Kinetics of GABA_B Receptor-Mediated Inhibition of Calcium Currents and Excitatory Synaptic Transmission in Hippocampal Neurons In Vitro

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Summary

The time courses of the γ -aminobutyric acid type B (GABA_B) receptor-mediated inhibition of excitatory synaptic transmission and of action potential-evoked calcium currents were studied in hippocampal neurons in vitro with step-like changes of a saturating baclofen concentration. Inhibition mediated by postsynaptic GABA_B receptors was excluded pharmacologically. Both presynaptic inhibition and reduction of calcium currents developed and declined exponentially with similar time constants of about 0.2 and 3 s, respectively. The close correlation of the time courses indicates that fast, G protein-mediated depression of voltage-gated calcium channels and thus direct reduction of the presynaptic calcium influx may contribute to the GABA_B receptor-induced inhibition of excitatory synaptic transmission in hippocampal neurons in vitro.

Introduction

Activation of the G protein-coupled subtype of γ -aminobutyric acid type B (GABA_B) receptors (for reviews see Bowery, 1989, 1993; Sivilotti and Nistri, 1991) inhibits excitatory synaptic transmission between hippocampal neurons (Scholz and Miller, 1991; Yoon and Rothman, 1991; Solis and Nicoll, 1992; Thompson and Gähwiler, 1992; for earlier publications see Nicoll et al., 1990). Postsynaptically, the GABA_B receptor-induced inhibition is due to a potassium-dependent, slow inhibitory postsynaptic potential (for reviews see Nicoll et al., 1990; Crunelli and Leresche, 1991; Sivilotti and Nistri, 1991). The presynaptic mechanisms of this short-term modulation, however, are still debated (Miller, 1990; Gage, 1992; Thompson et al., 1993). By coupling to G proteins (Morishita et al., 1990), GABA_B receptors affect a set of effectors including ion channels and cytoplasmic enzymes (for reviews see Bowery, 1989, 1993; Nicoll et al., 1990; Sivilotti and Nistri, 1991), each of which could contribute to presynaptic inhibition, as suggested by previous studies (Holz et al., 1989; Travagli et al., 1991; Thompson and Gähwiler, 1992). The GABA_B receptor-induced depression of voltage-gated calcium channels (for reviews see Carbone and Swandulla, 1989; Schultz et al., 1990; Anwyl, 1991; Scott et al., 1991; Bowery et al., 1993) would effectively inhibit transmitter release by reducing the presynaptic calcium influx. In hippocampal neurons, this mechanism of presynaptic inhibition has been sug-

gested by Scholz and Miller (1991), who demonstrated GABA_B receptor-induced inhibition of both voltage-activated calcium currents and evoked excitatory postsynaptic currents (EPSCs) in vitro. Evidently, this requires that the GABA_B receptor-induced reduction of voltage-activated calcium currents develops with identical kinetics as the presynaptic inhibition. To test whether this important, but previously not considered, condition was fulfilled, we resolved the time courses of the GABA_B receptor-induced inhibition of evoked EPSCs by means of superfusion techniques that allowed step-like changes of the baclofen concentration at cultured synapses. The calcium transients that trigger transmitter release from presynaptic terminals cannot be easily measured. Hence, we studied the time course of the GABA_B receptor-induced inhibition of whole-cell voltage-activated calcium currents. To investigate their modulation under physiological conditions, we imitated the natural stimulus of presynaptic calcium influx and used an action potential-like voltage command to evoke calcium currents, which lasted about 3 ms. Previously, neurotransmitter-induced inhibition of voltage-gated calcium channels was analyzed after depolarization of neurons for tens to hundreds of milliseconds, which had been shown to affect the GABA_B receptor-induced inhibition (Grassi and Lux, 1989). Our study revealed that the GABA_B receptor-induced inhibition of excitatory synaptic transmission and action potential-evoked calcium currents developed with closely matching time courses. Kinetic analysis thus supports the hypothesis that direct depression of presynaptic, voltage-gated calcium channels may contribute to presynaptic inhibition in hippocampal neurons in vitro.

Results

Baclofen-Induced Inhibition of Evoked EPSCs

To study excitatory synaptic transmission in cultured hippocampal neurons, EPSCs were evoked by extracellular stimulation of presynaptic neurons in the presence of the γ -aminobutyric acid type A receptor antagonist bicuculline methchloride (50 μ M). Postsynaptic GABA_B receptor-induced inhibition was suppressed by adding the lidocaine derivative QX-314, which blocks GABA_B receptor-induced potassium currents (Nathan et al., 1990; Andrade, 1991; Otis et al., 1993), intracellularly to postsynaptic neurons (Lambert and Wilson, 1993). This drug also suppressed voltage-activated sodium currents (see Experimental Procedures). Latencies of monosynaptic EPSCs (<5 ms) ranged from 1.4 to 4.5 ms (mean, 2.8 ± 0.8 ms; $n = 46$). Peak amplitudes of evoked EPSCs, which reversed at 0 mV ($n = 6$; Figure 1A), ranged from -20 to -922 pA at -70 mV holding potential (mean, 284 ± 230 pA; $n = 121$) and were reversibly blocked by 6,7-

Figure 1. Characterization of Extracellularly Evoked EPSCs

(A) EPSCs, evoked by extracellular stimulation in the presence of bicuculline methchloride (50 μM) extracellularly, reversed at 0 mV holding potential indicating the increase of a nonselective cationic conductance. The current-voltage curve was averaged from normalized EPSC peak amplitudes ($n = 6$; error bars indicate SD).

