GABA-Activated Cl^- Channels in Astrocytes of Hippocampal Slices

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We used kainic acid-lesioned hippocampal slices to examine glial responses to the inhibitory neurotransmitter GABA in a neuron-free environment. Slices were prepared from rats which received intracerebroventricular injections of kainic acid 1 month prior to experiments. Astrocytes (membrane potential averaged 81.4 ± 5.5 mV; $n = 46$; mean $\pm$ SD) were impaled in the CA3 region of the slice, which was completely depleted of neurons. GABA (1 mM) application by bath perfusion depolarized membrane potential from 1 to 5 mV. The GABA-induced depolarization was not affected by a tetrodotoxin (1 μM)/high- Mg^{2+} /low- Ca^{2+} solution. Changing the Cl^- equilibrium potential by reducing extracellular Cl^- greatly increased the GABA-induced depolarization. Muscimol mimicked the GABA response, while picrotoxin (0.1 mM), an antagonist of the GABA-activated Cl^- channel, resulted in a 60% blockade. The barbiturate, pentobarbital (0.1 mM), and the benzodiazepine agonist, flunitrazepam (1 mM), enhanced the depolarization by 60 and 40%, respectively. A blocker of glial GABA uptake, β -alanine (1 mM), did not affect the GABA-induced membrane depolarization, indicating that the depolarization is not caused by electrogenic uptake of the amino acid. The pharmacological properties of the GABA response of astrocytes from the hippocampal slice is similar to that previously described for cultured astrocytes from rat cerebral hemispheres. Our data suggest that GABA receptors, which are coupled to Cl^- channels, are also expressed by astrocytes in an intact tissue.

When the major inhibitory neurotransmitter in the CNS, GABA, is released, it binds to postsynaptic GABA_A receptors and opens Cl^- selective channels (Krnjevic, 1974; Bormann, 1988). The increase in the Cl^- permeability of the neuronal membrane shunts any current generated by excitation and is responsible for the inhibition of the postsynaptic neuron. Depending on the relation between resting membrane potential and Cl^- equilibrium potential, the neuronal membrane is either depolarized or hyperpolarized. In pyramidal cells of the hippocampus, GABA hyperpolarizes the cell when applied at the soma and depolarizes the cell when applied at the dendrites (Andersen et al., 1980).

Glial cells have been demonstrated to influence the size and time course of GABA-induced conductance changes in neurons by rapid removal of extracellular GABA from the synaptic region (e.g., Gallagher et al., 1983). Recent evidence from studies on cultured astrocytes and oligodendrocytes indicates that glial cells not only possess high-affinity uptake sites for GABA (Schousboe, 1981; Reynolds and Herschkovitz, 1984), but also a GABA receptor-coupled Cl^- channel (Kettenmann et al., 1984, 1987; Bormann and Kettenmann, 1989). Activation of GABA receptor-coupled Cl^- channels always induced a depolarization in glial cells because the intracellular Cl^- in these cells is elevated above passive distribution (Kettenmann et al., 1987; Hoppe and Kettenmann, 1989). The pharmacological properties of the glial GABA receptor were characterized in cultured astrocytes from rat cortex, and it shared many similarities with the neuronal GABA_A receptor. Glial GABA responses were blocked by the GABA_A receptor antagonists picrotoxin and bicuculline, and were mimicked by the agonist muscimol (Kettenmann and Schachner, 1985). The glial GABA response was enhanced, as in neurons, by pentobarbital and benzodiazepine agonists (Backus et al., 1988); however, unlike the effect on neurons, inverse benzodiazepine agonists increased GABA responses in astrocytes (Backus et al., 1988; Bormann and Kettenmann, 1989). GABA-induced depolarizations in glial cells *in situ* have also been observed (Krnjevic and Schwartz, 1967), but these depolarizations were speculatively attributed to the release of K^+ from surrounding neurons (Constanti and Galvan, 1978).

The aim of the present study was to identify glial GABA receptors in an intact tissue of the nervous system to verify that these receptors are not only expressed in cultured astrocytes, but that the expression of GABA receptor is a property of glial cells in the CNS.

