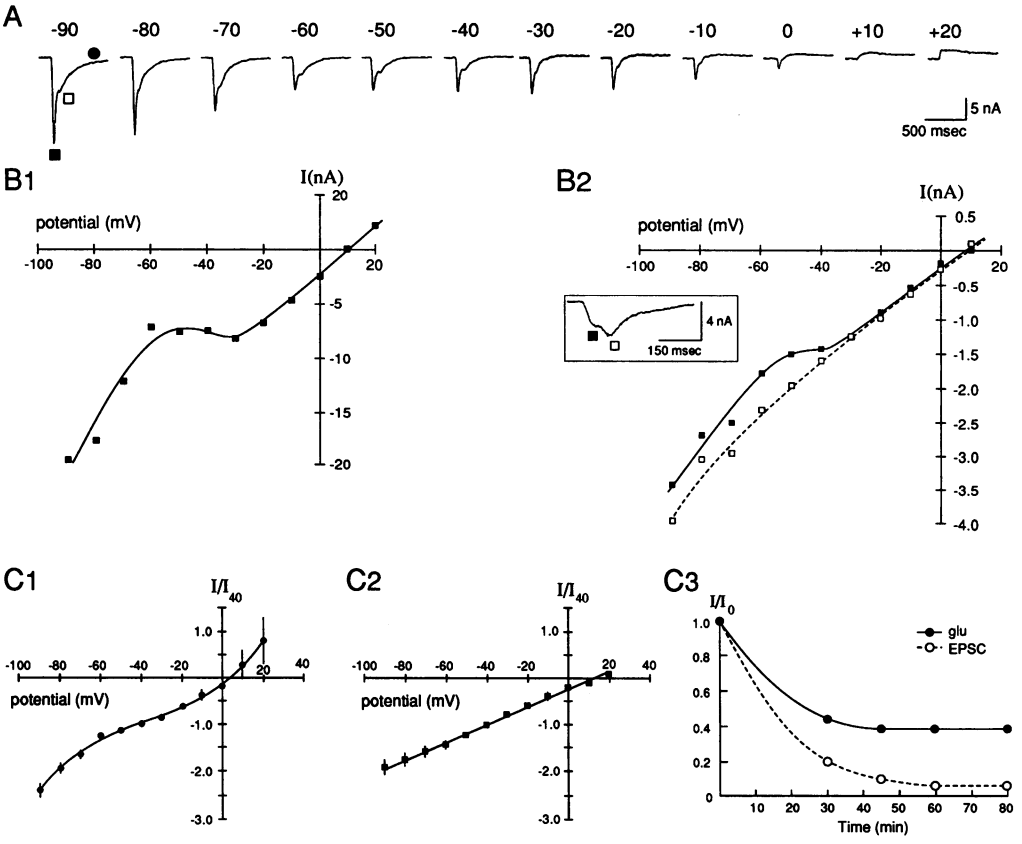

FIG. 3. The *I–V* relation for exogenous L-glutamate is also nonlinear. (*A*) Sample records of the response to 10 mM L-glutamate in a small L_{f_s} motor neuron at a series of potentials from −90 to +20 mV (*A*). The response has three components, a fast inward current (■), a slower inward current (□), and a small Cl^- current (●). (*B1*) *I–V* relation for the peak fast component versus voltage. On a different L_{f_s} motor neuron (*B2*) the slower inward current (□) was of similar amplitude to the fast (■) (see *Inset*) and had a linear *I–V* relation, whereas the fast component had a nonlinear relation. The Cl^- current evoked by L-glutamate was always much smaller than the fast and slower inward currents and appeared to have a slow onset (see record at +10 mV in *A*). The peak measures of the fast and slow inward currents would therefore have been relatively uncontaminated by this Cl^- current. (*C*) The *I–V* relation for the fast L-glutamate current can be made linear by lowering extracellular Mg^{2+}. A summary graph for the *I–V* relation of the L-glutamate response recorded in seven L_{f_s} motor neurons in normal sea water (*C1*) and from four L_{f_s} motor neurons in low Mg^{2+} sea water (*C2*). The response to L-glutamate and the synaptic current decrease in parallel during intracellular dialysis of the postsynaptic motor neuron (*C3*).

simulated the endogenous transmitter, we next examined the *I–V* relationships of the response to L-glutamate in L_{f_s} motor neurons. To evoke substantial inward currents, high doses (1–10 mM) of L-glutamate and very rapid application from a microperfusion system were required. These rather high doses may have been needed to increase the glutamate concentration sufficiently rapidly to obtain activation before the onset of receptor desensitization.