(B) EPSCs, evoked at -70 mV holding potential, were reversibly blocked by the selective antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM), indicating that postsynaptic non-NMDA glutamate receptors mediated the response.

(C) Superfusion of synapses with baclofen (10 μM), a selective GABA_B receptor agonist, reversibly inhibited evoked EPSCs. The inhibition was presynaptic, as the lidocaine derivative QX-314 (20 mM) blocked postsynaptic, GABA_B receptor-induced potassium currents.

dinitroquinoxaline-2,3-dione (DNQX; 10 μM; $93\% \pm 5\%$ inhibition; $n = 7$; Figure 1B), indicating that extracellularly evoked EPSCs were mediated by non-NMDA glutamate receptors. NMDA receptor-mediated components of EPSCs were blocked by extracellular magnesium (2 mM).

In 16 out of 17 synapses, application of the selective GABA_B receptor agonist R(+)-baclofen (baclofen; 10 μM) reversibly inhibited peak amplitudes of evoked EPSCs by 44–81% (mean, $67\% \pm 10\%$; Figure 1C). The presynaptic inhibition was mediated by GABA_B receptors, as it was suppressed by the selective receptor antagonist CGP 35348 (500 μM; mean of inhibition, $6\% \pm 4\%$; $n = 5$; Olpe et al., 1990), in line with previous reports (Solis and Nicoll, 1992; Thompson and Gähwiler, 1992). The involvement of G proteins in the GABA_B receptor-induced inhibition was tested by pretreating cultures with pertussis toxin (PTX; 500 ng/ml for at least 24 hr), which inactivates G_{ai} and G_{ao} subunits (Ui, 1984; Milligan, 1988). In PTX-treated cultures, the baclofen-induced inhibition of EPSCs was abolished (mean of inhibition, $3\% \pm 4\%$; $n = 6$). Thus, excitatory synaptic transmission between cultured hippocampal neurons was inhibited by GABA_B receptor-activated, PTX-sensitive G proteins as reported previously (Huston et al., 1990; Scholz and Miller, 1991; Yoon and Rothman, 1991). In other hippocampal preparations, however, the GABA_B receptor-induced inhibition of excitatory synaptic transmission persisted throughout PTX treatment (Dutar and Nicoll, 1988; Colmers and Pittman, 1989; Thompson and Gäh-

wiler, 1992). These discrepancies might be explained by cell type-specific differences in the activated G proteins or by the less effective PTX application in vivo compared with in vitro (Nicoll et al., 1990; Scott et al., 1991).

Kinetics of the GABA_B Receptor-Mediated, Presynaptic Inhibition of Evoked EPSCs

The onset of the GABA_B receptor-mediated inhibition of evoked EPSCs was studied with rapid application of a saturating baclofen concentration (50 μM) to cultured synapses (see Experimental Procedures). Step-like application was achieved by the pressure-driven extension of a laminar stream of agonist-containing solution positioned beneath the stimulated synapse, which was localized by the tip of the patch pipette delivering the extracellular stimulus (Figure 2A). The method allowed detection of the onset of inhibition at 50 ms (see Experimental Procedures). In the 10 synapses analyzed, rapid agonist application was proved with cadmium (50 μM), which instantly inhibits synaptic transmission by blocking presynaptic calcium currents (Pfrieger et al., 1992; Figure 3A). Figure 3B shows the time-resolved inhibition of excitatory synaptic transmission by cadmium and baclofen. The time courses of the presynaptic inhibition, induced by cadmium and baclofen, clearly differed. Cadmium suppressed EPSCs rapidly by a direct block of presynaptic calcium channels, whereas baclofen activated a multi-step signal transduction pathway involving GABA_B receptors and PTX-sensitive G proteins. The GABA_B

Figure 2. Superfusion Techniques for Rapid Changes of Agonist Concentrations at Cultured Neurons and Synapses

(A) Technique for fast agonist application to extracellularly stimulated synapses: A laminar stream of agonist-containing solution formed between two oppositely placed glass pipettes if positive and negative hydrostatic pressure was applied to the source and the suction pipette, respectively (top; broken line). A valve-controlled pressure increase in the source pipette extended the stream, thus superfusing the stimulated region (top; solid line). The speed of the concentration change was tested with the cadmium-induced block of HVA calcium currents elicited by voltage steps from -70 to 0 mV. In small neurons without neurites, fast application of cadmium ($100 \mu\text{M}$) blocked the HVA calcium current within maximally 50 ms (bottom). (B) Technique for fast removal of the agonist from a stimulated synapse: Under control conditions, the synapse was steadily superfused with the agonist (top; broken line). The superfusion was terminated rapidly by application of negative pressure to the agonist-containing source pipette, and the agonist was quickly displaced by the simultaneous extension of a nearby stream of agonist-free control solution (top; solid line). The block of the HVA calcium current by cadmium ($100 \mu\text{M}$) was alleviated within 50 ms in a small neuron without neurites (bottom).

