Cl\textsuperscript{−} Accumulation Does Not Account for the Depolarizing Phase of the Synaptic GABA Response in Hippocampal Pyramidal Cells

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Perkins, Katherine L. Cl\textsuperscript{−} accumulation does not account for the depolarizing phase of the synaptic GABA response in hippocampal pyramidal cells. J. Neurophysiol. 82: 768–777, 1999. It has been proposed that the depolarizing phase of the biphasic synaptic GABA response could be mediated by HCO\textsubscript{3}\textsuperscript{−} passing through GABA\textsubscript{A} channels after dissipation of the transmembrane Cl\textsuperscript{−} gradient due to intracellular Cl\textsuperscript{−} accumulation. To test this hypothesis, giant GABA-mediated postsynaptic currents (GPSCs) were recorded from pyramidal cells in slices of adult guinea pig hippocampus in the presence of 4-aminopyridine. GPSCs consisted of an early outward current (GABA\textsubscript{A} component) followed by a late inward current (GABA\textsubscript{D} component). Spontaneous outward inhibitory postsynaptic currents (IPSCs) occurred during the GABA\textsubscript{D} component of the GPSC. GPSCs that were evoked 1–12 s after the preceding GPSC (short interval, siGPSCs) showed no GABA\textsubscript{D} component even though in many cells the amplitude of the siGPSC was greater than the amplitude of the GABA\textsubscript{A} component of the preceding spontaneous GPSC. In addition, the siGPSC evoked during the GABA\textsubscript{D} component of a spontaneous GPSC was an outward current. To test whether the siGPSC lacked a GABA\textsubscript{D} component because it was generated predominantly at the soma, where less of an increase in [Cl\textsuperscript{−}], would occur, picrotoxin was applied to the soma of the pyramidal cell. To the contrary, this focal application of picrotoxin caused less of a reduction in the amplitude of the siGPSC than in the amplitude of the GABA\textsubscript{A} component of the GPSC. Furthermore when a GPSC and siGPSC were evoked 10 s apart using identical stimuli, the area under the outward current curve was sometimes greater for the siGPSC than for the GPSC, and yet the siGPSC had no inward component. This result indicates that even when the location of Cl\textsuperscript{−} entry was the same, more Cl\textsuperscript{−} could enter the cell during the siGPSC than during the outward component of the GPSC and yet not lead to an inward current. In addition, when the second of two identical stimuli was applied during the inward GABA\textsubscript{D} component of the first evoked GPSC, the GABA\textsubscript{A} response it generated was always outward, demonstrating that the equilibrium potential for GABA\textsubscript{A} responses did not become more positive than the holding potential during a GPSC. Finally, evoking GPSCs at a hyperpolarized potential revealed that the siGPSC actually lacked a GABA\textsubscript{D} conductance. These results disprove the Cl\textsuperscript{−} accumulation hypothesis of the synaptic depolarizing GABA response and suggest the possibility that a separate channel type may mediate the GABA\textsubscript{D} component of the GPSC.

INTRODUCTION

The major inhibitory neurotransmitter in the cerebral cortex is \textgamma-aminobutyric acid (GABA). GABA\textsubscript{A} receptor-channels mediate fast inhibition of the postsynaptic neuron. Chloride (Cl\textsuperscript{−}) is the major permeant ion of the GABA\textsubscript{A} receptor channel, but the channel is also permeable to bicarbonate ions (HCO\textsubscript{3}\textsuperscript{−}), with a permeability ratio of Cl\textsuperscript{−} to HCO\textsubscript{3}\textsuperscript{−} of 5:1 (Bormann et al. 1987; Kaila et al. 1993). In addition to mediating an inhibitory, hyperpolarizing response, GABA sometimes mediates an excitatory, depolarizing response in adult animals (Andersen et al. 1980; Michelson and Wong 1991; Staley et al. 1995). The synaptic GABA-mediated depolarizing response originally was described as part of a biphasic hyperpolarizing-depolarizing response in CA1 pyramidal cells in response to stimulation of the stratum radiatum in the presence of pentobarbital (Alger and Nicoll 1979, 1982a). Because the equilibrium potential for HCO\textsubscript{3}\textsuperscript{−} is much more depolarized than that of Cl\textsuperscript{−}, some investigators have hypothesized that the late, depolarizing part of the GABA response is mediated by HCO\textsubscript{3}\textsuperscript{−} (Grover et al. 1993; Staley et al. 1995; Voipio et al. 1995). According to one such hypothesis, a net inward current mediated by HCO\textsubscript{3}\textsuperscript{−} passes through GABA\textsubscript{A} receptor channels after the more permeant ion Cl\textsuperscript{−} has accumulated inside the cell during the early, hyperpolarizing part of the GABA response (Cl\textsuperscript{−} accumulation hypothesis) (Staley et al. 1995). Recently it has been determined that HCO\textsubscript{3}\textsuperscript{−} does carry, at least in part, the inward current underlying the ligand-mediated (cf. Kaila et al. 1997) synaptic depolarizing GABA response in CA3 pyramidal cells (Perkins and Wong 1996). At present, however, it has not been determined whether the depolarizing GABA response is mediated by the same receptor channels as the hyperpolarizing response. An alternative hypothesis is that a separate channel with a higher permeability to HCO\textsubscript{3}\textsuperscript{−} mediates the depolarizing GABA response (Perkins and Wong 1996, 1997). For these experiments, the convulsant 4-aminopyridine (4-AP) was used to elicit giant GABA-mediated postsynaptic currents (GPSCs) in pyramidal cells. These GPSCs reflect the synchronous release of GABA from presynaptic interneurons (Michelson and Wong 1991). The experiments reported here indicate that Cl\textsuperscript{−} accumulation does not account for the synaptic depolarizing GABA response and suggest that the hyperpolarizing and depolarizing GABA responses may be mediated by two different types of GABA channels.

