Rapid Activation of GABAergic Interneurons and Possible Calcium Independent GABA Release in the Mormyrid Electrosensory Lobe

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Han, Victor Z., Kirsty Grant, and Curtis C. Bell. Rapid activation of GABAergic interneurons and possible calcium independent GABA release in the mormyrid electrosensory lobe. J. Neurophysiol. 83: 1592–1604, 2000. The primary afferent fibers from the electrorceptors of mormyrid electric fish terminate centrally in the granular layer of the electrosensory lobe (ELL). This study examines the excitatory and inhibitory processes that take place in this layer using an in vitro slice preparation and field potentials evoked by stimulation of primary afferent fibers in the deep fiber layer of ELL. The postsynaptic response to stimulation of the afferent fibers was still present after blocking chemical transmission in three different ways: by adding glutamate receptor antagonists to the medium, by substituting a nominally calcium-free medium for normal medium, and by blocking calcium channels with cadmium. Blockade of chemical transmission was demonstrated by disappearance of control responses to parallel fiber stimulation. The continued presence of a postsynaptic response in the absence of chemical excitation is consistent with previous anatomic and physiological evidence for electrical synapses between afferent fibers and granular cells in ELL. Granular cell activation by primary afferent fibers was followed by a powerful, short-latency inhibition mediated by GABA and GABA_A receptors, as indicated by a large increase in the postsynaptic response to afferent fiber stimulation following application of the GABA_A receptor antagonist, bicuculline. Bicuculline caused a marked increase of the postsynaptic response even after chemical synaptic excitation had been blocked by glutamate receptor antagonists, by a calcium-free medium, or by cadmium. Thus activation of the inhibitory interneurons responsible for GABA release did not require chemical excitation. Nonchemical excitation of the inhibitory interneurons could be mediated either by electrical synapses between afferent fibers and inhibitory interneurons, or by nonsynaptic activation of the large GABAergic terminals that are known to be present on granular cells. The marked increase of the postsynaptic response caused by bicuculline in a calcium-free medium or in the presence of cadmium suggests that the release of GABA by inhibitory terminals was not entirely dependent on calcium influx. This effect of bicuculline on the postsynaptic response in a calcium-free medium or in the presence of cadmium was markedly reduced by prior addition of the GABA transporter antagonist, nipeptic acid. Thus calcium-independent release of GABA may occur in ELL and may be partly dependent on reversal of a GABA transporter. Rapid and powerful inhibition at the first stage in the processing of electrosensory information could serve to enhance the small differences in latency among afferent fibers that appear to encode small differences in stimulus intensity.

INTRODUCTION

The mormyrid electrosensory lobe (ELL) is a cerebellum-like structure and receives the primary afferent fibers from electrorceptors in the skin. The ELL is thus the first central stage in the processing of sensory input from cutaneous electrorceptors. The ELL, like many other sensory processing regions of the vertebrate brain, receives extensive input not only from the periphery but also from other central structures (Bell and Szabo 1986). This central input modulates the processing of sensory information in a dynamic and plastic manner (Bell et al. 1997b), and the mormyrid ELL is a good site for investigating the roles of such central inputs in sensory processing. Understanding sensory processing in ELL requires knowledge of the effects of both peripheral and central inputs to the structure. Primary afferent fibers from electrorceptors terminate on small granular cells in the granular layers of ELL (Bell et al. 1989). These small granular cells relay the electrosensory information on to larger cells of ELL that include the Purkinje-like medium ganglion cells and the two types of efferent cells, large ganglion and large fusiform cells. The physiology of the larger cells of ELL has been examined both in vivo (Bell et al. 1997a) and in vitro (Grant et al. 1998), but very little is known about the physiology of granular cells and about interactions in the granular layer that condition the electrosensory information received by the larger cells of ELL. This study uses field potentials and pharmacology to investigate responses and cellular interactions in the granular layer that are evoked by primary afferent stimulation. The focus is on a powerful and short-latency inhibition that takes place in this layer. All experiments were done in an in vitro slice preparation.

The ELL has six layers: molecular, ganglion, plexiform, granular, intermediate, and deep fiber (Fig. 1A) (Bell and Szabo 1986; Meek et al. 1999). Primary afferent fibers from electrorceptors terminate on granular neurons with mixed chemical and electrical synapses (Bell et al. 1989; Meek et al. 1999) (Fig. 1B, right). The same granular cells are also contacted by large γ-aminobutyric acid (GABA)–containing terminals that cover one-third to one-half of the small somas of granular neurons (Fig. 1, A and B). These terminals arise from large multipolar intermediate layer neurons (LMI cells) that have their cell bodies in the intermediate layer (Meek et al. 1999). The axons of LMI cells have local branches that terminate in the ELL granular layer near the cell body as well as branches that travel some distance to terminate in the ELL granular layer on both the ipsilateral and contralateral sides. The LMI cells are unusual in that their dendrites, which extend...
up into the overlying granular layer, become myelinated as they exit from the cell body. It has been suggested further that the dendrites retain their myelin as they branch and terminate on granular cells as presynaptic structures (Meek et al. 1999). Thus the large GABA-containing terminals on granular cells may arise from the dendrites as well as the axons of LMI cells.

