The Journal of Neuroscience, February 1, 1997, 17(3):1025–1032

$GABA_B$ Receptor-Mediated Inhibition of Tetrodotoxin-Resistant GABA Release in Rodent Hippocampal CA1 Pyramidal Cells

Wolfgang Jarolimek and Ulrich Misgeld

Institute of Physiology, University of Heidelberg, D-69120 Heidelberg, Germany

Tight-seal whole-cell recordings from CA1 pyramidal cells of rodent hippocampus were performed to study $GABA_B$ receptor-mediated inhibition of tetrodotoxin (TTX)-resistant IPSCs. IPSCs were recorded in the presence of TTX and glutamate receptor antagonists. (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs by a presynaptic action. The inhibition by (*R*)-(−)-baclofen was concentration-dependent, was not mimicked by the less effective enantiomer (*S*)-(+)-baclofen, and was blocked by the $GABA_B$ receptor antagonist CGP 55845A, suggesting a specific effect on $GABA_B$ receptors. The inhibition persisted in the presence of the Ca^{2+} channel blocker Cd^{2+}. There was no requirement for an activation of K^+ conductances by (*R*)-(−)-baclofen, because the inhibition of TTX-resistant IPSCs persisted in Ba^{2+} and Cd^{2+}. Because the time courses of TTX-resistant IPSCs were not changed by (*R*)-(−)-baclofen, there was no evidence for a selective inhibition of quantal release from a subgroup of GABAergic terminals. (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs in guinea pigs and Wistar rats, whereas the inhibition was much smaller in Sprague Dawley rats. In Cd^{2+} and Ba^{2+}, β-phorbol-12,13-dibutyrate and forskolin enhanced the frequency of TTX-resistant IPSCs. Only β-phorbol-12,13-dibutyrate reduced the inhibition by (*R*)-(−)-baclofen. We conclude that $GABA_B$ receptors inhibit TTX-resistant GABA release through a mechanism independent from the well known effects on Ca^{2+} or K^+ channels. The inhibition of quantal GABA release can be reduced by an activator of protein kinase C.

Key words: GABA; baclofen; $GABA_B$ receptors; quantal release; protein kinase C; adenylate cyclase; presynaptic; miniature IPSCs

Synaptically released GABA may activate presynaptic $GABA_B$ receptors that reduce subsequent GABA release. In pharmacological studies, the selective $GABA_B$ agonist baclofen potently reduces GABA release in various preparations (for review, see Misgeld et al., 1995).

Several mechanisms are presently discussed for the $GABA_B$ receptor-mediated autoinhibition. The issue of whether inhibition of GABA release by baclofen occurs independently of inhibition of Ca^{2+} influx or activation of K^+ conductance at the nerve terminals remains controversial. So far, no effects of baclofen on spontaneous tetrodotoxin (TTX)-resistant IPSCs have been detected in CA1 pyramidal cells in rat hippocampal slices (Cohen et al., 1992; Doze et al., 1995) or in CA3 pyramidal cells in organotypic slice cultures from rat (Scanziani et al., 1992). In the latter preparation, however, baclofen strongly reduces TTX-resistant EPSCs (Scanziani et al., 1992). On the other hand, in cultured rat midbrain neurons, baclofen reduces the frequency of both TTX-resistant IPSCs and EPSCs (Jarolimek and Misgeld, 1992). Furthermore, baclofen inhibits TTX-resistant IPSCs in thalamic slices (Ulrich and Huguenard, 1996).

A selective effect of baclofen on TTX-resistant EPSCs would suggest that GABA and glutamate release are differentially regulated by $GABA_B$ receptors (Thompson et al., 1993). Quantal GABA and glutamate release are regulated by various G-protein-coupled receptors in the hippocampus (Cohen et al., 1992; Scanziani et al., 1992, 1995; Scholz and Miller, 1992; Rekling, 1993; Thompson et al., 1993; Bijak and Misgeld, 1995; Lupica, 1995). Acetylcholine, for example, reduces both GABA and glutamate release (cf. Thompson et al., 1993; Scanziani et al., 1995), suggesting that a similar modulation of release can exist at both types of terminals. For an understanding of the regulation of GABA release and of the functional role of presynaptic $GABA_B$ receptors in the mammalian brain, it is important therefore to clarify the action of $GABA_B$ receptors on quantal GABA release.

In the course of experiments on $GABA_B$ receptor-mediated inhibition in guinea pig hippocampal slices, we surprisingly observed that (*R*)-(−)-baclofen reduced the frequency of IPSCs recorded in TTX and hence studied this effect in more detail. Because previous reports did not observe a baclofen-mediated inhibition of TTX-resistant IPSCs in CA1 neurons of rats (Cohen et al., 1992; Doze et al., 1995), we examined apparent discrepancies between our results and previous work.

Parts of this work have been published previously in abstract form (Jarolimek and Misgeld, 1996).

MATERIALS AND METHODS

The procedure for preparation and maintenance of hippocampal slices followed previously described methods (Misgeld et al., 1979). Guinea pigs (200–400 gm) were 6–9 weeks old; Wistar and Sprague Dawley rats (20–40 gm) were 2–3 weeks old. Slices were incubated in a solution containing (in mM): 127 NaCl, 5 KCl, 1.3 $MgSO_4$, 1.25 KH_2PO_4, 2.5 $CaCl_2$, 26 $NaHCO_3$, 10 glucose.

Patch-clamp recordings. Whole-cell patch-clamp recordings were made from CA1 pyramidal cells visualized through an upright microscope equipped with infrared light and differential interference optics (Stuart et al., 1993). Recordings were performed at room temperature (20–22°C). Slices were perfused at a rate of ~1.5 ml/min with a solution containing

Received Nov. 14, 1996; accepted Nov. 25, 1996.

This work was supported by the Sonderforschungsbereich 317/B13 of the Deutsche Forschungsgemeinschaft. We thank John F. X. O'Callaghan for helpful discussions and C. Heuser and A. Lewen for excellent technical support. The gift of baclofen, CGP55845A as well as CGP 37849, by Ciba Geigy, Basel, Switzerland, is highly appreciated.

Correspondence should be addressed to Dr. Ulrich Misgeld, Institute of Physiology, University of Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany.

(in mM): 130 NaCl, 2 KCl, 1.3 $MgSO_4$, 1.25 KH_2PO_4, 2.5 $CaCl_2$, 26 $NaHCO_3$, 10 glucose, pH 7.35. $BaCl_2$ (1 mM) was used in some experiments to block K^+ conductances. To avoid precipitation, $MgCl_2$ was substituted for $MgSO_4$, KH_2PO_4 was omitted, and KCl was increased to 3.25 mM to keep $[K^+]$ constant. All bath solutions were bubbled continuously with a mixture of 95% O_2/5% CO_2. Recordings were obtained in the presence of the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 10 μM), the NMDA receptor antagonist CGP37849 (1 μM), and TTX (1 μM). Cd^{2+} was used at a concentration of 100 μM to block Ca^{2+} currents. In fact, voltage steps from −65 to 0 mV did not elicit any Na^+ or Ca^{2+} current ($n = 4$). The nomenclature of baclofen in this paper follows that used in the chemical literature (cf. Froestl et al., 1995). The $GABA_B$ antagonist CGP 55845A, (*S*)-(+)-baclofen, and (*R*)-(−)-baclofen were kind gifts of Ciba Geigy, Switzerland. (*R*)-(−)-baclofen at a concentration of 10 μM was used throughout the study, except for the determination of concentration–response relationships.

