Unmyelinated Cutaneous Afferent Neurons Activate Two Types of Excitatory Amino Acid Receptor in the Spinal Cord of *Xenopus laevis* Embryos

Keith T. Sillar and Alan Roberts

Department of Zoology, University of Bristol, Bristol BS8 1UG, UK

The trunk and tail skin of *Xenopus laevis* embryos near the time of hatching is innervated by the mechanoreceptive free nerve endings of Rohon-Beard neurons, a homogeneous class of cutaneous primary afferent fibers. Rohon-Beard neurons have cell bodies and axons in the dorsal spinal cord, where they monosynaptically excite a population of dorsolaterally situated interneurons (Clarke and Roberts, 1984). EPSPs can be recorded in these dorsolateral interneurons following electrical stimulation of the unmyelinated neurites of Rohon-Beard neurons in the skin. The EPSPs are dual component, consisting of separate fast and slow potentials that are usually evoked synchronously and that closely resemble those described previously in *Xenopus* and lamprey motoneurons (Dale and Roberts, 1985; Dale and Grillner, 1986). The excitation of dorsolateral interneurons by Rohon-Beard neurons is reduced by the bath application of excitatory amino acid antagonists. Kynurenic acid suppresses both the fast and slow components of the EPSPs, while both (+/-)-2-amino-5-phosphonovaleric acid (APV) and 1 mM magnesium reduce the slow component but have little or no effect on the peak amplitude of the EPSPs. These data suggest that Rohon-Beard neurons release an excitatory amino acid neurotransmitter, which acts simultaneously at both *N*-methyl-D-aspartate (NMDA) and non-NMDA receptor types. This is the first direct demonstration of dual-component excitatory amino acid-mediated synaptic transmission from cutaneous primary afferent neurons in the vertebrate spinal cord. The bath application of the agonists NMDA, kainate, or quisqualate in salines containing 1 μ M TTX depolarized the interneurons and reduced their input resistance, which suggests that the interneurons possess all 3 types of excitatory amino acid receptor. Kynurenic acid strongly inhibits responses to NMDA and kainate, but is relatively less effective against the larger responses of quisqualate in this system.

The transmission of sensory information at the synapses of primary afferent neurons in the vertebrate spinal cord involves the release of neurotransmitters that evoke EPSPs in spinal neurons (Eccles, 1964), but the identity of the transmitter substance is

unknown for the majority of sensory cell categories. Neuropeptides such as substance P (Cuello and Kanazawa, 1978; Ljungdahl et al., 1978; Hunt et al., 1981), the nucleotide ATP (Jahr and Jessell, 1983), and excitatory amino acids like glutamate and aspartate (Davies and Watkins, 1981; Watkins and Evans, 1981; Jahr and Jessell, 1985; Jessell et al., 1986) are all candidates for a role in sensory transmission.

Excitatory amino acid neurotransmitters mediate neuronal excitation in diverse regions of the vertebrate CNS, including the cortex (Thomson et al., 1985), the hippocampus (Collingridge et al., 1983a, b), the caudate nucleus (Herrling et al., 1983) and the spinal cord (Davies et al., 1980; Davies and Watkins, 1981, 1983; Dale and Roberts, 1984, 1985; Dale, 1986; Dale and Grillner, 1986; Jessell et al., 1986). The endogenous transmitter, generally thought to be glutamate, aspartate, or a related substance, acts at up to 3 types of postsynaptic receptor, *N*-methyl-D-aspartate (NMDA), kainate, and quisqualate, named after their principal and selective agonists (Watkins and Evans, 1981).

Though they have often been implicated by circumstantial evidence, direct evidence for an involvement of excitatory amino acids in the transmission of sensory information is so far restricted to the synapse between Ia muscle spindle afferents and α -motoneurons in the rat spinal cord (Jessell et al., 1986). Here, L-glutamate is the likely transmitter, but its action does not appear to involve NMDA receptors except perhaps through polysynaptic pathways. Thus, evidence for a dual activation of both NMDA and non-NMDA receptors at the synapses of primary afferent neurons is still lacking. However, a role for both NMDA and non-NMDA receptors in synaptic transmission has recently been established in spinal motoneurons of both *Xenopus* embryos and lampreys. EPSPs evoked by intracellular stimulation of excitatory interneurons have 2 components with different time courses, a fast component, mediated by kainate/quisqualate receptors, and a slow component, mediated by NMDA receptors (Dale and Roberts, 1985; Dale and Grillner, 1986). In both animals there is strong evidence that the presynaptic neurons release a single transmitter that simultaneously activates both receptor types.

The skin of the trunk and tail of *Xenopus* embryos is innervated by a homogeneous population of mechanosensory afferents, called Rohon-Beard neurons. These have somata in the dorsal spinal cord (Hughes, 1957) and unmyelinated peripheral neurites that leave the spinal cord to form free nerve endings in the skin (Roberts and Hayes, 1977). The endings are sensitive to transient indentation of the skin, and they can also be specifically and unambiguously stimulated with short electrical cur-

Received May 11, 1987; revised Aug. 13, 1987; accepted Sept. 14, 1987.

This work was supported by the Medical Research Council (UK). We thank Mrs. Linda Teagle for expert technical assistance and Dr. Stephen R. Soffe for helpful discussions.

Correspondence should be addressed to Keith T. Sillar, Department of Zoology, University of Bristol, Woodland Road, Bristol BS8 1UG, UK.

