Relief of G-Protein Inhibition of Calcium Channels and Short-Term Synaptic Facilitation in Cultured Hippocampal Neurons

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G-protein inhibition of voltage-gated calcium channels can be transiently relieved by repetitive physiological stimuli. Here, we provide evidence that such relief of inhibition contributes to short-term synaptic plasticity in microisland-cultured hippocampal neurons. With G-protein inhibition induced by the GABA_B receptor agonist baclofen or the adenosine A1 receptor agonist 2-chloroadenosine, short-term synaptic facilitation emerged during action potential trains. The facilitation decayed with a time constant of ~100 msec. However, addition of the calcium channel inhibitor Cd²⁺ at 2–3 μ M had no such effect and did not alter baseline synaptic depression. As expected of facilitation from relief of channel inhibition, analysis of miniature EPSCs implicated presynaptic modulation, and elevating presynaptic Ca²⁺ entry blunted the facilitation. Most telling was the near occlusion of synaptic facilitation after selective blockade of P/Q- but not N-type calcium channels. This was as predicted from experiments using recombinant calcium chan-

nels expressed in human embryonic kidney (HEK) 293 cells; we found significantly stronger relief of G-protein inhibition in recombinant P/Q- versus N-type channels during action potential trains. G-protein inhibition in HEK 293 cells was induced via recombinant M2 muscarinic acetylcholine receptors activated by carbachol, an acetylcholine analog. Thus, relief of G-protein inhibition appears to produce a novel form of short-term synaptic facilitation in cultured neurons. Similar short-term synaptic plasticity may be present at a wide variety of synapses, as it could occur during autoreceptor inhibition by glutamate or GABA, heterosynaptic inhibition by GABA, tonic adenosine inhibition, and in many other instances.

Key words: short-term synaptic plasticity; facilitation; GABA; baclofen; G-protein inhibition; calcium channels; recombinant calcium channels; microcultures; cultured neurons; autapses; hippocampal neurons; adenosine; HEK 293 cells

Short-term synaptic plasticity may dramatically affect neuronal information transfer (Magleby, 1987; Zucker, 1989; Markram and Tsodyks, 1996; Sejnowski, 1996; Abbott et al., 1997; Tsodyks and Markram, 1997; Dobrunz and Stevens, 1999). Because neurotransmitter release depends supralinearly on presynaptic calcium entry through voltage-gated calcium channels, spike-to-spike changes in calcium currents could contribute substantially to synaptic plasticity. There has to date, however, been little evidence of this class of mechanisms (Borst and Sakmann, 1998; Cuttle et al., 1998; Forsythe et al., 1998). In particular, presynaptic calcium currents in well studied invertebrate neurons appear invariant from stimulus to stimulus (Smith and Zucker, 1980; Charlton et al., 1982; Wright et al., 1996). However, studies of vertebrate preparations provide growing evidence that during repetitive physiological stimuli, calcium channels may manifest progressive increases in current because of relief of G-protein-mediated inhibition. Specifically, in somatic and recombinant channels G-protein-mediated inhibition can be transiently re-

lieved by trains of voltage-clamp action potentials and strong depolarizations (Bean, 1989; Elmslie et al., 1990; Brody et al., 1997; Williams et al., 1997; Park and Dunlap, 1998; Tosetti et al., 1999), although there can be a tonic component of inhibition that is insensitive to depolarization in some preparations (Shapiro and Hille, 1993; Luebke and Dunlap, 1994). Such activity-dependent reversal of calcium channel inhibition could occur in presynaptic calcium channels as well, since at least the initial inhibition of neuronal calcium channels by G-proteins is known to underlie the suppression of neurotransmitter release produced when a wide variety of G-protein-coupled receptors are activated (Toth et al., 1993; Wu and Saggau, 1994, 1995; Dittman and Regehr, 1996; Takahashi et al., 1996). Although several groups have noted that this relief of inhibition potentially could cause a form of short-term synaptic facilitation (Elmslie et al., 1990; Shen and Horn, 1996; Brody et al., 1997), no explicit investigations of this proposal have been performed in synaptic preparations. Here, we tested for such a facilitation in single, cultured rat hippocampal neurons. When grown on glial microislands, the neurons form extensive synaptic connections onto themselves (“autapses”) (Van der Loos and Glaser, 1972), which have properties very similar to those of synapses between neurons (Bekkers and Stevens, 1991; Johnson and Yee, 1995; Goda and Stevens, 1996). Such autapses show large postsynaptic responses and thereby provide a convenient system for studying short-term plasticity, especially under conditions in which neurotransmission is reduced such as during G-protein-mediated inhibition and presynaptic calcium channel blockade.

It has been widely observed that short-term synaptic depression

Received Aug. 16, 1999; revised Nov. 8, 1999; accepted Nov. 9, 1999.

This work was supported by National Institutes of Health and National Science Foundation grants to D.T.Y. and a Medical Scientist Training Program fellowship to D.L.B. We thank C. Boyer and C. F. Stevens for the microisland culture protocol; A. Ghosh, C. Jahr, and C. Zorumski for initial advice on neuronal cell culture; SIBIA Neuroscience (human α_{1B}), T. Snutch, E. Perez-Reyes, and E. Peralta for clones; K. Elmslie for the generous gift of the ω Aga-IVA; and C. Aizenmann, H. Colecraft, D. DiGregorio, J. Dittmann, L. Jones, D. Linden, and P. Fuchs for helpful discussions and comments on this manuscript.