Materials and Methods

Hippocampal slices. Kainic acid (KA)-induced neuronal lesions were used to produce neuron-free areas in hippocampal slices. KA (2–3 μg) was injected bilaterally into the lateral ventricles of male Sprague-Dawley rats (150–200 gm). After injections, animals experienced repetitive seizures which gradually diminished in severity after a few hours. From 3 to 6 weeks after the injection, transverse hippocampal slices (500 μm) were prepared from the KA-treated rats. As has been shown before (Nadler et al., 1978), KA induced virtually complete neuronal degeneration in area CA3. In cresyl violet-stained tissue (Fig. 1), the loss of pyramidal cells and the proliferation of glial cells were evident.

Cell culture. Cultures of enriched astrocytes (>90%) were obtained from cerebral hemispheres of 0- to 2-d-old Sprague-Dawley rats according to McCarthy and De Vellis (1980), with modifications described by Keilhauer et al. (1985), and maintained in culture for 3–6 weeks. By a combination of immunocytochemical and electrophysiological techniques, it was previously shown that cells depolarized by GABA are glial fibrillary acidic protein-positive astrocytes (Kettenmann et al., 1984).

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Figure 1. GFAP labeled a LY-injected glial cell in a KA-lesioned hippocampal slice. *a*, Cresyl violet-stained cryostat section of a hippocampal slice from which the glial cell illustrated in *b*, *c* was recorded. There are no neurons visible in the CA3 region, which exhibits intense gliosis. The granule cell layer (GC) survived the KA lesion. A recording was obtained from a glial cell (in the area indicated by the arrow) which depolarized in response to GABA and was then injected with LY. The slice was cryostat sectioned (10–15 μ m), and the section containing the LY-stained cell body (*b*) was also immunohistochemically stained for GFAP (*c*). The adjacent section was stained with cresyl violet (*a*). GFAP-positive fibers are contained within the LY-stained cell (arrows), indicating that this cell was an astrocyte. The intensity of LY staining was greatly diminished by the diaminobenzidine reaction to visualize HRP. Scale bars: *a*, 200 μ m; *b* and *c*, 20 μ m.

Electrophysiological procedures. Hippocampal slices (500 μ m) or cell cultures were maintained in a recording chamber at 35°C (25–30°C for cell cultures) and were continuously perfused. Slices were submerged and perfused on one side. Cells were penetrated by 2 M K-acetate or KCl-filled electrodes (resistance 40–80 M Ω). A similar electrode recorded extracellular potentials which were subtracted from the intracellular recordings to determine the true transmembrane potential. For intracellular injection of Lucifer yellow (LY; Stewart, 1981), electrodes were filled with 4–6% LY (Sigma, USA); 2–4 nA min of hyperpolarizing current was sufficient to stain glial cells. Electrophysiological data were amplified by conventional electrophysiological equipment, stored on the floppy disk system of a digital oscilloscope (Nicolet, Madison, USA), and plotted on an XY-plotter.

Solutions. Slices or cultures were continuously perfused via pump or gravity-feed systems during recording. For slices, the control solution contained (in mM): NaCl, 124; KCl, 5; MgCl₂, 1.3; NaHCO₃, 26; CaCl₂, 2; glucose, 10 (pH 7.35). The standard salt solution for cell culture

contained (in mM): KCl, 5.4; NaCl, 116.4; NaH₂PO₄, 1.0; MgSO₄, 0.8; CaCl₂, 1.8; D-glucose, 5.6; NaHCO₃, 26.2. The TTX/high-Mg²⁺/low-Ca²⁺ solution was the control solution modified to include 1 μ M TTX, 10 mM Mg²⁺, and 0.2 mM Ca²⁺. NaCl (124 mM) was replaced with Na gluconate (124 mM) in experiments to reduce extracellular Cl[−]. The following substances were added to the salt solution: GABA (1, 10 mM), muscimol (1 mM), picrotoxin (0.1 mM), flunitrazepam (0.1–1 mM), pentobarbital (0.1 mM), β -alanine (1 mM).

