$GABA_B$ receptor-mediated presynaptic inhibition in guinea-pig hippocampus is caused by reduction of presynaptic Ca^{2+} influx

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1. The hypothesis that activation of $GABA_B$ receptors inhibits evoked synaptic transmission by reducing the presynaptic Ca^{2+} influx was tested using a recently developed technique for simultaneously recording the presynaptic Ca^{2+} transient ($[Ca^{2+}]_t$) and the field excitatory postsynaptic potential (fEPSP) evoked by a single electrical stimulus at CA3 to CA1 synapses of guinea-pig hippocampus.

2. The $GABA_B$ receptor agonist baclofen reversibly blocked, in a dose-dependant manner, both the fEPSP and the presynaptic $[Ca^{2+}]_t$ with similar time courses. During application of baclofen, the fEPSP was proportional to about the fourth power of the presynaptic $[Ca^{2+}]_t$, and the presynaptic fibre volley and the resting Ca^{2+} level did not change. These results are similar to those we previously observed following application of several voltage-dependent Ca^{2+} channel blockers, suggesting that baclofen inhibits the fEPSP by blocking the presynaptic Ca^{2+} influx.

3. The inhibition by baclofen of both the fEPSP and the presynaptic $[Ca^{2+}]_t$ was blocked by the $GABA_B$ receptor antagonist CGP 35348, consistent with the causal relationship between the $GABA_B$ receptor-mediated presynaptic inhibition of the $[Ca^{2+}]_t$ and the fEPSP.

4. The inhibition by baclofen of the $[Ca^{2+}]_t$ was partially occluded by application of the voltage-dependent Ca^{2+} channel blocker ω-conotoxin-GVIA (ω-CgTX-GVIA), but not ω-agatoxin-IVA (ω-AgaTX-IVA), suggesting that baclofen reduces the presynaptic $[Ca^{2+}]_t$ by blocking Ca^{2+} channels including the ω-CgTX-GVIA-sensitive type.

5. We conclude that baclofen inhibits evoked transmitter release by reducing presynaptic Ca^{2+} influx. These results, together with other findings showing that baclofen inhibits spontaneous transmitter release in cultured hippocampal slices by a mechanism downstream to Ca^{2+} influx, suggest that different mechanisms may underlie spontaneous and evoked transmitter release.

GABA is the most important inhibitory neurotransmitter in the central nervous system (Nicoll, Malenka & Kauer, 1990). Accumulated evidence suggests that activation of presynaptic $GABA_B$ receptors, a subtype of GABA receptors, inhibits transmitter release at many sites including the hippocampus, the neocortex and the dorsal raphe nucleus (for review see Nicoll *et al.* 1990; Thompson, Capogna & Scanziani, 1993). Activation of presynaptic $GABA_B$ receptors has also been shown to mediate heterosynaptic depression in the hippocampus (Isaacson, Solìs & Nicoll, 1993). This $GABA_B$ receptor-mediated presynaptic inhibition may regulate synaptic transmission and prevent excessive excitatory transmitter release (Thompson *et al.* 1993). Given such an important role, the mechanism for this presynaptic inhibition remains uncertain (Nicoll *et al.* 1990; Thompson *et al.* 1993).

Studies performed at neuronal somata have revealed that the $GABA_B$ receptor agonist baclofen inhibits a Ca^{2+} conductance (Scholz & Miller, 1991; Toselli & Taglietti, 1993; Pfrieger, Gottmann & Lux, 1994), and activates a K^+ conductance in hippocampal neurons (Newberry & Nicoll, 1984; Gähwiler & Brown, 1985; Gage, 1992). In principle, either direct inhibition of the Ca^{2+} conductance, or a decrease in amplitude or duration of an action potential secondary to an increase in the K^+ conductance would cause a decrease in the presynaptic Ca^{2+} current and hence inhibition of synaptic transmission. Indeed, baclofen has

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been reported to reduce presynaptic Ca^{2+} influx induced by a train of electrical stimulation as recorded with an extracellular ion sensitive electrode in the rat hippocampus (Heinemann, Hamon & Konnerth, 1984). However, it is not clear whether baclofen reduces presynaptic Ca^{2+} influx evoked by a single stimulus and whether the baclofen-mediated presynaptic inhibition can be accounted for by a reduction in Ca^{2+} influx during a presynaptic action potential. Furthermore, it is unknown whether the reduction in presynaptic Ca^{2+} influx is due to inhibition of Ca^{2+} channels or activation of K^+ channels.

Recently, it was found that baclofen, but not the Ca^{2+} channel blocker cadmium, reduces the frequency of miniature excitatory postsynaptic currents without affecting the distribution of their amplitudes in cultured hippocampal slices (Scanziani, Capogna, Gähwiler & Thompson, 1992). This finding indicates that spontaneous transmitter release may be inhibited by baclofen through a mechanism downstream to presynaptic Ca^{2+} influx. This evidence also raises the possibility that baclofen may inhibit evoked transmitter release by a similar mechanism.

Here, using a recently developed technique for simultaneously recording the presynaptic Ca^{2+} transient ($[Ca^{2+}]_t$) and the field excitatory postsynaptic potential (fEPSP) in hippocampal area CA1 (Saggau, Wu & Yeheskely, 1992; Wu & Saggau, 1994*a*), we examined whether activation of presynaptic $GABA_B$ receptors inhibits evoked synaptic transmission by reducing presynaptic Ca^{2+} influx.