receptor-induced inhibition rose with a delay of 110 – 200 ms (mean, 131 ± 46 ms; $n = 8$; Figure 3B), including the application delay of maximally 50 ms. Comparable delays were observed in the receptor-mediated induction of potassium currents and attributed to the initial activation of the G protein-dependent pathway (Inomata et al., 1989; Otis et al., 1993). In 5 out of 8 synapses, the GABA_B-induced inhibition, which varied from 39% to 85% (mean, $70\% \pm 14\%$; $n = 10$) when measured 1–2 s after application, rose single exponentially with time constants ranging from 110 to 200 ms with a mean of 173 ± 36 ms. In 3 out of 8 synapses, inhibition was established with an even faster time course, which could not be resolved owing to the system-limited resolution of 50 ms. To resolve the time course of recovery of EPSCs from steady-state GABA_B-induced inhibition ($50 \mu\text{M}$ baclofen applied for 10 – 20 s; inhibition by 47%–80%; mean, $61\% \pm 11\%$; $n = 8$), steady superfusion of synapses was rapidly terminated by a pulse of negative pressure in the source pipette, and additionally, baclofen was rapidly displaced from synapses by a stream of control solution (Figure 2B). In the 8 synapses analyzed, steady-state block of EPSCs by cadmium was relieved within 0.6 s (Figure 3C). Figure 3D depicts the decline of the cadmium- and GABA_B receptor-induced presynaptic inhibition after rapid removal of the blockers. The recovery of EPSCs from baclofen-induced inhibition started

with a delay of 1.1 – 2.1 s (mean, 1.7 ± 0.5 ; $n = 6$), including the time required for solution exchange. In 7 out of 8 synapses, the GABA_B receptor-induced presynaptic inhibition declined with single-exponential time courses and time constants ranging from 1.2 to 7.2 s (mean, 3.4 ± 1.9 s), yielding full recovery within several seconds. In summary, fast changes of the baclofen concentration at extracellularly stimulated synapses showed that after maximal activation of GABA_B receptors, the inhibition of EPSCs activated with a delay of 50 – 150 ms and rose exponentially with time constants between 110 and 200 ms. After removal of the agonist, the inhibition declined exponentially with a delay of 0.6 – 1.5 s and time constants ranging between 1 and 7 s. This time course of GABA_B receptor-induced presynaptic inhibition is corroborated by the similarly rapid paired-pulse depression of synaptic responses in hippocampal slices, which is also induced by presynaptic GABA_B receptors (Davies et al., 1990; Otis et al., 1993), implying that in hippocampal neurons GABA_B receptors inhibit presynaptic transmitter release through a fast signal-transducing pathway.

Voltage-Activated Calcium Currents Evoked by Action Potential-Like Voltage Commands

To study GABA_B receptor-mediated modulation of voltage-activated calcium currents under physiologi-

Figure 3. Time Course of the GABA_B Receptor-Mediated Inhibition of Evoked EPSCs after Fast Application and Removal of Baclofen

(A) Inhibition of evoked EPSCs 0.05 and 1.5 s after fast application of cadmium (50 μ M; open columns) and baclofen (50 μ M; closed columns); amplitudes were averaged from the 10 synapses analyzed (error bars indicate SD). In all synapses, the agonist was reliably applied within 50 ms, and cadmium did not contaminate the baclofen-induced inhibition as EPSCs were unaffected 50 ms after application of the agonist.

(B) The onset of the cadmium- (open squares) and the baclofen- (closed squares) induced inhibition of an evoked EPSC was resolved at 50 ms. The rise of the GABA_B receptor-induced inhibition was fitted with a single-exponential function (solid line) with a time constant of 200 ms (top). After fast application of baclofen, the pre-synaptic inhibition of the EPSC started with a delay and reached its maximum after 0.5 s (bottom).

(C) Cadmium- and GABA_B receptor-induced inhibition of EPSCs during steady-state superfusion and 0.05 and 0.55 s after fast removal of the blockers; amplitudes were averaged from the 8 synapses analyzed (error bars indicate SD). In all synapses, the agonist was removed within 0.6 s, and the baclofen-induced inhibition was not contaminated by cadmium.

(D) Decline of the inhibition of EPSCs after fast removal of cadmium and baclofen. The decline of the GABA_B receptor-induced inhibition was slower than the onset, but it could also be fitted with a single-exponential function (solid line) with a time constant of 2.8 s (top). After fast removal of baclofen, the EPSC recovered from presynaptic inhibition and reached its control amplitude after 4–5 s (bottom).