Intra-axonal recordings from primary afferent fibers near their terminals in ELL show synaptic potentials as well as orthodromic spikes from electoreceptors (Bell 1990). The synaptic potentials are probably due to synaptic input to postsynaptic granular cells that is observed inside the primary afferent because of the electrical synapses that the afferent makes with granular cells. The synaptic potentials include the following: excitatory postsynaptic potentials (EPSPs) evoked by a centrally originating corollary discharge signal associated with the motor command that drives the electric organ to discharge; EPSPs evoked by stimulation of electoreceptors near the one from which the recorded afferent arises that are due to convergence of different afferents onto the same granular cells; and an inhibitory postsynaptic potential (IPSP) that is evoked by stimulation of more distant electoreceptors. The IPSP is prominent and is probably mediated by the large GABAergic terminals of LMI cells on granular cells.

This study demonstrates that stimulation of afferent fibers from electoreceptors evokes a powerful, GABA-mediated inhibition in the granular layer of the mormyrid ELL. Primary afferent activation of the inhibitory interneurons that release the GABA is very rapid and does not depend on excitatory chemical synaptic transmission. In addition, the evoked release of GABA may be partly independent of external calcium.

METHODS

Slice preparation

Experiments were carried out using 26 fish of the mormyrid species Gnathonemus petersii. The fish were first deeply anesthetized with MS 222 (Sigma; concentration 1:10,000 in aquarium water). The brain was removed and cooled rapidly by immersion in an ice-cold balanced salt solution containing (in mM) 0 NaCl, 2.0 KCl, 1.25 KH₂PO₄, 24 NaHCO₃, 2.6 CaCl₂, 1.6 MgSO₄ 0.7H₂O, and 20 glucose [low sodium artificial cerebrospinal fluid (ACSF)], saturated with a gas mixture containing 95% O₂-5% CO₂. Three to four hundred micrometer-thick slices were cut in the frontal (transverse) plane using a custom-made horizontal rotating circular blade microtome, under the same ice-cold salt solution. After cutting, the slices were transferred to a bath containing equal parts of low sodium ACSF and normal ACSF (in mM: 124 NaCl, 2.0 KCl, 1.25 KH₂PO₄, 24 NaHCO₃, 2.6 CaCl₂, 1.6 MgSO₄ 0.7H₂O, and 20 glucose) at room temperature. The slices remained in this solution for 30 min and were then moved to a standard interface recording chamber where they were continuously superfused with normal ACSF at a flow rate of ~2
molecular layer. Where the cell bodies and basilar dendrites of medium ganglion and EPSP is generated but is positive in the ganglion and granular layers parallel fiber stimulation is negative in the molecular layer where the azole-4-propionic acid (AMPA) types. The field potential evoked by methyl-D-aspartate (NMDA) and efferent cells (Grant et al. 1998). This response is mediated by the monitor of excitatory chemical transmission. Parallel fiber stimuli following blocking agents of excitatory or inhibitory chemical syn-

Recording and stimulation

Field potentials were recorded in the granular layer of the medial zone of the electrosensory lobe (Fig. 2A) using glass microelectrodes with tip diameters of 3–5 μm and filled with 3 M NaCl (resistance 5–10 MΩ). Signals were recorded directly to a computer using the Axon Instruments interface and Axoscope software.

Electrical stimulation of the tissue was delivered through tungsten microelectrodes (A-M systems) insulated except at the tip and plated with gold to minimize electrode polarization. One stimulus electrode was placed in the middle of the molecular layer to activate parallel fibers (SM, Fig. 2A). The two other stimulus electrodes were placed in the intermediate (SI) or deep fiber (SD) layers to activate lateral GABAergic terminals and primary afferent fibers, respectively. The three stimulus electrodes were monopolar. Each stimulus electrode was paired with a second tungsten microelectrode in the recording chamber outside the brain slice that served as the indifferent electrode. Stimuli were 0.1 ms in duration and delivered at 0.5–1 Hz through a stimulus isolation unit. Stimulus intensities ranged from a few microamperes to 50 μA.

The field potential evoked by parallel fiber stimuli served as a monitor of excitatory chemical transmission. Parallel fiber stimuli evoke an EPSP in the apical dendrites of ELL medium ganglion and efferent cells (Grant et al. 1998). This response is mediated by the release of glutamate and by postsynaptic receptors of both the N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) types. The field potential evoked by parallel fiber stimulation is negative in the molecular layer where the EPSP is generated but is positive in the ganglion and granular layers where the cell bodies and basilar dendrites of medium ganglion and efferent cells act as a current source for the current sink in the molecular layer.

Pharmacology

All drugs were bath applied by adding them to the perfusate. The following blocking agents of excitatory or inhibitory chemical synapic transmission were tested: the non-NMDA glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 30 μM; RBI), the NMDA glutamate receptor antagonist d-2-amino-5-phenylpentanoate (AP5; 35 μM; RBI), the GABAA receptor antagonist bicuculline methiodide (30 μM; Sigma) the GABAβ receptor antagonist 2-hydroxysaclofen (500 μM; RBI), and the glycine receptor antagonist strychnine hydrochloride (15 μM; Sigma). In some experiments, the slices were bathed with ACSF in which the calcium ions were replaced by an equivalent increase in magnesium ions (“calcium-free” ACSF). Cadmium (100 μM) was used to block voltage-sensitive calcium channels, and tetrodotoxin (TTX; 0.5 μM) was used to block voltage-sensitive sodium channels.