METHODS

Recording of the fEPSP and the presynaptic $[Ca^{2+}]_t$ in the hippocampal slice

The method used has been described in detail elsewhere (Wu & Saggau, 1994*a*, *b*). Briefly, guinea-pigs (1–2 months old) were anaesthetized (by inhalation of methoxyflurane until irresponsive to foot pinch) and quickly decapitated. The brains were removed immediately and 400 μm transverse hippocampal slices were prepared and incubated at 30 °C in artificial cerebrospinal fluid comprising (mM): NaCl, 124; KCl, 5; $CaCl_2$, 2·5; $MgCl_2$, 1·2; $NaHCO_3$, 22; D-glucose, 10; gassed with 95% O_2 and 5% CO_2 to maintain a constant pH of 7·4. A small amount of 0·9 mM fura-2 AM (the acetoxymethyl ester form of fura-2) was pressure-injected into the slice in stratum radiatum of area CA1. About 1 h after injection, fluorescence emerging from an area with a diameter of about 150 μm in stratum radiatum of area CA1, approximately 500 μm from the injection site, was detected by a single photodiode. Calibration of Ca^{2+} signals was obtained by forming the ratio (360/380 nm) between two continuous recordings (100 ms) of fura-2 fluorescence intensity at two excitation wavelengths with an interval of 5–10 s. The background fluorescence (about 5–20% of the total fluorescence intensity) of the brain slice was measured before the injection of fura-2 AM and corrected before computing ratios. An extracellular recording electrode (2 M NaCl, 1–5 MΩ) was placed in the centre of the fluorescence recording area to record the fEPSP. The $[Ca^{2+}]_t$ and the fEPSP were evoked by a single electrical stimulation through a bipolar electrode positioned in the stratum radiatum close to the fura-2 injection site (see also Fig. 1 in Wu & Saggau, 1994*a*).

The amplitude of the $[Ca^{2+}]_t$ was measured as the difference between the maximal concentration and the resting level. The measured amplitude of the $[Ca^{2+}]_t$ is the averaged Ca^{2+} concentration for all the structures loaded with fura-2 in the recording area. However, not all of the loaded structures in the recording area may be stimulated (see also the discussion in Wu & Saggau, 1994*b*). Due to this consideration, the amplitude change of the $[Ca^{2+}]_t$ during application of drugs in each experiment was expressed as the percentage of the $[Ca^{2+}]_t$ before the drug application, and as such the absolute change of the $[Ca^{2+}]_t$ was normalized. Before calculating the initial slopes of fEPSPs, all traces of the electrical signals were corrected for contamination by the presynaptic volleys by subtracting responses obtained during routine application of the glutamate antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM) and aminophosphonovalerate (APV, 50 μM) at the end of each experiment. The measurement of the initial slope of the fEPSP should not be affected by population spikes or inhibitory components that occur later in the field recording. Averaging techniques ($n \leqslant 6$; interval, 10 s) were used to improve the signal-to-noise ratio. The effect of a drug on the fEPSP and the $[Ca^{2+}]_t$ was determined at the end of the drug application. Data in each experiment were normalized to baseline before any drug application, then pooled together and expressed as means ± S.E.M. Application of the above glutamate antagonists verified the presynaptic origin of the observed $[Ca^{2+}]_t$ by blocking the fEPSP but not the $[Ca^{2+}]_t$ (see Fig. 1 in Wu & Saggau, 1994*a*).

Drugs

Baclofen and CGP 35348 were gifts from Ciba-Geigy (Summit, NJ, USA). ω-CgTX (fraction GVIA) was purchased from Sigma (USA) and ω-AgaTX-IVA from Peptides International Inc. (Louisville, KY, USA) (Mintz & Bean, 1993).

RESULTS

Baclofen inhibits both the presynaptic $[Ca^{2+}]_t$ and the fEPSP

Addition of 50 μM baclofen, a $GABA_B$ agonist, resulted in rapid reduction of both the $[Ca^{2+}]_t$ and the fEPSP recorded in hippocampal area CA1 (Fig. 1*A* and *B*). This reduction was not accompanied by a change of either the presynaptic resting Ca^{2+} level (Fig. 1*A*) or the presynaptic fibre volley (Fig. 1*B*), and was reversible. The time courses of inhibition and recovery of both the $[Ca^{2+}]_t$ and the fEPSP were similar (Fig. 1*A*). The recovery often took more than 40 min (data not shown). Figure 1*C* summarizes the dose dependence of the inhibition after 10 min of exposure to baclofen (1–100 μM). At 10 μM, baclofen reduced the $[Ca^{2+}]_t$ to 80 ± 2% and the fEPSP to 41 ± 4% of baseline ($n = 6$). At 50 μM, baclofen approached its maximal effect by reducing the $[Ca^{2+}]_t$ to 67 ± 3% and the corresponding fEPSP to 17 ± 3% of baseline ($n = 7$). The percentage reductions of the fEPSP by baclofen are similar to other reports at this synapse (Lanthorn & Cotman, 1981; Ault & Nadler, 1982).

The relationship between the mean of the initial slope of the fEPSP (y) and the mean of the amplitude of the $[Ca^{2+}]_t$ (x) at each concentration was non-linear and could be approximated by a power function: $y = x^m$, where m is 4, and x and y are expressed as a percentage of their respective baselines. By replacing x in this equation with the measured mean $[Ca^{2+}]_t$ at each concentration of baclofen, the calculated fEPSP closely resembled the corresponding measured mean fEPSP (Fig. 1*C*). The exponent ($m = 4$) in this equation is similar to those obtained by applying either the non-specific voltage-dependent Ca^{2+} channel blocker cadmium ($m = 3{\cdot}6$) (Wu & Saggau, 1994*a*), or the specific blockers ω-CgTX-GVIA ($m = 3{\cdot}5$) and ω-AgaTX-IVA ($m = 4{\cdot}1$) (Wu & Saggau, 1994*b*). This suggests that the reduction of the $[Ca^{2+}]_t$ is sufficient to account for the inhibition of evoked synaptic transmission by baclofen.

The above equation was also used to examine closely the time course of the change in the $[Ca^{2+}]_t$ and fEPSP. The exponent m in this individual experiment was chosen such that the calculated fEPSP matched the observed fEPSP at the end of baclofen application, as judged by eye. As shown in Fig. 1*A*, the time course of the calculated fEPSP obtained with a value of m of 4·8 is similar to that of the measured fEPSP. Note that Fig. 1*A* shows data from an individual experiment, while Fig. 1*C* shows pooled data from a set of experiments; the value of the resulting exponent m was therefore 4. These results further suggest that baclofen inhibits the fEPSP by blocking the presynaptic $[Ca^{+}]_t$.