METHODS

Slice preparation

Experiments were done in adult guinea pig hippocampal slices. Guinea pigs (14–30 days old) were anesthetized with halothane and decapitated with a guillotine. One hippocampus was removed, and 300-\textmu m transverse slices were cut in oxygenated, ice-cold solution...
DEPOLARIZING GABA RESPONSE IS NOT DUE TO Cl\textsuperscript{−} ACCUMULATION

The access resistance ($R_i$) was estimated using the equation $R_i = \Delta V/A$, where $A$ is the amplitude of the capacitive current. Only recordings with an $R_i \leq 12$ MΩ were included in the analyses.

The liquid junction potential ($V_{ij}$) between the whole cell pipette solution and the bath solution was determined experimentally using the procedure of Neher (1992). Series resistance error ($V_i$) was calculated after the experiment using the equation $V_i = R_i \times I$. The $I$ used in the calculation was the baseline holding current at a given command potential, $V_{com}$. All potentials reported in this paper have been corrected for $V_i$ and $V_j$ using the equation $V_{m} = V_{com} - V_i - V_j$.

4-AP (50 μM) was used to elicit giant GPSCs in hippocampal pyramidal cells. 4-AP is particularly useful for this purpose because the GPSCs it elicits in a given cell type have a consistent time course (Perkins and Wong 1996); this allows comparison of particular components of the GPSC across events and across cells. Kaila and colleagues (Kaila et al. 1997; Smirnov and Kaila 1997) studied giant GABA-mediated events elicited by 40-pulse/100-Hz stimuli trains and reported the presence of a late component to the event that was a K\textsuperscript{+}-mediated, nonsynaptic inward current; the GPSCs elicited with 4-AP that are reported here and in two previous papers (Perkins and Wong 1996, 1997) do not contain and are not followed by this late K\textsuperscript{+} current.

The GPSCs elicited by 4-AP occurred spontaneously and also could be evoked by a stimulating electrode. When evoking GPSCs, single 50-μs stimuli were delivered using a bipolar tungsten electrode placed 0.3–1.5 mm away from the recording electrode. The response to a stimulus was considered to be a GPSC [or short interval GPSC (siGPSC), see following text] if it was at least half the amplitude of the preceding spontaneous GPSC.

**Focal application of picrotoxin**

To selectively apply picrotoxin (PiTX) to the soma of the recorded cell, a puffer pipette was positioned just below the surface of the slice and as near to the recording electrode as possible. Puffer solution was delivered by applying a constant pressure of 1–5 psi to the puffer pipette while the bath solution was superfused at 3–4 ml/min. Puffer pipettes were pulled from 1.5-mm-diam glass and had resistances of 6–8 MΩ when filled with puffer solution.

A two-way repeated-measures ANOVA was used to assess the effect of PiTX on the amplitudes of GPSCs and siGPSCs. To reduce variability in the GPSC amplitudes under the same condition between cells and the heterogeneity of variance between treatment groups, a transformation of the data were performed. A log_{10} transformation was chosen because the standard deviations of the treatment groups tended to be proportional to the means of the treatment groups.

**RESULTS**

**GPSCs and IPSCs**

As described previously (Perkins and Wong 1996), the GPSC recorded in pyramidal cells in the presence of GABA\textsubscript{B} blockers (Fig. 1) lasts 1.5–2 s and, at a holding potential of ~50 mV, consists of an early outward current (the GABA\textsubscript{A} component of the GPSC at potentials near rest (Perkins and Wong 1996). The HCO\textsubscript{3}\textsuperscript{−} equilibrium equation, CO\textsubscript{2}(dis) + H\textsuperscript{+} + HCO\textsubscript{3}\textsuperscript{−} ⇔ H\textsuperscript{2}CO\textsubscript{3}, describes the dependence of [HCO\textsubscript{3}\textsuperscript{−}] on [H\textsuperscript{+}] and [CO\textsubscript{2}]. Because the cell membrane is freely permeable to CO\textsubscript{2}(dis) and because the partial pressure of CO\textsubscript{2} is kept constant, to change the intracellular equilibrium concentration of HCO\textsubscript{3}\textsuperscript{−}, the pH of the intracellular solutions also must be changed. The pH corresponding to a given concentration of HCO\textsubscript{3}\textsuperscript{−} was calculated using the Henderson-Hasselbalch equation as described in Perkins and Wong (1996).