The glutamate current consisted of up to three components. One was small in amplitude, sustained, and reversed close to the chloride equilibrium potential (Fig. 3*A*, ●). Since this response was probably due to activation of a chloride channel, a known inhibitory action of L-glutamate in molluscs (10–12) and one that is presumably not related to the fast excitatory synaptic actions of the sensory neurons, we will not consider this component further. The other two components consisted of inward currents, one fast, the other slightly slower, each of which exhibited rapid desensitization. The relative size of the two inward currents was somewhat variable; in most cases they were of similar amplitude (Figs. 3*B2*, *Inset*, and 4*H*) but occasionally one or other component predominated (Figs. 3*A* and 4 *E* and *F*). In examples where the fast component was greater than the slower component, so that the peak of the fast current was relatively uncontaminated by the slower current, the *I–V* relation was very similar to that of the EPSC having a plateau region between −40 and −70 mV (Fig. 3 *A* and *B1*). Where the slower component was of similar size to the fast component (Fig. 3*B2*, *Inset*), the peak of the fast current was still nonlinear (Fig. 3*B2*); however the plateau region was less marked presumably due to contamination of the fast current by the slower current, the peak of which had a linear *I–V* relation (Fig. 3*B2*). When the data from all experiments were normalized to the current evoked at −40 mV and averaged, the mean *I–V* relation for the peak of the fast component of the glutamate current exhibited a flattening over the range −40 to −70 mV and a reversal potential between 0 and 10 mV (Fig. 3*C1*). Recordings of the responses to L-glutamate in low Mg^{2+} sea water showed that the *I–V* relation of the peak of the fast L-glutamate current became linear (Fig. 3*C2*). Thus, L-glutamate evoked two distinct inward currents, one of which had characteristics that were very similar to those of the synaptic current.

If L-glutamate is the sensory neuron transmitter, all of the neurons innervated by sensory cells should possess similar receptors to L-glutamate. We therefore also studied the effect of L-glutamate on another follower cell of the sensory neurons, the motor neuron L7. L7 exhibited multicomponent responses to L-glutamate very similar to those seen in the L_{f_s} motor neurons (n = 5, Fig. 4*H*). In addition, small motor cells grown in isolation *in vitro* exhibited identical responses to glutamate (n = 3, data not shown), eliminating the remote possibility that L-glutamate might cause the release of the endogenous transmitter from the sensory neuron terminals.