cal conditions, voltage-gated calcium channels were activated by an action potential-like voltage command (further referred to as spike command) instead of the traditional, rectangular voltage steps. The spike command was derived from action potentials evoked in hippocampal neurons by background synaptic activity (Figure 4A, inset). Since voltage steps that are applied with the whole-cell patch clamp are filtered owing to the series resistance and the membrane capacitance (Armstrong and Gilly, 1992; for values see Experimental Procedures), we found it necessary to test how effectively the rapidly rising and decaying spike command could be applied to the somatic membrane and compared the applied spike command with the actual somatic membrane potential by double whole-cell recordings from the same cell. A double whole-cell recording of a spike command applied to a medium-sized neuron ($\phi \approx 30$ μ m) with neurites is depicted in the upper panel of Figure 4A. The applied spike command was slowed and diminished. In 7 large

neurons ($\phi \approx 50$ μ m), comprising at least one neurite, peaks of the actual membrane potential were delayed by 340–600 μ s (mean, 430 ± 90 μ s) and decreased by 16%–34% (mean, $21 \pm 6\%$; or 23 ± 7 mV) relative to peaks of the spike command. In 6 small neurons ($\phi \approx 10$ –20 μ m) without neurites, the delay was 150–250 μ s (mean, 200 ± 40 μ s) with amplitude errors ranging from 3% to 11% (mean, $6 \pm 3\%$ or 7 ± 4 mV). The double whole-cell recording demonstrated that the action potential-like voltage pulse could be effectively applied to the soma at least in small and medium-sized neurons, in which the slowing and the diminution of the spike potential were tolerable.

Spike-evoked calcium currents through voltage-gated channels were isolated by subtracting leak and capacitive currents evoked in the presence of 100 μ M cadmium (Figure 4A, lower panel). In 5 neurons analyzed with double whole-cell recordings, the delay, between the peaks of the actual membrane potential and the leak-corrected calcium influx, ranged from

Figure 4. Action Potential-Evoked Calcium Currents

(A) Application of an action potential-like voltage command to a neuron clamped at -70 mV holding potential. Inset: an action potential recorded from a hippocampal neuron (broken line) and the derived spike command (solid line). Double whole-cell recording (voltage- and current-clamp) from a medium-sized hippocampal neuron (top) to compare the applied spike command (broken line) with the actual membrane potential (solid line). The calcium current evoked by the applied spike command was obtained by subtracting the leak and capacitive current recorded in the presence of 100 μ M cadmium (bottom).

(B) Spike-evoked calcium currents were reversibly inhibited during superfusion with baclofen (10 μ M).

0.82 to 1.26 ms (mean, 0.99 ± 0.17 ms; spike command peak, $+40$ mV). This delay could be attributed to the activation characteristics of voltage-gated calcium channels (Brown et al., 1984). The shape of the spike-evoked calcium influx clearly resembled that reported in previous studies, which also used action potentials to activate calcium currents (Spencer et al., 1989; McCobb and Beam, 1991; Scroggs and Fox, 1992). In 72 neurons, the time-to-peak values (10%–90%) ranged between 0.46 and 1.23 ms (mean, 0.83 ± 0.16 ms), and the half amplitude influx lasted from 0.78 to 2.74 ms (mean, 1.38 ± 0.30 ms). Amplitudes of the calcium influx, measured as time integral over the whole current, ranged from 0.31 to 11.13 nC (mean, 3.0 ± 2.3 nC).

Baclofen-Induced Inhibition of Spike-Evoked Calcium Currents

Activation of $GABA_B$ receptors induces G protein-mediated inhibition of voltage-activated calcium currents in hippocampal (Scholz and Miller, 1991) and other neurons (Grassi and Lux, 1989; Mintz and Bean, 1993). We confirmed this effect for currents that were evoked with a spike command. As the spike depolarized neurons for about 3 ms, we could study the $GABA_B$ receptor-induced inhibition without voltage-dependent relief, which develops with time constants beyond 4 ms (Grassi and Lux, 1989; Elmslie et al., 1990; Kasai, 1992). In 20 out of 22 hippocampal neurons, baclofen (10 μ M applied for 5–20 s) inhibited spike-evoked calcium influx reversibly by 21%–84% (mean, $47\% \pm 14\%$; Figure 4B). Again, as for evoked EPSCs, the amount of inhibition varied, suggesting that individual neurons were differently equipped with the $GABA_B$ -induced signaling pathway. The inhibition was mediated by $GABA_B$ receptors, as CGP 35348 (500 μ M) significantly reduced the amount of inhibition to $28\% \pm 15\%$; $n = 12$; $p < 0.01$). PTX-sensitive G proteins mediated the $GABA_B$ -induced inhibition of calcium currents, as in PTX-treated cultures (500 mg/ml; for at least 24 hr). The baclofen-induced inhibition was completely abolished (mean, $4\% \pm 5\%$; $n = 8$) in line with previous studies (Holz et al., 1986; Scholz and Miller, 1991). Thus, $GABA_B$ receptors reduced the spike-evoked calcium influx in cultured hippocampal neurons by activation of PTX-sensitive G proteins.

Kinetics of the $GABA_B$ Receptor-Mediated Inhibition of Spike-Evoked Calcium Currents

The $GABA_B$ receptor-mediated, presynaptic inhibition of EPSCs, which rose and declined exponentially within one and several seconds, respectively, could be caused by the direct depression of presynaptic calcium channels. If this were true, the time courses of the two $GABA_B$ receptor-induced effects should be similar. Thus, we determined the time course of the inhibition of spike-evoked calcium currents with fast application and removal of baclofen as described above. Calcium influx was evoked with a stimulus interval of 0.2 s, and under control conditions, it decreased by $12.8\% \pm 5.6\%$ ($n = 8$) after 30 stimuli. With barium as charge carrier, the decrease was significantly reduced to $4.0\% \pm 2.3\%$ ($n = 5$), indicating calcium-dependent inactivation of calcium channels. The small decrease left with barium was probably due to a voltage-dependent component of inactivation, since it was significantly reduced to $1.4\% \pm 0.6\%$ ($n = 5$) with a stimulus interval of 0.6 s. Thus, spike-evoked calcium currents evoked at 5 Hz decreased owing to voltage- and calcium-dependent inactivation of calcium channels (Morad et al., 1988; Carbone and Swandulla, 1989; Fryer and Zucker, 1993).