Copyright © 1988 Society for Neuroscience 0270-6474/88/041350-11\$02.00/0

A *Xenopus laevis* stage 37/38

B

Figure 1. *A*, *Xenopus laevis* preparation at stage 37/38 showing the approximate locations of recording and stimulating electrodes. *B*, Schematic diagram of the spinal cord showing the location and morphology of a Rohon-Beard sensory neuron and a dorsolateral sensory interneuron (after Roberts and Clarke, 1982). The dendrites of the interneuron project from the soma into the dorsolateral tracts (DT) within which run the central axons of Rohon-Beard neurons. The spinal cord is approximately 50–100 μ m in diameter at this stage in development. Dorsal is at the top, rostral is to the left. NC, neural canal; MZ, marginal zone.

rent pulses to the skin (Clarke et al., 1984). Such stimulation evokes monosynaptic excitation of a population of sensory interneurons located dorsolaterally in the spinal cord (Clarke and Roberts, 1984). The simplicity in the organization of sensory pathways in the embryo thus offers a favorable opportunity to investigate the type of neurotransmitter released by an identified class of cutaneous sensory neuron in a vertebrate spinal cord. Previous immunohistochemical studies have revealed that the Rohon-Beard neurons show substance P-like immunoreactivity (Clarke et al., 1984; Gallagher and Moody, 1985). This evidence suggests that substance P could be the transmitter released by Rohon-Beard neurons, particularly in view of their many sim-

ilarities to mammalian C fibers. In the present paper we show that the monosynaptic excitation of dorsolateral interneurons in response to skin stimulation is mediated by 2 types of excitatory amino acid receptor. The postsynaptic potentials consist of 2 components, one mediated by kainate/quisqualate receptors and the other by NMDA receptors. These results suggest that substance P is not the primary transmitter released by Rohon-Beard neurons, though they do not preclude the possibility that substance P or a related peptide may serve some other, perhaps neuromodulatory role in this system.

Materials and Methods

All experiments were performed on stage 37/38 *Xenopus laevis* embryos (Nieuwkoop and Faber, 1956; Fig. 1) removed from their egg membranes, paralyzed in 70–100 μ M *d*-tubocurarine chloride (Sigma), and pinned on their sides with micropins through the notochord to the Sylgard surface of a Perspex table. The table, mounted in a 2 ml preparation bath, could be rotated about its long axis to facilitate dissection and recording. The preparation (Fig. 1A) was continuously superfused (ca. 5 ml/min) with frog Ringer's solution (containing 115 mM NaCl, 2.5 mM KCl, 5 mM CaCl₂, 2.4 mM NaHCO₃, buffered to pH 7.4) at room temperature. For experiments involving the bath application of excitatory amino acid agonists in the presence of TTX, a modified saline was used to ensure stable calcium concentrations. The saline contained 120 mM NaCl, 2.5 mM KCl, 5 mM CaCl₂, 15 mM NaHCO₃ (pH 7.6), and was continuously bubbled with 5% CO₂, 95% O₂. The activity of neurons recorded in this saline prior to TTX application was indistinguishable from that described elsewhere in the text.

The trunk skin was peeled back and removed, using fine mounted needles, to reveal the myotomes. Extracellular recordings of the activity of motoneuron axons were made with glass suction electrodes placed on the intermyotome clefts (cf. Kahn and Roberts, 1982). To obtain intracellular recordings, parts of the myotomes overlying the rostral spinal cord, from the otic capsules to about midtrunk level, were removed. Neurons in the dorsal half of the spinal cord were impaled, usually following capacity overcompensation, with electrodes filled with 3 M potassium acetate (pH 7.4) and having DC resistances of 150–350 M Ω . These electrodes were made on a Campden Instruments 753 microelectrode puller. Lower-resistance electrodes, which were suitable for recording from most other types of spinal neuron, were found to be inadequate for stable recordings from dorsolateral interneurons.

The peripheral neurites of Rohon-Beard neurons were stimulated electrically via glass suction electrodes placed on the tail skin (cf. Clarke et al., 1984; Fig. 1). Current pulses (0.5 or 1 msec duration) were used to evoke EPSPs in the interneurons and would often initiate episodes of fictive swimming. Unless otherwise stated, 1 μ M strychnine sulfate was used to abolish swimming responses and to ensure that inhibitory inputs to the interneurons (Clarke and Roberts, 1984) were blocked (Sillar, 1986), so that EPSPs could be studied in isolation.

The experiments were performed in magnesium-free control salines containing 5 mM calcium and 70–100 μ M curare. Pharmacological agents were added to the perfusate, either directly or by switching to a separate bottle of saline containing the agent. Excitatory amino acid antagonists (+/-)-2-amino-5-phosphonopivalic acid (APV) and kynurenic acid were obtained from Cambridge Research Biochemicals and Sigma, respectively. The excitatory amino acid agonists kainate, quisqualate, and NMDA were applied in similar fashion in salines containing 0.1 μ M TTX to block impulse activity and 1 μ M strychnine to block spontaneous inhibitory potentials (Sillar and Softe, 1987). These agents were supplied by Sigma.

Conventional recording and amplification techniques were used throughout. Signals were stored on a 4-channel Racal tape recorder and permanent records were made using a Gould 1401 digital oscilloscope coupled to an X-Y plotter. The majority of the results presented here were selected from 71 dorsolateral interneurons (see Results for method of identification) recorded in 57 embryos. Additional information from other spinal cord cell categories is also presented. These data were obtained by intracellular recording from 5 motoneurons, 10 Rohon-Beard neurons, and 17 dorsally located "rhythmic neurons." Presumed motoneurons were identified on the basis of their very ventral position in the cord (cf. Softe and Roberts, 1982). Criteria for the identification of other cell types are given in the text.

Figure 2. *A–C*, Three types of response recorded in dorsally located spinal cord neurons when electrical stimulation of the skin evokes an impulse in the peripheral neurite of a Rohon-Beard neuron. *A*, In Rohon-Beard neurons, the impulse arises at a latency of about 3 msec from a flat baseline and is not preceded by any synaptic excitation (Record supplied by Dr. J. D. W. Clarke). *B*, Dorsolateral interneurons receive short (4–10 msec)-latency EPSPs in response to skin stimulation that, when large enough, elicits an impulse. During the swimming activity that follows, the interneuron is inhibited (*starts at arrow*). *C*, Rhythmic neurons fire once per cycle during swimming (see text). They never fired impulses at short latency to skin stimulation. The initial response of these neurons to skin stimulation consists of a longer-latency (12–20 msec) IPSP, which is occasionally preceded by a small depolarization. Recordings in *A–C* were made in strychnine-free saline.