Data analysis

Data were analyzed off-line using Clampfit (Axon Instruments) and Origin (Microcal) software. All of the responses illustrated in the figures are averages of 15 individual responses. Responses in the granular layer to primary afferent stimulation consist of an initial positive-negative wave due to activation of the presynaptic fibers and a subsequent negative wave due to activation of postsynaptic granular cells (Fig. 2B; see RESULTS). The amplitude of the postsynaptic component was measured by calculating the area between the baseline and the response trace in the averaged record, beginning at the transition between pre- and postsynaptic components of the response at a latency of ~2 ms and ending 10–25 ms later (see shaded area in Fig. 2B, trace 5). (The recorded response varied in different slices, and the delay of the endpoint used in measuring the area varied accordingly.) Means ± SD of these areas were calculated across different replicates of the same experiment in different slices and were compared using Student’s t-test. Differences with P values smaller than 0.01 were judged to be significant.

When a conditioned-test paradigm was used, as in measuring paired-pulse depression (Figs. 7 and 8) or lateral inhibition (Figs. 9 and 10), the average response to the conditioning stimulus alone (control) was subtracted from the average response to conditioning plus test stimuli to obtain the effect of the test stimulus in isolation. The response to the test stimulus was expressed as a percent of the response to the same stimulus in the absence of a conditioning stimulus. Results are plotted as means ± SD.

FIG. 2. Field potentials evoked by primary afferent stimulation. A: diagram of the ELL slice preparation showing the tip of the recording electrode in the granular layer (wide gray line). Stimulating electrodes in the molecular layer (SM) to activate parallel fibers, in the intermediate layer (SI) to activate interneurons, and in the deep fiber layer (SD) to activate primary afferents. ELL, electrosensory lobe; Egp, eminentia granularis posterior. B: field potentials evoked in the different layers of ELL by low-intensity stimulation of the deep fiber layer (SD). This activation of primary afferent fibers evoked a response with two negative components in the granular layer: a brief early component (n pre), which is thought to be the presynaptic afferent fiber volley, and a long-lasting late component (n post), which is considered to be the postsynaptic response of granular layer cells. Note that n pre is maximal in the intermediate layer and that n post is largely confined to the granular cell layer (from Grant et al. 1998). C: field potentials recorded in the granular layer in response to a train of deep layer stimuli delivered at 200 Hz. Only the 1st stimulus evokes a large postsynaptic response (single arrow). A more slowly rising negativity following the initial fast presynaptic component is visible in response to stimulus 2–4 (double arrow) (from Grant et al. 1998).
RESULTS

Field potentials in the granular layer evoked by primary afferent fiber stimulation

The field potential recorded in the granular layer in response to weak stimulation (3–10 μA) of the deep fiber layer has two negative components (Fig. 2B). The initial component has been identified as the incoming presynaptic volley (“n pre“ in Fig. 2B) and the later component as the postsynaptic response of granular cells (“n post“ in Fig. 2B), based on their latencies, spatial distribution, and abilities to follow high-frequency stimulation (Grant et al. 1998).

The responses to weak stimulation of the deep fiber layer may be attributed to activation of primary afferent fibers (Grant et al. 1998). Although there are other types of fibers in the deep fiber layer besides primary afferents (Meek et al. 1999), these other fiber types are either unmyelinated or much smaller in diameter than the primary afferents, and thus are likely to have a higher threshold. In addition, the laminar distribution of field potentials (Fig. 2B) is consistent with the laminar termination pattern of primary afferents but not with the termination pattern of the other fiber types. Primary afferent fibers terminate on cells in the granular layer and do not extend beyond the boundary between the granular and plexiform layers (Bell and Szabo 1986; Meek et al. 1999). The region in which the postsynaptic response (n post) is observed thus corresponds anatomically to the region in which primary afferent fibers terminate.

The postsynaptic response shows marked paired-pulse depression (see Paired-pulse depression of responses to deep fiber layer stimulation and Fig. 7) and does not follow frequencies >100 Hz (Grant et al. 1998). The latter is illustrated in Fig. 2C by the almost complete lack of a postsynaptic response to all but the first stimulus in a train of stimuli at 200 Hz. Primary afferent fibers make electrical synapses on granular cells (Bell et al. 1989; Meek et al. 1999), and an electrotonic EPSP would be expected to follow much higher rates of stimulation than 100 Hz. Perhaps an electrotonic EPSP contributes to the slowly rising negativity that follows the presynaptic volley in later responses (double arrows in Fig. 2C). The larger portion of the postsynaptic response, that does not follow high frequencies, is possibly due to postsynaptic sodium spikes. It is not due to chemical EPSPs or calcium spikes because it is still present when calcium inflow is prevented in calcium-free medium (Fig. 5Ab) or by blocking calcium channels with cadmium (Fig. 5Bb). The poor following capacity of the larger portion of the postsynaptic response could be due to refractoriness of the postsynaptic spike.

Pharmacology of field potentials in granular layer

A pharmacological approach was used to understand the functional organization of primary afferent projections and synaptic transmission in the granular layer.

The presence of large GABAergic terminals on the granular cells that receive primary afferent input (Fig. 1B) suggests that GABAergic inhibition plays an important role in shaping the spatial and temporal properties of granular cell responsiveness. Bath application of the GABA_A receptor blocker bicuculline markedly increased both the amplitude and duration of the negative field potential corresponding to the postsynaptic response (Figs. 3B and 4B), confirming that the initial excitation of granular cells is normally followed by a powerful, primary afferent–evoked inhibition, mediated via GABA_A receptors. The difference between responses in the absence and in the presence of bicuculline was highly significant (n = 28 slices, P << 0.001), with the average response under bicuculline being 193% of the control response.