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Figure 1. Short-term synaptic plasticity was changed during calcium channel inhibition by G-proteins but not by Cd^{2+} . **A**, EPSCs measured in microisland cultures of hippocampal neurons. After a somatic voltage-clamp stimulus (*stim.*; top), voltage-gated and capacitive currents were followed by inward synaptic currents (*a*). Responses in $2\ \mu\text{M}$ NBQX (*n*) were subtracted from total currents (*a*) to isolate synaptic currents (*a-n*), which were integrated over a 2–20 msec window after the stimulus (Q_{EPSC}). **B**, Diary plot of Q_{EPSC} for the first (solid diamonds) and second (open squares) stimuli of 50 Hz stimulus trains. Cd^{2+} concentration was titrated so that the initial EPSC amplitude was similar to that during G-protein-mediated inhibition induced by $10\ \mu\text{M}$ baclofen. **C**, Isolated synaptic currents during 50 Hz trains showing short-term plasticity at baseline (control; left), during G-protein-mediated inhibition (baclofen; middle), and in Cd^{2+} (right). **D**, Normalized Q_{EPSC} averages across 12 cells. In all cells included in the average, Cd^{2+} blockade was titrated to match the extent of baclofen inhibition. Left, Normalization by the first Q_{EPSC} in control (solid squares) for each cell. Baclofen (open circles) inhibited the first Q_{EPSC} by $75 \pm 4\%$, and Cd^{2+} (gray triangles; typically $2\text{--}3\ \mu\text{M}$) inhibited by $71 \pm 4\%$. Right, Normalization by the first Q_{EPSC} in each condition to facilitate comparison of short-term synaptic plasticity. Plasticity was significantly different between baclofen and control and between baclofen and Cd^{2+} for the second and all subsequent responses ($p < 0.005$) but was not different between Cd^{2+} and control ($p > 0.27$).

(STD) is attenuated by an overall reduction of neurotransmitter release (Lev-Tov and Pinco, 1992; Barnes-Davies and Forsythe, 1995; Isaacson and Hille, 1997; Varela et al., 1997; Brenowitz et al., 1998) such as during G-protein-mediated inhibition. These effects on synaptic efficacy have been attributed to release-dependent mechanisms such as vesicle depletion (Zucker, 1989). By contrast, we have found that G-protein-mediated inhibition reduces STD or converts it to relative facilitation, in a manner that appears distinct from release-dependent mechanisms but consistent with relief of G-protein-mediated inhibition of calcium channels.

MATERIALS AND METHODS

Cell culture. Hippocampal neurons were cultured on glial microislands essentially as reported (Furshpan et al., 1986; Bekkers and Stevens, 1991). Briefly, a 0.15% agarose solution was spread uniformly on glass coverslips; then 2 mg/ml poly-D-lysine plus 3 mg/ml collagen (Cellprime; Collagen Corporation, Palo Alto, CA) in 8.5 mM acetic acid was airbrushed (Aztek, Rockford, IL) spattered to form 50- to 750- μm -diameter "islands" of adhesive substrate. Cultured rat astrocytes were plated at a density of 6,000–24,000 cells/ml in Minimal Essential Medium (MEM) with Earle's salts (Life Technologies, Gaithersburg, MD), 10% fetal calf serum, 20 mM glucose, 0.5% N2 supplement (Life Technologies), 0.5% penicillin/streptomycin stock, and phenol red. After 4–6 d at 37°C in a 5% CO_2 atmosphere, the astrocytes spread out over the microislands but did not grow on the agarose. Neurons were isolated by trituration of papain-digested, CA1- and CA3-enriched hippocampi from 1- to 2-d-old Sprague Dawley rats. Between 2,000 and 28,000 cells/ml were added to the plates containing astrocytes, and they were cultured in MEM with Earle's salts plus 25 mM HEPES (Life Technologies), 10% horse serum (Life Technologies), 20 mM glucose, 1% N2 supplement, 1 mM sodium pyruvate, 0.5% penicillin/streptomycin stock, and 0.875 mg/ml biotin. The day after plating, 35 μM 5-fluoro-2-deoxyuridine (Sigma, St. Louis, MO) with 75 μM uridine was added. The culture medium was not changed for up to 21 d *in vitro*.

Electrophysiology. Recordings were made after 7–21 d *in vitro*. Whole-cell patch-clamp pipets with a resistance of 3–4 M Ω were pulled from borosilicate glass and filled with (in mM): 137 Kgluconate, 12 NaCl, 10 HEPES, 4 EGTA, 0.5 CaCl_2 , 4 MgATP, and 0.3 LiGTP, pH 7.2 with KOH. The standard extracellular solution contained (in mM): 145 NaCl, 5.4 KCl, 2 CaCl_2 , 1 MgCl_2 , and 10 HEPES, pH 7.4 with NaOH, adjusted to 310 mOsm with 15–25 mM glucose. For early experiments, the intracellular solution contained 145 Kgluconate and 4 NaCl but was otherwise unchanged. No differences were apparent because of these changes, and results have been pooled. All chemicals were from Sigma except for baclofen, 2-chloroadenosine (2-CADO), and 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(f)quinoxaline-7-sulfonamide (NBQX) from Research Biochemicals (Natick, MA), $\omega\text{Ctx-GVIA}$ from Alomone Labs (Jerusalem, Israel), and $\omega\text{Aga-IVA}$ (a gift from Dr. K. Elmslie, Tulane University). A $-14\ \text{mV}$ junction potential (pipet minus bath) was corrected before sealing. All recordings were made at room temperature ($23\text{--}25^\circ\text{C}$). Filtering was at 5 kHz, sampling was at 25 kHz, and the holding potential was $-80\ \text{mV}$. Series resistance was typically 6–10 M Ω and compensated 50–85% when possible. EPSCs stabilized in 3–5 min after break-in, and data traces were acquired every 15–30 sec. EPSC integrals followed the same trends as peak EPSCs but showed less variability from sweep to sweep and were more sensitive to the contribution of slightly asynchronous release events (data not shown). EPSC records have been displayed after subtraction of NBQX-insensitive currents (Fig. 1A), and 1–3 msec around each stimulus has been blanked for clarity. NBQX-insensitive currents were unchanged in baclofen, 2-CADO, or $2\text{--}3\ \mu\text{M}$ Cd^{2+} (data not shown). Rundown or decreases in EPSC amplitude over tens of minutes occurred in some cells but did not significantly affect short-term synaptic plasticity (data not shown). When EPSC amplitudes in two conditions were compared, measurements were bracketed or made within 5–10 min of each other. Minis were recorded without series resistance compensation and were bandpass filtered between 10 and 1000 Hz; 500–800 sweeps of minis were obtained per cell. Mini amplitudes were measured in a semiautomatic manner using custom software in Matlab and confirmed by visual inspection. Cd^{2+} ($2\ \mu\text{M}$) had no effect on minis; their amplitudes were clearly altered by changing the baseline potential, and NBQX eliminated them entirely (data not shown). In 4