Results

Criteria for identification of dorsolateral interneurons

In order to investigate the neurotransmitter released by the primary afferent Rohon-Beard neurons in the spinal cord, we have made intracellular recordings from postsynaptic neurons that are excited by skin stimulation. To facilitate such recordings, the microelectrodes were aimed, under visual control, using a dissecting microscope (75 $\times$), at the dorsal quarter of the cord (Fig. 1), a region in which sensory interneurons are known to occur (Clarke and Roberts, 1984). The criteria used for the identification of “dorsolateral interneurons” followed those adopted by Clarke and Roberts (1984) in the same preparation. In outline: dorsolateral interneurons receive EPSPs at a latency of 4–10 msec in response to skin stimulation, which can sum to evoke a nonovershooting impulse at a latency of 6–15 msec (Fig. 2*B*). During swimming, these interneurons are inhibited by rhythmic IPSPs. These criteria distinguish them from the 2 other types of neuron that were also encountered in the same regions of the spinal cord (Fig. 2, *A*, *C*). Rohon-Beard sensory

Figure 3. EPSPs in dorsolateral interneurons. *A*, Stimulation of the ipsilateral tail skin at increasing intensity (*at arrow*) evokes a series of EPSPs that increases in amplitude in 4 or 5 discrete steps before spike threshold is reached. Note that the latency of the EPSPs is less than 10 msec. *B*, A threshold EPSP in a different interneuron. Two sweeps are superimposed, just above and just below the threshold, for the first response. The EPSP was evoked in an all-or-none fashion.

neurons fire an all-or-none overshooting impulse to a threshold skin stimulus (Fig. 2*A*). Also, they are not active and receive no obvious synaptic input during swimming activity. Rhythmic cells receive an IPSP to ipsilateral skin stimulation at a latency of 12–20 msec. The IPSP is occasionally preceded by an initial depolarization at slightly shorter latency (Fig. 2*C*). However, rhythmic cells did not fire impulses in response to skin stimulation except at long latency during the episodes of fictive swimming that sometimes ensued.

Rohon-Beard-evoked excitation of dorsolateral interneurons

Electrical current pulses to the skin of 1 msec excited the peripheral neurites of Rohon-Beard neurons, as described by Clarke et al. (1984). Such stimulation evoked EPSPs in dorsolateral interneurons that could be graded in a manner consistent with the sequential excitation of several Rohon-Beard neurons (Fig. 3*A*). On the basis of similar results and careful latency measurements, Clarke and Roberts (1984) concluded that Rohon-Beard central axons excite dorsolateral interneurons monosynaptically. Usually 4 or 5 increments in EPSP amplitude could be distinguished before spike threshold was reached, indicating that the area of skin beneath the stimulating electrode contained the processes of a similar number of Rohon-Beard neurons (cf. Roberts and Hayes, 1977; Clarke and Roberts, 1984). In contrast to Clarke and Roberts (1984), we observed few, if any, later polysynaptic components on the falling phases of the EPSPs, provided that swimming was not evoked.

The EPSPs recorded in the present study were generally of much longer duration (around 200 msec; see below) than those

Figure 4. Dependence of long-duration EPSPs on stable resting potentials. *A*, Approximately 1 min after penetration, a dorsolateral interneuron showed a resting potential of -60 mV. Skin stimuli above (*i*) and below (*ii*) spike threshold evoked responses of approximately 40 msec duration. *B*, Three minutes after penetration, the resting potential of this interneuron stabilized at -76 mV. Now suprathreshold *i* and subthreshold *ii* stimuli evoked responses lasting about 200 msec.

recorded in the previous study of dorsolateral interneurons (Clarke and Roberts, 1984). The most likely explanation for this is that the present results were obtained using microelectrodes that inflicted less damage upon penetration. Our recordings were stable for long periods (up to 2 hr), during which time the interneurons maintained resting potentials of over 55 mV (mean, 79.3) and would fire impulses (>50 mV) in response to depolarizing current pulses or skin stimulation. In support of this explanation, the EPSPs recorded early in a penetration could be very short (50 msec or less; Fig. 4*A*) and then increase in duration as the resting potential increased and stabilized. On other occasions, the EPSPs never exceeded about 50 msec. In these cases the neurons would not usually fire impulses to injected current or skin stimulation. These recordings were often lost in less than 15 min and were not used for the analysis of EPSPs or for pharmacological manipulations.

Characteristics of threshold EPSPs

As stimulus intensity was increased, the first EPSP recorded in dorsolateral interneurons appeared at a discrete and repeatable

Figure 5. Time course and amplitude of all-or-none threshold, skin-evoked EPSPs. *A*, *i-iii*. Examples of threshold EPSPs in 3 different interneurons. The EPSPs were generally large and of long duration. *B*, *C*, Distribution of the amplitudes and durations of 50 threshold EPSPs from 13 dorsolateral interneurons.

stimulus intensity, at a constant latency, and in an all-or-none fashion (Fig. 3*B*). This suggests that each threshold EPSP results from a single presynaptic impulse in a single Rohon-Beard neuron, and provides additional evidence for monosynaptic connections between these 2 cell types. In general, these threshold, presumed-unitary EPSPs are large (Figs. 5–7), with a mean amplitude of 8.3 ± 3.5 mV ($n = 50$). If the thresholds of some Rohon-Beard neurons are similar, certain of the largest-threshold EPSPs could have resulted from the recruitment of 2 or more presynaptic neurons. However, the fact that the smallest-threshold EPSP we recorded was approximately 3 mV suggests that each Rohon-Beard neuron produces large, unitary EPSPs. Clarke and Roberts (1984) detected EPSPs as small as 1.5 mV, again suggesting that less stable penetrations with lower-impedance electrodes caused damage to the cells.