The activation kinetics of the $GABA_B$ receptor signaling to voltage-gated calcium channels were investi-

Figure 5. Time Course of the GABA_B Receptor-Mediated Inhibition of Spike-Evoked Calcium Currents after Fast Application and Removal of Baclofen

(A) Inhibition of spike-evoked calcium currents 0.05 and 1.5 s after fast application of cadmium (50 μ M; open columns) and baclofen (50 μ M; closed columns); amplitudes were averaged from the 8 neurons analyzed (error bars indicate SD). In all neurons, the agonist was reliably applied within 50 ms, and cadmium did not contaminate the baclofen-induced inhibition. (B) Onset of the cadmium- (open squares) and the GABA_B receptor-induced (closed squares) inhibition of a spike-evoked calcium current resolved at 50 ms. The rise of the baclofen-induced inhibition was fitted with a single-exponential function (solid line) with a time constant of 230 ms (top). After fast application of baclofen, the inhibition of the spike-evoked calcium current started with a delay and reached its maximum after 0.5 s (bottom).

(C) Cadmium- and baclofen-induced inhibition during steady-state superfusion and 0.05 and 0.65 s after fast removal of the blockers; mean of the 9 neurons analyzed (error bars indicate SD). The agonist was removed effectively within 0.7 s, and contamination of the baclofen-induced inhibition by cadmium was excluded. (D) Decline of the cadmium- and GABA_B receptor-induced inhibition of spike-evoked calcium currents after fast removal of the blockers. The decline of the baclofen-induced inhibition was fitted with a single-exponential function (solid line) with a time constant of 1.3 s (top). After fast removal of baclofen, the calcium current recovered from inhibition and reached its control amplitude after 4 s (bottom).

gated by fast application of baclofen (50 μ M) to small and medium-sized neurons. Agonist application within 50 ms was proved with cadmium in the 12 neurons analyzed (Figure 5A). The distinct time courses of the cadmium- and the baclofen-induced inhibition of spike-evoked calcium currents are depicted in Figure 5B. In 8 out of 12 neurons, the GABA_B receptor-induced inhibition of the spike-evoked calcium influx started with a delay of 100–200 ms (mean, 131 ± 46 ms; $n = 8$), rose exponentially, and amounted 25%–56% (mean, $40\% \pm 10\%$; $n = 12$) 1–2 s after application. The activation time constants ranged from 130 to 290 ms with a mean of 220 ± 60 ms ($n = 6$). As for the EPSCs, we observed inhibition of calcium currents with time courses too fast to be resolved quantitatively ($n = 2$). Thus, the GABA_B receptor-mediated inhibition of evoked EPSCs and of spike-evoked calcium currents activated with closely matching time courses (Table 1), indicating that the onset of presynaptic inhibition could be determined by the depression of presynaptic calcium channels.

We next studied whether this also held true for the recovery from presynaptic inhibition and analyzed

the decline of the GABA_B receptor-mediated inhibition of spike-evoked calcium currents. Figure 5D depicts the different decays of the GABA_B receptor- and the cadmium-induced inhibition of a spike-evoked calcium current. In 9 out of 11 neurons, in which rapid removal was proven (Figure 5C), steady-state inhibition (44%–80%; mean, $59\% \pm 11\%$; $n = 11$) declined exponentially after a delay of 0.5–0.85 s (mean, 0.7 ± 0.1 s), with time constants varying from 1.2 to 5.0 s (mean, 2.2 ± 1.2 s). These values were in a similar range as those we found for the presynaptic inhibition (Table 1), indicating that not only the onset, but also the decline, of the presynaptic inhibition could be determined by the GABA_B receptor-induced inhibition of spike-evoked calcium currents.

Absence of GABA_B Receptor-Evoked Potassium Currents in Hippocampal Neurons In Vitro

In hippocampal slices, GABA_B receptors also activate potassium currents (Gähwiler and Brown, 1985; Otis et al., 1993), which could mediate presynaptic inhibition of EPSCs. We examined, therefore, whether activation of GABA_B receptors induced potassium

Table 1. Time Constants of the GABA_B Receptor-Induced Inhibition

Inhibition of	Delay (s)		τ (s)	
	Min-Max	Mean $\pm$ SD (n)	Min-Max	Mean $\pm$ SD (n)
EPSCs	On 0.10–0.20	0.13 $\pm$ 0.05 (8)	0.11–0.20	0.17 $\pm$ 0.04 (5)
	Off 1.1–2.1	1.7 $\pm$ 0.5 (6)	1.2–7.2	3.4 $\pm$ 1.9 (7)
Calcium currents	On 0.10–0.20	0.13 $\pm$ 0.05 (8)	0.13–0.28	0.22 $\pm$ 0.06 (6)
	Off 0.5–0.9	0.7 $\pm$ 0.1 (9)	1.2–5.0	2.2 $\pm$ 1.2 (9)

currents in cultured hippocampal neurons. Baclofen (10 or 50 μ M) was applied to neurons, clamped at -30 mV holding potential with GTP (2 mM) and ATP (1 mM) added intracellularly to optimize conditions for G protein activation. In only 7 out of 42 cells, superfusion with baclofen induced small outward currents (mean amplitude, 13 ± 7 pA). Thus, we found that in the majority of hippocampal neurons in vitro, GABA_B receptors did not couple to potassium channels. A similar lack of GABA_B receptor-induced potassium currents has been reported previously in embryonic neurons (Harrison, 1990). It may be due to absent expression of necessary signal-transducing proteins in immature neurons (Janigro and Schwartzkroin, 1988) or due to a culture-conditioned defect in the signaling pathway.