mm Ca^{2+} plus 100 μM 4-aminopyridine (4-AP) experiments, extracellular Mg^{2+} was removed, extracellular NBQX was used at 5 μM , and intracellular Na^+ was 4 mM. For toxin experiments, 1 mg/ml bovine serum albumin and 0.1 mg/ml cytochrome C were added to the bath. $\omega\text{Ctx-GVIA}$ rapidly and irreversibly reduced initial EPSCs, but $\omega\text{AgA-IVA}$'s effects reversed partially over tens of minutes, so all measurements were obtained within a few minutes of toxin application.

Transfection of human embryonic kidney 293 cells. Human embryonic kidney (HEK) 293 cells were transiently transfected using calcium phosphate precipitation with the following calcium channel clone cDNAs: rat brain α_{1A} (Starr et al., 1991) or human brain α_{1B} (Williams et al., 1992) plus $\alpha_2\delta$ (Tomlinson et al., 1993) and one of three β subunits: β_{2a} (Perez-Reyes et al., 1992), β_3 (Castellano et al., 1993a), or β_4 (Castellano et al., 1993b). M2 receptor cDNA (Peralta et al., 1987) was included as well. Methods were otherwise as described (Brody et al., 1997).

Statistical analysis. All p values for pairwise comparisons represent two-tailed, paired t test results or two-tailed, unequal variance t test results as appropriate; $p > 0.05$ was considered not significant. Error values ($\pm$) were SEs. Approximate confidence intervals for fit parameters were calculated using the "locally linear hypothesis" (Pandit and Wu, 1990). t tests between pairs of fit parameters were performed using approximate SDs calculated in the same manner.

RESULTS

Changes in short-term synaptic plasticity after activation of G-protein-coupled receptors

To record postsynaptic currents, we delivered brief voltage-clamp stimuli via a somatic, whole-cell patch pipet (Fig. 1*A*, *top*). A propagating action potential was generated, with momentary loss of voltage-clamp control because of large sodium and potassium currents corresponding to initial inward and outward currents, respectively. Subsequently, inward current flowed through AMPA-type glutamate receptors (Fig. 1*A*, *a*) that were recorded with good voltage-clamp control. Subtraction of responses obtained with the AMPA-antagonist NBQX (Fig. 1*A*, *n*) isolated synaptic currents (Fig. 1*A*, *a-n*), which were integrated to quantitate EPSCs (Fig. 1*A*, Q_{EPSC}). Evidence of good voltage-clamp control during EPSCs included an extrapolated EPSC reversal potential near 0 mV and unchanged short-term synaptic depression when the sizes of the EPSCs were reduced using subsaturating concentrations of NBQX (data not shown) (Bekkers and Stevens, 1991).

We tested for changes in short-term synaptic plasticity attributable to relief of G-protein-mediated inhibition by comparing synaptic responses in the absence and presence of G-protein-coupled receptor agonists. In control conditions, autapses showed STD in response to 50 Hz trains of stimuli, as illustrated by the decrement of Q_{EPSC} between the first and second responses (Fig. 1*B*) and exemplar records (Fig. 1*C*, *left*). Population data uniformly confirmed such baseline depression (Fig. 1*D*, *solid squares*). By contrast, the pattern of short-term synaptic plasticity was qualitatively different after application of the GABA_B agonist baclofen (10 μM). Initial EPSCs were inhibited by $75 \pm 4\%$, and the depression converted to relative facilitation (Fig. 1*C*, *middle*) or appeared markedly reduced (Fig. 1*D*, *open circles*), as would be predicted for relief of G-protein-mediated inhibition of presynaptic calcium channels. These effects were not unique to inhibition via GABA_B receptors, because nearly identical inhibition and changes in short-term plasticity were seen with 1 μM 2-CADO, which also activates G-proteins via adenosine A1 receptors (data not shown).