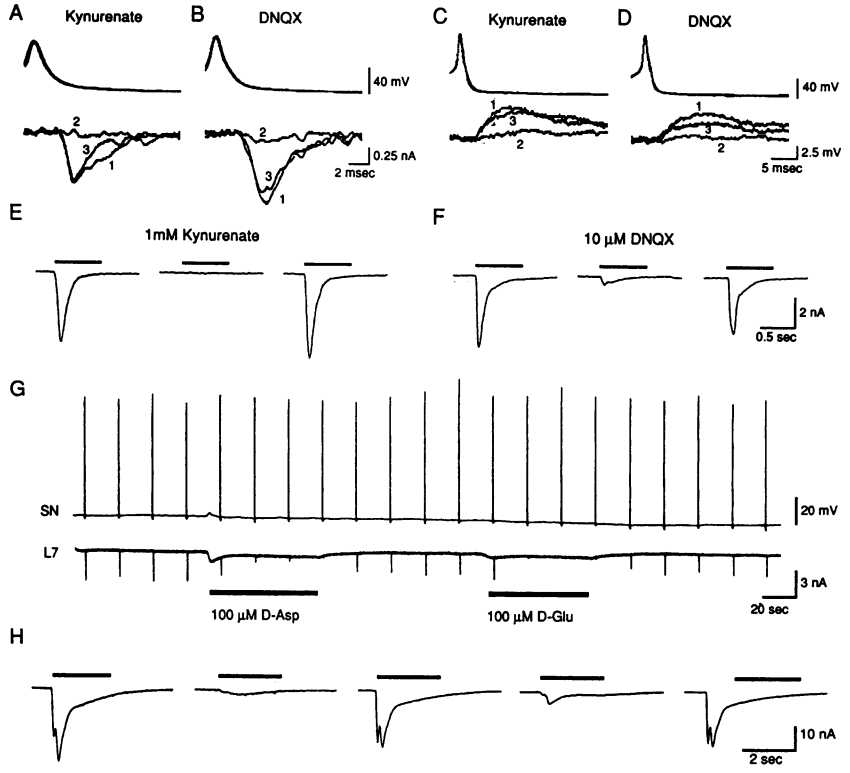

FIG. 4. Antagonism of the natural synaptic and glutamate responses. Kynurenate (1 mM) and 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM) greatly reduced the EPSC recorded in L_{f_s} motor neurons under voltage clamp in culture (*A* and *B*) and current clamp in the ganglion (*C* and *D*). The numbers indicate the traces during the control (1), drug application (2), and wash (3). The sensory neuron was stimulated once every 10 sec in *A* and *B* and once every 2 min in *C* and *D*. Kynurenate and DNQX were also able to block the responses to 10 mM L-glutamate (applied during bars in *E* and *F*). Synapses formed onto motor neuron L7 have similar properties. A slow record showing the effect of 100 μM D-aspartate and 100 μM D-glutamate (applied during bars) on EPSCs evoked in L7 (*G*, upper trace, sensory neuron; lower trace, L7). L7 also exhibited a multicomponent response to 10 mM L-glutamate (*H*, applied during bars) that was similar to that of the L_{f_s} motor neurons and was also blocked by the antagonists D-aspartate and D-glutamate (*H*).

The Current Produced by L-Glutamate Is Washed Out of the Motor Cell by Dialysis, in Parallel with the Synaptic Current. To test further whether L-glutamate does indeed activate the postsynaptic receptor utilized by the endogenous transmitter, we exploited whole-cell recordings to dialyze the postsynaptic neuron while measuring the response to L-glutamate and the natural synaptic current. Over a period of 1–4 hr, we observed a gradual diminution of the EPSC produced by stimulating the sensory neuron once every 15 min (a sufficiently long interval to avoid significant homosynaptic depression), which was paralleled by a decrease in the response to L-glutamate (Fig. 3*C3*, $n = 4$). This parallel washout of the L-glutamate and synaptic currents suggests that both use a receptor with similar properties and that this postsynaptic receptor may need to be phosphorylated to function. The lack of complete washout of the response to L-glutamate may have been due to the presence of extrasynaptic receptors, perhaps located on processes distal to the soma and thus more resistant to washout.

Antagonists of the Response to L-Glutamate Block the Synaptic Response. We next searched for agents that could block the synaptic current and the response to L-glutamate. We