Discussion

This study revealed that in hippocampal neurons in vitro, the GABA_B receptor-induced inhibition of evoked EPSCs and of spike-evoked calcium currents rose and declined with very similar kinetics, indicating that presynaptic GABA_B receptors may inhibit excitatory synaptic transmission by depressing voltage-gated calcium channels and thus decreasing the calcium influx that triggers transmitter release. The fast kinetics of the GABA_B receptor-induced inhibition of spike-evoked calcium currents indicated that the signal transduction involves a tight, membrane-delimited coupling of GABA_B receptor-activated G proteins to voltage-gated calcium channels.

Presynaptic Inhibition Due to Depression of Voltage-Gated Calcium Channels

Presynaptic GABA_B receptors inhibit excitatory synaptic transmission between hippocampal (Dutar and Nicoll, 1988; Forsythe and Clements, 1990; Thompson and Gähwiler, 1992) as well as other neurons of the CNS (Howe et al., 1987; Seabrook et al., 1990; Alford and Grillner, 1991). This inhibition could be effectively brought about by the G protein-mediated depression of voltage-gated calcium channels, which has been demonstrated in neurons from different regions of the nervous system (Grassi and Lux, 1989; Mintz and Bean, 1993). In hippocampal neurons, however, the coupling of GABA_B receptors to calcium channels has been questioned owing to an early study (Gähwiler and Brown, 1985), and consequently, presynaptic inhibition in the hippocampus has been attributed to

other GABA_B receptor-induced effects (Thompson et al., 1993). In line with Scholz and Miller (1991), we show that in cultured hippocampal neurons, GABA_B receptors markedly reduced voltage-activated calcium currents and that the inhibition was mediated by PTX-sensitive G proteins. The expression of this GABA_B receptor-induced effect in cultured hippocampal neurons suggested that depression of voltage-gated calcium channels could have caused the presynaptic inhibition of evoked EPSCs. We thus examined the time courses of the baclofen-induced presynaptic inhibition and the reduction of spike-activated calcium currents after step-like changes of the agonist concentration and found a good agreement of the kinetic properties of both effects, as these developed and declined with exponential kinetics and similar time constants (Table 1). Thus, the kinetic data support the hypothesis that in hippocampal neurons in vitro, the depression of presynaptic, voltage-gated calcium channels may be one mechanism of the GABA_B receptor-induced inhibition of synaptic transmission.

Clearly, our data do not rule out that other GABA_B receptor-induced effects contribute to presynaptic inhibition of synaptic transmission. GABA_B receptor-induced potassium currents (Gähwiler and Brown, 1985; Nicoll et al., 1990; Sivilotti and Nistri, 1991; Gage, 1992; Bowery, 1993) could inhibit evoked EPSCs by hyperpolarizing presynaptic terminals and thus reducing the action potential, since these currents activate similarly fast (Otis et al., 1993) as the presynaptic inhibition reported here and are similarly sensitive to PTX (Andrade et al., 1986) and CGP 35348 (Solis and Nicoll, 1992). The absence of whole-cell somatic potassium currents which we observed does not rule out that, in presynaptic terminals, GABA_B receptors couple to potassium channels. However, compared with the GABA_B receptor-induced inhibition of evoked EPSCs, potassium currents activated by postsynaptic GABA_B receptors decrease 10-fold more rapidly (Otis et al., 1993). Similarly, Otis et al. (1993) reported that paired-pulse inhibition of synaptic transmission also decreases more slowly than the GABA_B receptor-induced potassium current. Assuming that presynaptically and postsynaptically induced potassium currents have similar kinetics, this indicates that presynaptic inhibition is mediated by additional mechanisms with relatively slow decrease. In comparing pharmacological sensitivities, previous authors concluded that GABA_B receptor-induced potassium currents do not cause presynaptic inhibition of excitatory synaptic

transmission (Thompson and Gähwiler, 1992; Lambert and Wilson, 1993), but rather inhibitory synaptic transmission (Thompson and Gähwiler, 1992). The baclofen-induced increase of a voltage-dependent potassium current (Saint et al., 1990) does not appear to contribute to presynaptic inhibition, since, in contrast with inhibition of evoked EPSCs, this effect is insensitive to PTX (see Gage, 1992). In addition to their effects on ionic currents, GABA_B receptors could cause presynaptic inhibition by inhibition of the adenylate cyclase (Jenks et al., 1991; Travagli et al., 1991). Furthermore, there is increasing evidence that G protein-coupled receptors (Man-Son-Hing et al., 1989; Scholz and Miller, 1992; Reklung, 1993), including the GABA_B type (Scanziani et al., 1992), may cause presynaptic inhibition by interfering directly with the release apparatus. If the latter signal transduction pathways contribute to presynaptic inhibition, their time courses should meet those we described here.