Recording pipettes were filled with (in mM): 130 CsCl, 10 NaCl, 0.25 $CaCl_2$, 5 EGTA, 10 HEPES, 10 glucose, 4 Mg^{2+}-ATP, 5 QX-314. Resistance to bath was 4–6 MΩ before seal formation. QX-314 was included in the patch pipette solution to block $GABA_B$ receptor-activated K^+ channels (Nathan et al., 1990). To allow equilibration between the pipette solution and the cell, recording of TTX-resistant IPSCs was started 10–15 min after rupturing the membrane. Once equilibrium was achieved, even large concentrations of (*R*)-(−)-baclofen did not activate a K^+ current. Membrane currents were measured with a discontinuous single-electrode voltage-clamp amplifier (npi, Tamm, Germany) at a holding potential of −65 mV. All drugs were bath-applied from a wide-bore pipette used as a common outlet for different solutions. Because the pipette was positioned close to the slice and the chamber volume was small (~400 μl), a complete exchange of the external solution took <30 sec. All inorganic salts were of analytical grade from Merck (Darmstadt, Germany). All drugs, except CNQX (Tocris Cookson, Bristol, England) and TTX and QX-314 (Biotrend, Köln, Germany), were from Sigma (Deisenhofen, Germany)

Data acquisition and analysis. Spontaneous synaptic currents were filtered at 1.3–2.0 kHz with a 4-pole Bessel filter, sampled between 2 and 10 kHz using pClamp software (Axon Instruments, Foster City, CA), and stored additionally on a DAT recorder. Spontaneous synaptic currents were detected by a program written in our laboratory. The program detects an event if the difference between a "running average" (data averaged for 2 msec) and data points 2 msec later exceeds a given threshold (8–14 pA). The program searches for the data point for which the difference from the baseline is maximal, and no further increase in the difference is detectable within a certain time period (usually set to 2 msec). This allows the exclusion of local maxima that arise from noise fluctuations. Events that are superimposed but have a latency of >2 msec were detected and measured separately. Superposition of events, however, was rare. Even in cells in which TTX-resistant IPSCs occurred at a high frequency (7 Hz), <10% of the events had a peak-to-peak difference of 20 msec or less ($n = 5$ cells). The amplitude of an event is calculated as the difference between the peak value (average of 1.0 msec of data starting from the largest deflection) and the baseline (average of 2 msec of data preceding the first deflection from baseline). Each event was inspected visually before being accepted. The 10–90% rise time was the time difference between two data points closest in amplitude to the value calculated by adding or subtracting 10% of the event amplitude from the baseline or peak, respectively. The resulting list of event amplitudes, times, and rise times was used for constructing frequency and amplitude histograms. Events chosen for analysis of kinetic parameters arose from baseline without an inflection during the rising phase, and no additional event occurred during the decay phase. For fitting model exponentials to the decay phase of spontaneous currents, the simplex (mono-exponential) or the Chebychev (double-exponential) algorithms of the pClamp software were used. To decide whether a mono- or double-exponential function provided a better fit for a given decay phase, all decay phases were fitted with a double-exponential function. The criteria for a fit being mono-exponential were that the faster decay time constant (τ_1) differed from the slower decay time constant (τ_2) by <10%, or the amplitude coefficient of τ_2 was <15% of the coefficient of τ_1.

Drug effects were calculated as changes in the frequency of TTX-resistant IPSCs. The frequency was determined from the number of events within 60–180 sec epochs before the drug application (control), 1 or 2 min (for baclofen) or >4 min (for all other treatments) after commencement of drug application, and 7–10 min after start of wash (recovery). Cumulative amplitude or frequency distributions were compared with the Kolmogoroff–Smirnoff test. Two distributions were considered to be significantly different when $p < 0.01$. The paired Student's *t* test was used to determine statistically significant changes in kinetic parameters. Numerical values are given as mean ± SD when data are derived from a given cell. Data from several cells are presented as mean ± SEM.

RESULTS

In the presence of TTX (1 μM) and glutamate receptor antagonists, spontaneous synaptic inward currents were recorded at a holding potential of −65 mV in CA1 pyramidal cells of guinea pig hippocampal slices (Fig. 1*A*). Because of their sensitivity to the $GABA_A$ antagonists bicuculline or picrotoxin (20 μM; $n = 4$), these currents were TTX-resistant IPSCs. Bath-applied (*R*)-(−)-baclofen reversibly inhibited TTX-resistant IPSCs (Fig. 1*A*). (*R*)-(−)-baclofen (10 μM) reduced the frequency by 50% on average but did not affect the amplitude distribution of TTX-resistant IPSCs (Fig. 1*B*, Table 1), suggesting a presynaptic reduction of transmitter release. The frequency of TTX-resistant IPSCs was reduced by (*R*)-(−)-baclofen in a concentration-dependent manner (Fig. 1*C*). The EC_{50} calculated from the Hill equation was 1.8 μM. Ten micromolar (*R*)-(−)-baclofen was an almost saturating concentration (Fig. 1*C*) and hence was used in all further experiments.

The (*R*)-(−)-baclofen-induced inhibition of TTX-resistant IPSCs was a $GABA_B$ receptor-mediated effect. The less potent enantiomer of baclofen (*S*)-(+)-baclofen (10 μM) did not reduce the frequency or amplitude of TTX-resistant IPSCs (Table 1). Furthermore, the (*R*)-(−)-baclofen-mediated inhibition of TTX-resistant IPSCs was fully antagonized by the high-affinity $GABA_B$ antagonist CGP55845A (Fig. 1*D*, Table 1). CGP55845A itself did not change the frequency or amplitude distributions of TTX-resistant IPSCs (Table 1), indicating that $GABA_B$ receptors were not activated tonically by quantal GABA release.

(*R*)-(−)-baclofen effects are independent of Ca^{2+} and K^+ conductances

In a recent study on rat hippocampal CA1 neurons, baclofen reduced TTX-resistant IPSCs recorded in 20 mM extracellular $[K^+]$ ($[K^+]_o$) but did not affect TTX-resistant IPSCs in 5 mM $[K^+]_o$ (Doze et al., 1995). The inhibition of TTX-resistant IPSCs in high $[K^+]_o$ was likely caused by an inhibition of Ca^{2+} influx into depolarized terminals, because Cd^{2+} occluded the effect of baclofen. Cd^{2+} (100 μM) also reduced the amplitude of TTX-resistant IPSCs recorded at low $[K^+]_o$ in slices [Llano and Gerschenfeld, 1993; Doze et al., 1995; this study (Table 1)]. In addition, we observed an apparent reduction in frequency that may have resulted, however, from a loss of events that became too small to detect. To exclude an inhibition of Ca^{2+} influx as a possible mechanism for a diminution of TTX-resistant IPSCs, we applied a high concentration of Cd^{2+} (100 μM). Under this condition, (*R*)-(−)-baclofen still inhibited TTX-resistant IPSCs (Fig. 2*A*) in all cells (7 of 7). The inhibition was reversible on washout (Fig. 2*A*). As in control, (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs in Cd^{2+}, without an effect on the amplitude distribution (Fig. 2*B*). The effect of (*R*)-(−)-baclofen on TTX-resistant IPSCs was quantitatively similar in the presence and absence of Cd^{2+} (Table 1). Thus, (*R*)-(−)-baclofen reduces quantal GABA release by a mechanism independent of Ca^{2+} influx through Cd^{2+}-sensitive Ca^{2+} channels.