Threshold EPSPs, like compound EPSPs, were usually long in duration, lasting for up to 260 msec (mean, 165.7 ± 71.1

Figure 6. Variability in the rise time of skin-evoked EPSPs. *A*, *i–iii*, Three threshold EPSPs evoked in one interneuron at the same intensity and position of stimulation. The EPSPs usually had a time to peak of 2–3 msec but occasionally a slower rise time of 12–20 msec was observed (e.g., *ii*). Note that in *A*, *i*, the EPSP has a fast rise and fall (cf. Fig. 7, *A*, *iii*, and text). *B*, Distribution of rise times in 50 threshold EPSPs recorded in 13 interneurons (same population as illustrated in Fig. 5).

msec, $n = 50$; Fig. 5). However, they were clearly distributed in 2 populations and about 30% had durations of 50–70 msec (Fig. 5C). Most EPSPs had times to peak of about 2–3 msec (Fig. 6, *A*, *B*), but in some the rise time was considerably longer (Fig. 6*A*, *ii*, *B*).

Dual-component threshold EPSPs

The occurrence of threshold EPSPs with fast or slow rise times and short or long durations reflects the presence of at least 3 types of response. The most frequently recorded threshold EPSP had a mixed time course, with a fast rise and slow fall (Figs. 5*A*; 7*A*, *i*). However, fast rise and fall- or slow rise and fall-threshold EPSPs were also observed. On 3 occasions these 3 types of response were recorded in a single interneuron at a constant stimulus intensity and position (e.g., Figs. 6*A*, 7*A*). Since the threshold EPSPs probably result from a single presynaptic Rohon-Beard impulse, a possible explanation for this observation is that the most common mixed form of EPSP is a summation of separate fast and slow components. Since the

Figure 7. Fast, slow, and mixed components of threshold EPSPs. *A*, *i–iii*, The 3 forms of EPSP recorded in a single interneuron at a constant stimulus intensity and position near response threshold. The commonest form of EPSP (*i*) had a fast rise (ca. 2–3 msec to peak) and slow fall (ca. 200 msec duration). The second form of EPSP (*ii*) had a slow rise (13 msec to peak) and fall time. The third EPSP (*iii*) had a fast rise and fast fall time. *B*, The fast and slow EPSPs had latencies identical to the mixed EPSP and might therefore derive from the same presynaptic spike.

2 components had identical latencies to onset (Fig. 7*B*), the possibility that the dual-component EPSPs could arise from the simultaneous activation of 2 different presynaptic neurons with very similar thresholds therefore seems unlikely. On raising the stimulus above that for a threshold, apparently unitary response, compound EPSPs were always of mixed time course and fast- or slow-component potentials alone were never seen.

Pharmacology of EPSPs

An explanation for EPSPs with different rise times and durations has been presented for dual-component potentials recorded in *Xenopus* and lamprey motoneurons (Dale and Roberts, 1985; Dale and Grillner, 1986). For *Xenopus* motoneurons, it was shown that the separate fast and slow components of EPSPs evoked by a single presynaptic neuron result from the activation of 2 types of excitatory amino acid receptor on the postsynaptic neuron. Activation of one type, the NMDA receptor, led to a slow rise and fall EPSP, while activation of non-NMDA receptors produced a fast rise and fall EPSP. In view of the striking similarities to the EPSPs described here in dorsolateral interneurons, we have used excitatory amino acid antagonists to test whether these 2 types of receptor are activated by Rohon-Beard neurons.

The bath application of kynurenic acid (at 0.5–2 mM), a broad-spectrum antagonist of both NMDA and non-NMDA receptors (Perkins and Stone, 1982; Ganong et al., 1983; Herrling, 1984) (though see later), resulted in a reversible reduction in all the

Figure 8. Kynurenic acid reduces EPSPs in dorsolateral interneurons. *A*, A 2 mM amount of kynurenic acid reversibly reduces the amplitude of skin-evoked EPSPs. Prior to bath application of kynurenic acid, a stimulus just subthreshold for spike initiation evoked a compound EPSP of about 36 mV (not shown). Stimulus intensity was then set slightly higher, at a level just sufficient to evoke an impulse in the interneuron (top trace). The amplitude of the response after 100 msec (at arrow) was 7.7 mV. In kynurenic acid, the peak amplitude of the response and the amplitude at 100 msec were reduced progressively over a 3 min period (middle 3 traces). The bottom trace shows recovery of the EPSP 3 min after returning to control saline. *B*, Graph of the experiment shown in *A*. EPSP amplitudes at the peak (○) and at 100 msec (▲) are plotted as a percentage of the amplitude before treatment with kynurenic acid. Note that the initial peak amplitude and the amplitude at 100 msec are reduced at comparable rates and to similar degrees.

EPSPs evoked by skin stimulation (Figs. 8, and 10). The peak amplitude of compound EPSPs (fast component) and the EPSP amplitude 100 msec after onset (slow component) were reduced to very similar extents (Fig. 8, *A*, *B*). These effects were consistent: At 1 mM the peak amplitudes of compound EPSPs were reduced by $61 \pm 7\%$ ($n = 4$) and the EPSP at 100 msec by $74 \pm 2.5\%$ ($n = 2$); at 2 mM the peaks of the EPSPs were reduced by $81 \pm 5.6\%$ ($n = 2$) and the EPSPs at 100 msec by $86 \pm 2\%$ ($n = 2$). A similar result was obtained with a graded series of stimuli at increasing intensity (see Fig. 10*A*). The compound EPSPs within the series, comprising the summation of several smaller monosynaptic EPSPs, were all reduced in a similar and continuously graded way.

An involvement of NMDA receptors in the response to skin stimulation was revealed by the bath application of the specific antagonist, APV. A total of 30 or 50 μ M APV reversibly reduced the amplitude of the EPSPs at 100 msec, but had less effect on

Figure 9. APV selectively blocks the slow NMDA component of skin-evoked EPSPs. *A*, A skin stimulus in control saline evoked a compound EPSP of 34 mV and triggered a spike in an interneuron (top trace). The same stimulus after 6 min in 50 μ M APV still evoked an impulse in the cell, but the amplitude of the EPSP at 100 msec (at arrow) was reduced from 9.7 mV to about 1 mV. The compound EPSP (measured by slightly reducing the stimulus intensity to just below spike threshold) fell by only 23%. *B*, Graph of the experiment shown above (measured as in Fig. 8). Note that the EPSP peak amplitude is slightly reduced while the amplitude at 100 msec is reduced to an extent similar to that observed in kynurenic acid (Fig. 8).

the amplitude of the early fast component (Figs. 9, *A*, *B*; 10*B*). A total of 50 μ M APV reduced the initial peak EPSP by only $25 \pm 8.3\%$ ($n = 3$), while 30 μ M APV had a smaller effect, reducing the EPSPs by $17 \pm 5.2\%$ ($n = 3$). In contrast, 30 or 50 μ M APV had a much larger effect on the amplitude of the EPSPs after 100 msec, reducing them by $97.5 \pm 4.8\%$ ($n = 6$).