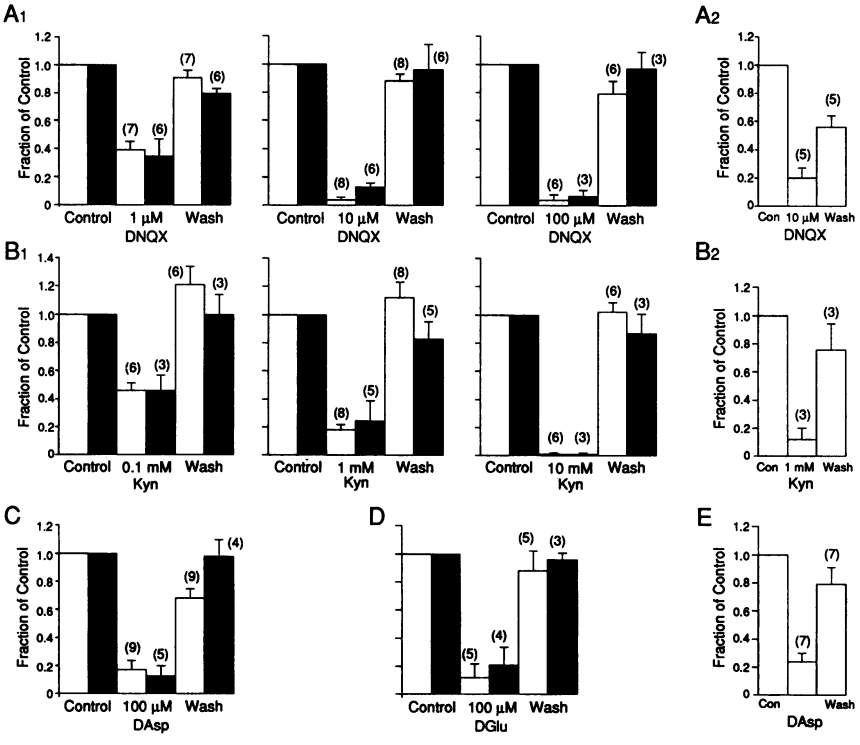

FIG. 5. The antagonists block the responses to the natural transmitter and L-glutamate in a dose-dependent manner. Summary bar graphs show the extent of blockade by DNQX at synapses in culture (*A1*) and in the ganglion (*A2*), kynurenate in culture (*B1*) and in the ganglion (*B2*), D-aspartate in culture (*C*), D-glutamate in culture (*D*), and (1–10 mM) D-aspartate in the ganglion (*E*). The error bars represent one standard error of the mean and the numbers above each bar represent the number of experiments. The open bars depict the synaptic response and the solid bars depict the response to 10 mM glutamate.

found four antagonists: kynurenic acid, DNQX, D-aspartate, and D-glutamate. Kynurenic acid and DNQX act as *N*-methyl-D-aspartate (NMDA) and non-NMDA antagonists in the vertebrate central nervous system, whereas the two D-amino acids are partial agonists of vertebrate glutamate receptors. All four antagonists were effective at blocking the synaptic current and the response to L-glutamate in a dose-dependent and reversible manner (Figs. 4 and 5). Furthermore, the antagonists were effective at synapses formed onto the small L_{f_s} motor neurons and motor neuron L7, confirming that similar receptors are present on the different sensory neuron targets (Fig. 4). Although neither kynurenic acid nor DNQX had any agonist activity on the motor neurons, D-aspartate and D-glutamate acted as partial agonists (Fig. 4). In addition, unlike the situation with kynurenic acid or DNQX, the potency of the two D-amino acids as antagonists seemed to vary, being strong in the summer months of June and July (Fig. 5) but weaker in October (data not shown). The phosphono-substituted analogues of the D-amino acids (D,L-aminophosphonobutyric acid and D,L-aminophosphovaleric acid) were ineffective as antagonists; however, NMDA, although possessing no agonist action, acted as an antagonist at 5 mM (data not shown).

We next tested whether our antagonists could block transmission at sensory synapses in the abdominal ganglion. Kynurenic acid (1 mM) and DNQX (10 μM) were highly effective at blocking the synaptic transmission (Figs. 4 *C* and *D* and 5 *A2* and *B2*) and, once again, had no agonist activity. By contrast, higher levels of D-aspartate (1–10 mM) were required (Fig. 5*E*) possibly due to the presence of avid glutamate-uptake mechanisms in the ganglion. At these higher levels D-aspartate caused depolarization of the motor cells and an increase in the spontaneous frequency of synaptic inputs to these cells.