Subtraction of averaged traces obtained in the presence of bicuculline from those obtained in its absence showed that the GABAergic inhibition began at a short latency of only 1 ms after the peak of the presynaptic volley (Fig. 3C). Adding the glycine receptor antagonist strychnine to the bath did not cause a significant change in the postsynaptic response (11 slices, P = 0.27; not illustrated). Thus glycine does not seem to be involved as a transmitter at this initial stage of processing in ELL.

Primary afferent terminals are excitatory (Bell 1990) and form mixed chemical-electrical synapses on granular neurons (Fig. 1B) (Bell et al. 1989; Meek et al. 1999). Glutamate is the most common excitatory transmitter, and antagonists of glutamate receptors were therefore applied to examine the role of chemical transmission in the excitatory response evoked in the granular layer by primary afferent fiber stimulation (Fig. 4, D–F). Application of CNQX, an antagonist of the AMPA type of glutamate receptors, caused a reduction in the postsynaptic response to deep fiber layer stimulation (Fig. 4D). Subsequent addition of the NMDA receptor antagonist, AP5, caused a slightly additional reduction that was particularly evident in the late components of the response (Fig. 4E). On average, the two glutamate receptor antagonists together reduced the control response (Figs. 3B and 4B).
response by 30% relative to the control response recorded in normal ACSF (Fig. 4, A and C) and the difference between responses in the absence and in the presence of the antagonists was statistically significant \((n = 8\) slices, \(P \ll 0.001\)). Responses to molecular layer stimulation (SM in Fig. 4), recorded simultaneously, were completely blocked by addition of the two antagonists (Fig. 4E), indicating that the drugs penetrated the slice and were effective in blocking glutamatergic synaptic transmission. The reduction of the postsynaptic response to deep fiber layer stimulation in the presence of the glutamate receptor antagonists indicates that the effect of primary afferent activation on granular cells is due in part at least to glutamate-mediated chemical transmission.

When bicuculline was added to the bath solution in the presence of CNQX and AP5, despite the existing blockade of glutamate receptors, there was nevertheless a large increase in the postsynaptic response (Fig. 4F). The increase caused by bicuculline in the eight slices tested was significant \((P = 0.003)\) and averaged 207%. This increase was less, however, than that observed in the absence of the glutamate antagonists when only bicuculline was added to the bath (Fig. 4B). The prominent postsynaptic response in the presence of the glutamate receptor antagonists and bicuculline indicates that synaptic transmission from primary afferent fibers to granular cells also takes place by some mechanism in addition to glutamate-mediated chemical transmission, with the most probable such mechanism being electrical transmission via the morphologically demonstrated gap junctions.

The marked increase in the postsynaptic response caused by bicuculline in the presence of the glutamate receptor antagonists also suggests that primary afferent activation continued to evoke the release of GABA despite the blockade of chemical excitatory transmission. This in turn suggests that excitation of the inhibitory interneurons responsible for GABA release can also take place by some other means than glutamate-mediated chemical synaptic transmission.

**Role of calcium in generating the postsynaptic response**

Transmitter release is generally believed to depend on influx of calcium through voltage-gated calcium channels. The role of chemically mediated transmission was therefore investigated further by replacing calcium in the medium with an equimolar concentration of magnesium, and by blocking voltage-gated calcium channels with cadmium (100 \(\mu M\)). The primary afferent volley evoked by stimulation in the deep fiber layer continued to evoke a clear postsynaptic response, both in nominally calcium-free medium (Fig. 5Aa) and in the presence of cadmium (Fig. 5Bb). The continued presence of a postsynaptic response in the absence of calcium, or when calcium channels are blocked by cadmium, provides further evidence for a role of electronic EPSPs in the excitation of granular cells by primary afferent fibers. The simultaneous disappearance of responses to parallel fiber stimulation (SM; Fig. 5, Ab and Bb) confirmed the effective blockade of excitatory chemical transmission under these conditions.

Addition of bicuculline to the bath still caused a marked increase in the amplitude and duration of the postsynaptic responses to stimulation of the deep fiber layer in a calcium-free medium (Fig. 5Ac) or in the presence of cadmium (Fig. 5Bc). Bicuculline caused an average increase of 220% \((n = 5\) slices, \(P \ll 0.001)\) in the postsynaptic response recorded in a calcium-free medium and an average increase of 272% \((n = 9\) slices, \(P \ll 0.001)\) in the postsynaptic response recorded in the presence of cadmium. The effects of a calcium-free medium and of bicuculline on responses to molecular layer and deep fiber layer stimuli were reversed after returning to normal ACSF (Fig. 5Ad). The effects of cadmium on responses to parallel fiber stimuli did not reverse, however, at least within a period of 1 h after removing the cadmium (Fig. 5Bd). As expected, addition of the sodium channel blocker, TTX, abolished both the pre- and postsynaptic components of the response to primary afferent stimulation (Fig. 5Ae).