It has been shown that magnesium ions exert a voltage-dependent block of the NMDA receptor ion channel (Mayer et al., 1984; Nowak et al., 1984). The bath application of 1 mM magnesium had little or no effect on the peak amplitude of the EPSPs, but significantly reduced the amplitude of the slow component and the half-fall time of the EPSPs (Fig. 11). Threshold EPSPs were reduced in total duration from over 200 msec to approximately 50 msec (Fig. 11*A*), suggesting that the remaining EPSP resulted entirely from the activation of non-NMDA receptors. These effects were also obvious in the responses to a graded series of stimuli at increasing intensity (Fig. 11*B*). The maximum durations of the compound EPSPs were all reduced to the same degree.

The above results, taken together with the effects of APV and

Figure 10. Comparison of the effects of kynurenic acid and APV on skin-evoked EPSPs. *A*, *i–iii*, Kynurenic acid reversibly reduces the amplitude and duration of EPSPs in a graded series of stimuli at increasing intensity. *B*, *i–iii*, APV reduces the duration of EPSPs in a similar graded series, but has little effect on the initial peak amplitude of the EPSPs.

kynurenic acid, demonstrate that the EPSPs in dorsolateral interneurons result from the activation of both NMDA and non-NMDA receptor types by a transmitter released from the Rohon-Beard neurons.

Effects of excitatory amino acid agonists

The blockade of Rohon-Beard-evoked EPSPs by the specific NMDA receptor antagonist APV and by the broad-spectrum excitatory amino acid antagonist kynurenic acid strongly suggests that the dorsolateral interneurons possess at least 2 types of excitatory amino acid receptor. Dale and Roberts (1984) have reported that *Xenopus* embryo motoneurons have receptors for NMDA, kainate, and quisqualate. To determine whether the dorsolateral interneurons possess a similar complement of receptors, intracellular recordings have been made from cells in salines containing 0.1 μM TTX to block spike mediated synaptic transmission (Soffe, 1987) and 1 μM strychnine sulfate to block TTX-resistant spontaneous IPSPs (Sillar and Soffe, 1987). Depolarizing current pulses, sufficient to evoke impulses, were injected intracellularly during TTX application. When all impulses had failed, it was assumed that TTX had blocked impulse activity throughout the cord. Trains of hyperpolarizing current pulses (8 at 1 Hz every 30 sec) were then injected to monitor changes in cell input resistance during agonist application. Since the saline used in this section was modified from that of Dale and Roberts (1984) (see Materials and Methods), some experiments were also performed on motoneurons to check whether the responses to bath-applied agonists were similar. The results in this and the following section are taken from 8 dorsolateral

interneurons and 5 motoneurons recorded intracellularly in 13 preparations.

All 3 agonists depolarized the interneurons and motoneurons and decreased their input resistance (Fig. 12, Table 1). At 30 or 60 μM, the responses to NMDA were similar in magnitude in these 2 types of neuron and were comparable to the effects of 5 μM kainate. However, 4–8 μM quisqualate evoked much larger depolarizations, associated with a more dramatic decrease in input resistance. The effects of quisqualate were also far larger than those reported previously for *Xenopus* motoneurons by Dale and Roberts (1984). In summary, the bath application of excitatory amino acid agonists has shown that the dorsolateral interneurons, like motoneurons, possess all 3 types of excitatory amino acid receptor.

Table 1. Excitatory amino acid agonist-evoked depolarizations and conductance changes in dorsolateral interneurons and motoneurons

Agonist	Amplitude (mV)		
	Dorsolateral interneurons (n = 8)	Motoneurons (n = 5)	% Conductance change (n = 8)
NMDA	35.8 ± 15.6 (6)	28.7 ± 3.0 (4)	12.3 ± 14.6 (4)
Kainate	21.2 ± 4.2 (5)	30.6 ± 7.8 (5)	22.3 ± 6.0 (7)
Quisqualate	65.5 ± 7.2 (4)	61.5 ± 9.2 (4)	59.2 ± 14.0 (6)

n = Number of neurons tested. Numbers in parentheses are numbers of applications of agonist from which the mean and SD were calculated. Agonist concentrations: NMDA, 30–60 μM; kainate, 5 μM; quisqualate, 4–8 μM.

Figure 11. Magnesium ions block the slow components of mixed EPSPs. *A, i–iii*, Four consecutive and superimposed threshold EPSPs before, during, and after the bath application of 1 mM magnesium. In the presence of magnesium, the slow component of these mixed EPSPs is considerably reduced and they resemble the fast EPSPs occasionally observed in the absence of magnesium (see, e.g., Figs. 6*A*, 7). The half-fall time of the EPSPs (*closed arrow*) is thus considerably reduced. *B*, A graded series of stimuli before, during, and after the application of 1 mM magnesium. The maximum duration of the EPSPs (*open arrows*) was reversibly reduced from about 220 to about 70 msec. The initial peak EPSP amplitude was reduced to a much smaller extent. The effects of magnesium are comparable to the effects of APV (cf. Figs. 9, 10).