GFAP staining. After recordings were obtained from cells injected with LY, slices were fixed in 4% paraformaldehyde in phosphate buffer (0.1 M) for 24 hr. Following infiltration with 20% sucrose, sections (10–15 μ m) were cut on a cryostat and observed on a fluorescence microscope to determine which section(s) had the LY-stained cell. Immunohistochemical staining for glial fibrillary acidic protein (GFAP) was accomplished using a Vectastain kit (Vector Lab.) and a monoclonal antibody for GFAP (Labsystems, Helsinki). The final visualization of the HRP was accomplished using a diaminobenzidine reaction. This, however,

Figure 2. GABA- and muscimol-induced membrane depolarization. *A*, A stable intracellular recording was obtained from a glial cell in a KA-lesioned slice. GABA (1 mM) was applied as indicated by bar and reversibly depolarized glial membrane potential. The resting membrane potential is indicated at the beginning of the trace. *B*, In another cell, muscimol (1 mM), an agonist, was applied as indicated and also reversibly depolarized the glial cell.

greatly diminished the LY fluorescence. Adjacent sections were stained with cresyl violet to examine the extent of the KA-induced lesion.

Results

Identification of astrocytes in the hippocampal slice

Hippocampal slices were obtained from rats which had received an injection of KA 3–6 weeks prior to the experiment. Cells were impaled in the CA3 area, which was free of neurons as described previously (MacVicar et al., 1987). Neurons were rarely impaled (e.g., only in 3 out of several hundred impalements; in these cases, the slice was then no longer used because it contained viable neurons). Stable penetration of glial cells resulted in the recording of a very negative resting membrane potential (81.4 ± 5.5 mV; mean $\pm$ SD; $n = 46$) with no apparent spike or postsynaptic potential activity.

GABA-induced depolarizations in the slice

In all cells examined, application of GABA (1 or 10 mM) for 1 min depolarized the membrane potential reversibly (Fig. 2*A*). The maximum depolarization induced by 1 or 10 mM GABA was 1.6 ± 0.7 mV ($\pm$ SD; $n = 10$), or 3.5 ± 0.9 ($\pm$ SD; $n = 9$), respectively. In 5 experiments, we verified that this GABA-induced depolarization also occurred when synaptic and spike activities in the slices were minimized by superfusing a “low- Ca^{2+} /TTX/high- Mg^{2+} solution” (see Materials and Methods; data not shown); under these conditions the mean depolarization induced by 1 mM GABA was 1.5 ± 0.7 ($\pm$ SD; $n = 5$).

In three out of three experiments we observed that the GABA-induced depolarization could be reversed (Fig. 3) when the membrane potential was depolarized to approximately -15 mV during a sustained application of 50 mM extracellular KCl (Fig. 3; in “TTX solution/high Mg^{2+} /low Ca^{2+} ”). Furthermore, altering the equilibrium potential for Cl^- (E_{Cl}) by replacing extracellular Cl^- with gluconate, an impermeant anion, increased the GABA

Figure 3. Reversal of GABA depolarizations in high K^+ . A stable intracellular recording was obtained from a glial cell in 5 mM K^+ , then K^+ was increased to 50 mM by equimolar replacement of Na^+ with K^+ . When membrane potential was approximately -15 mV (after 10 min), GABA (1 mM) was bath-applied and caused a membrane hyperpolarization. Membrane potential was still slowly drifting and is indicated by the dotted line. Extracellular K^+ was then decreased to 5 mM, and GABA now caused a depolarization as membrane potential was slowly recovering to control levels.

(1 mM)-induced depolarization from 1.6 to 7 mV ($n = 4$; Fig. 4*A*). The above experiments suggest that GABA is activating a Cl^- conductance and the normal E_{Cl} in glial cells is less negative than membrane potential. Six cells from these experiments were stained by injecting -2 nA of LY for 2.0 min. As described earlier (Gutnick et al., 1981; Connors et al., 1984), the dye spread to adjacent cells indicating electrical coupling. Some cells were subsequently identified as astrocytes by immunohistochemically staining the slice for GFAP, a specific glial marker (Fig. 1).