The large increases in postsynaptic responses caused by bicuculline, in calcium-free medium and in the presence of cadmium, suggest that GABA release continues to be evoked...
even though chemical excitatory chemical transmission is blocked. This result is consistent with the results obtained in the presence of glutamate receptor antagonists, in suggesting that excitation of the inhibitory interneurons responsible for GABA release does not require chemical synaptic transmission. In addition, and more surprisingly perhaps, the results also suggest that some of the evoked GABA release at these synapses is calcium independent because the GABA appears to have been released in a calcium-free medium and when voltage-gated channels were blocked with cadmium.

Calcium-independent release of transmitter has been observed at other synapses, and evidence has been obtained that transporter molecules have a role in such release (Attwell et al. 1993; Schwartz 1987). The proposed mechanism is one in which the normal direction for the transport of transmitter, from outside to inside, is reversed when the inside of the cell becomes depolarized or experiences a large increase in sodium ion concentration. We therefore tested the effects of the GABA transporter blocker, nipecotic acid (NA) in nine slices (Fig. 6). Addition of NA in a calcium-free medium caused a small but not statistically significant increase in the postsynaptic response to deep fiber layer stimulation \( (n = 9 \text{ slices}) \). The effect of subsequent addition of bicuculline to such slices was variable. Addition of bicuculline under these conditions yielded a reduction in the postsynaptic response in three slices, no change in one slice, some enhancement but clearly less than that observed in the absence of NA in three slices, and enhancement similar to that observed without NA in two slices. The average increase caused by the addition of bicuculline to a calcium-free medium in the presence of NA caused an average increase of 9% in the postsynaptic response, a change that was not significant \( (n = 9 \text{ slices}, P = 0.7) \). This small increase is in contrast to the highly significant increase of 220% caused by bicuculline in a calcium-free medium that did not contain NA, as described above. The results suggest a possible role of GABA transporters in the calcium-independent release of GABA in ELL.

**Paired-pulse depression of responses to deep fiber layer stimulation**

As reported previously (Grant et al. 1998 and above), most of the postsynaptic response to deep fiber layer stimulation does not follow repetitive stimulation at frequencies of \( >100 \text{ Hz} \). The interaction between successive stimuli was examined in more detail with a paired-pulse protocol. The amplitude of the presynaptic volley was reduced for stimulus intervals of

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**FIG. 5.** Blockade of chemical synaptic transmission by removal of \( \text{Ca}^{2+} \) or by addition of cadmium does not prevent bicuculline effect. A: effects of \( \text{Ca}^{2+}\)-free/high-Mg\( ^{2+} \) bath solution. Aa: control responses to parallel fiber (SM) and primary afferent (SD) stimulation. Ab: response to SM disappeared in calcium-free ACSF indicating blockade of chemical transmission, but the postsynaptic component to SD increased slightly. Ac: the postsynaptic response to SD in calcium-free ACSF was markedly enhanced by bicuculline. Ad: washing in normal ACSF restored parallel fiber responses to SM and returned the response to SD toward control values. Ae: addition of the sodium channel blocker TTX abolished all responses. B: effects of adding Cd\( ^{2+} \) to the bath solution. Ba: control responses to SM and SD. Bb: cadmium-blocked response to SM disappeared in the presence of cadmium but the postsynaptic response to SD did not. Bc: postsynaptic response (to SD) in the presence of cadmium was greatly increased by bicuculline. Bd: response to SD returns to control level after return to normal ACSF, but response to SM does not.

**FIG. 6.** Possible role of GABA transporter in calcium independent GABA release. A: control responses to SM and SD stimulation. B: calcium-free ACSF and nipecotic acid had little effect on response to SD. C: with nipecotic acid in the bath, however, bicuculline did not cause the marked enhancement of the SD response seen previously. D: responses to SM and SD after wash out with normal ACSF. E: in normal ACSF, bicuculline alone caused the customary increase in the SD response.
<10 ms (Fig. 7B, bottom graph), due presumably to primary afferent fiber refractoriness. However, the postsynaptic component of the response showed a more prolonged depression (Fig. 7, A and B), which lasted between 25 and 40 ms in the 15 different slices that were tested.

This depression of the second response in the paired-pulse protocol could be due to release of GABA by the first stimulus, and this possibility was first tested by applying bicuculline to the bath. Surprisingly, however, the addition of bicuculline greatly increased the degree and duration of the depression (Fig. 7, C and D), rather than reducing it. Under bicuculline, paired-pulse depression of the postsynaptic response lasted for >200 ms (Figs. 7D and 8) in the 12 different slices that were tested, in marked contrast to the 25–40 ms of depression observed in the absence of bicuculline. The brief, paired-pulse depression of the presynaptic component was not affected by bicuculline (Fig. 7D, bottom), however, indicating that the increased duration of paired-pulse depression caused by bicuculline is a postsynaptic phenomenon.

Bicuculline blocks GABA_A receptors but does not affect GABA_B receptors. Inhibition due to activation of GABA_B has a long duration of action, suggesting that they might be involved in the long-lasting depression after a single stimulus. Accordingly, the role of GABA_B receptors was tested with the GABA_B antagonist 2-hydroxy-saclofen. The increase in paired-pulse depression observed with bicuculline in the bath
that the same drug caused in paired-pulse depression (Fig. 7, C and D; see DISCUSSION). Blockade of the inhibitory effect of a lateral stimulus by bicuculline was observed in all eight slices in which the effects of the drug were tested.

However, the inhibitory effect of a lateral stimulus was still present in a calcium-free medium (Fig. 10B) in six of nine slices tested. Addition of bicuculline to the calcium-free medium blocked lateral inhibition in all six of these slices (Fig. 10C), just as it did in normal medium. These results indicate that lateral inhibition at this first stage of the system is mediated by GABA and suggest that the GABA may be released, in part at least, by a calcium-independent mechanism.