The solution used in the majority of the experiments was the 102 mM HCO\textsubscript{3}−/pH 8.0 solution, which contained (in mM) 102 KCO\textsubscript{3}, 28 KOH, 5 NaCl, 5 TEA-Cl, 10 HEPES, 4 EGTA, 5 N-(2,6-dimethyl phenylcarbamoylmethyl)-triethylammonium bromide (QX-314; RBI, Natick, MA), and 4 Mg-ATP. The 49 mM HCO\textsubscript{3}−/pH 7.7 solution contained (in mM) 49 KCO\textsubscript{3}, 76 KOH, 5 KCl, 5 NaCl, 5 TEA-Cl, 10 HEPES, 4 EGTA, 5 QX-314, and 4 Mg-ATP. The pH of solutions containing HCO\textsubscript{3}− was adjusted with methanesulfonic acid while bubbling with 95% O\textsubscript{2}-5% CO\textsubscript{2} gas. In addition, solutions containing HCO\textsubscript{3}− were continuously bubbled with 95% O\textsubscript{2}-5% CO\textsubscript{2} gas for ≥1 h immediately before filling the recording pipette. A third solution, used for only one recording, contained (in mM) 125 CsOH, 10 NaCl, 10 HEPES, 2 EGTA, 5 QX-314, and 4 Mg-ATP and was adjusted to pH 7.3 using methanesulfonic acid. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated.

As noted above, the recording pipette solutions contained QX-314 and TEA. Intracellular QX-314 blocks voltage-dependent sodium currents (Connors and Prince 1982) and the hyperpolarization-activated current $I_h$ (Perkins and Wong 1995). Intracellular TEA blocks several voltage-dependent K\textsuperscript{+} currents (Chen and Wong 1992). Suppression of these voltage-dependent conductances facilitated the study of synaptic events. In addition, QX-314 blocks the GABA\textsubscript{B} component of the GPSC (Perkins and Wong 1996).

**Whole cell recording**

Electrophysiological recordings were carried out in the whole cell voltage-clamp configuration (Hamill et al. 1981) on CA3 and CA1 pyramidal cells using a List EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany) and pClamp software (Axon Instruments, Foster City, CA). Whole cell electrode resistances ranged from 2 to 5 MΩ when filled with intracellular recording solution. Seals were established using the patch-slice method of Blanton et al. (1989). No series resistance or slow capacitance compensation was used during the experiment.

The capacitive current response to a 5-mV voltage step ($\Delta V$) was recorded in all cells and periodically retested during the experiment.
component) followed by a late inward current (the GABA_D component). The GABA_D component corresponds to the depolarizing GABA response in current-clamp recordings (Michelson and Wong 1991). Many small spontaneous inhibitory postsynaptic currents (IPSCs) appear in these recordings. These IPSCs are outward at potentials more positive than about −60 mV and inward when the membrane potential is hyperpolarized to the point that the GABA_A component of the GPSC is outward (Fig. 1A). At potentials at which the GABA_A component of the GPSC is outward, small outward IPSCs even can be observed during the inward GABA_D component of the GPSC (Fig. 1B; n = 39 cells). The occurrence of these outward GABA-mediated IPSCs during the inward GABA_D component of the GPSC indicates that the GABA_A equilibrium potential remains more negative than the holding potential during a GPSC, at least at the location where the IPSCs are generated. This finding suggests that the transmembrane Cl⁻ gradient may not dissipate sufficiently during a GPSC to account for its transition to inward current. Alternatively, the synapses mediating the GABA_D component of the GPSC and those mediating the IPSCs may be localized to different parts of the cell. One might hypothesize that the synapses mediating the GABA_D component of the GPSC are localized to the dendrites (Alger and Nicoll 1979, 1982a,b; Andersen et al. 1980; Thalmann et al. 1981) and that the dissipation of the transmembrane Cl⁻ gradient is localized there.

**Evoking siGPSCs**

The data discussed above demonstrated that the transmembrane Cl⁻ gradient does not dissipate over the entire cell during a GPSC to the point that all GABA-mediated synaptic responses are inward. To test whether the transmembrane Cl⁻ gradient dissipates sufficiently at the site of generation of the GPSCs to cause the late component to be inward, GPSCs were evoked at short intervals after a spontaneous GPSC. The GPSCs evoked after a short interval will be referred to as siGPSCs. Assuming for now that the spontaneous GPSC and the siGPSC are generated at the same cellular location, the Cl⁻ accumulation hypothesis predicts that the siGPSC will develop into an inward current more quickly due to residual Cl⁻ accumulation from the previous GPSC. To test this prediction, CA3 and CA1 pyramidal cells were held at a potential at which the GPSC consisted of an outward current followed by an inward current, and the spontaneous IPSCs were outward, usually −40 to −50 mV. A siGPSC was evoked with a stimulating electrode 5–12 s after a spontaneous GPSC. Instead of converting from outward current to inward current more quickly than usual, the siGPSC consisted of only an outward current (n = 20 of 20 cells; Fig. 2, top). GPSCs evoked after a 30- to 60-s interval had normal outward and inward components (n = 20 of 21 cells, Fig. 2, bottom).