Alternative hypotheses to relief of G-protein-mediated inhibition of calcium channels

In addition to the possibility of dynamic changes in Ca^{2+} influx through calcium channels, a number of more traditional explanations

could account for the effects of baclofen on synaptic plasticity. The most prominent among these involves release-dependent short-term depression mechanisms such as vesicle depletion, postsynaptic receptor desensitization, and autoreceptor inhibition by glutamate (Zucker, 1989), which may underlie short-term depression in many synapses. For example, if vesicle depletion were occurring, the baclofen-induced reduction in overall presynaptic Ca^{2+} influx through calcium channels would decrease fractional depletion of releasable vesicles per action potential, thereby diminishing short-term synaptic depression. To exclude this possibility, we titrated the calcium channel blocker Cd^{2+} into the bath to mimic the initial reduction of release probability produced by baclofen (Fig. 1*B*). Like G-protein-mediated inhibition, Cd^{2+} in effect reduces channel open probability. Unlike G-protein-mediated inhibition, however, Cd^{2+} blockade appears to be voltage independent over a physiological voltage range (Leonard et al., 1987) and remains invariant with repetitive stimulation of calcium channels (see Discussion). Despite a substantial reduction in transmitter release by Cd^{2+} , the short-term synaptic depression was unaffected in almost all cells (Fig. 1*C*, *right*, *D*, *gray triangles*). In rare recordings (3 of 29), there was somewhat less depression in Cd^{2+} . However, baclofen or 2-CADO always produced even greater changes in short-term plasticity than did Cd^{2+} (data not shown). These results excluded a contribution of release-dependent mechanisms to the changes in synaptic plasticity produced by baclofen. Instead, our unpublished results suggest that baseline depression in this preparation may reflect action potential propagation failure at axonal branch points (Parnas, 1972; Hatt and Smith, 1976; Macagno et al., 1987; Streit et al., 1992; Wall, 1995; Debanne et al., 1997).

A remaining conventional explanation for the baclofen-induced changes in plasticity was that facilitation caused by residual calcium bound to presynaptic effectors was potentiated with lowered calcium entry (Rahamimoff, 1968; Stanley, 1986). This possibility seemed unlikely because our pipet solution contained a high concentration of EGTA, which would chelate residual Ca^{2+} (Hochner et al., 1991; Atluri and Regehr, 1996). It appeared that this EGTA effectively diffused to the presynaptic terminals, because after whole-cell access was obtained, the rate of spontaneous miniature EPSCs was initially high but declined over 3–5 min. Furthermore, bath-applied EGTA AM had no effect on short-term synaptic plasticity (data not shown). Most importantly, facilitation from residual calcium was ruled out by the invariance of short-term synaptic plasticity with the addition of Cd^{2+} (Fig. 1*D*).

Having excluded major known forms of synaptic plasticity that could have produced our results, we next considered novel mechanisms other than relief of G-protein-mediated inhibition. One possibility was that baclofen and 2-CADO attenuated the release-independent short-term depression mechanism present at baseline. To explore this hypothesis, we examined short-term plasticity as the duration (d) between pairs of stimuli was varied (Fig. 2). Recovery from depression in control proceeded in two rising exponential phases with time constants of ~ 6 and ~ 5000 msec (Brody and Yue, unpublished observations). Mennerick and Zorumski (1995) have observed a similar time course previously. Most importantly, in control, there was little change in short-term plasticity for interstimulus intervals between 20 and 420 msec (Fig. 2*B*, *solid squares*). In baclofen, however, the second EPSC was larger than the first EPSC at short intervals, but smaller at longer intervals (Fig. 2*B*, *dashed line*, *open circles*). Thus there appeared to be a distinct component of facilitation in baclofen

Figure 2. Recovery of synaptic responses between two stimuli ($S1$, $S2$) in control and in baclofen. *A*, Stimulus protocol (top) and single-sweep exemplars in baclofen. *B*, Averages across cells. Little change in short-term depression in control (solid squares) is seen during the time intervals examined (20–420 msec). In baclofen (open circles), there was additional facilitation at short intervals, which decayed exponentially (dashed line) with a 96 msec time constant (95% confidence interval, 65–180 msec). Statistical significance levels: $p < 0.005$ (**) and $p < 0.01$ (*). The eight cells contributing to the average recovery data are different from those used in Figure 1.

that decayed rapidly ($\tau = 96$ msec), leaving the subsequent recovery from depression indistinguishable from that of the control over the remaining period in which comparisons were made ($d = 170$ – 420 msec). These results would be difficult to reconcile with simple attenuation of depression. They fit well, however, with reestablishment of G-protein-mediated inhibition after its relief by the first stimulus (Lopez and Brown, 1991; Zamponi and Snutch, 1998), all superimposed on unchanged baseline depression.

To exclude novel postsynaptic mechanisms underlying the baclofen-induced facilitation, we analyzed miniature EPSCs (minis), which arise from the asynchronous release of single synaptic vesicles. Changes in postsynaptic receptor sensitivity and dendritic attenuation that might produce synaptic plasticity would be apparent as changes in mini amplitudes (Wyllie et al., 1994; Isaac et al., 1996; Carroll et al., 1998). During baseline periods (Fig. 3*A*), baclofen did not change either the mean or distribution of mini peak amplitudes (Table 1; Fig. 3*B*; Kolmogorov–Smirnov test, $p > 0.2$). A particular advantage of single-neuron cultures is that all minis arise from the same presynaptic cell as evoked EPSCs. In other preparations, minis may reflect release events at numerous synapses outside of the subset that contributes to evoked EPSCs. Therefore, if there were a change in the amplitude of minis originating from this subset, the change might be poorly resolved in the amplitude histogram for minis arising from all synapses. Instead, in single-neuron cultures, the lack of change in mini amplitudes is sufficient to exclude novel postsynaptic mechanisms as the basis for the baclofen effect on evoked EPSCs.