The $GABA_B$ antagonist CGP 35348 blocks the effects of baclofen

Consistent with the causal relationship between the baclofen-mediated decrease of the $[Ca^{2+}]_t$ and the fEPSP, the effects of baclofen (10 μM) on both the $[Ca^{2+}]_t$ and fEPSP were abolished by CGP 35348 (500 μM, $n = 4$; Fig. 2), a selective $GABA_B$ receptor blocker (Olpe *et al.* 1990). CGP 35348 by itself had no significant effect on either the $[Ca^{2+}]_t$ or the

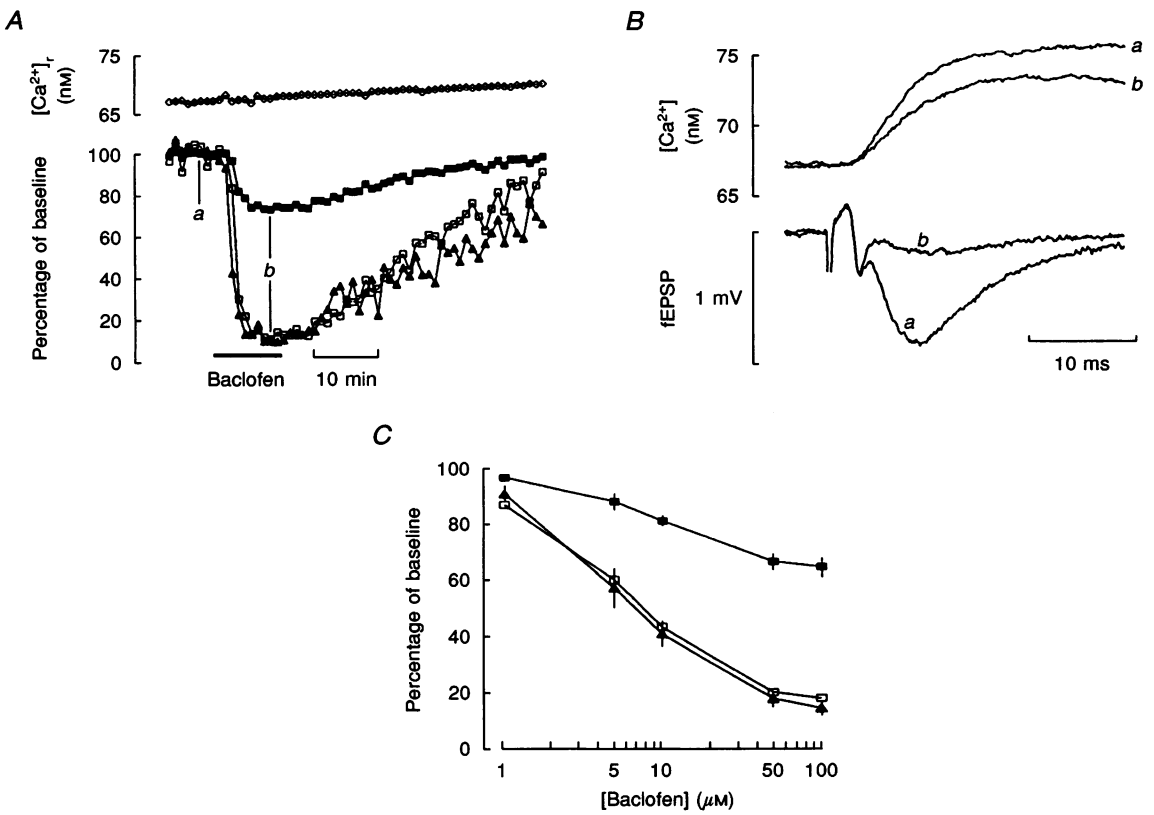

Figure 1. Baclofen decreases both the presynaptic $[Ca^{+}]_t$ and the fEPSP

A, a typical experiment showing the change of the resting Ca^{2+} level ($[Ca^{2+}]_r$), the amplitude of the $[Ca^{2+}]_t$ (■) and the initial slope of the fEPSP (▲) during bath application of 50 μM baclofen (indicated by the horizontal line). The calculated fEPSP (□; y) was obtained from the equation: $y = x^{4{\cdot}8}$, where x is the measured amplitude of the presynaptic $[Ca^{2+}]_t$. The exponent m of 4·8 was chosen such that y matched the observed fEPSP at the end of the baclofen application. *B*, sampled recordings taken at the times indicated in *A*. *C*, dose-dependent block by baclofen. Observations (means ± S.E.M.) from 3–7 slices at each concentration are normalized. The calculated fEPSP slope (y) was obtained from the equation: $y = x^4$, where x is the amplitude of the presynaptic $[Ca^{2+}]_t$. Symbols are as shown in *A*.

fEPSP. These results confirm that $GABA_B$ receptor activation is required to cause the observed reduction in both the $[Ca^{2+}]_t$ and the fEPSP.