**DISCUSSION**

This study used pharmacological tools and field potentials to analyze synaptic transmission in the granular layer of the mormyrid ELL, where the primary afferent fibers from electroreceptors terminate. The following aspects are discussed: 1) synaptic transmission at the mixed chemical-electrical synapse between primary afferent fibers and granular cells, 2) activation of GABAergic interneurons, 3) paired-pulse depression, 4) calcium-independent release of GABA in the ELL granular layer, 5) comparison with gymnotid electric fish, and 6) functional implications of the large and rapid release of GABA for processing of electrosensory information.

**Synaptic transmission at the mixed chemical-electrical synapse between primary afferent fibers and granular cells**

The present findings are consistent with previous anatomic (Bell et al. 1989; Meek et al. 1999) and physiological (Bell 1990) evidence for electrical transmission between primary afferent fibers and granular cells of ELL. Thus a postsynaptic response was still present in the granular layer after manipulations that blocked chemical excitatory transmission. These manipulations included the following: the addition of the glutamate receptor blockers, CNQX and AP5, to the medium; the addition of cadmium to the medium; and substitution of calcium-free medium for normal medium. In each case, the effective blockade of chemical excitatory transmission was indicated by disappearance of the postsynaptic response to parallel fiber stimulation.

The postsynaptic response to primary afferent stimulation in the granular layer was much reduced by addition of the glutamate receptor antagonists to the medium (Fig. 4E), and in fact became obvious only after the further addition of bicuculline to the bath (Fig. 4F). The reduction caused by glutamate receptor antagonists indicates that glutamate-mediated excitatory chemical transmission contributes significantly to transmission at the primary afferent to granular cell synapse. Such a contribution is consistent with morphological evidence for chemical as well as electrical transmission at this synapse (Bell et al. 1989; Meek et al. 1999).

Somewhat surprisingly, the postsynaptic response to primary afferent stimulation was not much affected by substitution of a calcium-free medium for normal medium (Fig. 5Ab) or by addition of cadmium to the medium (Fig. 5Bb). These manipulations blocked chemical excitatory transmission, as indicated by disappearance of the parallel fiber responses, and would therefore be expected to reduce the postsynaptic re-
sponses to afferent stimulation just like the glutamate receptor antagonists. Perhaps the substitution of calcium-free medium and the addition of cadmium both increased the excitability of granular cells, compensating for the reduction in synaptic current. The well-known enhancement of neuronal excitability that is present in low calcium (Hille 1992) may not have been fully opposed by substitution with equimolar magnesium. In addition, manipulations that interfere with calcium influx could also interfere with calcium-activated potassium channels or with release of inhibitory transmitter. These latter possibilities have been used to explain the finding that hippocampal cells in slices are depolarized and more excitable in a medium with low calcium and equimolar substitution with magnesium or manganese (Jefferys and Haas 1982; Taylor and Dudek 1982) and might also explain an increased excitability of ELL granular cells under similar conditions.

**FIG. 9.** Bicuculline blocks lateral inhibition. A: stimulus at a site in the intermediate layer (SI) 300 μm lateral to the recording site, evoked almost no response (left, top trace), but caused a clear reduction (lateral inhibition) in responses to SD (left, bottom traces). Graphs on the right show the normalized areas of pre- and postsynaptic responses to the SD stimulus as a function of the interstimulus interval (SI-SD). Error bars show standard deviation for averaged responses (n = 15). B: addition of bicuculline enhanced the SD response (as noted previously), but in the presence of this GABA receptor blocker, lateral inhibition was no longer evident (bottom traces on left; graphs of SD response amplitudes on right). C: lateral inhibition was partially restored after removing bicuculline.
The postsynaptic response to primary afferent stimulation in the ELL granular layer was dramatically increased after addition of the GABA receptor antagonist, bicuculline, but was not much affected by addition of the glycine receptor antagonist, strychnine. Thus GABA release and GABA receptors appear to have important roles at this first stage of electrosensory processing in ELL, but glycine release and glycine receptors do not.

The marked increase in the postsynaptic response caused by bicuculline suggests that GABA release is normally evoked by primary afferent stimulation and that such release causes a large and rapid inhibition of granular cells. The latency of the GABA-mediated inhibition, determined by subtracting the pre-bicuculline control response from the response under bicuculline, was 1 ms. This is very short for the presumed disynaptic pathway from primary afferents through GABAergic inhibitory interneurons to granular cells, considering that delays at chemical synapses in cold-blooded vertebrates at room temperature are \( \sim 0.5 \) ms (Katz and Miledi 1965). However, our results suggest that chemically mediated excitation of the GABAergic interneuron may not be necessary for GABA release, because bicuculline still causes a marked increase in the postsynaptic response when chemical synaptic transmission has been blocked by glutamate receptor antagonists, by a calcium-free medium, or by the presence of cadmium. How might GABA release be evoked under such circumstances?

The granular cells that receive primary afferent input from the periphery also receive very large GABA-containing synaptic terminals that arise from the axons, and possibly the presynaptic myelinated dendrites also, of large multipolar intermediate layer neurons (LMI cells) (Bell et al. 1989; Meek et al. 1999). These multipolar interneurons are therefore a prime candidate for the source of the GABA that is released in the granular layer by stimulation of afferent fibers. Two mechanisms for activating LMI cells without chemical excitatory transmission may be suggested: nonsynaptic activation of LMI cell terminals following granular cells excitation and electrical synapses between primary afferent fibers and LMI cell dendrites.