Furthermore, baclofen did not alter average mini amplitudes during a time window stretching from 20 to 100 msec after an

Figure 3. Analysis of minis. *A*, Stimulus protocol (top) for acquiring minis at baseline and after an evoked EPSC, with sample records in control (middle) and baclofen (bottom). Minis were well resolved after decay of full-sized EPSCs. Full-sized EPSCs have been truncated for clarity. *B*, Distributions of mini sizes at baseline were no different between control (solid line) and baclofen (dashed line; Kolmogorov–Smirnov test, $p > 0.2$). Plotted on the y-axis is the cumulative probability that peak mini amplitudes were smaller than the values on the x-axis. *C*, Mini sizes versus time after EPSC-producing stimuli are shown. Mini sizes increased slightly with increasing times after stimuli (solid lines; slope, 0.056 pA/msec in control, $p < 0.001$; slope, 0.044 pA/msec in baclofen, $p < 0.002$). Slopes are not different between control and baclofen ($p = 0.56$). All data in *B* and *C* are from one representative cell.

evoked EPSC (Table 1; Fig. 3*C*), despite the persistence of baclofen-induced facilitation throughout this period (Fig. 2*B*). Amplitudes did increase with time after the evoked EPSCs ($\sim 20\%$ in 80 msec), but the increases were indistinguishable between control and baclofen (Fig. 3*C*). This increase could in no way account for the facilitation that occurred only in baclofen and decayed over the same time interval (Fig. 2*B*). Thus, these results ruled out a major postsynaptic component as the basis of the facilitation in baclofen.

A final class of alternative mechanisms we examined concerned the possibility that baclofen unmasked activity-dependent but

Table 1. Characteristics of minis

	Baseline size (pA)	After stimulus size (pA)	Baseline rate (Hz)
Control	15.22 $\pm$ 1.64	15.08 $\pm$ 2.11	1.62 $\pm$ 0.50
Baclofen	14.77 $\pm$ 1.36	16.13 $\pm$ 2.26	0.67 $\pm$ 0.21*

Baseline minis were recorded during 1 sec epochs before an EPSC-evoking stimulus. After stimulus minis were recorded between 20 and 100 msec after a 2 msec voltage step that evoked full-sized EPSC. Values are given as means $\pm$ SE ($n = 7$ cells). There were no statistically resolvable differences in the amplitudes of minis either at baseline or after stimuli, but baclofen did change the rate of spontaneous minis (* $p < 0.05$ vs control).

Figure 4. Elevation of presynaptic calcium entry with 4 mM Ca^{2+} plus 100 μM 4-AP (4Ca/4-AP) blunted inhibition by baclofen or 2-CADO and occluded changes in short-term plasticity. *A*, Synaptic currents showing an increase in EPSC amplitudes in 4Ca/4-AP (middle) and mild effects of 20 μM baclofen in 4Ca/4-AP (right). Averages of 10, 13, and 10 sweeps (left, middle, right, respectively) from the same cell are shown. *B*, Q_{EPSC} averages across cells. Data from each cell are normalized by the first Q_{EPSC} in control (2 mM Ca^{2+} , 1 mM Mg^{2+} , no 4-AP) before averaging. 4Ca/4-AP ($n = 6$; solid squares) increased initial EPSCs 3.47 (± 0.73)-fold. 4Ca/4-AP plus 20 μM baclofen or 2 μM 2-CADO ($n = 3$ of each averaged together; open circles) inhibited EPSCs by 34 $\pm 6.7\%$ on average relative to 4Ca/4-AP, and 4Ca/4-AP plus 3 μM Cd^{2+} ($n = 6$; gray triangles) reduced EPSCs by 42 $\pm 6.6\%$ relative to 4Ca/4-AP. *C*, Same data normalized to the first Q_{EPSC} in each condition to facilitate comparison of short-term synaptic plasticity. No significant differences ($p > 0.5$ for all responses) between Cd^{2+} (gray triangles) and baclofen or 2-CADO (open circles).

Ca^{2+} -independent changes in maximal release probability, downstream of calcium entry. If such a mechanism accounted for the facilitation in baclofen, then this facilitation would be insensitive to raising overall presynaptic calcium entry. Alternatively, if relief of calcium channel inhibition explains the facilitation, it should be blunted with saturation of the Dodge–Rahamimoff relation between calcium influx and vesicle release (Dodge and Rahamimoff, 1967; Reid et al., 1998). In this case, proportionate spike-to-spike increases in presynaptic calcium currents would enhance neurotransmitter release to a lesser extent when absolute calcium current magnitudes are larger. To test this, we increased presynaptic calcium entry mainly (see Materials and Methods) by raising extracellular Ca^{2+} from 2 to 4 mM and adding 100 μM 4-AP to broaden the presynaptic action potentials (Fig. 4*A*, 4Ca/4-AP) (Llinas et al., 1976; Wheeler et al., 1996; Hjelmstad et al., 1997). In 4Ca/4-AP, initial EPSCs were on average 3.47-fold larger than in control (Fig. 4*B*), and baclofen, 2-CADO, and

Cd^{2+} all inhibited the EPSCs less effectively (compare Fig. 4*B* with Fig. 1*D*, left). Most importantly, in 4Ca/4-AP, baclofen or 2-CADO caused only small changes in short-term plasticity (Fig. 4*C*), thereby excluding progressive changes in maximal release probability as the mechanism of facilitation. These results support other mechanisms, including relief of G-protein-mediated inhibition, presynaptic action potential changes, and spike-to-spike increases in the calcium affinity of the release apparatus.