ω-CgTX-GVIA partially occludes the effect of baclofen on the $[Ca^{2+}]_t$

The presynaptic $[Ca^{2+}]_t$ at CA3 to CA1 synapses has been found to have three pharmacologically distinct components: a ω-CgTX-GVIA-sensitive component, a ω-AgaTX-IVA-sensitive component and an unidentified component insensitive to ω-CgTX-GVIA, ω-AgaTX-IVA and nifedipine (Wu & Saggau, 1994*b*). If baclofen preferentially affects one type of Ca^{2+} channel, the extent of inhibition of the $[Ca^{2+}]_t$ by baclofen would differ before and after application of a specific blocker of that channel. Because the effects of ω-CgTX-GVIA and ω-AgaTX-IVA are mostly persistent, 1 μM of each of the two blockers was applied for only 18 min to achieve a maximal effect (Wu & Saggau, 1994*b*). After 10–15 min of washout, 50 μM baclofen was applied for 10 min (Figs 3*A* and 4*A*). As measured immediately before baclofen application, the N-type Ca^{2+} channel blocker ω-CgTX-GVIA (Olivera *et al.* 1990) reduced the $[Ca^{2+}]_t$ to 61 ± 3% and the fEPSP to 26 ± 3% ($n = 5$) of baseline. Subsequent baclofen application further reduced the $[Ca^{2+}]_t$ to 47 ± 3% and the fEPSP to 3 ± 1% of baseline ($n = 5$; Fig. 3*A*, *B* and *C*). In Fig. 3*A*, the time course of the calculated fEPSP obtained with a value of m of 3·9 matches that of the measured fEPSP well, supporting the causal relationship between the presynaptic $[Ca^{2+}]_t$ and the fEPSP. The exponent m in this experiment was chosen when the calculated fEPSP best fitted the observed fEPSP at the end of ω-CgTX-GVIA and baclofen application, as judged by eye. Baclofen (50 μM) reduced the $[Ca^{2+}]_t$ by an additional 14 ± 2% (relative to the total $[Ca^{2+}]_t$ before any drug application; $n = 5$) when ω-CgTX-GVIA-sensitive Ca^{2+} channels were completely blocked by a saturating concentration (1 μM) of ω-CgTX-GVIA (Wu & Saggau, 1994*b*). As we have shown above (Fig. 1*C*), 50 μM baclofen alone inhibited the $[Ca^{2+}]_t$ by 33 ± 3% ($n = 7$). Thus the blocking effect of baclofen on the $[Ca^{2+}]_t$ with preapplication of ω-CgTX-GVIA was significantly reduced compared with that without any drug preapplication (Student's t test, $P < 0.01$; Fig. 3*D*). These data indicate that the baclofen-sensitive $[Ca^{2+}]_t$ (33 ± 3%; $n = 7$) has two components: (1) ω-CgTX-GVIA insensitive (14 ± 2%; $n = 5$), and (2) ω-CgTX-GVIA sensitive (19 ± 4%) (the percentages refer to the total $[Ca^{2+}]_t$ before any drug application). The ω-CgTX-GVIA-sensitive component (19 ± 4%) was obtained by subtracting the ω-CgTX-GVIA-insensitive component (14 ± 2%; $n = 5$) from the total baclofen-sensitive $[Ca^{2+}]_t$ (33 ± 3%; $n = 7$) and by pooling the standard error values.

ω-AgaTX-IVA does not occlude the effect of baclofen on the $[Ca^{2+}]_t$

With the same experimental protocol as shown in Fig. 3, the P-type Ca^{2+} channel blocker ω-AgaTX-IVA (Mintz & Bean, 1993) reduced the $[Ca^{2+}]_t$ to 74 ± 4% and the corresponding fEPSP to 35 ± 5% of baseline. Subsequent application of baclofen further reduced the $[Ca^{2+}]_t$ to 40 ± 4% and the corresponding fEPSP to 2 ± 2% of baseline ($n = 4$; Fig. 4*A*, *B* and *C*). The time course of the change in the presynaptic $[Ca^{2+}]_t$ and the fEPSP is also similar, as shown in Fig. 4*A* in which the calculated fEPSP obtained with a value of m of 4·5 matches the measured fEPSP well. The exponent m in this figure was obtained in the same way as in Fig. 3. Baclofen (50 μM) blocked the $[Ca^{2+}]_t$ by an additional 34 ± 2% (relative to the value before any drug application; $n = 4$) when ω-AgaTX-IVA-sensitive Ca^{2+} channels were blocked by 1 μM ω-AgaTX-IVA. As mentioned earlier, 50 μM baclofen alone reduced the $[Ca^{2+}]_t$ by 33 ± 3% ($n = 7$). The baclofen-sensitive $[Ca^{2+}]_t$ was not significantly different with or without ω-AgaTX-IVA preapplication (t test, $P > 0.5$; Fig. 4*D*), suggesting that baclofen does not inhibit ω-AgaTX-IVA-sensitive Ca^{2+} channels.

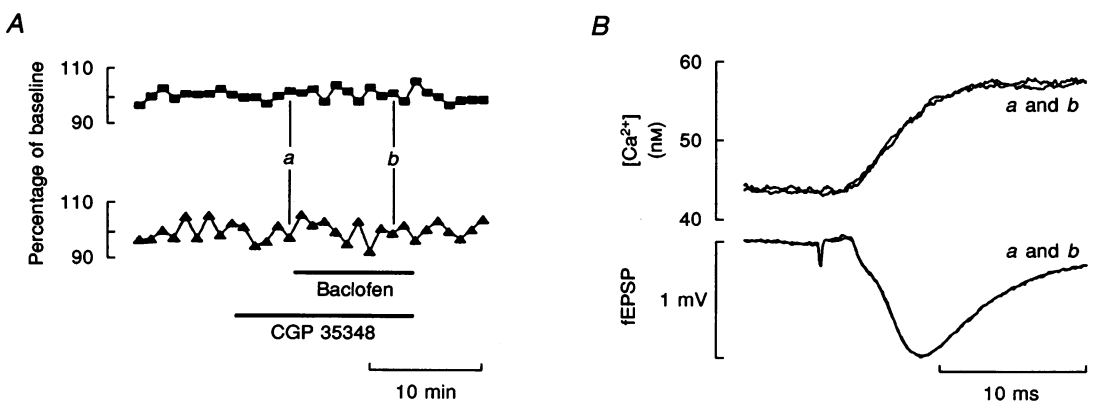

Figure 2. The $GABA_B$ antagonist CGP 35348 blocks the inhibitory effect of baclofen

A, in the presence of 500 μM CGP 35348 (indicated by the horizontal line), neither the amplitude of the $[Ca^{2+}]_t$ (■) nor the initial slope of the fEPSP (▲) changed during application of 10 μM baclofen. CGP 35348 by itself affected neither the $[Ca^{2+}]_t$ nor the fEPSP. *B*, sampled recordings taken at the times indicated in *A* are superimposed.