Despite the presence of QX-314 and Cs^+ in the patch pipette so that no change in somatic K^+ conductance was visible, a K^+

Table 1. Effects of baclofen and other agents on TTX-resistant IPSCs

		Frequency (Hz)		Amplitude (pA)		
Agent	Condition	Control	Agent	Conrol	Agent	*n*
Guinea pig						
10 μM (−)Baclofen	Control solution	4.8 ± 0.5	2.5 ± 0.3*	41.6 ± 3.0	38.3 ± 3.1	14
10 μM (+)Baclofen	Control solution	2.8 ± 0.6	2.7 ± 0.6	35.3 ± 7.0	35.9 ± 8.9	4
0.5 μM CGP55845A	Control solution	3.4 ± 0.6	3.3 ± 0.6	32.9 ± 4.7	31.7 ± 4.3	9
10 μM (−)Baclofen	0.5 μM CGP55845A	3.2 ± 0.4	3.1 ± 0.4	41.9 ± 6.0	41.5 ± 7.6	6
100 μM Cd^{2+}	Control solution	3.3 ± 0.3	2.6 ± 0.3$^+$	35.1 ± 4.3	24.4 ± 2.2$^+$	10
10 μM (−)Baclofen	100 μM Cd^{2+}	3.4 ± 0.3	1.6 ± 0.3*	22.1 ± 2.8	22.1 ± 2.6	7
10 μM (−)Baclofen	1 mM Ba^{2+}, 100 μM Cd^{2+}	4.2 ± 0.6	2.6 ± 0.3*	26.6 ± 2.0	25.2 ± 1.3	6
10 μM (±)Baclofen	Control solution	3.3 ± 0.7	2.2 ± 0.5*	39.3 ± 5.3	38.8 ± 5.1	7
Sprague Dawley rat						
10 μM (−)Baclofen	100 μM Cd^{2+}	1.5 ± 0.1	1.1 ± 0.1^x	23.4 ± 1.1	23.5 ± 1.2	8
Wistar rat						
10 μM (−)Baclofen	100 μM Cd^{2+}	2.0 ± 1.0	0.9 ± 0.4*	23.8 ± 1.4	23.7 ± 2.0	6

Numbers for frequency and amplitude are mean values of averages ($n > 100$ events) from different cells ± SEM. *n* is the total number of cells tested with each agent. In addition to the drugs listed, TTX (1 μM), CNQX (10 μM), and CGP 37849 (1 μM) were present throughout (control solution). Differences in amplitude or frequency within a single cell were tested by the Kolmogoroff–Smirnoff test ($p < 0.01$*). Cd^{2+} (100 μM) significantly reduced amplitude and frequency of TTX-resistant IPSCs in 7 of 10 cells ($^+$). (*R*)-(−)-baclofen significantly reduced the amplitude of TTX-resistant IPSCs in only 3 of 8 cells (^{x}).

conductance increase in the dendrites not detected by a somatic recording electrode might have contributed to the inhibition by (*R*)-(−)-baclofen. To exclude a postsynaptic $GABA_B$ receptor-mediated K^+ conductance increase, the K^+ channel blocker Ba^{2+} (1 mM) was used. Because Ba^{2+} is also a charge carrier for voltage-activated Ca^{2+} channels, Cd^{2+} (100 μM) was also applied. In the presence of Ba^{2+} and Cd^{2+}, (*R*)-(−)-baclofen still reduced the frequency of TTX-resistant IPSCs, and the magnitude of the reduction was similar to the effect observed in the absence of the channel blockers (Table 1). Again, there was no significant change in the amplitude of TTX-resistant IPSCs (Table 1). Thus, (*R*)-(−)-baclofen does not reduce quantal GABA release by a postsynaptic mechanism.

Inhibition of TTX-resistant IPSCs by $GABA_B$ receptors does not change their kinetics

In CA1 neurons of rat hippocampal slices, evoked IPSCs with a slow decay are more sensitive to baclofen than rapidly decaying IPSCs (Pearce et al., 1995). To determine whether (*R*)-(−)-baclofen affects only a subpopulation of TTX-resistant IPSCs, we analyzed their kinetic parameters in the absence and presence of (*R*)-(−)-baclofen. In rat hippocampal CA1 neurons, the decay of electrically evoked IPSCs has been fitted with a mono-exponential (Roepstorff and Lambert, 1994) or double-exponential function (Pearce, 1993). In guinea pigs, a mono-exponential function fitted well the decay phase of most TTX-resistant IPSCs (Fig. 3*A, inset*). The mean τ value in control solution (15.2 ± 2.0 msec; 5 cells) was not significantly different from the mean τ value in the presence of (*R*)-(−)-baclofen (16.2 ± 2.2 msec; $p > 0.1$) (Fig. 3*A*). The distribution of τ was unchanged by (*R*)-(−)-baclofen, and there was no correlation between τ and the amplitude of individual events in the absence or presence of (*R*)-(−)-baclofen (Fig. 3*C*). A detailed analysis of the fits revealed that the decay phase of a portion of TTX-resistant IPSCs was better fitted by a double-exponential function. The fraction of TTX-resistant IPSCs with a double-exponential decay was similar in the presence and absence of (*R*)-(−)-baclofen (0.36 ± 0.11, 0.34 ± 0.04; $n = 5$). The mean τ values for mono-exponential fits (control, 14.6 ± 2.0 msec; (*R*)-(−)-baclofen, 16.0 ± 2.4 msec; $p > 0.1$) and τ_1 and τ_2 values for double-exponential fits were also not significantly different (control: τ_1, 5.4 ± 0.5 msec, τ_2, 26.0 ± 2.7 msec; (*R*)-(−)-baclofen: τ_1, 5.8 ± 0.4 msec, τ_2, 26.6 ± 2.3 msec). Furthermore, the rise time of TTX-resistant IPSCs was unaffected by (*R*)-(−)-baclofen. The mean rise time (10–90%) in control (1.35 ± 0.06 msec; $n = 5$ cells) was similar to the value in the presence of (*R*)-(−)-baclofen (1.40 ± 0.06 msec; $p > 0.1$) when measured in the same cells. Rise time distributions in both conditions were indistinguishable (Fig. 3*B*), and there was also no correlation between the rise time and the amplitude of single events in the presence of (*R*)-(−)-baclofen (Fig. 3*D*). The analysis of rise time and decay time constants did not provide any evidence for the existence of groups of TTX-resistant IPSCs with a differential sensitivity to (*R*)-(−)-baclofen.

Inhibition of TTX-resistant IPSCs by $GABA_B$ receptors exists in different rodents

Because in hippocampal CA1 neurons of Sprague Dawley rats 10 μM (±)-baclofen did not inhibit TTX-resistant IPSCs in 5 mM $[K^+]_o$ (Doze et al., 1995), we first investigated possible experimental differences that could account for the discrepancy in results. We tested a 1:1 mixture of (*S*)-(+)- and (*R*)-(−)-baclofen (each 5 μM). The mixture had a smaller effect than 10 μM (*R*)-(−)-baclofen, as expected from the concentration–response curve of the active enantiomer and the lack of effect of the inactive enantiomer (Table 1). Second, we tested whether a difference in species was responsible for the discrepancy. We recorded TTX-resistant IPSCs from CA1 pyramidal cells of Sprague Dawley rats and studied the effects of (*R*)-(−)-baclofen in the presence of Cd^{2+}. In contrast to the effects in guinea pigs, 10 μM (*R*)-(−)-baclofen only slightly reduced the frequency of TTX-resistant IPSCs in Sprague Dawley rats (Table 1). In five of eight pyramidal cells of Sprague Dawley rats, there was no significant reduction of the frequency of TTX-resistant IPSCs (Fig. 4*A,B*). In the three cells (from three animals) that exhibited a significant reduction, the effect amounted to 32%. Thus, $GABA_B$ receptor-mediated inhibition may be more difficult to detect in Sprague Dawley rats than in guinea pigs, particularly if a nonsaturating agonist concentration is used. Sprague Dawley rats, however, are exceptional among rodents with respect to another G-protein-mediated effect (Salin et al., 1995). To test for species versus strain differences, we therefore recorded TTX-resistant IPSCs in Cd^{2+} from another rat