Blockade of N- versus P/Q-type channels results in predicted differential effects on short-term synaptic plasticity

To generate a highly discriminating test of whether relief of G-protein-mediated inhibition underlies the facilitation, we hoped to exploit a clear intrinsic difference between the two types of presynaptic calcium channels that trigger transmitter release (Takahashi and Momiyama, 1993; Wheeler et al., 1994; Reuter, 1995; Reid et al., 1997). One such distinction was apparent between recombinant P/Q-type (α_{1A}) and N-type (α_{1B}) channels expressed in HEK 293 cells along with M2 muscarinic acetylcholine receptors (Fig. 5). M2 receptor activation by carbachol, an acetylcholine analog, inhibited P/Q- and N-type channels to comparable extents (27 ± 5 and 36 $\pm 8\%$, respectively). However, 50 Hz trains of voltage-clamp action potential waveforms (APWs) relieved more of the inhibition of P/Q-type (Fig. 5*A,C*, left, *D*) than of N-type (Fig. 5*B,C*, right, *D*) channels. Voltage pulses to +100 mV relieved inhibition nearly completely in both channel types (92% for N-type; 95% for P/Q-type). Thus the differing behavior of channel types during trains reflected distinctions in the kinetics of relief and not in the voltage-dependent nature of the inhibition. Furthermore, these distinctions between P/Q- and N-type channels were not unique to the particular combination of auxiliary subunits used in these experiments. The stronger relief of G-protein-mediated inhibition shown by P/Q-type channels was qualitatively preserved when either the β_{1B} or β_3 subunit was substituted for the β_{2A} subunit used in the experiments shown in Figure 5 (data not shown), after taking into account the overall changes in baseline inactivation properties seen with these other subunits (Patil et al., 1998). Assuming that the contrasting features of M2 receptor-mediated inhibition of P/Q- and N-type channels in HEK 293 cells would hold true for baclofen-induced channel inhibition in hippocampal neurons, we made the following specific prediction: if relief of G-protein-mediated inhibition produces the facilitation seen in baclofen, then the facilitation should be larger for transmission mediated exclusively by P/Q-type channels than for transmission mediated by N-type channels.

To test this key prediction, we compared the effects of baclofen during selective blockade of N-type channels with those during blockade of P/Q-type channels. Synaptic transmission via P/Q-type channels was isolated with $\omega\text{Ctx-GVIA}$, a peptide toxin that selectively blocks N-type channels (Dunlap et al., 1995; Mori et al., 1996). During steady-state $\omega\text{Ctx-GVIA}$ blockade (Fig. 6*A*), baclofen still inhibited transmission by 70 $\pm 4\%$ ($n = 7$), and short-term depression was reduced or converted to facilitation (Fig. 6*A,C*, left, *D*), as in the absence of toxins (Fig. 1). In separate cells, N-type channel-mediated transmission was isolated with $\omega\text{Aga-IVA}$, a peptide inhibitor of both P- and Q-type channels (Dunlap et al., 1995; Mori et al., 1996). After $\omega\text{Aga-IVA}$ blockade (Fig. 6*B*), baclofen inhibited neurotransmission by an additional 73 $\pm 4\%$ ($n = 6$). In contrast to the results with isolated P/Q-type channels (Fig. 6*A*), baclofen caused little change in short-term plasticity when only N-type channels triggered trans-

Figure 5. Recombinant P/Q-type calcium channels show more relief of G-protein-mediated inhibition than do N-type channels. Cloned calcium channels were expressed in HEK 293 cells along with M2 receptors, and calcium currents were evoked using 50 Hz trains of voltage-clamped APWs that started from a base potential of -80 mV, peaked at $+30$ mV, and lasted 1.5 msec at half-amplitude. The waveforms were based on those recorded in the calyx of Held (Borst et al., 1995) but were scaled in amplitude and duration to the parameters mentioned above. The half-width duration of 1.5 msec was approximately threefold longer than originally recorded in the calyx, to accord with the duration of action potentials measured in cultured neuron somata, whose half-widths averaged ~ 1.42 msec (Brody and Yue, unpublished observations). **A**, P/Q-type channel currents (α_{1A} β_{2A} α_{2D}) showing progressive increases in current (arrow) during G-protein-mediated inhibition by $50 \mu\text{M}$ carbachol (CCh). Little change in the calcium currents in control solutions during APW trains (top). **B**, N-type channel records (α_{1B} β_{2A} α_{2D}) showing little relief of G-protein-mediated inhibition (arrow). **C**, Peak currents (I_{PEAK}) averaged across cells in control (solid squares) or carbachol (open circles). Normalization is by the first peak current in each cell before averaging. **D**, Ratios of carbachol to control peak currents ($I_{\text{CCh}}/I_{\text{control}}$) providing direct comparison of relief of G-protein-mediated inhibition between P/Q-type channels (open triangles) and N-type channels (solid diamonds). Normalization is by the first ratios in each cell before averaging.

mission (Fig. 6*B,C, right, D*). The distinction between the effects of baclofen with the two toxins was actually greater than might be expected from our studies of recombinant channels, even after factoring in a standard third to fourth power relationship between Ca^{2+} current and EPSCs. Quantitative differences between the extents of facilitation in the cultured neurons and HEK 293 cells could reflect differences in the nature of the G-proteins or in the parameters of action potentials. These parameters may change during repetitive presynaptic firing but were held fixed in experiments with recombinant channels. Overall, the remarkable agreement of these results with our prediction strongly supported the hypothesis that relief of G-protein-mediated inhibition of calcium channels underlies the short-term synaptic facilitation observed in the presence of baclofen.

The experiments summarized in Figure 6 merit an important technical clarification. To conserve on expenditure of toxins, we stopped toxin application before baclofen exposure (e.g., Fig. 6*A,B*). Nonetheless, the baclofen experiments can be interpreted as if steady-state toxin blockade were still in effect, because these experiments were always performed within an ~ 5 min window shortly after toxin application, and control experiments showed almost no reversal of blockade over this time span. In such 5 min windows, there was $-0.1 \pm 0.96\%$ recovery of pret toxin currents after cessation of $\omega\text{Ctx-GVIA}$ ($n = 4$ cells) and $-1.89 \pm 2.3\%$ recovery after $\omega\text{Aga-IVA}$ ($n = 3$ cells). Hence, the conclusions based on results in Figure 6 are no different from those that would be drawn using continuous toxin application.