Morphological work indicates that the somas of LMI cells are contacted by only a few excitatory terminals and that none of these terminals appear to originate from primary afferent fibers (Meek et al. 1999). Moreover, the dendrites of LMI cells become myelinated as they exit from the soma, and it is possible that the dendritic branches retain their myelin until they terminate as large presynaptic endings on granular cells. If the entire dendritic arbor of these cells is indeed myelinated, there would be little if any opportunity for excitatory synaptic input to the dendrites. The possible absence of excitatory synaptic input on the soma and dendrites of LMI cells has lead to the suggestion that the large LMI terminals on granular cells might be excited directly and nonsynthetically following granular cell excitation by input from primary afferent fibers (Meek et al. 1999).

Two mechanisms may be suggested for nonsynaptic excitation of the terminals: ephaptic excitation in which current generated by the granular cells passes through the synaptic cleft following granular cells excitation and electrical synapses between primary afferent fibers and LMI cell dendrites. The possible absence of excitatory synaptic input on the soma and dendrites of LMI cells has led to the suggestion that the large LMI terminals on granular cells might be excited directly and nonsynthetically following granular cell excitation by input from primary afferent fibers (Meek et al. 1999). Two mechanisms may be suggested for nonsynaptic activation of the calyx type afferent terminals on type I vestibular hair cells (Goldberg 1996). Alternatively, parts of the dendritic arbor of LMI cells may be free of myelin and contacted by electrical or mixed chemical-electrical synapses from primary afferent fibers or granular cells. More detailed knowledge of LMI and granular cells is needed to distinguish these different means of exciting LMI cells without chemical excitatory transmission.

**Paired-pulse depression**

The postsynaptic granular layer response to the second of two identical fiber layer stimuli showed was depressed for \( \sim 30 \) ms after the first stimulus. The duration of the paired-pulse depression was greatly enhanced by addition of the GABA antagonist, bicuculline. This long duration of paired-pulse de-
pression after addition of bicuculline appeared to be due in part to activation of \( \text{GABA}_A \) receptors, because the depression was reduced by the further addition of the \( \text{GABA}_B \) receptor antagonist, 2-hydroxy-saclofen. The prolongation of paired-pulse depression following addition of bicuculline is somewhat surprising because one would suppose that GABA release and activation of \( \text{GABA}_A \) receptors after the first stimulus would contribute to depression of the response to the second stimulus. Results similar to ours have also been obtained in other systems, however. In the frontal cortex (Kang 1995) and hippocampus (Higgins and Stone 1993), bicuculline increased paired-pulse depression, and this depression was partially reduced by \( \text{GABA}_B \) antagonists. \( \text{GABA}_B \) antagonists were also found to reduce paired-pulse depression in the olfactory bulb (Keller et al. 1998).

The blockade of \( \text{GABA}_A \) receptors with bicuculline results in a large increase in the response of granular cells to afferent stimuli, as indicated by the large increase in the postsynaptic response. The increased granular cell response under bicuculline would result in a longer refractory period for these cells and thus lead to a longer duration of paired-pulse depression. In addition, the increased granular cell response could result in a large increase in the amount of GABA released by LMI cells, supposing that granular cell excitation activates LMI cell terminals by synaptic or nonsynaptic mechanisms, as described in the preceding section. Increased GABA release would result in strong activation of \( \text{GABA}_B \) receptors.

A single stimulus to the intermediate layer, at a site lateral to the recording point in the granular layer where a postsynaptic response to afferent stimulation was recorded, caused a reduction in the postsynaptic response that lasted \( \sim 30 \) ms (Fig. 9A). Bicuculline blocked this lateral inhibitory effect (Fig. 9B). This blockade is in contrast to the enhancement of paired-pulse depression caused by the same drug. The lateral inhibitory stimulus did not excite the granular cells at the recording site and may have caused the release of only a small amount of GABA that was sufficient to activate \( \text{GABA}_A \) receptors but not sufficient to activate \( \text{GABA}_B \) receptors. \( \text{GABA}_B \) receptors are known to require release of greater amount of GABA for activation than \( \text{GABA}_A \) receptors (Nicoll et al. 1990), due perhaps to location of \( \text{GABA}_B \) receptors outside the synaptic cleft.

**Calcium independent release of GABA in the ELL granular layer**

Bicuculline caused a marked increase in the postsynaptic response to afferent stimulation in calcium-free medium and also after addition of cadmium to the medium. These results imply that GABA release was evoked when entry of calcium into the presynaptic terminal was either prevented or greatly reduced. Such calcium-independent release of GABA is unusual but has been demonstrated in the distal retina (Schwartz 1987), in the striatum (Bernath and Zigmond 1988), in the hippocampus (During et al. 1995), and in the cerebellum (Rossi and Hamann 1998). The suggested mechanism in each case is a reversal in the normal direction of the GABA transporter; instead of carrying GABA into the cell, as normally occurs, the transporter carries GABA out of the cell. Reversal of the transport direction occurs when the terminal is depolarized or contains an elevated concentration of sodium (for review see Attwell et al. 1993). Nipecotic acid is an antagonist of the GABA transporter and has been shown to block calcium-independent release of GABA in several of the above systems. Similarly, in the ELL, nipecotic acid prevented or reduced the marked increase in the granular layer response to afferent stimulation caused by bicuculline, under conditions in which calcium entry was blocked or reduced (Fig. 6). Some might argue that the rapid onset of GABA release following primary afferent activation precludes a mechanism that involves reversal of the GABA transporter. However, experiments with the glutamate transporter have shown that the initial phase of transporter action can be activated within less than a millisecond when transmitter concentrations are high (Wadiche and Kavanaugh 1998), and the same might be true of the GABA transporter. Thus calcium-independent release of GABA in the ELL granular layer may be present and may be dependent at least in part on the GABA transporter.