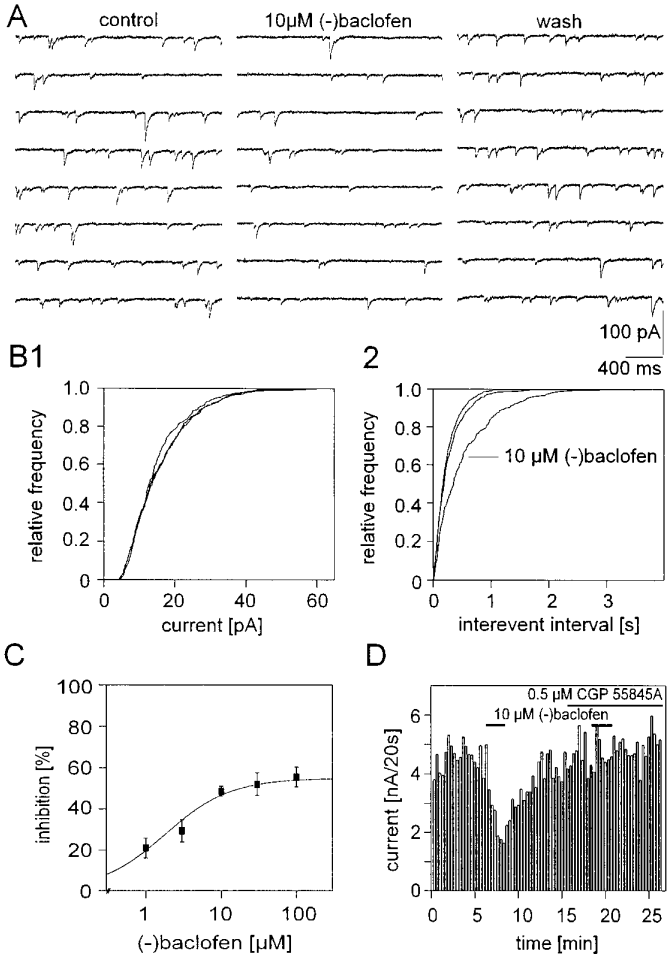

Figure 1. Effect of (*R*)-(−)-baclofen on TTX-resistant IPSCs. *A*, Eight consecutive traces (2 sec each) showing TTX-resistant IPSCs before (*left*), 1 min after start of (*R*)-(−)-baclofen application (*middle*), and 7 min after commencing wash (*right*). *B*, Cumulative amplitude (*B1*) and frequency (*B2*) distributions are plotted for control, (*R*)-(−)-baclofen effect, and wash. The amplitude distributions were unchanged. The frequency distribution was shifted to the right by (*R*)-(−)-baclofen. The number of events used for the cumulative plots was 495 for control, 232 during (*R*)-(−)-baclofen, and 445 during wash. *C*, Concentration-dependent reduction of the frequency of TTX-resistant IPSCs by (*R*)-(−)-baclofen. Effects were calculated as the ratio (expressed in percent) of the frequency in the presence of a given (*R*)-(−)-baclofen concentration to the mean frequency before and after (*R*)-(−)-baclofen application. *Squares* are mean values from 5–14 cells for each concentration. Error bars represent SEM. The *solid line* is a fit (least-square method) to the Hill function, assuming a Hill coefficient of 1.0 and a maximal reduction of the frequency to 45% of the control. The calculated EC_{50} is 1.78 μM. *D*, Block of the (*R*)-(−)-baclofen effect by a high-affinity $GABA_B$ antagonist (CGP 55845A). The plot of the sum of the amplitudes sampled for 20 sec against time shows that (*R*)-(−)-baclofen inhibited TTX-resistant IPSCs. The effect was blocked by the $GABA_B$ antagonist CGP55845A. The antagonist by itself had no effect. *Horizontal bars* indicate the drug application periods.

strain (Wistar). (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs in hippocampal CA1 pyramidal cells of Wistar rats (Fig. 4*C,D*). Again, there was no effect on the amplitude of TTX-resistant IPSCs (Fig. 4*D*, Table 1). The inhibition by (*R*)-(−)-baclofen of the frequency of TTX-resistant IPSCs in Wistar rats was significant in six of six cells and quantitatively similar to the effect in guinea pigs (Table 1).

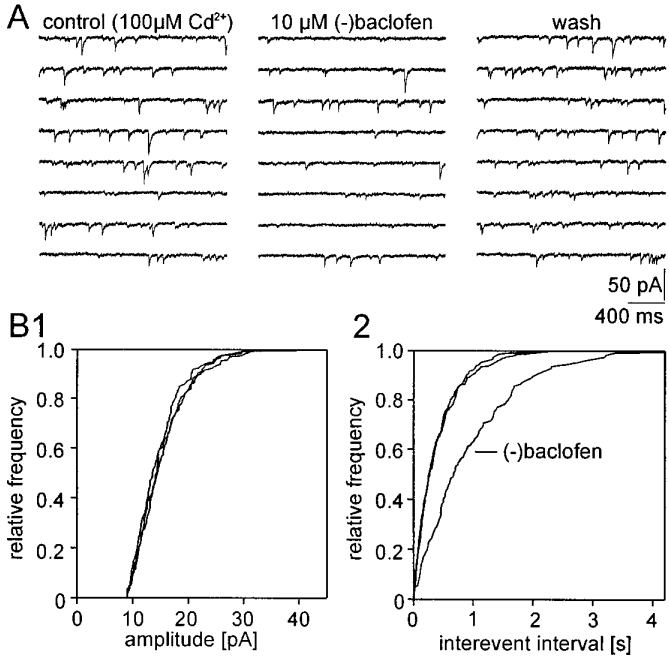

Figure 2. (*R*)-(−)-baclofen-induced reduction of the frequency of TTX-resistant IPSCs in the presence of Cd^{2+}. *A*, Display of data as in Figure 1*A, B*. (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs in 100 μM Cd^{2+}. The effect of (*R*)-(−)-baclofen was fully reversible. *B*, (*R*)-(−)-baclofen did not affect the amplitude of TTX-resistant IPSCs (*B1*) but reduced their frequency (*B2*). The number of events used for the cumulative plots was 309 for control, 126 during (*R*)-(−)-baclofen, and 296 for recovery.

Protein kinase C (PKC) activator reduces $GABA_B$ receptor-mediated inhibition of quantal GABA release

In rat hippocampal CA1 pyramidal cells, activation of PKC reduces the baclofen-induced diminution of evoked IPSCs, although the depressant effect on the postsynaptic $GABA_B$ response is much stronger (Pitler and Alger, 1994). Because the inhibition of quantal release could be one mechanism by which $GABA_B$ receptors reduce GABA release, we studied the effects of PKC activators on the $GABA_B$ receptor-mediated inhibition of TTX-resistant IPSCs. Cd^{2+} and Ba^{2+} were continuously present to avoid interference with Ca^{2+} or K^+ conductances. As reported elsewhere (Capogna et al., 1995), β-phorbol-12,13-dibutyrate (1 μM) increased the mean frequency of TTX-resistant IPSCs not altering amplitude distribution (Figs. 5, 6). (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs significantly less in the presence of β-phorbol-12,13-dibutyrate (Fig. 6*B*), indicating that activation of PKC reduces $GABA_B$ receptor-mediated inhibition of quantal GABA release. Forskolin (20 μM), an indirect activator of protein kinase A (PKA), also enhanced the frequency of TTX-resistant IPSCs recorded in Cd^{2+} and Ba^{2+}. After stimulation of PKA by forskolin, (*R*)-(−)-baclofen strongly reduced the frequency of TTX-resistant IPSCs (Figs. 5*C,D*, 6*B*). Quantitatively, this effect was identical to the effect of (*R*)-(−)-baclofen in the absence of forskolin (Fig. 6*B*). On average, the increase in the frequency of TTX-resistant IPSCs was larger with β-phorbol-12,13-dibutyrate than with forskolin (Fig. 6*A*); however, when only cells with similar increases in frequency were considered (compare Fig. 5; β-phorbol-12,13-dibutyrate: 208 ± 22%, $n = 3$; forskolin: 189 ± 15%, $n = 3$), the effects of (*R*)-(−)-baclofen were still significantly different (β-phorbol-12,13-dibutyrate: 78 ± 8%,