DISCUSSION

This report is the first that specifically implicates a contribution of relief of G-protein-mediated inhibition to synaptic plasticity. This form of relative facilitation is distinct from the mechanisms of short-term facilitation that have been described in other prepa-

rations. Presynaptic calcium channels in the calyx of Held exhibit a Ca^{2+} entry-dependent form of facilitation that contributes to short-term synaptic plasticity (Borst and Sakmann, 1998; Cuttle et al., 1998), but this effect may involve the action of Ca^{2+} -calmodulin (Lee et al., 1999) and does not require G-protein activation. Others have shown that STD can be attenuated by an overall reduction of neurotransmitter release, yielding a relative facilitation (Lev-Tov and Pinco, 1992; Barnes-Davies and Forsythe, 1995; Isaacson and Hille, 1997; Varela et al., 1997). However, these effects on STD were attributable to release-dependent mechanisms such as vesicle depletion (Zucker, 1989). At an avian calyceal synapse, such attenuation of STD can in fact result in frank facilitation of synaptic responses during high-frequency stimulation, with concomitant increases in the likelihood of firing postsynaptic action potentials (Brenowitz et al., 1998). By contrast, in hippocampal autapses, we have found that G-protein-mediated inhibition reduces STD or converts it to relative facilitation, in a manner that is consistent with relief of G-protein-mediated inhibition of calcium channels but not with Ca^{2+} -dependent facilitation or attenuation of release-dependent depression.

Have there been previously reported hints of a mechanism such as that observed here? At the same avian synapses, conversion of paired-pulse depression to facilitation by baclofen inhibition could have resulted in part from relief of G-protein-mediated inhibition, because comparable Cd^{2+} -mediated inhibition occluded depression but produced less facilitation (Otis and Trussell, 1996). Also, in rat hippocampal slice cultures, adenosine inhibited transmission and increased paired-pulse facilitation. Although lowering $[\text{Ca}^{2+}]_o$ and increasing $[\text{Mg}^{2+}]_o$ inhibited transmission more than did adenosine, this maneuver increased paired-pulse facilitation less than did adenosine (Debanne et al.,

Figure 6. Synaptic facilitation during G-protein-mediated inhibition is larger for transmission mediated by P/Q-type channels than for transmission mediated by N-type channels. **A**, Blocking N-type channels to isolate neurotransmission via P/Q-type calcium channels is shown. *Top*, First Q_{EPSC} before and during blockade by $\omega\text{Ctx-GVIA}$, a selective blocker of N-type calcium channels. Average inhibition was $63 \pm 11\%$ ($n = 7$), and average time to half-block was 37 ± 13 sec ($n = 6$). Baclofen further inhibited synaptic transmission. *Middle*, Synaptic currents evoked by a 50 Hz train in $\omega\text{Ctx-GVIA}$ (single sweep) are shown. *Bottom*, Baclofen application during toxin blockade still shifted short-term plasticity toward facilitation (average of 19 sweeps; same cell as above). **B**, Blocking P/Q-type channels to isolate neurotransmission via N-type channels. *Top*, Blockade by $\omega\text{Aga-IVA}$, a selective blocker of P/Q-type calcium channels, is shown. Average inhibition was $79 \pm 5\%$ ($n = 6$), and average time to half-block was 210 ± 71 sec ($n = 6$). *Inset*, Expanded y-axis shows inhibition by baclofen after toxin block. *Middle*, Synaptic currents evoked by a 40 Hz train in $\omega\text{Aga-IVA}$ (single sweep) are shown. *Bottom*, During toxin blockade, baclofen inhibited EPSCs but had little effect on short-term synaptic plasticity (average of 19 sweeps; same cell as above). **C**, Effects of baclofen on short-term plasticity after $\omega\text{Ctx-GVIA}$ block (left) and after $\omega\text{Aga-IVA}$ block (right). Normalized Q_{EPSC} averages across cells in control solution after toxin application (solid squares) and in baclofen after toxin application (open circles) are shown. After $\omega\text{Ctx-GVIA}$ block, short-term plasticity in baclofen was significantly different from control ($p < 0.01$) for stimuli 4 and 6–10. After $\omega\text{Aga-IVA}$ block, plasticity in baclofen was not significantly different from control ($p > 0.05$) except on stimuli 6, 8, and 9 ($p = 0.008$ – 0.04). **D**, Direct comparison of changes in short-term plasticity in baclofen after $\omega\text{Ctx-GVIA}$ block (open triangles) and after $\omega\text{Aga-IVA}$ block (solid diamonds). Ratios of baclofen to control normalized Q_{EPSC} data from **C** (baclofen/post-toxin) are averaged across cells. Differences are significant ($p < 0.05$) for stimuli 3–10.

1996). Finally, in frog sympathetic ganglia, synaptic depression caused by acetylcholine autoinhibition was prominent during 5 Hz stimulation, but not at 20 Hz (Shen and Horn, 1996). The authors proposed that 20 Hz stimulation could have relieved the inhibition of presynaptic calcium channels, whereas 5 Hz action potentials did not. One important difference between some previous results and ours is that the baseline depression in cultured hippocampal neurons was not detectably release dependent. This unusual finding has been investigated in a separate set of experiments, which supports axonal branch-point failure (Parnas, 1972; Hatt and Smith, 1976; Macagno et al., 1987; Streit et al., 1992; Wall, 1995; Debanne et al., 1997) as the underlying mechanism for the short-term synaptic depression (Brody and Yue, unpublished observations). Regardless of the mechanism, the release-independent nature of the baseline depression clarified the interpretation of changes in short-term plasticity during G-protein-mediated inhibition of calcium channels.