DISCUSSION

L-Glutamate appears to mimic the endogenous transmitter in four ways. (*i*) It produced a fast inward current that had a similar reversal potential to the natural transmitter, indicating a similar ionic selectivity for the glutamate channel and synaptic channel. (*ii*) The *I–V* relationship of the EPSC and the L-glutamate current has a characteristic plateau region between −40 mV and −70 mV that seems to depend on extracellular Mg^{2+}. (*iii*) The synaptic current and that evoked by L-glutamate are affected similarly by dialysis of the postsynaptic neuron. (*iv*) A common set of antagonists blocks the synaptic current as well as the response to glutamate. This strongly suggests that a glutamate-like molecule is the endogenous transmitter of the sensory neurons. Since L-glutamate is the only naturally occurring amino acid or related substance to mimic the natural excitatory postsynaptic potential of the several we have examined (which include L-aspartate, L-homocysteate, γ-aminobutyric acid, glycine, α-ketoglutarate, fumarate, *N*-acetylglutamate, and a variety of α- and γ-linked glutamate-containing di- and tripeptides), we favor L-glutamate itself as the transmitter. However, until release of L-glutamate is demonstrated, definitive proof is lacking.

The glutamate receptor in *Aplysia*, which mediates the synaptic currents, has interesting similarities with the NMDA receptor of vertebrates. The *Aplysia* receptor undergoes weak voltage-dependent block by Mg^{2+}. In addition, the antagonists we have used also act on the NMDA receptor. This suggests that the channel pore and the transmitter recognition site of the *Aplysia* receptor may have some homology to those of the NMDA receptor. It is therefore tempting to suggest that this *Aplysia* receptor could represent an invertebrate homologue of the vertebrate NMDA receptor. If the *Aplysia* channel does indeed represent an evolutionary precursor of the NMDA channel, its further modification toward the vertebrate NMDA channel may have occurred during evolution from marine invertebrates, whose ionic composition of blood closely corresponds to sea water, to organisms capable of regulating their internal ionic environment. Sea water contains 55 mM Mg^{2+}, which therefore constitutes a significant ion for carrying current. By contrast, in organisms that evolved toward land-living forms (e.g., fresh water invertebrates, insects, and vertebrates), Mg^{2+} would cease to be of value for carrying current due to its relatively low concentration in blood and its high intracellular concentrations. Under these circumstances, evolutionary pressures may have favored accentuation of the voltage-dependent Mg^{2+} blockade. Interestingly, a receptor even closer to the NMDA receptor has been discovered in crayfish visual interneurons (extracellular Mg^{2+}, 2.6 mM), which is subject to a very strong blockage by Mg^{2+} (13). Among the higher invertebrates, land snails and insects may have receptors with yet stronger similarities to the vertebrate NMDA receptor.

We thank Drs. A. B. MacDermott and S. A. Siegelbaum for helpful comments on the manuscript, C. Lam for assistance with the art work, H. Ayers and A. Krawetz for typing the manuscript, and the Royal Society for support (N.D.).

1. Kandel, E. R. (1976) *Cellular Basis of Behavior* (Freeman, San Francisco).
2. Castellucci, V. F. & Kandel, E. R. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 5004–5008.
3. Castellucci, V. F. & Kandel, E. R. (1976) *Science* **194**, 1176–1178.
4. Watkins, J. C. & Evans, R. H. (1981) *Annu. Rev. Pharmicol. Toxicol.* **21**, 165–206.
5. Collingridge, G. L., Kehl, S. J. & McLennan, H. (1983) *J. Physiol. (London)* **334**, 33–46.
6. Rayport, S. G. & Schacher, S. (1986) *J. Neurosci.* **6**, 759–763.
7. Dale, N. & Kandel, E. R. (1990) *J. Physiol. (London)* **421**, 203–222.
8. Nowak, L., Bregestovski, P., Ascher, P., Herbert, A. & Prochiantz, A. (1984) *Nature (London)* **307**, 462–465.
9. Mayer, M. L., Westbrook, G. L. & Guthrie, P. B. (1984) *Nature (London)* **309**, 261–263.
10. Kejoe, J. (1978) *Nature (London)* **274**, 866–869.
11. Szczepaniak, A. C. & Cottrell, G. A. (1973) *Nature New Biol.* **241**, 62–64.
12. Yarowsky, P. J. & Carpenter, D. O. (1976) *Science* **192**, 807–809.
13. Pfeiffer-Linn, C. & Glantz, R. M. (1991) *Synapse* **9**, 35–42.