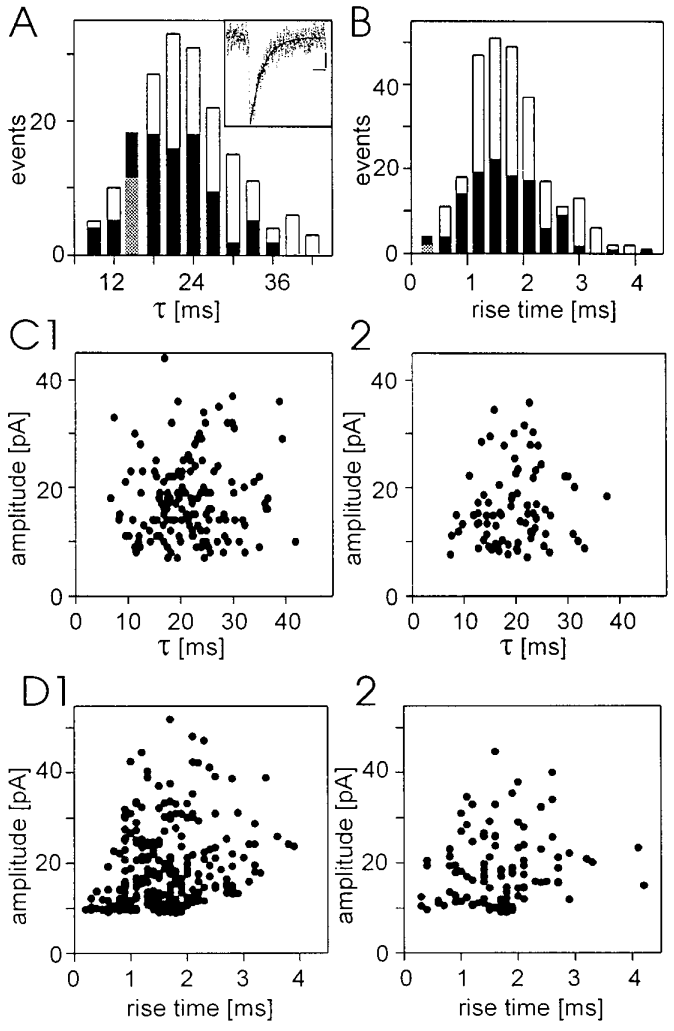

Figure 3. Kinetics of TTX-resistant IPSCs in (*R*)-(−)-baclofen. *A*, Distributions of the mono-exponential decay time constant τ in control solution (mean ± SD, 21.5 ± 7.0 msec; $n = 177$) and in the presence of (*R*)-(−)-baclofen (19.5 ± 6.4 msec; $n = 99$) were not significantly different ($p > 0.1$, paired Student's *t* test), whereas the frequency was reduced. *Inset* shows a mono-exponential fit (time constant, τ, 18.06 msec; amplitude coefficient, *A*, 26.8). Calibration: 5 pA, 20 msec. *White* and *stippled bars* are control data; *black bars* represent data obtained in (*R*)-(−)-baclofen. *B*, Distribution of the rise time (10–90%) of TTX-resistant IPSCs recorded in control solution (mean ± SD, 1.63 ± 0.68 msec; $n = 267$) and in the presence of (*R*)-(−)-baclofen (1.69 ± 0.70 msec; $n = 113$) were also not significantly different ($p > 0.1$). *C*, Plot of the amplitude of individual TTX-resistant IPSCs against their τ value in the absence (*C1*) and presence (*C2*) of (*R*)-(−)-baclofen. (*R*)-(−)-baclofen did not change the relation between the amplitude of TTX-resistant IPSC and τ. *D*, Plot of the rise time of individual TTX-resistant IPSCs against their peak amplitude in the absence (*D1*) and presence (*D2*) of (*R*)-(−)-baclofen. (*R*)-(−)-baclofen did not change the relation between rise time and amplitude when measured for individual events.

$n = 3$; forskolin: 58 ± 10%, $n = 3$; $p < 0.05$, unpaired Student's *t* test).

DISCUSSION

Presynaptic $GABA_B$ receptors in the CA1 region of the rodent hippocampus inhibit quantal GABA release, and this inhibition is reduced by an activator of PKC. Inhibition of quantal release may participate in the autoinhibition of GABA release in the hippocampus, in addition to the well-known $GABA_B$ receptor-mediated inhibition of Ca^{2+} channels and activation of K^+ conductances.

Characterization of (*R*)-(−)-baclofen-induced inhibition of quantal GABA release

(*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs in CA1 pyramidal cells of the rodent hippocampus by an action on $GABA_B$ receptors. The pharmacological identification rests on the specific action of agonists and antagonists. We found that the active (*R*)-(−) but not the inactive (*S*)-(+) enantiomer of baclofen reduced TTX-resistant IPSCs. Furthermore, the inhibition was blocked by a high-affinity $GABA_B$ antagonist, CGP 55845A (Froestl et al., 1992; Jarolimek et al., 1993). The EC_{50} for the baclofen-mediated inhibition of TTX-resistant IPSCs (1.8 μM) is similar to the EC_{50} described for various presynaptic baclofen effects. Typical concentrations of baclofen used for presynaptic inhibition of IPSCs in CA1 neurons range from 1 to 50 μM (Lambert et al., 1991; Doze et al., 1995; Pearce et al., 1995), although a high-affinity effect of baclofen (EC_{50} 100 nM) has also been found (Pearce et al., 1995).

Baclofen inhibits Ca^{2+} channels (for review, see Dolphin, 1995), and this effect may contribute substantially to the potent presynaptic inhibition of action potential-dependent GABA release in the hippocampus (Pitler and Alger, 1994; Doze et al., 1995). In our study on CA1 neurons of the rodent hippocampus, (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs also after blockade of Ca^{2+} channels by Cd^{2+}. Thus, inhibition of Ca^{2+} channels is not the only mechanism by which GABA can inhibit its own release. We tested (*R*)-(−)-baclofen in the presence of both TTX and Cd^{2+}, because it has been shown that spontaneous synaptic activity recorded in TTX acquires Cd^{2+}-sensitivity when $[K^+]_o$ is raised to 20 mM in cultured neurons (Finch et al., 1990; Jarolimek and Misgeld, 1991) and in brain slices (Doze et al., 1995). Doze et al. (1995) found that baclofen reduced the frequency of TTX-resistant IPSCs in 20 mM $[K^+]_o$ but not in 5 mM $[K^+]_o$. Because Cd^{2+} occluded the baclofen effect in 20 mM $[K^+]_o$, they concluded that the effect of baclofen on TTX-resistant IPSCs exclusively reflected an action on Ca^{2+} channels. In cultured midbrain neurons, (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs in both 5 and 20 mM $[K^+]_o$. Because the effect was much stronger in 20 mM $[K^+]_o$, we suggested that in 20 mM $[K^+]_o$, (*R*)-(−)-baclofen acts through an inhibition of both Ca^{2+} channels and quantal release (Jarolimek and Misgeld, 1992).

Activation of a postsynaptic K^+ conductance by (*R*)-(−)-baclofen could mimic an inhibition of TTX-resistant IPSCs; however, there was neither an (*R*)-(−)-baclofen-induced postsynaptic K^+ conductance increase nor a reduction of the amplitude of TTX-resistant IPSCs, and the inhibition of TTX-resistant IPSCs persisted in the presence of the K^+ channel blocker Ba^{2+} (and Cd^{2+}). This suggests that $GABA_B$ receptors reduce quantal GABA release by a presynaptic, Cd^{2+}- and Ba^{2+}-insensitive mechanism.