Pharmacology of GABA-induced depolarizations

We tested the effect of an agonist, an antagonist, and 2 modulators of neuronal GABA_A receptors to compare the GABA responses in hippocampal astrocytes with those in neurons and cultured glial cells. Muscimol, (1 mM), a GABA_A receptor agonist that is not taken up in brain slices (Korn and Dingledine, 1986), mimicked the action of GABA by inducing a maximum depolarization of 2.7 ± 1.8 mV (Fig. 2*B*; $n = 5$). Picrotoxin (0.1 mM), which has been shown to block the GABA-coupled Cl^- channel in neurons (Barker et al., 1983), reversibly reduced the GABA-induced depolarization in the astrocytes by 40% ($n = 4$; Fig. 4*B*) compared with a control obtained from the same cell. Pentobarbital, a barbiturate, reversibly enhanced the GABA-induced depolarization by 60% ($n = 9$; Fig. 5); a similar action on the neuronal GABA_A receptor has been reported (Study and Barker, 1981). Flunitrazepam (1 mM), a benzodiazepine receptor agonist, enhanced the GABA response by 40% ($n = 4$; Fig. 5), showing further similarities to the neuronal GABA_A receptor (Bormann and Sakmann, 1984). A blocker of GABA uptake, β -alanine (1 mM) (Shon and Kelly, 1974; Bowery et al., 1979; Schousboe, 1981), did not affect the size of the GABA-induced depolarization (data not shown; $n = 3$). The pharmacological properties of the GABA receptor in astrocytes from hippocampus are thus similar to those of the GABA_A receptor in neurons and the GABA receptor in cultured astrocytes.

Figure 4. GABA-induced membrane depolarizations are dependent on the Cl^- gradient and blocked by picrotoxin. During an intracellular recording from a glial cell in a KA-lesioned slice, GABA (1 mM) was applied as indicated by bar. Three responses to GABA were obtained from the same cell and were superimposed. The individual applications were separated by at least 10 min to avoid progressive desensitization. First, as a control, GABA was applied. For the next application, the experimental solution (gluconate-low Cl^- or picrotoxin) was applied for 2–10 min and the effect of GABA was again tested in the presence of the experimental solution. Resting membrane potential was not affected by either Cl^- replacement or picrotoxin. After a 20 min wash with normal bathing solution, GABA was again applied. *A*, Reducing extracellular Cl^- by replacing most Cl^- with an impermeant anion, gluconate, greatly enhanced the depolarization from GABA (1 mM) application. Note also that the depolarization in gluconate quickly begins to desensitize. This is also observed during GABA applications on cultured glial cells. *B*, Picrotoxin (0.1 mM) reversibly decreased the GABA-induced depolarization. Therefore, the glial GABA response is altered by both changing the $[\text{Cl}^-]$ and by pharmacologically blocking the Cl^- channel with picrotoxin.

Comparison of glial GABA responses in situ and in vitro

The GABA-induced depolarizations in astrocytes from the rat hippocampal slice were much smaller in size than those described for cultured astrocytes from rat cortex (Kettenmann et al., 1984). This difference can be attributed to 2 aspects: first, the diffusion of applied GABA to astrocytes is slower in the slice than in culture because of the thickness of the slice; second, there may be a difference in E_{Cl^-} . The contribution from the former was directly tested. To obtain an estimate of the velocity of GABA diffusion in the slice, we increased the extracellular K^+ concentration from 5 to 50 mM and recorded the resulting membrane depolarization of an astrocyte (not shown). The membrane potential depolarized by 60 mV and reached a plateau within 4.5 min; 90% of the depolarization was reached within 3.5 min. We then compared GABA- and K^+ -induced depolarization in cultured astrocytes and varied the velocity of application by varying the speed of bath perfusion. The exchange rate of the bath was monitored by recording the time course of the depolarization induced by an increase in the extracellular K^+ concentration. An exchange rate of at least 90% of the bathing solutions within 15 sec was required to elicit GABA(1mM)-induced depolarizations, which were larger than 10 mV. A reduction of the perfusion rate from 90% exchange/10 sec to 90% exchange/60 sec in culture to mimic the slow diffusion of GABA into the slice reduced the GABA-induced depolarization from >20 mV to <5 mV (Fig. 6). We conclude that slow application of GABA results only in small depolarizations even at optimal GABA concentrations.