Considering only the data of the type illustrated in Fig. 2, it could be hypothesized that the cell was able to extrude the Cl⁻ that entered during the preceding GPSC by the time of the siGPSC and that the siGPSC was not big enough to cause sufficient Cl⁻ accumulation for the development of an inward current. However, in some cases a siGPSC was evoked 1–2 s after the onset of the preceding spontaneous GPSC. In these cases (n = 6 cells), the siGPSC still consisted of only outward current. Figure 3 illustrates that even when a siGPSC was evoked during the GABA_D component of a spontaneous GPSC, the siGPSC was outward. If dissipation of the Cl⁻ driving force were responsible for the late inward current, then the siGPSC stimulated during the GABA_D component of the GPSC would have been inward.

Another result also sheds doubt on the Cl⁻ accumulation hypothesis: the siGPSC evoked 1–12 s after a spontaneous GPSC was sometimes of greater amplitude than the preceding spontaneous GPSC (Fig. 4; n = 11 of 24 cells) and yet still had no inward current component. Integration of the outward current revealed that more net charge flowed during the siGPSC than during the outward current component of the preceding
spontaneous GPSC. For example, $Q = 4.6 \times 10^{-10}$ C for the siGPSC shown in Fig. 4, and $Q = 2.7 \times 10^{-10}$ C for the outward component of the spontaneous GPSC shown in Fig. 4. Overlap of the spontaneous GPSC with its corresponding siGPSC in Fig. 4B illustrates that more negative charge entered the cell during the siGPSC than during the GABA A component of the GPSC. This finding is counter to the Cl$^{-}$ accumulation hypothesis. If sufficient Cl$^{-}$ had accumulated during the outward component of the spontaneous GPSC to cause it to turn inward, then the siGPSC also should have turned inward after a similar (or even lesser) amount of Cl$^{-}$ current flow, and it did not.

**Effect of focal somatic picrotoxin**

Although the above findings suggest that Cl$^{-}$ accumulation does not account for the late inward component of the GPSC, there is an explanation that could reconcile this data with the Cl$^{-}$ accumulation hypothesis: perhaps the synapses responsible for the siGPSC are largely somatic whereas the synapses responsible for the spontaneous GPSC are largely dendritic. This hypothesis of a differential site of generation proposes, first, that the spontaneous GPSC would involve either dendritic synapses only or both somatic and dendritic synapses with Cl$^{-}$ accumulation in the dendrites leading to the appearance of the late inward component. Second, the siGPSC instead would involve primarily synapses on the soma, where less of an increase in [Cl$^{-}$]$_{i}$ would occur as a result of Cl$^{-}$ entry, due to the lower surface/volume ratio of the soma as compared with the dendrites (and due to the reservoir of solution in the patch electrode); thus no net inward current would develop. This somatic location would explain how the siGPSC evoked during the GABA A component of the spontaneous GPSC (Fig. 3) could be outward (less [Cl$^{-}$]$_{i}$ increase in the soma than in the dendrites during the spontaneous GPSC) and would explain the lack of a GABA D component to the siGPSC even though more Cl$^{-}$ entered the cell during the siGPSC (Fig. 4; less of an increase in [Cl$^{-}$]$_{i}$ because the active synapses are somatic). If

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**FIG. 2.** GPSC evoked after a short interval (siGPSC) has no late inward component. siGPSC evoked −7 s after a spontaneous GPSC (top) had no late inward component; whereas the GPSC evoked 35 s after a spontaneous GPSC (bottom) had a late inward component. Recordings were from a single CA3 pyramidal cell held at −49 mV and were made using the 102 mM HCO$_{3}^{-}$/pH 8.0 intracellular solution.

**FIG. 3.** siGPSC evoked during the GABA A component of a spontaneous GPSC is an outward current. Recording was from a CA3 pyramidal cell held at −48 mV. Stimulus artifact is visible as a vertical line. Intracellular solution was the 102 mM HCO$_{3}^{-}$/pH 8.0 solution.

**FIG. 4.** siGPSC can be larger than the preceding spontaneous GPSC and still have no late inward current component. **A:** siGPSC evoked after an −10-s interval had a greater amplitude than the GABA A component of the preceding spontaneous GPSC and yet had no late inward current component. **B:** GPSC and siGPSC shown in A are shown here overlapped to emphasize that a greater net charge flowed during the siGPSC than during the outward component of the spontaneous GPSC. Recording was from a CA3 pyramidal cell held at −45 mV. Intracellular solution was the 102 mM HCO$_{3}^{-}$/pH 8.0 solution.
this hypothesis is correct, blocking somatic GABA synapses should cause a greater reduction in the amplitude of the siGPSC than in the amplitude of the GABA\textsubscript{A} component of the spontaneous GPSC.