Specificity of antagonists

Both APV and magnesium have been shown to specifically block NMDA receptors on *Xenopus* embryo neurons (Dale and Roberts, 1984; S. R. Soffe, personal communication), as well a variety of other vertebrate neurons (e.g., Dale and Grillner, 1986). However, the broad-spectrum antagonist kynurenic acid, which is thought to block all types of excitatory amino acid receptor (Perkins and Stone, 1982; Ganong et al., 1983; but see Miller and Coleman, 1985), has not been tested previously in the *Xenopus* embryo preparation. In 5 motoneurons and 8 dorsolateral interneurons in 13 embryos, 0.5–2 mM kynurenic acid reduced the depolarizations evoked by 30 μ M NMDA and 5 μ M kainate by 82% or more (Fig. 12, *A, B*). However, kynurenic acid only reduced the depolarizations evoked by 4–8 μ M quisqualate by $8.8 \pm 1\%$ and only caused a small recovery in cell input resistance. This was true also for 2 μ M quisqualate, which evoked a smaller depolarization of 35 mV in one motoneuron and which was decreased by only 25% in 1 mM kynurenic acid. Thus, while kynurenic acid reduced the depolarizations evoked by all 3 agonists, it had a stronger effect against kainate and NMDA than against quisqualate.

Discussion

This paper presents evidence that cutaneous afferent neurons release an excitatory amino acid neurotransmitter at their central synapses onto sensory interneurons in the spinal cord. The

transmitter activates NMDA and non-NMDA receptors to produce dual-component EPSPs.

Origin of EPSPs

We have recorded intracellularly from dorsolateral interneurons in the spinal cord of *Xenopus* embryos and evoked EPSPs in them by stimulating the skin electrically. Direct evidence that this form of stimulation excites the peripheral neurites of mechanosensory Rohon-Beard neurons innervating the skin was presented by Clarke et al. (1984). The previous evidence that the central axons of Rohon-Beard neurons make monosynaptic contact with dorsolateral interneurons was based mainly on the short latency of the EPSPs (Clarke and Roberts, 1984). Our results add further support for a monosynaptic connection, since the EPSPs evoked by a just-suprathreshold skin stimulus were recruited at a short and constant latency and in an all-or-none fashion. The unitary characteristics of the EPSPs at a threshold stimulus intensity strongly suggest that they result from single presynaptic impulses in single Rohon-Beard neurons. As the stimulus intensity was increased above threshold, the EPSP usually increased in 4 or 5 discrete steps, and both unitary and compound EPSPs had smooth falling phases (Figs. 2–7, 11). These observations suggest that the compound suprathreshold EPSPs are produced by a simple summation of the unitary EPSPs evoked when up to 4 or 5 Rohon-Beard neurons are stimulated simultaneously. The density of Rohon-Beard neu-

Figure 12. Effects of kynurenic acid on agonist-evoked depolarizations. Depolarizations evoked in dorsolateral interneurons by the bath application of 30 μ M NMDA (*A*) or 5 μ M kainate (*B*) were powerfully blocked by 1 mM kynurenic acid. Responses to quisqualate (*C*) at 8 μ M were much larger (see Table 1), and kynurenic acid had only a small antagonistic effect on these responses. Experiments were performed in the presence of 0.1 μ M TTX and 1 μ M strychnine.

rites beneath the skin is quite compatible with our 50- μ m-tip-diameter electrode exciting this number of neurites (Roberts and Hayes, 1977). When stimulating currents were high enough to evoke swimming, later, presumably polysynaptic, potentials were seen on the falling phases of the EPSPs, as reported by Clarke and Roberts (1984).

It has recently been shown that, in addition to the monosynaptic EPSPs from Rohon-Beard cell activation, dorsolateral interneurons also receive frequent spontaneous EPSPs in salines containing TTX (Roberts and Sillar, 1987; Sillar and Soffe, 1987). The spontaneous EPSPs, which are also abolished by kynurenic acid, have time courses similar to those of the spike-mediated fast, slow, and mixed EPSPs described in the present paper (K. T. Sillar, unpublished observation). This feature makes the possibility of a polysynaptic pathway being activated in response to a threshold skin stimulus extremely unlikely.

Dual-component EPSPs

Both threshold and compound EPSPs usually have a fast rise and slow fall time and last around 200 msec (e.g., Fig. 5*A*).

Reasons that the slow fall EPSPs were not seen by Clarke and Roberts (1984) were given in Results. The occurrence of separate fast and slow threshold potentials in some interneurons suggests that the commonest EPSP, with a fast rise and slow fall, is the summation of separate fast- and slow-component EPSPs. Our inference that the 2 components of the mixed EPSPs arise from a single presynaptic Rohon-Beard impulse is based on the observation that each component has the same threshold and latency, and also on the precedent that similar dual-component EPSPs have been described previously at central synapses onto motoneurons in the *Xenopus* embryo (Dale and Roberts, 1985) and in the lamprey (Dale and Grillner, 1986) spinal cord. The inference is also reinforced by pharmacological properties that are common to all of these dual-component EPSPs (see below).

Sensory transmitter

All the EPSPs evoked in dorsolateral interneurons by skin stimulation were reduced by the broad-spectrum excitatory amino acid antagonist kynurenic acid (Figs. 8, 10). We conclude that the transmitter released by Rohon-Beard neurons is an excitatory amino acid. The selective antagonism of the slow components, but not of the initial fast components, of the EPSPs by APV (Figs. 9, 10) and magnesium ions (Fig. 11) provides evidence that the transmitter acts at both NMDA and non-NMDA receptors. The bath application of the excitatory amino acid agonists kainate, quisqualate, and NMDA has shown that the postsynaptic dorsolateral interneurons possess all 3 types of receptor (Fig. 12, Table 1) and that kynurenic acid strongly antagonized the depolarizations evoked by kainate and NMDA. However, kynurenic acid was relatively less effective against quisqualate.

It has been shown previously that Rohon-Beard neurons have substance P-like immunoreactivity (Clarke et al., 1984; Gallagher and Moody, 1985). However, although they may indeed contain substance P (or a related tachykinin), we have shown that the primary transmitter released in the spinal cord acts at excitatory amino acid receptors. Thus, the present results also suggest that 2 different transmitter systems may coexist within a single class of cutaneous afferent neuron. Preliminary experiments, using the bath application of substance P or Eledoisin-related peptide (at 10 μ M), support the notion that substance P does not play a major role in fast synaptic transmission in this system (K. T. Sillar, unpublished observations). Neither substance P nor Eledoisin produced any discernible polarization of intracellularly recorded spinal neurons or significantly modified their responses to skin stimulation. This does not, however, rule out the possibility that substance P or a related peptide could have long-term central effects or that it may play some other, perhaps neuromodulatory, role in the periphery.