Baclofen differentially inhibits three components of the $[Ca^{2+}]_t$

The presynaptic $[Ca^{2+}]_t$ was found to be mediated by three pharmacologically distinct types of Ca^{2+} channels (Wu & Saggau, 1994*b*). As shown in Table 1 (Control group) similar results were obtained in the present study: ω-CgTX-GVIA-sensitive Ca^{2+} channels contributed 39 ± 3% ($n = 5$; Fig. 3*C*) of the total presynaptic $[Ca^{2+}]_t$; ω-AgaTX-IVA-sensitive channels contributed 26 ± 4% ($n = 4$; Fig. 4*C*); and some unidentified type(s) of Ca^{2+} channel resistant to ω-CgTX-GVIA and ω-AgaTX-IVA contributed 35 ± 4%, which was calculated by subtracting the means of ω-CgTX-GVIA- and ω-AgaTX-IVA-sensitive components from the total (100%) with a pooled standard error. If a reduction in the $[Ca^{2+}]_t$ is secondary to activation of K^+ channels rather than due to direct inhibition of Ca^{2+} channels, baclofen would be expected to inhibit the different components of the $[Ca^{2+}]_t$ by a similar percentage. Therefore, it is important to know what percentage of each of these three components of the presynaptic $[Ca^{2+}]_t$ is inhibited by baclofen.

As determined by applying a saturating concentration (50 μM) of baclofen (Figs 1*C* and 3*D*), the baclofen-sensitive $[Ca^{2+}]_t$ (33 ± 3%; $n = 7$) has two components: (1) ω-CgTX-GVIA sensitive (19 ± 4%), and (2) ω-CgTX-GVIA insensitive (14 ± 2%; $n = 5$) (the percentage refers to the

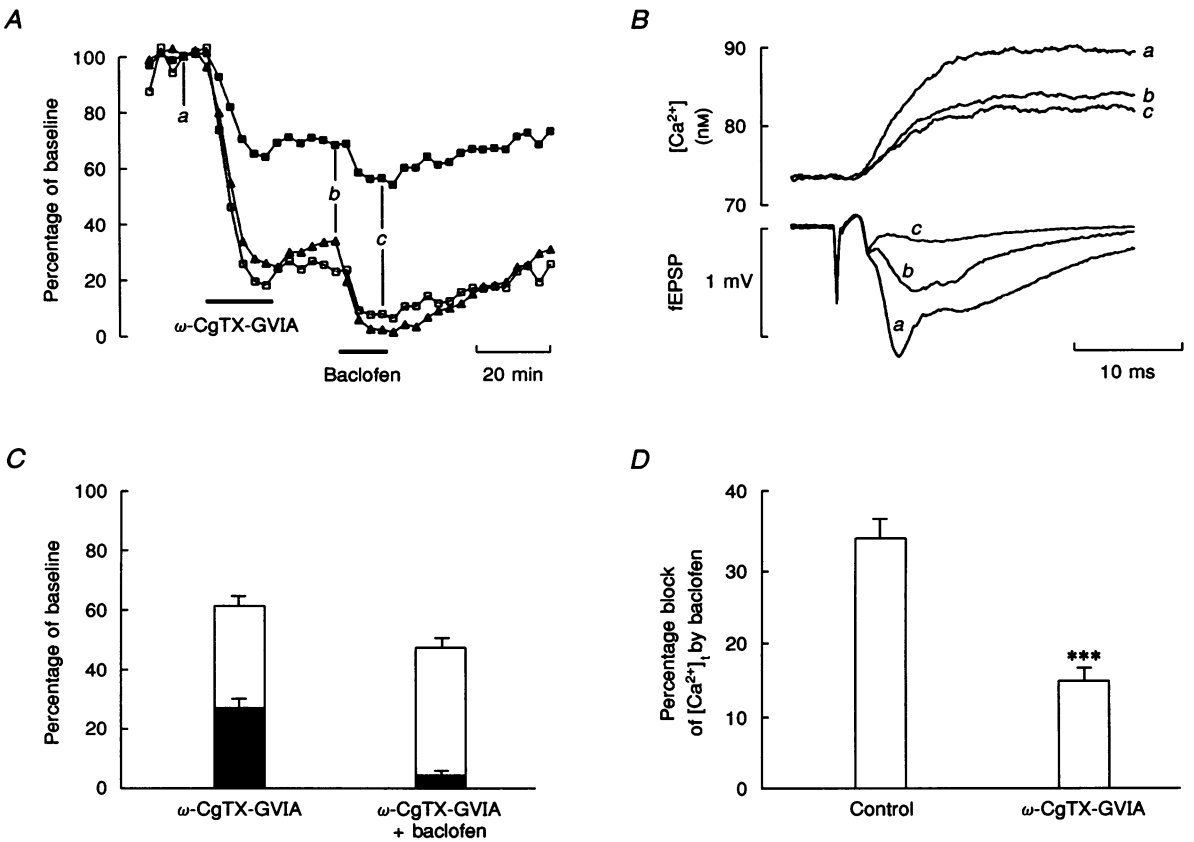

Figure 3. ω-CgTX-GVIA partially occludes the inhibitory effect of baclofen on the $[Ca^{2+}]_t$

A, a typical experiment showing the changes of the amplitude of the $[Ca^{2+}]_t$ (■) and the initial slope of the fEPSP (▲) during sequential application of 1 μM ω-CgTX-GVIA and 50 μM baclofen (indicated by the horizontal lines). The calculated fEPSP (□; y) was obtained from the equation: $y = x^{3.9}$, where x is the measured amplitude of the presynaptic $[Ca^{2+}]_t$. The exponent m of 3·9 was chosen such that y matched the observed fEPSP at the end of ω-CgTX-GVIA and baclofen application. *B*, sampled recordings taken at the times indicated in *A*. *C*, inhibition of both the $[Ca^{2+}]_t$ (□) and the fEPSP (■) during sequential application of 1 μM ω-CgTX-GVIA and 50 μM baclofen is summarized from data from 4 experiments. Data are normalized to baseline before ω-CgTX-GVIA application, and expressed as means ± S.E.M. Because of the persistent blocking effect of ω-CgTX-GVIA, the subsequent 10 min baclofen application was regarded as coaction of these two substances (ω-CgTX-GVIA + baclofen). *D*, comparison of the effects of baclofen on $[Ca^{2+}]_t$ before (Control) and after ω-CgTX-GVIA application. Data in the control group were obtained from Fig. 1*C*, while data in the ω-CgTX-GVIA group were obtained by subtracting the summated effect of ω-CgTX-GVIA and baclofen from the effect of ω-CgTX-GVIA. Both groups of data are expressed as a percentage of the baselines before any drug application, and plotted as means ± S.E.M. The baclofen-sensitive $[Ca^{2+}]_t$ was significantly reduced after ω-CgTX-GVIA application (*** $P < 0.01$; t test).