Discussion

Presence of glial GABA receptors in situ

Previous studies have already shown that glial cells in the intact tissue were depolarized by GABA (Krnjevic and Schwartz, 1967; Constanti and Galvan, 1978). It was, however, suggested that the depolarization is caused by either electrogenic uptake of GABA (e.g., Krnjevic, 1984) or by the increase of extracellular K^+ concentration released from adjacent neurons (Constanti and Galvan, 1978). In these experiments, however, the CA3 neurons have been lesioned by a KA injection. The GABA-induced depolarization was not blocked by inhibiting any possible residual neuronal activity or synaptic transmission with a combination of TTX, raised extracellular Mg^{2+} , and reduced Ca^{2+} . For cultured astrocytes, it has been unequivocally demonstrated that the GABA-induced depolarization is caused by the opening of Cl^- channels activated by GABA (Kettenmann et al., 1987; Bormann and Kettenmann, 1989). GABA responses in glial cells in the slice could be enhanced by the barbiturate pentobarbital and blocked by the GABA_A receptor antagonist picrotoxin. In addition, the blocker of glial GABA uptake, β -alanine (Shon and Kelly, 1974; Bowery et al., 1979), did not affect the GABA-induced depolarization, indicating that electrogenic uptake did not cause the depolarization. Also, muscimol, a GABA_A agonist which is not taken up in brain slices (Johnston et al., 1978; White and Snodgrass, 1979; Korn and Dingledine, 1986) depolarized glial cells in this study. Therefore, the pharmacological properties of glial GABA responses in the KA-lesioned slice are in line with those described for cultured astrocytes and are thus

Figure 5. Effect of pentobarbital and flunitrazepam on the GABA-induced depolarization. GABA alone was applied for the control response; the experimental solutions (pentobarbital or flunitrazepam) were applied for 2 min and the response to GABA was then tested again. *A*, In the presence of 0.1 mM pentobarbital (PB), the GABA-induced depolarization was enhanced compared with the control. *B*, Similar to the response in *A*, the addition of flunitrazepam (1 mM) enhanced the amplitude of the GABA-induced depolarization. Effects of both drugs were reversible upon wash.

similar to the properties of the neuronal GABA_A receptor. This suggests that the expression of GABA_A receptors in astrocytes is not induced by the culture conditions, but is an intrinsic property of these cells. We cannot exclude, however, that the lack of neurons and the reactive gliosis might affect the expression of channels in astrocytes.

Sensitivity of the glial GABA receptor

In this study we have used higher concentrations of GABA than similar studies performed on neurons or on cultured astrocytes and have detected only small changes in the resting membrane potential of astrocytes in the slice. While the single-channel properties of the GABA receptors in neurons and cultured astrocytes are similar (Bormann and Kettenmann, 1989), several properties of glial cells in an intact tissue make it difficult to detect membrane depolarizations upon GABA receptor activation. First, glial cells are strongly electrically coupled, thus forming a large syncytium (Gutnick et al., 1981; Kettenmann and Ransom, 1988). When GABA is applied, it first activates receptors located on the surface of the slice. However, because of the extensive coupling, the depolarization is strongly attenuated by the glial cells located deeper in the slice. Subsequently, when GABA has penetrated further into the slice, receptors on the surface are inactivated and thus attenuate the depolarization of the glial cells deeper in the slice. The extensive coupling also results in small membrane potential changes when substances are applied iontophoretically, since only a relatively small membrane area of the whole syncytium can be activated as it has been done in cortical glial cells (Krnjevic and Schwartz, 1967). Second, the input resistance of glial cells is much lower than

Figure 6. Influence of the bath perfusion speed on the size of the GABA-induced depolarization in cultured astrocytes. Membrane potential (V_m) was recorded from a cultured astrocyte from rat cortex. In *A*, GABA (1 mM) was applied as indicated by bar, and in *B*, with the same speed of bath perfusion, $[K^+]$ was increased from 5.4 to 50 mM. In *C* and *D*, the velocity of bath perfusion was reduced and GABA (*C*) resulted in a smaller depolarization. The slowed perfusion speed is reflected in the slower depolarization induced by the increase in $[K^+]$ (*D*).