Recent evidence from a number of vertebrate preparations indicates that excitatory amino acid transmitters play an important and perhaps general role in the transmission of sensory information, in addition to being involved in a variety of higher integrative processes. In the rat spinal cord it has been shown that the fast EPSPs in α -motoneurons following specific stimulation of 1a muscle spindle afferents are blocked by kynurenic acid (Jessell et al., 1986). The EPSPs were not affected by the specific NMDA receptor antagonist APV, suggesting that the endogenous transmitter (γ -glutamate or a related compound) acts at kainate and/or quisqualate receptors. However APV blocked later components of the excitation of motoneurons, indicating that NMDA receptors may be activated by some

interposed interneurons in a polysynaptic pathway. A similar situation may occur in the responses of ventrobasal thalamus neurons to natural stimulation of vibrissal follicle afferents in the rat (Salt, 1986). Until now, the evidence for an involvement of excitatory amino acid transmitters in cutaneous sensory pathways was indirect. For example, synaptic transmission between dorsal root ganglion and dorsal horn neurons in culture is also blocked by excitatory amino acid antagonists, although NMDA receptors do not appear to be involved (Jahr and Jessell, 1985).

At present we can only speculate as to the functional role of dual-component EPSPs at the first synapse in the embryo's skin sensory system. The slow components of similar potentials in the embryo's motor system have been proposed as generating a tonic background depolarization during swimming. The summation of long-duration NMDA receptor-mediated synaptic potentials at normal swimming frequencies is likely to underlie the maintenance of sustained motor responses to brief trigger stimuli (Roberts et al., 1984). If such summation also occurred during repetitive skin stimulation, then it could play an important role in boosting the responsiveness of the swimming system to skin sensory inputs. Each Rohon-Beard neuron fires one or a few impulses to a transient local indentation of the skin within its receptive field (Clarke et al., 1984). Since the receptive fields of neighboring Rohon-Beard neurons are overlapping, a brisk stroke to the skin could create both temporal and spatial summation of EPSPs in the sensory interneurons. The long duration of the EPSPs due to NMDA receptor activation, would enhance the summation of skin inputs, since the probability of firing a postsynaptic neuron is likely to increase if the delay between successive presynaptic impulses is less than the duration of the slow component of the EPSP. Thus, a possible role for dual-component synaptic transmission at these sensory synapses could be to prime the system in response to stimuli below the threshold for the initiation of swimming.

The blockade of the NMDA receptor ion channel by low concentrations of magnesium (Fig. 11; Mayer et al., 1984; Nowak et al., 1984) calls into question the behavioral validity of the above hypothesis if magnesium is present in the extracellular environment. However the blockade is voltage-dependent, operating maximally near neuronal resting potential. Hence it is possible that fast EPSPs produced by Rohon-Beard inputs to the dorsolateral interneurons, such as would occur during brisk strokes to the skin, could depolarize the postsynaptic neurons sufficiently to transiently remove the magnesium block of the NMDA receptor ion channels. This, in turn, would facilitate the temporal and spatial summation of Rohon-Beard inputs. Recently, voltage-jump experiments have shown that the blocking and unblocking kinetics of the NMDA receptor ion channels in magnesium is extremely rapid, such that current flow is close to its steady state within 1–2 msec (Mayer and Westbrook, 1985).