Previous studies have shown that presynaptic $GABA_B$ receptor-mediated effects are heterogenous at different GABAergic nerve terminals in the hippocampus. A portion of evoked IPSCs is reduced only slightly (Pearce et al., 1995) or not at all (Lambert and Wilson, 1993) by baclofen, whereas another portion of IPSCs recorded in the same cells is highly sensitive to baclofen. In CA1 neurons of rat hippocampus, evoked IPSCs with a slow decay are more sensitive than fast decaying IPSCs (Pearce et al., 1995). The analysis of the rise time and decay time constants of TTX-resistant

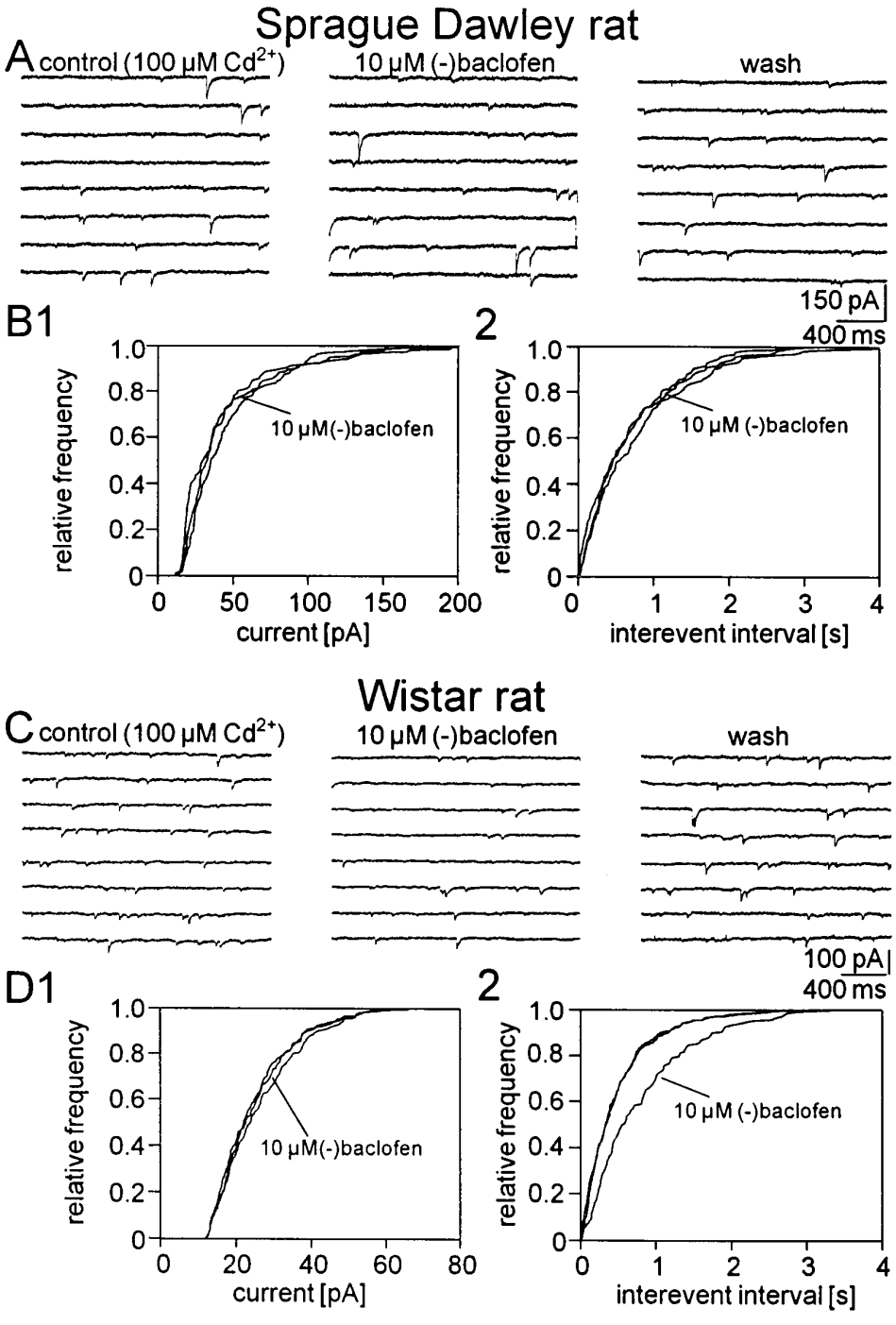

Figure 4. (*R*)-(−)-baclofen does not reduce the frequency of TTX-resistant IPSCs in a CA1 neuron of a Sprague Dawley rat but inhibits TTX-resistant IPSCs in a CA1 neuron of a Wistar rat. All recordings were performed in Cd^{2+} (100 μM). *A*, Eight consecutive traces (2 sec each) showing TTX-resistant IPSCs before (*left*) and 90 sec after start of the (*R*)-(−)-baclofen application (*middle*), and 7 min after commencing wash (*right*). *B*, Cumulative amplitude (*B1*) and frequency (*B2*) distributions are plotted for control, (*R*)-(−)-baclofen effect, and wash. (*R*)-(−)-baclofen did not significantly reduce the frequency or amplitude of TTX-resistant IPSCs in 100 μM Cd^{2+} in this cell. The number of events used for the cumulative plots was 180 for control, 162 during (*R*)-(−)-baclofen, and 160 for recovery. *C, D*, (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs in Wistar rats, whereas the amplitude distribution was unchanged. The number of events used for the cumulative plots was 248 for control, 151 during (*R*)-(−)-baclofen, and 240 for recovery.

IPSCs in the guinea pig hippocampus did not reveal such a divergence on the level of quantal GABA release.

Various transmitters are known to inhibit quantal release of glutamate and GABA (for references, see introductory remarks). The $GABA_B$ receptor agonist baclofen was reported to inhibit TTX-resistant EPSCs (Scanziani et al., 1992) but not TTX-resistant IPSCs in the rat hippocampus (Cohen et al., 1992; Scanziani et al., 1992; Doze et al., 1995). $GABA_B$ receptors, however, do inhibit TTX-resistant IPSCs in thalamic neurons of rat slices (Ulrich and Huguenard, 1996) and cultured rat midbrain neurons (Jarolimek and Misgeld, 1992). This might indicate that $GABA_B$ receptor subtypes or different second messenger systems are involved in different brain regions of different species. The data of this study, however, indicate that (*R*)-(−)-baclofen inhibits quantal GABA release also in the hippocampus.

The findings of the present study contradict a previous study in which baclofen did not inhibit TTX-resistant IPSCs in hippocampal CA1 neurons of Sprague Dawley rats (Doze et al., 1995). We found two reasons for an explanation of the discrepancy. We used an almost saturating concentration of the active enantiomer of baclofen ((*R*)-(−)-baclofen) to elicit maximal effects, whereas the less effective racemate was used in the previous study. The racemate had significantly smaller effects in guinea pig slices than the almost saturating concentration of (*R*)-(−)-baclofen. The baclofen-mediated reduction in the frequency of TTX-resistant IPSCs was qualitatively similar in guinea pigs and in Wistar and