GABA_B Receptors Inhibit Voltage-Gated Calcium Channels through a Fast Pathway

The molecular components involved in the GABA_B receptor-induced depression of voltage-gated calcium channels have been studied previously. There is a considerable amount of evidence that beneath G proteins, neither cytoplasmic second messengers nor protein kinases mediate the signaling (Green and Cottrell, 1988; Diversé-Pierluzzi and Dunlap, 1993; for reviews see also Dolphin, 1990; Schultz et al., 1990; Anwyl, 1991) and indication (Bean, 1989; Grassi and Lux, 1989; Kasai, 1992) that G proteins mediate calcium channel inhibition by a membrane-delimited pathway (Brown, 1993). The fast onset of the GABA_B receptor-induced inhibition of spike-evoked calcium currents we describe supports this conclusion. The GABA_B receptor-mediated inhibition developed similarly fast as noradrenergic and the fast component of muscarinic inhibition (Bernheim et al., 1991) and even faster than glutamatergic inhibition (Swartz and Bean, 1992) of voltage-activated calcium currents. The fast actions of these neurotransmitters on calcium channels appear not to involve readily diffusible second messengers. Furthermore, signal transduction between GABA_B receptors and voltage-gated calcium channels activates similarly fast as the membrane-delimited signaling between muscarinic receptors and potassium channels (Yatani et al., 1987; Inomata et al., 1989). In several but not all neurons, calcium currents and EPSCs were found maximally depressed within 150 ms. Similarly rapid inhibition of calcium currents by other neurotransmitters has been previously reported (Bean, 1989; Jones, 1991; Pollo et al., 1992), but these very fast effects were inconsistently observed. Other studies on time courses of receptor-induced calcium current inhibition, however, reported slower onset rates even though solution exchanges (≤ 100 ms) were similarly fast (Boland and Bean, 1993; Sahara and Westbrook, 1993). This may indicate different signaling mechanisms.

After removal of baclofen, the GABA_B receptor-induced inhibition of spike-evoked calcium currents decayed with time constants of several seconds. Decreases with similar time courses have been reported for inhibition of voltage-activated calcium currents by different G protein-coupled receptors (Bernheim et al., 1991; Jones, 1991; Swartz and Bean, 1992; Pollo et al., 1992). The muscarinic potassium current decays with a faster time course (Nargeot et al., 1982; Breitwieser and Szabo, 1988), and the decay of the GABA_B receptor-induced potassium current has a clearly faster component (Otis et al., 1993). However, in the case of G protein-mediated current induction, the deactivation might be caused by a fast, channel-intrinsic process. Since the coupling between GABA_B receptors and voltage-gated calcium channels probably does not involve soluble second messengers, the signal transduction could be rate limited by the G protein activation and deactivation cycle (Birnbaumer et al., 1990), as previously suggested for the muscarinic activation of potassium channels (Breitwieser and Szabo, 1988).

Experimental Procedures

Cell Culture and Drugs

Hippocampi from embryonic rats (embryonic day 19) were triturated with fire-polished pasteur pipettes without prior enzymatic treatment in chilled Earl's buffered salt solution, supplemented with penicillin, streptomycin, and fungizone, but with no calcium added (GIBCO, Life Technologies, Eggenstein, Germany). Dissociated cells were plated on polyornithine/laminin-coated culture dishes (density = $4 \cdot 10^4$ to $6 \cdot 10^4$ cells per cm^2) and cultured with 5% CO₂ in Eagle's basal medium, which was supplemented with horse serum (10%), L-glutamine, glucose, insulin, penicillin, and streptomycin (GIBCO). After 3 days in vitro, proliferation of glial cells was minimized by adding cytosine- β -D-arabino-furanoside hydrochloride (10 μM) (Sigma Chemie, Deisenhofen, Germany) for 24 hr to the culture medium. Cultures were viable up to 14 days and used after at least 6 days. Baclofen was purchased from Research Biochemicals Inc. (Köln, Germany). The selective GABA_B receptor antagonist CGP 35348 was a kind gift of CIBA-GEIGY (Basel, Switzerland). For treatment with PTX (RBI), the toxin was added to the culture medium for at least 24 hr at a concentration of 500 ng/ml.