To test the possibility that, in contrast to the GPSC, the siGPSC reflects the activation of predominantly somatic synapses, a puffer pipette containing 100 \textmu{}M PiTX was situated in the CA3 cell body layer near the recording electrode (see METHODS). For this experiment, CA3 pyramidal cells were chosen in which the siGPSC evoked after a 5- to 12-s interval was of greater amplitude than the GABA\textsubscript{A} component of the preceding spontaneous GPSC. After recording control spontaneous GPSCs and siGPSCs, PiTX was delivered to the soma of the recorded cell. The presence of fast green in the puffer solution allowed visualization of the spread of PiTX. The affected area included the cell body layer around the electrodes, the local stratum oriens at least two-thirds of the way to the fimbria, the local stratum lucidum, and sometimes the proximal edge of the local stratum radiatum.

This focal application of PiTX (Fig. 5) reduced the amplitude of the GABA\textsubscript{A} component of the spontaneous GPSC by 56 \pm 6\% (mean \pm SE, n = 3 cells) and the amplitude of the siGPSC by 37 \pm 8\% (n = 3 cells). The amplitude of the GABA\textsubscript{D} component of the spontaneous GPSC showed no consistent change as a result of PiTX application to the soma (0 \pm 8\%, n = 3 cells). The siGPSC still had no inward component in PiTX. The effect of PiTX was largely reversible (Fig. 5). After washout, the amplitude of the GABA\textsubscript{A} component of the spontaneous GPSC recovered to 89 \pm 9\% of control, the siGPSC amplitude was 91 \pm 7\% of control, and the amplitude of the GABA\textsubscript{D} component of the spontaneous GPSC was 95 \pm 11\% of control. Notably, rather than reducing the amplitude of the siGPSC to a greater extent than the amplitude of the GABA\textsubscript{A} component of the GPSC, the focal application of PiTX actually reduced the amplitude of the GABA\textsubscript{A} component of the spontaneous GPSC to a greater extent. A two-way repeated-measures ANOVA revealed that PiTX significantly reduced the amplitudes of the outward component of the spontaneous GPSC and the siGPSC (P < 0.05) and that the PiTX had a greater effect on the amplitude of the outward component of the spontaneous GPSC than on the amplitude of the siGPSC (P < 0.05; a log\textsubscript{10} transformation of the data were used—see METHODS).

These results indicate that the siGPSC was not generated more somatically than the GABA\textsubscript{A} component of the spontaneous GPSC. Notably, in the presence of focal somatic PiTX, the siGPSC was still greater in amplitude than the GABA\textsubscript{A} component of the preceding spontaneous GPSC, and yet had no late inward current. This result indicates that even when GABA-mediated Cl\textsuperscript{−} entry is occurring exclusively in dendrites, which should be the site with a greater potential for [Cl\textsuperscript{−}]\textsubscript{i} increase, more Cl\textsuperscript{−} can enter the cell during a siGPSC than during the preceding spontaneous GPSC and still not result in an inward current.

**Evoking pairs of GPSCs with identical stimuli**

The preceding results suggest that Cl\textsuperscript{−} accumulation cannot account for the depolarizing phase of the biphasic synaptic GABA response. However, one might imagine a scenario in which these data still could be reconciled with the Cl\textsuperscript{−} accumulation hypothesis: even though the spontaneous GPSC and the evoked siGPSC both are generated partly in the dendrites, perhaps they are segregated to different areas of the dendrite or to different dendritic branches. In this case, one might propose that the siGPSC evoked during the GABA\textsubscript{D} component of a spontaneous GPSC was outward because it was generated in a different location where no Cl\textsuperscript{−} had accumulated. In addition, to explain why a greater amount of Cl\textsuperscript{−} can enter during a siGPSC than during the previous spontaneous GPSC and yet not result in an inward current, one might propose that a greater amount of Cl\textsuperscript{−} entry is required to cause a sufficient rise in [Cl\textsuperscript{−}], at those dendritic sites involved in the siGPSC. Furthermore, one would have to propose that a lesser amount of Cl\textsuperscript{−} flows into the cell during a siGPSC (because the siGPSC does
not have an inward component) than during a GPSC evoked with an identical stimulus (which does have an inward component, see Fig. 2).

As a final test of the Cl\textsuperscript{−} accumulation hypothesis, pairs of GPSCs were evoked at varying intervals with identical stimuli and recorded from a CA3 pyramidal cell. The stimulating electrode was placed 0.3–0.6 mm away from the recording electrode. To assess whether the stimuli directly activated the interneurons generating the GPSC, the delay between the beginning of the stimulus artifact and the beginning of the GPSC (and siGPSC) was measured. The delay was measured for six stimuli in each of the three cells. The values in the three cells were 1.02 ± 0.04 ms (mean ± SD), 1.08 ± 0.06 ms, and 1.8 ± 0.1 ms. The delay values for GPSCs and siGPSCs were pooled. An illustration of the short delay is in Fig. 6C. These short delay times indicate that monosynaptic connections were evoked by the stimuli, suggesting that the second of two identical stimuli will result in activation of the same synapses, or a subset of those synapses, on the recorded cell.