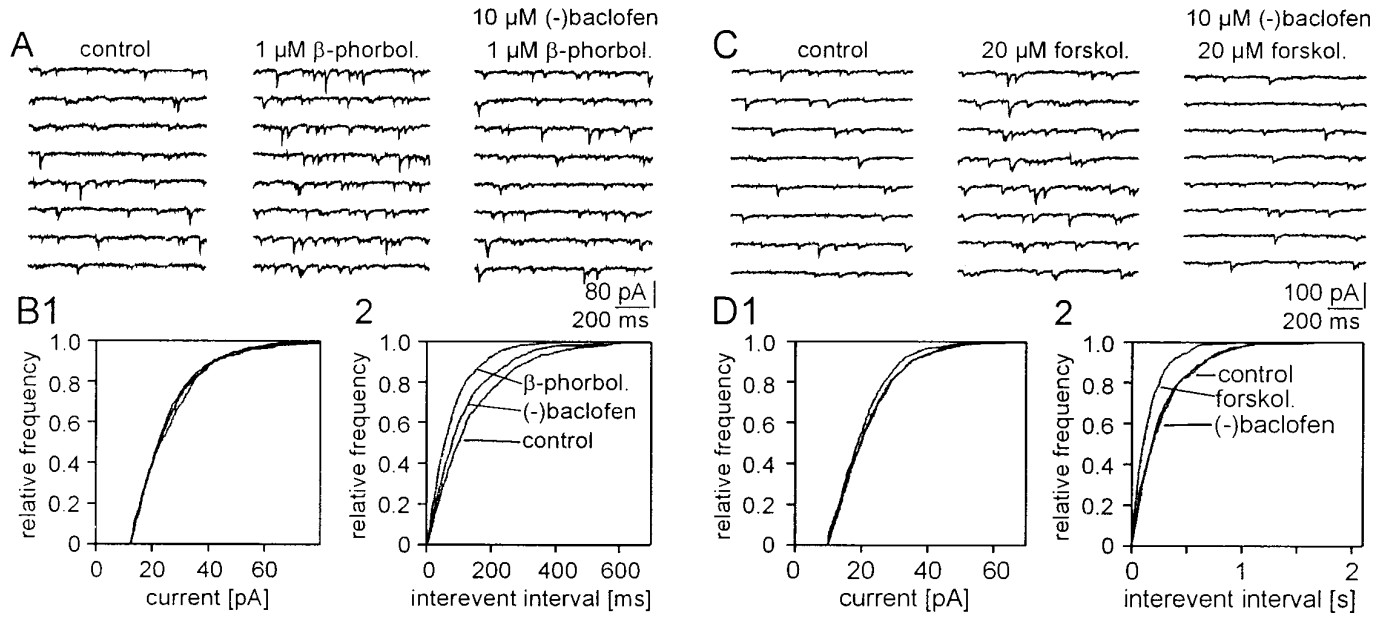

Figure 5. Effects of (*R*)-(−)-baclofen on TTX-resistant IPSCs on stimulation of β-phorbol-12,13-dibutyrate (*β-phorbol.*) or forskolin (*forskol.*). TTX-resistant IPSCs were recorded in Ba^{2+} (1 mM) and Cd^{2+} (100 μM). *A*, $GABA_B$ receptor-mediated inhibition of TTX-resistant IPSCs in the presence of β-phorbol-12,13-dibutyrate. Consecutive traces (1 sec each) show that β-phorbol-12,13-dibutyrate (*β-phorbol.*; 1 μM) strongly increased the frequency of TTX-resistant IPSCs. (*R*)-(−)-baclofen reduced the frequency of TTX-resistant IPSCs. *B*, Cumulative amplitude and frequency distributions of TTX-resistant IPSCs reveal that neither (*R*)-(−)-baclofen nor β-phorbol-12,13-dibutyrate affected the amplitude distribution; however, β-phorbol-12,13-dibutyrate increased and (*R*)-(−)-baclofen thereafter reduced the frequency of TTX-resistant IPSCs. The number of events used for the cumulative plots was 443 for control, 780 for β-phorbol-12,13-dibutyrate, and 541 during β-phorbol-12,13-dibutyrate and (*R*)-(−)-baclofen. *C*, $GABA_B$ receptor-mediated inhibition of TTX-resistant IPSCs in the presence of forskolin (20 μM). Consecutive traces (1 sec each) show that forskolin increased the frequency of TTX-resistant IPSCs. (*R*)-(−)-baclofen in the presence of forskolin still reduced the frequency. *D*, Cumulative amplitude and frequency distributions of TTX-resistant IPSCs. Neither forskolin nor (*R*)-(−)-baclofen (*traces* are superimposed) significantly affected the amplitude distribution (*D1*). Forskolin increased the frequency of TTX-resistant IPSCs; (*R*)-(−)-baclofen reduced it (*D2*). The number of events used for the cumulative plots was 364 for control, 652 for forskolin, and 374 during forskolin and (*R*)-(−)-baclofen.

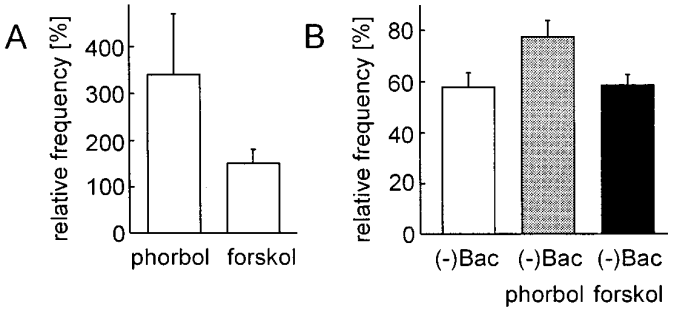

Figure 6. Graphic representation of the effects of β-phorbol-12,13-dibutyrate, forskolin, and (*R*)-(−)-baclofen on TTX-resistant IPSCs. All recordings were performed in the presence of Cd^{2+} (100 μM) and Ba^{2+} (1 mM). *A*, β-phorbol-12,13-dibutyrate (*phorbol*; 1 μM) or forskolin (*forskol*; 20 μM) increased the frequency of TTX-resistant IPSCs. *B*, In control (*white column*) and in the presence of forskolin (*forskol*), (*R*)-(−)-baclofen (*(−)Bac*) strongly reduced the frequency of TTX-resistant IPSCs. In the presence of β-phorbol-12,13-dibutyrate, the reduction was significantly smaller (*phorbol*; Student's *t* test; $p < 0.05$). *Columns* represent mean effect for all cells ($n = 4$ for *A*; $n = 6$ for *B*). Error bars represent SEM.

Sprague Dawley rats; however, the reduction by a saturating concentration was quantitatively smaller in Sprague Dawley rats than in guinea pigs or Wistar rats. The application of an agonist concentration near the EC_{50} of the active enantiomer in combination with the use of slices from "insensitive" Sprague Dawley rats together may explain the negative finding of the previous study. In contrast to the presynaptic inhibition of evoked EPSCs by κ-opioids that is found in hippocampal slices of various rodents but not in those of Sprague Dawley rats (Salin et al., 1995), presynaptic inhibition of quantal GABA release by $GABA_B$ receptors exists also in thalamic (Ulrich and Huguenard, 1996) and hippocampal terminals of the latter species. The inconsistency and faintness of the effect in hippocampal terminals of Sprague Dawley rats may depend then on a different stage of regulation of $GABA_B$ receptor-mediated inhibition among different rodent strains, e.g., on a regulation through phosphorylating and dephosphorylating processes.

Regulation of $GABA_B$ receptor-mediated inhibition of quantal GABA release

The mechanism by which $GABA_B$ receptors reduce quantal transmitter release is unknown. Many proteins that are involved in the release cascade possess potential phosphorylation sites (Südhof, 1995), which could be the target of second messengers activated by $GABA_B$ receptors. Activation of PKA or PKC enhances quantal GABA release (Capogna et al., 1995; Sciancalepore and Cherubini, 1995; this study), indicating that phosphorylation may play an essential role in the tuning of quantal release. Phosphorylation by PKC not only enhances transmitter release but also reduces the inhibition of quantal GABA release by baclofen. In contrast, activation of PKA by forskolin does not reduce the baclofen effect, although it also enhances transmitter release. Thus, the autoinhibition of GABA release is controlled by PKC in a synergistic way, i.e., a reduction in the effectiveness of the inhibitor and an increased transmitter release. In the CA1 region of the hippocampus, activation of PKC causes a strong depression

of the postsynaptic $GABA_B$ response and a smaller inhibition of the baclofen-induced reduction of evoked IPSCs (Pitler and Alger, 1994). The $GABA_B$ receptor-mediated inhibition of IPSCs is probably caused by an inhibition of Ca^{2+} influx and of quantal release. Because the $GABA_B$ receptor-mediated inhibition of Ca^{2+} currents may be PKC insensitive (Diversé-Pierluissi and Dunlap, 1993), the PKC-sensitive reduction of evoked release could be caused by the inhibition of quantal GABA release.