Table 1. Differential inhibition of three distinct components of the $[Ca^{2+}]_t$ by baclofen

	Control (%)	Baclofen (%)	Baclofen/control (%, mean)
ω-CgTX-GVIA sensitive	39 ± 3(5)	19 ± 4	49
ω-AgaTX-IVA sensitive	26 ± 4(4)	—	—
Unidentified	35 ± 4	14 ± 2(5)	40
Total	100	33 ± 3(7)	33

The control group shows the percentages (means ± S.E.M.; number of experiments (n) in parentheses) of three components of the $[Ca^{2+}]_t$. The baclofen group shows the percentages of baclofen-mediated block of each component of the $[Ca^{2+}]_t$ relative to the total $[Ca^{2+}]_t$ before any drug application. The baclofen/control group shows the mean percentages of baclofen-mediated block of each component of the $[Ca^{2+}]_t$ relative to the corresponding control value, i.e. 49% = 19/39%, 40% = 14/35%. Baclofen inhibits ω-AgaTX-IVA-sensitive $[Ca^{2+}]_t$ by an insignificant percentage. The percentages not followed by n in the control and baclofen groups were obtained by subtraction. See Results for a detailed description.

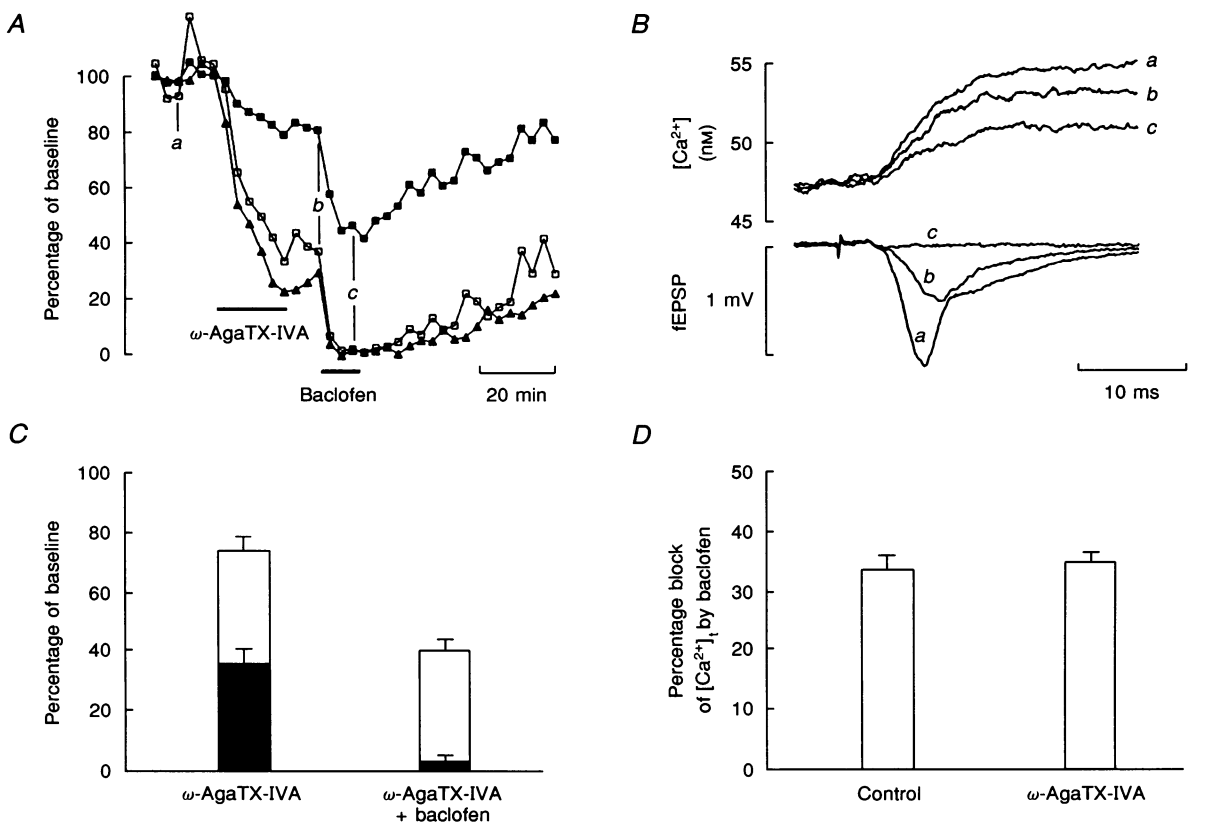

Figure 4. ω-AgaTX-IVA does not occlude the inhibitory effect of baclofen on the $[Ca^{2+}]_t$

Data processing in *A–D* is the same as in *A–D* of Fig. 3. *A*, a typical experiment showing the changes of the $[Ca^{2+}]_t$ (■) and the fEPSP (▲) during sequential application of 1 μM ω-AgaTX-IVA and 50 μM baclofen (indicated by the horizontal lines). The calculated fEPSP (□; y) was obtained from the equation: $y = x^{4.5}$, where x is the measured amplitude of the presynaptic $[Ca^{2+}]_t$. The exponent m of 4·5 was chosen such that y matched the observed fEPSP at the end of ω-AgaTX-IVA and baclofen application. *B*, sampled recordings taken at the times indicated in *A*. *C*, inhibition of both the $[Ca^{2+}]_t$ (□) and the fEPSP (■) during sequential application of 1 μM ω-AgaTX-IVA and 50 μM baclofen is summarized from data from 4 experiments. *D*, comparison of the blocking effect of baclofen on the amplitude of the $[Ca^{2+}]_t$ before (Control) and after ω-AgaTX-IVA application.