that of neurons. This results in a smaller membrane depolarization when comparable numbers of receptors are activated. Third, the normal E_{Cl} in glial cells in the slice preparations may be closer to resting membrane potential. Shifting E_{Cl} to a more depolarized potential by reducing extracellular Cl^- greatly enhanced the amplitude of the depolarization. These 3 factors may explain why GABA responses can be easily detected in neurons, but not in glial cells of a slice preparation. Compared with cultured astrocytes, responses are smaller because of the slow diffusion of the transmitter in the slice when applied by bath exchange. We obtained an estimate of the time course for diffusion in the slice during a solution change by increasing extracellular K^+ and recording the membrane depolarization of an astrocyte. Diffusion into the slice greatly reduces the rate at which agents can be introduced onto cells. Thus, activating receptors by slowly increasing the concentration in the slice led to a small depolarization of the glial cell, in contrast to a step-like solution change causing a large depolarization in culture (see Fig. 6). Considering these less-than-optimal conditions for detection of GABA responses, we conclude that glial cells could express GABA receptors in significant amounts and that they could be functionally relevant. Receptor activation leads only to small depolarization, but could locally induce large Cl^- currents (Kettenmann et al., 1988). The presence of GABA_A receptors and GABA-activated Cl^- currents in glial cells may have widespread implications for GABA-binding studies and GABA-activated Cl^- flux studies. In most biochemical studies in the CNS, it has been assumed that GABA-activated Cl^- channels

Figure 7. Hypothetical function of glial GABA receptors. The diagram displays the presumptive spatial arrangement of glial and neuronal GABA receptors necessary to explain the role of astrocytes in the Cl⁻ homeostasis. The close vicinity of an astrocytic process at a synapse to a neuron is assumed. At lower right is a synaptic region marked by the synaptic vesicles. This part illustrates that GABA is released from the presynaptic terminal and activates GABA receptors at the neuronal postsynaptic membrane. The GABA receptor Cl⁻ channel opens and triggers a Cl⁻ flux from the extracellular space into the neuron. GABA also activates astrocytic GABA receptors, which leads to an efflux of Cl⁻ from the glial cell into the extracellular space. Movements of other ions, particularly K⁺, may also occur, and extracellular [K⁺] could also increase due to a K⁺ efflux from GABA-depolarized astrocytes.

are expressed only on neurons. This work indicates that there should be more caution attributing a purely neuronal site for some neurotransmitters. There is also evidence for glutamate (kainate type; MacVicar et al., 1988) and muscarinic (S. A. Crichton and B. A. MacVicar, unpublished observations) receptors on glial cells in the KA-lesioned hippocampal slice.

Function of glial GABA receptors

The possibility that GABA receptors are expressed by glial cells in an intact tissue leads to the question of their functional role. These receptors could serve as a signal from an inhibitory neuron to an adjacent glial cell. Furthermore, we present, in the following, a hypothesis by which glial cells could be enabled to regulate the extracellular Cl⁻ concentration in the vicinity of a GABAergic synapse (Fig. 7).

We assume that glial GABA receptors are concentrated at processes or endfeet facing the synaptic cleft of a GABAergic synapse. When neuronal GABA receptors are activated, it leads to an influx of Cl⁻ into those neurons which hyperpolarize upon application of GABA. The Cl⁻ influx leads, as a consequence, to a decrease in extracellular [Cl⁻] as described by Müller et al. (1988). In contrast, activation of glial GABA receptors leads to an efflux of Cl⁻. Glial cells could thereby be enabled to regulate [Cl⁻]. This mechanism of extracellular Cl⁻ regulation could serve to stabilize [Cl⁻] in the synaptic cleft at times of high inhibitory activity and supports the concept of glial cells being regulatory units of extracellular ion concentrations. Since the efflux of Cl⁻ is also accompanied by an efflux of K⁺ (Kettenmann et al., 1988), a rise in extracellular [K⁺] should be observed during GABA application. An efflux of K⁺ has been observed during a KA-

induced depolarization of glial cells (MacVicar et al., 1988). This could lead to a K⁺-induced depolarization of neurons, and indeed, hyperpolarizing IPSPs are followed by a depolarization (Diesz and Prince, 1989). GABA-evoked depolarizations of neurons could reflect a glial-generated [K⁺] increase.

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