References

- Clarke, J. D. W., and A. Roberts (1984) Interneurons in the *Xenopus* embryo spinal cord: Sensory excitation and activity during swimming. *J. Physiol. (Lond.)* 354: 345–362.
- Clarke, J. D. W., B. P. Hayes, S. P. Hunt, and A. Roberts (1984) Sensory physiology, anatomy and immunohistochemistry of Rohon-Beard neurones in embryos of *Xenopus laevis*. *J. Physiol. (Lond.)* 348: 511–525.
- Collingridge, G. L., S. J. Kehl, and H. McLennan (1983a) The antagonism of amino acid induced excitations of rat hippocampal neurones *in vitro*. *J. Physiol. (Lond.)* 334: 19–31.
- Collingridge, G. L., S. J. Kehl, and H. McLennan (1983b) Excitatory amino acids and synaptic transmission in Schaffer collateral-commissural pathway of the rat hippocampus. *J. Physiol. (Lond.)* 334: 33–46.
- Cuello, A. C., and I. Kanazawa (1978) The distribution of substance P immunoreactive fibers in the rat central nervous system. *J. Comp. Neurol.* 178: 129–156.
- Dale, N. (1986) Excitatory synaptic drive for swimming mediated by amino acid receptors in the lamprey. *J. Neurosci.* 6: 2662–2675.
- Dale, N., and S. Grillner (1986) Dual-component synaptic potentials in the lamprey mediated by excitatory amino acid receptors. *J. Neurosci.* 6: 2653–2661.
- Dale, N., and A. Roberts (1984) Excitatory amino acid receptors in *Xenopus* embryo spinal cord and their role in the activation of swimming. *J. Physiol. (Lond.)* 348: 527–543.
- Dale, N., and A. Roberts (1985) Dual-component amino-acid-mediated potentials: Excitatory drive for swimming in *Xenopus* embryos. *J. Physiol. (Lond.)* 363: 35–59.
- Davies, J., and J. C. Watkins (1981) Pharmacology of glutamate and aspartate antagonists on cat spinal neurones. In *Glutamate as a Neurotransmitter*, G. Di Chiara and G. L. Chessa, eds., pp. 275–284, Raven, New York.
- Davies, J., and J. C. Watkins (1983) Role of excitatory amino acid receptors in mono- and polysynaptic excitation in the cat spinal cord. *Exp. Brain Res.* 49: 280–290.
- Davies, J., R. H. Evans, A. A. Francis, A. W. Jones, and J. C. Watkins (1980) Excitatory amino acid receptors in the vertebrate CNS. In *Neurotransmitters and their Receptors*, V. Z. Littauer, ed., pp. 333–347, Wiley, New York, London.
- Eccles, J. C. (1964) *The Physiology of Synapses*, Springer-Verlag, New York.
- Gallagher, B. C., and S. A. Moody (1985) Soc. Neurosci. Abstr. 11: 1063.
- Ganong, A. H., T. H. Lanthorn, and C. N. Cotman (1983) Kynurenic acid inhibits synaptic and amino acid-induced responses in the rat hippocampus and spinal cord. *Brain Res.* 273: 170–174.
- Herrling, P. L. (1984) Evidence that the cortically evoked e.p.s.p. in cat caudate neurones is mediated by non-NMDA excitatory amino acid receptors. *J. Physiol. (Lond.)* 353: 98P.
- Herrling, P. L., R. Morris, and T. E. Salt (1983) Effects of excitatory amino acids and their antagonists on membrane and action potentials of cat caudate neurones. *J. Physiol. (Lond.)* 339: 207–222.
- Hughes, A. F. W. (1957) The development of the primary sensory system in *Xenopus laevis* (Daudin). *J. Anat.* 91: 323–338.
- Hunt, S. P., J. S. Kelly, P. C. Emson, J. R. Kimmel, R. J. Miller, and J.-Y. Wu (1981) An immunohistochemical study of neuronal populations containing neuropeptides or γ -amino-butyrate within the superficial layers of the rat dorsal horn. *Neuroscience* 6: 1883–1898.
- Jahr, C. E., and T. M. Jessell (1983) ATP excites a subpopulation of rat dorsal horn neurones. *Nature* 304: 730–733.
- Jahr, C. E., and T. M. Jessell (1985) Synaptic transmission between dorsal horn neurones in culture: Antagonism of monosynaptic EPSPs and glutamate excitation by kynurenate. *J. Neurosci.* 5: 2281–2289.
- Jessell, T. M., K. Yoshioka, and C. E. Jahr (1986) Amino acid receptor-mediated transmission at primary afferent synapses in rat spinal cord. *J. Exp. Biol.* 124: 239–258.
- Kahn, J. A., and A. Roberts (1982) The central nervous origin of the swimming motor pattern in embryos of *Xenopus laevis*. *J. Exp. Biol.* 99: 185–196.
- Ljungdahl, A., T. Hökfelt, and G. Nilsson (1978) Distribution of substance P-like immunoreactivity in the central nervous system of the rat. I. Cell bodies and nerve terminals. *Neuroscience* 3: 861–943.
- Mayer, M. L., and G. L. Westbrook (1985) The action of *N*-methyl-D-aspartic acid on mouse spinal neurones in culture. *J. Physiol. (Lond.)* 361: 65–90.
- Mayer, M. L., G. L. Westbrook, and P. B. Guthrie (1984) Voltage-dependent block by Mg^{++} of NMDA responses in spinal cord neurones. *Nature* 309: 261–263.
- Miller, R. F., and P. A. Coleman (1985) Kynurenic acid distinguishes between quisqualate and kainic acid receptors in the mudpuppy retina. *Soc. Neurosci. Abstr.* 15: 105.
- Nieuwkoop, P. D., and J. Faber (1956) *Normal Tables of Xenopus laevis* (Daudin), North-Holland, Amsterdam.
- Nowak, L., P. Bregestovski, P. Ascher, A. Herbert, and A. Prochiantz (1984) Magnesium gates glutamate-activated channels in mouse central neurones. *Nature* 307: 462–465.

- Perkins, M. N., and T. W. Stone (1982) An iontophoretic investigation of the actions of convulsant kynurenines in and their interaction with the endogenous excitant quinolinic acid. *Brain Res.* 247: 184–187.
- Roberts, A., and J. D. W. Clarke (1982) The neuroanatomy of an amphibian embryo spinal cord. *Phil. Trans. R. Soc. [Biol.]* 296: 195–212.
- Roberts, A., and B. P. Hayes (1977) The anatomy and function of free nerve endings in an amphibian skin sensory system. *Proc. R. Soc. [Biol.]* 196: 415–429.
- Roberts, A., and K. T. Sillar (1987) Unmyelinated skin afferent neurones, Rohon-Beard cells, release an excitatory amino acid in the spinal cord of *Xenopus laevis* embryos. *J. Physiol. (Lond.)* 388: 54P.
- Roberts, A., N. Dale, and S. R. Soffe (1984) Sustained responses to brief stimuli: Swimming in *Xenopus* embryos. *J. Exp. Biol.* 112: 321–335.
- Salt, T. E. (1986) Mediation of thalamic sensory input by both NMDA receptors and non-NMDA receptors. *Nature* 322: 263–265.
- Sillar, K. T. (1986) Glycine-mediated phase-dependent gating of sensory input during swimming in *Xenopus* embryos. *J. Physiol. (Lond.)* 382: 181P.
- Sillar, K. T., and S. R. Soffe (1987) Spontaneous tetrodotoxin-resistant inhibitory and excitatory potentials in *Xenopus* embryo spinal cord neurones. *J. Physiol. (Lond.)* 388: 55P.
- Soffe, S. R. (1987) Ionic and pharmacological properties of reciprocal inhibition in *Xenopus* embryo motoneurones. *J. Physiol. (Lond.)* 382: 463–473.
- Soffe, S. R., and A. Roberts (1982) Activity of myotomal motoneurones during fictive swimming in frog embryos. *J. Neurophysiol.* 48: 1274–1278.
- Thomson, A. M., D. C. West, and D. Lodge (1985) An *N*-methylaspartate receptor-mediated synapse in rat cerebral cortex: A site of action of ketamine? *Nature* 313: 479–481.
- Watkins, J. C., and R. H. Evans (1981) Excitatory amino acid neurotransmitters. *Annu. Rev. Pharmacol. Toxicol.* 21: 165–206.