The evoked release of GABA is not measured directly in our experiments but is inferred on the basis of the large increase in postsynaptic responses that occurs when the GABA receptor antagonist, bicuculline, is added to the medium. It might be argued, however, that the effect of bicuculline is not due to blocking the effect of the evoked GABA release, but is instead due to blocking the effect of GABA that is present in the slice in the absence of afferent activation, due to tonic rather than evoked release. Blocking the tonic inhibition caused by GABA with bicuculline would make the granular layer cells more excitable, causing them to respond more vigorously to afferent input.

Two of our results suggest that GABA release in the ELL granular layer is evoked, and that the bicuculline effect cannot be explained by a blockade of tonic GABAergic inhibition. First, the fact that a \( \text{GABA}_B \) antagonist reduced the depression of the postsynaptic response caused by a preceding stimulus implies that GABA release was evoked by the first stimulus. Second, the fact that bicuculline blocked the inhibition caused by a stimulus to the intermediate layer, just lateral to the recording site, means that GABA release was evoked by the lateral stimulus. Moreover, the lateral inhibitory effect was still present in calcium-free medium and was still blocked by bicuculline (Fig. 10).

The marked effects of bicuculline on the postsynaptic response in a calcium-free medium or in the presence of cadmium are evidence that the evoked release of GABA may occur in a calcium-independent manner in the granular layer of the mormyrid ELL. Field potentials are subject to a variety of interpretations, however, and the clear demonstration of calcium-independent transmitter release and analysis of its mechanisms will require intracellular recording from the cellular elements involved in the process.

**Comparison with gymnotid electric fish**

The electrosensory systems of gymnotid electric fish from South America and mormyrid fish from Africa have a surprisingly large number of similar features, given that the two electrosensory systems almost certainly evolved independently (Finger et al. 1986). Such similarities are present at the initial stages of central processing of electrosensory information in ELL. Thus the synapses made by primary afferent fibers in gymnotids have the morphology of mixed chemical-electrical...
synapses, like those in mormyrids (Maler et al. 1981). In addition, the primary afferent fibers in gymnotids activate large inhibitory neurons in the deeper layers, which are known as ovoid cells. These ovoid cells have large widely branching axons that terminate both ipsilaterally and contralaterally on granular neurons and other cell types of ELL (Bastian et al. 1993; Maler and Mugnaini 1994) and are therefore similar to the inhibitory large multipolar interneurons (LMI cells) of mormyrids. Thus afferent activation in both systems appears to result in a powerful and widespread inhibition at this first stage of electroreceptive processing.

Differences between the two types of fish are also present at this first stage of processing. First, in vitro studies of the gymnotid ELL show that the excitatory effect of primary afferents on postsynaptic cells is largely blocked by glutamate receptor antagonists, indicating that transmission is mainly chemical, in spite of the morphological evidence of mixed synapses (Berman and Maler 1998). However, our results in mormyrids showed that synaptic excitation still occurs when chemical transmission is blocked by glutamate receptor antagonists blockers, by calcium-free medium, or by cadmium. The recording of large synaptic potentials inside primary afferents in mormyrids (Bell 1990) also argues for the importance of chemical transmission in these fish. Second, the inhibition caused by ovoid cells in gymnotids has a very slow onset (Bastian 1993) and appears to be mediated by receptors of the GABA<sub>B</sub> type (Berman and Maler 1998). This again is in contrast to the mormyrid ELL, where the primary afferent-induced inhibition was shown to have a very rapid onset and to be mediated by GABA<sub>A</sub> receptors.

Functional implications of the large and rapid release of GABA for processing of electrosensory information

Electric fish generate an electric organ discharge (EOD) and sense nearby objects by the distortions that such objects cause in the pattern of self-generated electrical current that flows through their skin, a process known as active electrolocation. In mormyrid electric fish, the magnitude of current flowing through different regions of the skin is sensed by mormyromast electroreceptors. The response latency of afferent fibers from these electroreceptors is exquisitely sensitive to the magnitude of the current (Szabo and Hagiwara 1967). Behavioral (Hall et al. 1995) results suggest that this response latency, as measured from the time of a centrally originating corollary discharge signal associated with the motor command that drives the EOD, is the code for stimulus intensity. The behavioral results indicate that fish are sensitive to changes in response latency as small as 0.1 ms.

Strong and rapid lateral inhibition of the granular cells, mediated by the laterally spreading processes of large multipolar interneurons, as described in this paper, would enhance small differences in latency between the responses of electroreceptor afferents from adjacent skin regions and would minimize repetitive firing in response to the EOD. Responses of granular cells would therefore convey a sharpened electrical image to second-order cells in ELL.

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REFERENCES