When the stimuli were 8–12 s apart, the second evoked GPSC of the pair (the siGPSC) was of the same or shorter amplitude as the preceding evoked GPSC and never had an inward component (n = 3 cells, Fig. 6). The integral of the siGPSC was compared with the integral of the outward current component of the first evoked GPSC of a pair (the GPSC) to compare Cl\textsuperscript{−} entry between the two. In at least one case in every cell (n = 3 cells), the area under the siGPSC was ≥5% larger than the area under the GPSC, and yet the siGPSC had no inward component. For example, $Q = 3.0 \times 10^{-10}$ C for the siGPSC shown in Fig. 6, and $Q = 2.5 \times 10^{-10}$ C for the outward component of the GPSC shown in Fig. 6. Overlapping the two traces (Fig. 6B) illustrates that the integral for the siGPSC was greater than the integral of the outward component of the GPSC because the siGPSC was longer in duration, perhaps because no GABAD component interrupted it. Assuming that Cl\textsuperscript{−} entry was occurring at the same sites on the cell following each stimulus, if Cl\textsuperscript{−} accumulation had been the cause of the reversal to inward current during the first evoked GPSC, then the siGPSC should have also become inward, but did not. When the interval between the two GPSCs was 45–60 s, the second GPSC of a pair had a normal GABAD component (n = 3 cells).

A second set of experiments was carried out using pairs of identical stimuli. In this case, the second stimulus was delivered 2 s after the first, which was during the GABAD component of the first evoked GPSC. In every case (n = 14 trials in 3 cells), the evoked current, while smaller than a GPSC, was always outward (Fig. 7). If Cl\textsuperscript{−} accumulation had been the cause of the reversal to inward current during the first evoked GPSC, then the siGPSC should have also become inward, but did not. When the interval between the two GPSCs was 45–60 s, the second GPSC of a pair had a normal GABAD component (n = 3 cells).

Time course of conductance change revealed by hyperpolarization

The previous experiments indicate that Cl\textsuperscript{−} accumulation cannot account for the GABAD component of the GPSC. The data also suggest that evoking a GPSC after a short interval may selectively activate the GABA\textsubscript{A} component of the GPSC to the exclusion of the GABAD component. From the data illustrated in Fig. 6B, it is apparent that while the duration of the siGPSC was longer than that of the outward component of the GPSC, overall, the duration of the siGPSC was shorter. Possible explanations for the apparent lack of a GABAD component to the siGPSC are that the siGPSC had no GABAD component...
Conductance or that the conductance underlying the GABA_D component of the GPSC was present but either the cell was at the GABA_D reversal potential or the GABA_D current was masked by an outward current. To determine whether the siGPSC has a GABA_D conductance, the time course of the conductance change associated with the siGPSC and the GPSC was compared.

Recordings from both CA3 and CA1 pyramidal cells were used in this set of experiments. Either 5–10 s or 30–60 s after a spontaneous GPSC at −50 mV, the membrane potential was hyperpolarized by 15–35 mV. Six hundred milliseconds after the hyperpolarization, a GPSC (or siGPSC) was evoked using a stimulating electrode (Fig. 8). The evoked GPSCs and siGPSCs were entirely inward, as expected. The GPSC evoked after 30–60 s lasted 1.5–2 s, the duration of a normal GPSC. The siGPSC evoked after 5–10 s had a shorter duration (0.7–0.9 s) than the normal GPSC, even though the amplitudes of the GABA_A components of the GPSC and the siGPSC were comparable (Fig. 8, n = 8). The duration of the siGPSC evoked at the hyperpolarized potential was comparable with the duration of the siGPSC evoked at −50 mV (Fig. 8, dotted trace). The fact that the duration of the siGPSC was shorter both at −50 mV and at more hyperpolarized potentials indicates that the conductance underlying the siGPSC is also shorter in duration than that underlying the GPSC. The siGPSC has no inward component at −50 mV because the siGPSC is actually missing the GABA_D conductance.

Subtraction of the siGPSC evoked at a hyperpolarized potential (which is missing the GABA_D component) from the GPSC evoked at the same hyperpolarized potential after a longer interval (which has a GABA_D component) revealed the time course of the GABA_D component of the GPSC. The siGPSC and the subtracted trace are plotted together in Fig. 8B for comparison. The siGPSC should show the time course of the GABA_A component, and the subtracted trace should show the time course of the GABA_D component. The peak of the GABA_A component occurred at just under 100 ms; whereas the peak of the GABA_D component did not occur until 350–400 ms after the stimulus. There was significant overlap between the GABA_D component and the falling phase of the GABA_A component.

**DISCUSSION**

Previous experiments showed that changing [HCO_3^-] has a much greater effect on the reversal potential of the GABA_D component of the GPSC than on the reversal potential of the GABA_A component (Perkins and Wong 1996). Those experiments did not determine the reason for this difference. At that time, the two hypotheses proposed to explain the greater effect of [HCO_3^-] on the reversal potential of the GABA_D component were that the Cl^- driving force had dissipated during the GABA_A component of the GPSC, leaving a net HCO_3^- current through GABA_A channels (Cl^- accumulation hypothesis), or that the GABA_D response was mediated by a separate set of channels which showed a greater permeability to HCO_3^-.

The data presented in this paper favor the latter hypothesis.