total $[Ca^{2+}]_t$ before any drug application). As shown in Table 1 (Baclofen group), baclofen- and ω-CgTX-GVIA-sensitive $[Ca^{2+}]_t$ is 19 ± 4% of the total $[Ca^{2+}]_t$ before any drug application. Thus, baclofen inhibited the ω-CgTX-GVIA-sensitive $[Ca^{2+}]_t$ by a mean value of 49% (19/39%) (Table 1, Baclofen/control group). As shown in Fig. 4, baclofen did not significantly inhibit ω-AgaTX-IVA-sensitive $[Ca^{2+}]_t$ (Table 1); thus the baclofen-sensitive but ω-CgTX-GVIA-insensitive $[Ca^{2+}]_t$ (14 ± 2% of total $[Ca^{2+}]_t$) must be mediated by the unidentified type(s) of Ca^{2+} channel (Table 1, Baclofen group). Baclofen inhibited the unidentified $[Ca^{2+}]_t$ by a mean value of 40% (14/35%) (Table 1, Baclofen/control group).

DISCUSSION

Fluorometric recording of the presynaptic $[Ca^{2+}]_t$

As we discussed previously (Wu & Saggau, 1994*a*, *b*), several lines of evidence strongly suggest that the presynaptic $[Ca^{2+}]_t$ serves as an indicator of the local Ca^{2+} concentration in terminals responsible for synaptic transmission. First, although the optical recording area covers presynaptic terminals and axons close to terminals, voltage-dependent Ca^{2+} channel blockers are usually found to be in terminals (Robitaille, Adler & Charlton, 1990; Cohen, Jones & Angelides, 1991; Zucker, Delaney, Mulkey & Tank, 1991). Second, the time course and the amplitude of the presynaptic $[Ca^{2+}]_t$ we recorded (Wu & Saggau, 1994*a*) are very similar to those recorded from single mossy fibre terminals in hippocampal area CA3 (Regehr, Delaney & Tank, 1994). The onset of the presynaptic $[Ca^{2+}]_t$ differs from that of the presynaptic volley by less than 1 ms (Wu & Saggau, 1994*a*). Third, application of various voltage-dependent Ca^{2+} channel blockers including cadmium (Wu & Saggau, 1994*a*), ω-CgTX-GVIA and ω-AgaTX-IVA (Wu & Saggau, 1994*b*) reveals that: (1) the changes of the $[Ca^{2+}]_t$ and the fEPSP are of similar time course during application of these toxins, (2) the block of both the presynaptic $[Ca^{2+}]_t$ and the fEPSP is dose dependent, and (3) the fEPSP varies approximately as the fourth power of the presynaptic $[Ca^{2+}]_t$ or Ca^{2+} influx, similar to the results found at squid giant synapses in which the EPSP is about the third power of the presynaptic Ca^{2+} current (Augustine, Charlton & Smith, 1985).

As we mentioned in a previous paper (Wu & Saggau, 1994*b*), the amplitudes of the presynaptic $[Ca^{2+}]_t$ recorded with fura-2 were less than 30–40 nM, much lower than the Ca^{2+} concentration (of the order of 100 μM) estimated to trigger transmitter release (Llinás, Sugimori & Silver, 1992; Von Gersdorff & Matthews, 1994). The recorded lower amplitude of the $[Ca^{2+}]_t$ may be due to the following considerations. First, the $[Ca^{2+}]_t$ recorded with fura-2 is the spatially averaged Ca^{2+} concentration in the optical recording area covering the terminals as well as axons, and as such may greatly underestimate the Ca^{2+} concentration near the inner surface of the presynaptic membrane where transmitter is released. Second, since the Ca^{2+} dissociation constant of fura-2 is about 200 nM as measured *in vitro* (Grynkiewicz, Poenie & Tsien, 1985), fura-2 will be essentially saturated at a Ca^{2+} concentration of a few micromoles. This will cause an underestimation of the Ca^{2+} concentration close to Ca^{2+} channel mouths. Third, endogenous Ca^{2+} buffers may compete with fura-2 in binding Ca^{2+} and thus reduce the $[Ca^{2+}]_t$ recorded with fura-2. Finally, the measured $[Ca^{2+}]_t$ is the averaged Ca^{2+} concentration for all the structures loaded with fura-2 in the recording area, but not all of the loaded structures may be stimulated.

Since the average Ca^{2+} level might not be linearly related to the local Ca^{2+} level, the power relationship we measured between the fEPSP and the $[Ca^{2+}]_t$ might not closely reflect the relationship between transmitter release and the local Ca^{2+} level responsible for this release. Nevertheless, the measured power relationship is useful when comparing the effects of baclofen with various voltage-dependent Ca^{2+} channel blockers.

Activation of $GABA_B$ receptors inhibits synaptic transmission by reducing the presynaptic $[Ca^{2+}]_t$

Baclofen inhibited both the presynaptic $[Ca^{2+}]_t$ and the fEPSP in a dose-dependent manner without affecting either the presynaptic fibre volley or the resting Ca^{2+} level; these results are similar to those obtained following application of several voltage-dependent Ca^{2+} channel blockers including cadmium, ω-CgTX-GVIA and ω-AgaTX-IVA (Wu & Saggau, 1994*a*, *b*). During application of baclofen, the decrease of the fEPSP and presynaptic $[Ca^{2+}]_t$ had similar time courses, and the fEPSP was about the fourth power of the $[Ca^{2+}]_t$. The inhibition by baclofen of both the fEPSP and $[Ca^{2+}]_t$ was blocked by the $GABA_B$ antagonist CGP 35348. These results strongly suggest that baclofen, via activation of $GABA_B$ receptors, inhibits the fEPSP by reducing the presynaptic $[Ca^{2+}]_t$ at CA3 to CA1 synapses of the hippocampus.