Electrophysiological Recording

Currents were recorded using the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) with an EPC 7 amplifier (List-Medical, Darmstadt, Germany) and the pCLAMP hard- and software (Axon Instruments, Inc., Foster City, CA). Patch pipettes of borosilicate glass had resistances between 3 and 10 M Ω . Experiments were carried out at 18°C–22°C. EPSCs were evoked by extracellular stimulation of presynaptic axons (Figure 2A). Short (200 μs) current pulses from an isolated stimulator (Hi-Med) were delivered through a patch pipette, which was filled with a mixture (1:1) of extracellular solution and 1 M NaCl and grounded through the bath reference. EPSCs were recorded at a holding potential of -70 mV in 125 mM NaCl, 5 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES (pH 7.4 with 50 μM bicuculline methchloride or -iodide [Sigma] added). The intracellular recording solution contained 110 mM KCl, 0.25 mM CaCl₂, 10 mM EGTA, and 10 mM HEPES (pH 7.4). Voltage-activated sodium currents, elicited in postsynaptic neurons by the extracellular stimulation, disturbed evoked EPSCs and were blocked with the quaternary lidocaine derivative QX-314 (20 mM; RBI) added to the intracellular recording solution. Whole-cell current-clamp recordings from hippocampal pyramidal neurons confirmed

that QX-314 (20 mM) suppressed sodium-dependent action potentials within 2 min after breaking the patch ($n = 5$; data not shown) as previously reported (Connors and Prince, 1982; Isaac and Wheal, 1993). For the kinetic analysis, EPSCs were evoked with a stimulus interval of 0.3 s, which decreased peak amplitudes by $51\% \pm 18\%$ ($n = 10$) after 10 stimuli, with a stable amplitude thereafter.

Voltage-activated calcium currents were recorded in a sodium-free extracellular solution (130 mM tetraethylammonium chloride, 5 mM CaCl_2 , 10 mM HEPES [pH 7.4]), with the intracellular solution containing 100 mM CsCl, 0.25 mM CaCl_2 , 10 mM EGTA, 20 mM tetraethylammonium chloride, and 10 mM HEPES (pH 7.4 with 1 mM Mg-ATP and 2 mM Na-GTP added). Estimates of series resistances were gained according to Armstrong and Gilly (1992) by analyzing currents that were evoked by depolarizing voltage steps (-70 to -60 mV; 50 ms). As the measurements were done in the solutions used for calcium current recording, membrane resistances were high ($1\text{--}7$ G Ω ; mean, 4 ± 2 G Ω ; $n = 7$). Series resistances were calculated from the fastest time constant of the decay of the capacitive current component (Jackson, 1992) divided by the membrane capacitance ($6.1\text{--}26.2$ pF; mean, 16.2 ± 5.9 pF; $n = 18$). Values ranged from 6 to 20 M Ω (mean, 11.4 ± 4.2 M Ω ; $n = 18$). The membrane capacitance and the series resistance (for maximally 50%) were compensated with the analog circuits of the patch-clamp amplifier. The spike command used to activate voltage-gated calcium channels was derived from action potentials evoked by spontaneous synaptic activity in high density cultures. In the inset in Figure 4A, a recorded action potential (broken line) and the derived spike command are depicted. From the holding potential (-70 mV), the command potential rose within 1.75 ms to a threshold of -35 mV, reached the peak value ($+40$ mV) within 450 μ s, and repolarized to -70 mV within 1.8 ms.

Superfusion Techniques for Fast Application and Removal of Receptor Agonists

The superfusion technique, originally introduced by Streit and Lux (1989), was modified to allow fast application (Leßmann et al., 1992) and removal of receptor agonists. For fast application, a laminar stream of agonist-containing solution, which formed between two oppositely placed glass pipettes ($\phi \approx 20$ and 50 μ m) under positive (source pipette) and negative (suction pipette) hydrostatic pressure, was positioned close to the region to be superfused (Figure 2A). A valve-controlled pressure increase in the source pipette rapidly expanded the stream, thus superfusing the nearby cell or synapse. For fast removal of the agonist, a pulse of negative pressure in the source pipette terminated steady superfusion. Additionally, the agonist was rapidly displaced by simultaneously extending a nearby stream of agonist-free control solution, delivered from a second source pipette (Figure 2B). The performance of the superfusion system was tested by blocking high voltage-activated calcium currents by cadmium in neurons with few and short neurites (Figures 2A, bottom, and 2B, bottom). The kinetics of the cadmium-induced block should reliably measure the efficiency of application and removal. After fast application of cadmium, the delay of block onset, the system dead time, ranged from 8 to 14 ms with a mean value of 10 ± 2 ms ($n = 14$). High voltage-activated (HVA) calcium currents were blocked by more than 85%, 50 ms after cadmium application (mean, $94\% \pm 7\%$; $n = 9$; Figure 2A, bottom). The efficiency of removal was tested by removing steady-state cadmium block from HVA calcium currents. Current amplitudes recovered to at least 80% of control (mean, $89\% \pm 7\%$; $n = 15$) 50 ms after removal of cadmium (Figure 2B, bottom). Before the kinetics of the GABA_B -induced inhibition were analyzed, fast solution changes at individual synapses and neurons were tested with cadmium. The positions of the superfusion pipettes were adjusted until inhibition of EPSCs and calcium currents was fully established or removed by application of cadmium or control solution. Thereafter, the baclofen-containing delivery barrel of the multibarreled system was switched on. Prior to baclofen application to cells, washout for several seconds ascertained complete removal of cadmium.

To analyze the onset of the baclofen-induced inhibition at the maximally possible resolution (50 ms), we used the following application and stimulation paradigm: After control EPSCs or calcium currents were evoked with stimulus intervals of 0.3 and 0.2 s, respectively, baclofen was applied 50 ms before the first test stimulus with the application pulse lasting for 1 s. In each subsequent trial, the interval between the baclofen application and the first test stimulus was prolonged by 50 ms. The time course of inhibition was obtained by combining these trials.

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