Cl^- accumulation does not account for the late inward current component of the GPSC

Cl^- accumulation has been shown to occur in hippocampal pyramidal cells due to repeated applications of exogenous GABA (Huguenard and Alger 1986), due to repetitively evoked IPSCs (Ling and Benardo 1995) and due to a rise in extracellular [K^+] as a result of a 10-pulse/100-Hz train of stimuli (inhibition of Cl^-/K^+ cotransport) (see Fig. 7 in Kaila et al. 1997). The data in this paper demonstrate, however, that Cl^- accumulation cannot account for the GABA_D component of the GPSC in hippocampal pyramidal cells. If the GABA_A equilibrium potential (E_GABA_A) had shifted during the GABA_A component of the GPSC to the point where E_GABA_A was more positive than the holding potential, either due to Cl^- entry through GABA_A channels or due to an inhibition of Cl^-/K^+ cotransport (see Kaila et al. 1997), then the GABA-mediated synaptic current evoked during the GABA_D component of a GPSC should have been an inward current instead of the outward current which was observed (Figs. 3 and 7) (see also Alger and Nicoll 1979, 1982a). In addition, if the Cl^- accumulation hypothesis were correct, then siGPSCs that reached the magnitude of a normal GPSC would then have turned inward; instead, siGPSCs reached a greater magnitude than the preceding GPSC without exhibiting an inward current (Figs. 4 and 6). The experiments using pairs of identical stimuli demonstrated that Cl^- does not accumulate during a GPSC to the point that E_GABA_A is more positive than holding potential even right at the site of GPSC generation: Cl^- accumulation cannot account for the late inward current component of the GPSC.

Why is the GABA_D component of the GPSC an inward current?

Cl^- accumulation cannot account for the late inward current component of the GPSC. The most obvious remaining possibility is that the GABA_A and GABA_D components of the
GPSC are mediated by two different channels, the GABA_A channel and the hypothetical GABA_D channel (which may simply be a particular isoform of the GABA_A channel). On the basis of ion permeability experiments (Perkins and Wong 1996), the GABA_A channel would have a higher permeability ratio of HCO$_3^-$: Cl$^-$ than the GABA_A channel.

Initially, it seems that a second viable hypothesis is that the GABA_A component and the GABA_D component of the GPSC account for earlier experiments, which showed that changing 1986; Müller et al. 1989). However, this hypothesis cannot account for earlier experiments, which showed that changing the HCO$_3^-$ concentration inside the cell ([HCO$_3^-$]) had a greater effect on the reversal potential of the GABA_D component of the GPSC than on the reversal potential of the GABA_A component of the GPSC (Perkins and Wong 1996). The Goldman equation predicts that the opposite result would have been obtained if the steady-state level of Cl$^-$ were higher at the part of the cell where the GABA_A component of the GPSC was initiated. The reasoning is as follows. Call the site where the GABA_D response originates the D compartment and the site where the GABA_A response originates the A compartment. Because the GABA_D component of the GPSC reverses at a more depolarized potential than the GABA_A component of the GPSC, the D compartment would be maintained at a higher level of Cl$^-$ than the A compartment. The siGPSC at both −50 and −84 mV had a shorter time course than the siGPSC evoked after 60 s and a lower time course than the GPSC evoked after a longer interval. Recording was from a single CA3 pyramidal cell. Intracellular solution was the 49 mM HCO$_3^-$/pH 7.7 solution.

Note that the denominator stays constant because the extracellular solution is constant. The lower the [Cl$^-$] is, the more the value of [HCO$_3^-$] dominates the numerator and the more effect changing [HCO$_3^-$] has on $E_{rev}$. For example, if compartment A has a [Cl$^-$] of 5 mM and compartment D has a [Cl$^-$] of 15 mM, at a [HCO$_3^-$] of 19 mM, the reversal potential of the GABA_A response would be −72 mV and the reversal potential of the GABA_D response would be −52 mV. If then the [HCO$_3^-$] was changed to 80 mM, the GABA_A response would reverse at −49 mV and the GABA_D response would reverse at −39 mV. Thus in this example, changing the [HCO$_3^-$], from 19 to 80 mM caused a 23-mV change in the GABA_A reversal potential and only a 13-mV change in the GABA_D reversal potential.

A third hypothesis is that GABA_A channels may be modified during a GABA response—perhaps as a result of prolonged contact with GABA—so that they become more permeable to HCO$_3^-$. To explain why the duration of the GABA-mediated conductance is much longer when a GABA_D component is present, the process that is responsible for conversion to a more HCO$_3^-$-permeable channel also would change the channel kinetics. A similar hypothesis has been proposed to explain the...
ability of neuroactive steroids to induce a depolarizing GABA response (Burg et al. 1998). To explain the lack of an inward current component to the siGPSC, this hypothesis would further require that the process, whatever it is, takes several seconds to recover after a previous period of activation. If this hypothesis were correct, however, and the same channels were responsible for both the GABA_A and GABA_D components of the GPSC, one would have expected that when PiTX blocked a portion of the GABA_A component of the spontaneous GPSC, it also would have blocked a portion of the accompanying GABA_D component, but it did not (Fig. 5). It is not sufficient to propose that the hypothetical conversion renders the channel insensitive to PiTX, because it has been shown that the depolarizing response to GABA may actually be more sensitive to PiTX than the hyperpolarizing response (Alger and Nicoll 1982b).