Different mechanisms underlying inhibition of evoked and spontaneous transmitter release

Presynaptic mechanisms downstream to Ca^{2+} influx were found to be involved in baclofen-mediated inhibition of spontaneous transmitter release in cultured hippocampal slices (Scanziani *et al.* 1992). As stated above, these mechanisms must play a very minor role, if any, in the $GABA_B$-mediated inhibition of evoked transmitter release in the hippocampal area CA1. As with baclofen, adenosine, an important endogenous presynaptic modulator in the central nervous system (Nicoll *et al.* 1990), was found to inhibit evoked synaptic transmission primarily by reducing the presynaptic $[Ca^{2+}]_t$ at CA3 to CA1 synapses of hippocampus (Wu & Saggau, 1994*c*), and to inhibit spontaneous transmitter release by a mechanism subsequent to presynaptic Ca^{2+} influx at the neuromuscular junction

and in cultured hippocampal slices (Silinsky, 1984; Scanziani *et al.* 1992). These results suggest that the mechanisms underlying modulation of spontaneous transmitter release are substantially different from those modulating evoked transmitter release. Interestingly, it was found that separate mechanisms may also underlie evoked and spontaneous transmitter release at the *Drosophila* neuromuscular junction (Littleton, Stern, Schulze, Perin & Bellen, 1993). These findings suggest that caution should be used when extrapolating results from the study of spontaneous transmitter release into evoked release, such as in the field of long-term potentiation (for review see Stevens, 1993).

Differential inhibition of presynaptic Ca^{2+} channel types by baclofen

Previously, we have demonstrated that about 40% of the total $[Ca^{2+}]_t$ is ω-CgTX-GVIA sensitive, more than 20% is ω-AgaTX-IVA sensitive, and less than 40% of the $[Ca^{2+}]_t$ is insensitive to these two blockers and the L-type channel blocker nifedipine (Wu & Saggau, 1994*b*,*c*). A similar percentage block of the $[Ca^{2+}]_t$ by ω-CgTX-GVIA (1 μM) and ω-AgaTX-IVA (1 μM) was also obtained in this study (Figs 3*C* and 4*C*; Table 1, Control). As shown in Table 1, on average, baclofen inhibited the ω-CgTX-GVIA-sensitive $[Ca^{2+}]_t$ by 49% and the unidentified $[Ca^{2+}]_t$ by 40%. Baclofen did not significantly inhibit the ω-AgaTX-IVA-sensitive $[Ca^{2+}]_t$ (Fig. 4; Table 1). Differential inhibition of pharmacologically distinct components of the presynaptic $[Ca^{2+}]_t$ by baclofen suggests that inhibition of the presynaptic $[Ca^{2+}]_t$ is not secondary to modulation of K^+ channels. These results suggest that baclofen mediates presynaptic inhibition of evoked synaptic transmission by inhibiting Ca^{2+} channels including ω-CgTX-GVIA-sensitive N-type and some unidentified type(s) of channel at presynaptic terminals of CA3 pyramidal neurons of hippocampus. These results corroborate previous findings that baclofen inhibits Ca^{2+} currents at somata of cultured hippocampal pyramidal neurons (Scholz & Miller, 1991; Toselli & Taglietti, 1993; Pfrieger *et al.* 1994) with a similar time course to that of baclofen-mediated inhibition of synaptic transmission (Pfrieger *et al.* 1994).

The fEPSP was found to be approximately the fourth power of the presynaptic $[Ca^{2+}]_t$ during application of various voltage-dependent Ca^{2+} channel blockers (Wu & Saggau, 1994*a*, *b*). Thus, the fraction of the $[Ca^{2+}]_t$ remaining after application of an effective voltage-dependent Ca^{2+} channel blocker is usually much larger than the remaining fraction of the fEPSP. This makes the direct measurement of the $[Ca^{2+}]_t$ a better alternative to the fEPSP for detecting partial occlusion of the inhibitory effects of a drug by an effective voltage-dependent Ca^{2+} channel blocker. For example, in the present study, ω-CgTX-GVIA reduced the $[Ca^{2+}]_t$ to 61% (mean; as are all the following percentages) and the fEPSP to 26% of baseline (Table 1). Subsequent baclofen (50 μM) application further reduced the $[Ca^{2+}]_t$ to 47% and the fEPSP to 3% of baseline. Assuming that subsequent baclofen (50 μM) application reduces the $[Ca^{2+}]_t$ by an additional 33% of the initial baseline (before ω-CgTX-GVIA application) as in the control condition without preapplication of w-CgTX-GVIA, i.e. the $[Ca^{2+}]_t$ was reduced to 28% (61–33%) of the initial baseline, the fEPSP will be reduced to 0·61% ($0{\cdot}28^4$) of the initial baseline based on the fourth power relationship between the fEPSP and the presynaptic $[Ca^{2+}]_t$. Similarly, if subsequent application of baclofen reduces the $[Ca^{2+}]_t$ by an additional 14% of the initial baseline as shown in Fig. 3, i.e. the $[Ca^{2+}]_t$ was reduced to 47% of the initial baseline, the fEPSP will be reduced to 4·88% ($0{\cdot}47^4$) of the initial baseline. If we could only record the fEPSP, it would be difficult to differentiate fEPSPs that are between 0·16 and 4·88% of the initial baseline. As a consequence, the variation in measuring such small fEPSPs would be critical in determining the occlusion. Obviously, it is much easier to detect the difference in the $[Ca^{2+}]_t$ between 20 and 47% of the initial baseline.

In summary, we found that activation of presynaptic $GABA_B$ receptors by their agonist baclofen inhibits evoked synaptic transmission primarily by reducing the presynaptic $[Ca^{2+}]_t$ at hippocampal CA3 to CA1 synapses. The reduced $[Ca^{2+}]_t$ was due to inhibition of presynaptic ω-CgTX-GVIA-sensitive Ca^{2+} channels and some unidentified Ca^{2+} channels resistant to both ω-CgTX-GVIA and ω-AgaTX-IVA.

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Acknowledgements

We thank Drs D. Johnston and R. Gray for kindly allowing us to use some of their equipment. We appreciate Dr R. H. Thalmann for critical comments on the manuscript and generously providing some drugs including baclofen and CGP 35348. We thank S. Sinha, B. Avery and A. Bullen for critical and helpful comments on the manuscript. The computer software for data acquisition and analysis was developed by S. S. Patel (Baylor College of Medicine).

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Received 29 July 1994; accepted 7 December 1994.