Conclusion

$GABA_B$ receptors, like other G-protein-coupled receptors, are functionally heterogenous. They can couple to K^+ channels, to Ca^{2+} channels, or directly to the release cascade. For many receptors that could be characterized by their sequence structure, the functional diversity is associated with the existence of receptor subtypes. Receptor subtypes may be expected, therefore, for $GABA_B$ receptors. Activation of $GABA_B$ receptors inhibits quantal GABA release in the CA1 region of the rodent brain and is regulated by PKC. Various neurotransmitters with a G-protein requirement, including GABA, modulate quantal transmitter release (cf. introductory remarks). The dual mode of action, i.e., modulation of voltage-gated channels and quantal release, may represent a common feature of modulation of transmitter release at synapses mediating fast synaptic transmission.

REFERENCES

Bijak M, Misgeld U (1995) Adrenergic modulation of hilar neuron activity and granule cell inhibition in the guinea-pig hippocampal slice. Neuroscience 67:541–550.

Capogna M, Gähwiler BH, Thompson SM (1995) Presynaptic enhancement of inhibitory synaptic transmission by protein kinases A and C in the rat hippocampus *in vitro*. J Neurosci 15:1249–1260.

Cohen GA, Doze VA, Madison DV (1992) Opioid inhibition of GABA release from presynaptic terminals of rat hippocampal interneurons. Neuron 9:325–335.

Diversé-Pierluissi M, Dunlap K (1993) Distinct, convergent second messenger pathways modulate neuronal calcium currents. Neuron 10:753–760.

Dolphin AC (1995) Voltage dependent calcium channels and their modulation by neurotransmitters and G proteins. Exp Physiol 80:1–36.

Doze VA, Cohen GA, Madison DV (1995) Calcium channel involvement in $GABA_B$ receptor-mediated inhibition of GABA release in area CA1 of the rat hippocampus. J Neurophysiol 74:43–53.

Finch DM, Fisher RS, Jackson MB (1990) Miniature excitatory synaptic currents in cultured hippocampal neurons. Brain Res 518:257–268.

Froestl W, Mickel SJ, Von Sprecher G, Bittiger H, Olpe HR (1992) Chemistry of new $GABA_B$ antagonists. Pharmacol Commun 2:52–56.

Froestl W, Mickel SJ, Hall RG, Von Sprecher G, Strub D, Baumann PA, Brugger F, Gentsch C, Jaekel J, Olpe HR, Rihs G, Vassout A, Waldmeier PC, Bittiger H (1995) Phosphinic acid analogues of GABA. 1. New potent and selective $GABA_B$ agonists. J Med Chem 38:3279–3312.

Jarolimek W, Misgeld U (1991) Reduction of $GABA_A$ receptor-mediated inhibition by the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3,-dione in cultured neurons of rat brain. Neurosci Lett 121:227–230.

Jarolimek W, Misgeld U (1992) On the inhibitory actions of baclofen and γ-aminobutyric acid in rat ventral midbrain culture. J Physiol (Lond) 451:419–443.

Jarolimek W, Misgeld U (1996) $GABA_B$ receptor-mediated inhibition of quantal GABA release in CA1 pyramidal cells of guinea pig hippocampal slices.Pflügers Arch 431:R81.

Jarolimek W, Demmelhuber J, Bijak M, Misgeld U (1993) CGP 55845A blocks baclofen, γ-aminobutyric acid and inhibitory postsynaptic potassium currents in guinea pig CA3 neurons. Neurosci Lett 154:31–34.

Lambert NA, Wilson WA (1993) Heterogeneity in presynaptic regulation of GABA release from hippocampal inhibitory neurons. Neuron 11:1057–1067.

Lambert NA, Harrison NL, Teyler TJ (1991) Baclofen-induced disinhibition in area CA1 of rat hippocampus is resistant to extracellular Ba^{2+}. Brain Res 547:349–352.

Llano I, Gerschenfeld HM (1993) Inhibitory synaptic currents in stellate cells of rat cerebellar slices. J Physiol (Lond) 468:177–200.

Lupica CR (1995) δ and μ enkephalins inhibit spontaneous GABA-mediated IPSCs via a cyclic AMP-independent mechanism in the rat hippocampus. J Neurosci 15:737–749.

Misgeld U, Sarvey JM, Klee MR (1979) Heterosynaptic postactivation potentiation in hippocampal CA 3 neurons: long-term changes of the postsynaptic potentials. Exp Brain Res 37:217–229.

Misgeld U, Bijak M, Jarolimek W (1995) A physiological role for $GABA_B$ receptors and the effects of baclofen in the mammalian central nervous system. Prog Neurobiol 46:423–462.

Nathan T, Jensen MS, Lambert JDC (1990) The slow inhibitory postsynaptic potential in rat hippocampal CA1 neurones is blocked by intracellular injection of QX-314. Neurosci Lett 110:309–313.

Pearce RA (1993) Physiological evidence for two distinct $GABA_A$ responses in rat hippocampus. Neuron 10:189–200.

Pearce RA, Grunder SD, Faucher LD (1995) Different mechanisms for use-dependent depression of two $GABA_A$-mediated IPSCs in rat hippocampus. J Physiol (Lond) 484:425–435.

Pitler TA, Alger BE (1994) Differences between presynaptic and postsynaptic $GABA_B$ mechanisms in rat hippocampal pyramidal cells. J Neurophysiol 72:2317–2327.

Rekling JC (1993) Effects of met-enkephalin on GABAergic spontaneous miniature IPSPs in organotypic slice cultures of the rat hippocampus. J Neurosci 13:1954–1964.

Roepstorff A, Lambert JDC (1994) Factors contributing to the decay of the stimulus-evoked IPSC in rat hippocampal CA1 neurons. J Neurophysiol 72:2911–2926.

Salin PA, Weisskopf MG, Nicoll RA (1995) A comparison of the role of dynorphin in the hippocampal mossy fiber pathway in guinea pig and rat. J Neurosci 15:6939–6945.

Scanziani M, Capogna M, Gähwiler BH, Thompson SM (1992) Presynaptic inhibition of miniature excitatory synaptic currents by baclofen and adenosine in the hippocampus. Neuron 9:919–927.

Scanziani M, Gähwiler BH, Thompson SM (1995) Presynaptic inhibition of excitatory synaptic transmission by muscarinic and metabotropic glutamate receptor activation in the hippocampus: are Ca^{2+} channels involved? Neuropharmacology 34:1549–1557.

Scholz KP, Miller RJ (1992) Inhibition of quantal transmitter release in the absence of calcium influx by a G-protein-linked adenosine receptor at hippocampal synapses. Neuron 8:1139–1150.

Sciancalepore M, Cherubini E (1995) Protein kinase A-dependent increase in frequency of miniature GABAergic currents in rat CA3 hippocampal neurons. Neurosci Lett 187:91–94.

Stuart GJ, Dodt H-U, Sakmann B (1993) Patch-clamp recordings from the soma and dendrites of neurons in brain slices using infrared video microscopy. Pflügers Arch 423:511–518.

Südhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375:645–653.

Thompson SM, Capogna M, Scanziani M (1993) Presynaptic inhibition in the hippocampus. Trends Neurosci 16:222–227.

Ulrich D, Huguenard JR (1996) $GABA_B$ receptor-mediated responses in GABAergic projection neurones of rat nucleus reticularis thalami *in vitro*. J Physiol (Lond) 493:845–854.