Why does the siGPSC lack the GABA_D component?

The GABA_D component of the GPSC is absent or greatly reduced in the siGPSCs (Figs. 2, 4, 6, and 8). The experiment in which GPSCs were evoked at hyperpolarized potentials after different intervals was particularly informative in this regard (Fig. 8). It demonstrated that the apparent absence of a GABA_D component to the siGPSC at −50 mV was not due to the fact that the GABA_D current was at its reversal potential and was also not due to its being masked by an outward GABA-mediated current. That experiment revealed the time course of the GABA-mediated conductance change and demonstrated that the GABA_D component of the GPSC was actually absent from the siGPSC.

The lack of a GABA_D component to the siGPSCs can be attributed to the short interval after which they are evoked because GPSCs evoked with an identical stimulus, but after 30–60 s, have a normal GABA_D component. Other than the above-described hypothesis of a refractory channel conversion process, there are three possible explanations for this lack of a GABA_D conductance to the siGPSC. The first is that the hypothetical GABA_D channels are desensitized after the GPSC and take some time to recover from desensitization. This explanation may be unlikely: earlier experiments showed that the depolarizing component of biphasic responses to a brief application of 1 mM GABA solution to a CA1 pyramidal cell could last >4 s (Wong and Watkins 1982), which is much longer than the duration of the GABA_D component of the GPSC. The other two explanations involve a presynaptic mechanism. One possibility is that less GABA is released in response to the second of two closely timed stimuli and that the hypothetical GABA_D channels are extrasynaptic (Alger and Nicoll 1982b) or located at the periphery of synapses. In this case, less GABA release would result in a failure of GABA to diffuse to extrasynaptic sites and thus in a failure to activate the GABA receptor channels which are more permeable to HCO_3^-. The second explanation involving a presynaptic mechanism is that separate classes of presynaptic interneurons (or separate axon collaterals from the same presynaptic neurons) innervate exclusively either GABA_A channels or GABA_D channels on pyramidal cells. A similar segregation of receptors has been proposed for GABA_A and GABA_B receptors (reviewed in Nurse and Lacaille 1997) and for GABA_A,fast and GABA_A,slow receptors (Banks et al. 1998). In this scenario, the class of pre-synaptic neurons (or axon collaterals) innervating GABA_D channels would take longer to recover after a GPSC than the class innervating GABA_A channels.

Location of the synaptic GABA_D response

The results of focal application of PiTX to the somata of CA3 pyramidal cells demonstrated that both the GABA_A and GABA_D components of the GPSC can be elicited in the dendrites, confirming an earlier study that used current source density analysis in the CA1 region (Lambert et al. 1991). Exposing the soma and proximal dendrites to PiTX reduced the GABA_A response while having little effect on the GABA_D response, suggesting that synaptic GABA_D responses are generated preferentially on the distal dendrites of CA3 pyramidal cells. A dendritic origin of GABA_D responses in CA1 pyramidal cells has been suggested by many earlier studies (e.g., Alger and Nicoll 1979; Andersen et al. 1980; Thalmann et al. 1981). Because the GABA_A response to exogenously applied GABA is more sensitive to PiTX than the GABA_A response (Alger and Nicoll 1982b), PiTX presumably would have blocked the GABA_A component of the GPSC if it had originated on the soma. Thus the results presented here suggest that synapses containing GABA_A channels exist on both the soma and the dendrites of CA3 pyramidal cells; whereas synapses containing the hypothetical GABA_D channels exist predominantly, or even exclusively, on the dendrites.

Hypothetical GABA_D channel

These experiments disprove the Cl^- accumulation hypothesis and suggest that the GABA_D response could be mediated by a different channel than the GABA_A response. The data illustrated in Fig. 8B demonstrate that the GABA_A response has slower rise and decay times than the GABA_A response; thus if the GPSC is mediated by two different channels, the hypothetical GABA_D channel might be predicted to have slower channel kinetics. Earlier ion permeability experiments (Perkins and Wong 1996) indicate that this hypothetical GABA_D channel should have a greater permeability to HCO_3^- than the GABA_A channel. In addition, the GABA_D receptor channel would be expected to have a lower affinity for GABA (Wong and Watkins 1982) and perhaps a greater sensitivity to PiTX and bicuculline (Alger and Nicoll 1982b). Furthermore GABA_D channels on pyramidal cells would be expected to be localized to the dendrites. The fact that giant GABA_A-mediated responses (siGPSCs) can occur independent of GABA_D responses suggests, in addition, that if two different channel types exist, they would be segregated either to different synapses or to different subsynaptic sites on hippocampal pyramidal cells. The hypothetical GABA_D channel would very likely not only mediate the GABA_D component of the GPSC in pyramidal cells but also the HCO_3^- dependent (Lamsa and Kaila 1997), GABA-mediated excitatory transmission among interneurons (Michelson and Wong 1991).

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