Another key aspect of our approach concerns the use of Cd^{2+} to test for release-dependent mechanisms by reducing presynaptic Ca^{2+} influx. Both G-protein-mediated inhibition and Cd^{2+} blockade of calcium channels in effect reduce channel open probability without altering unitary current amplitude; Cd^{2+} at low doses produces a millisecond flickering block of calcium channels (Lansman et al., 1986), whereas G-protein-mediated inhibition of neuronal calcium channels slows the activation of channels (Carabelli et al., 1996; Patil et al., 1996). An advantageous distinction between G-protein-mediated inhibition and Cd^{2+} blockade is that the former can be relieved during repetitive activity (e.g., Fig.

5A) but Cd^{2+} blockade remains invariant during analogous trains of APWs (Fig. 7). Here, the patterns of short-term changes in recombinant P/Q- or N-type currents did not change after robust blockade by Cd^{2+} . The invariance of Cd^{2+} blockade during repetitive activity probably arises from the relative voltage independence of Cd^{2+} blockade of neuronal channels over a physiological range of voltages (Leonard et al., 1987). When voltage-dependent unblock of Cd^{2+} in neuronal calcium channels has been observed (Thevenod and Jones, 1992), it was not apparent for voltages up to +40 mV and manifested clearly only with stronger (unphysiological) depolarization.

Changing extracellular calcium and/or magnesium concentrations to test for release-dependent mechanisms may not be equivalent to Cd^{2+} blockade. Altering Ca^{2+} or Mg^{2+} alters unitary channel conductance (Church and Stanley, 1996), which may have distinct effects on calcium concentrations in presynaptic microdomains. Also, changes in these divalent cations may alter membrane excitability by surface-charge-screening effects (Frankenhaeuser and Hodgkin, 1957), which appears to affect various ion channels inhomogeneously (Green and Andersen, 1991). The low doses of Cd^{2+} required to block calcium channels do not produce detectable surface-charge effects, as gauged by the voltage required for either half-activation of calcium channels (Leonard et al., 1987) or half-inactivation of sodium channels (Hanck and Sheets, 1992). Furthermore, the micromolar concentrations of Cd^{2+} used in this study had no measurable effect on action potentials measured in hippocampal somata, with half-width durations of 1.42 ± 0.16 msec in control versus 1.39 ± 0.14 msec in

Figure 7. Recombinant P/Q- and N-type calcium channels show activity-independent blockade by Cd^{2+} . Cloned calcium channels were expressed in HEK 293 cells, and calcium currents were evoked using 50 Hz trains of action potential waveforms, as in Figure 5. *A*, P/Q-type channel currents (α_{1A} β_4 $\alpha_2\delta$) were partially blocked by $2 \mu\text{M}$ Cd^{2+} . There were no apparent changes in the amplitudes of the currents with repetitive stimuli either in control or in Cd^{2+} . *B*, N-type channels coexpressed with a β subunit favoring inactivation (α_{1B} β_3 $\alpha_2\delta$) were also partially blocked by $2 \mu\text{M}$ Cd^{2+} , and there were no changes in the extent of inactivation during Cd^{2+} blockade. Scale bars apply to both *A* and *B*. Displayed are averages of four sweeps (control) and eight sweeps (Cd^{2+}) for both *A* and *B*. *C*, Normalized peak current amplitudes from P/Q-type channel records, averaged from the same cell shown in *A*. No significant differences between control (solid squares) and Cd^{2+} (open circles). *D*, Normalized peak currents from N-type channel records, averaged from the same cell shown in *B*. The analysis in *C* and *D* confirmed the activity-independent nature of Cd^{2+} blockade of both neuronal calcium channel types.

$2 \mu\text{M}$ Cd^{2+} ($n = 5$ cells; $p > 0.40$). These distinctions are relevant to the present results, because changing Ca^{2+} and/or Mg^{2+} does affect short-term synaptic plasticity in this preparation (Mennerick and Zorumski, 1995), but in a complex manner that is inconsistent with the presence of release-dependent depression (Brody and Yue, unpublished observations). Thus, the use of Cd^{2+} allowed us to exclude unambiguously several potential alternative explanations for the effects of baclofen on short-term synaptic plasticity.

Although baclofen did not show discernible postsynaptic effects (Fig. 3; Table 1), it did reduce the rate of spontaneous EPSCs from 1.62 Hz in control to 0.67 Hz in baclofen (Table 1). In other preparations, presynaptic inhibition has similar effects (Scanziani et al., 1992; Dittman and Regehr, 1996), which have been hypothesized to involve an inhibitory effect on the release machinery downstream of Ca^{2+} entry. Although this effect merits future investigation, it is unlikely to contribute to the relative facilitation produced by baclofen. First, a tonic decrease in overall release probability, such as might be inferred from the decrease in mini frequency, would not be expected to give rise to facilitation in the autapse preparation, because of the evidence favoring release-independent short-term depression (i.e., Fig. 1, the experiments with Cd^{2+}). Second, effects on the release machinery downstream of Ca^{2+} entry could not account for the near occlusion of baclofen-induced facilitation after blockade of P/Q-type channels (Fig. 6).

One final alternate mechanism that we have not considered is the potential interaction of baclofen with the rab protein pathway (Simons and Zerial, 1993; Fischer von Mollard et al., 1994). Rab proteins are small GTP-binding proteins that may catalyze GTP hydrolysis in the course of facilitating vesicle docking and/or

priming (Sudhof, 1995). Activation of G-protein-coupled receptors by baclofen could in principle decrease local GTP concentrations and thereby reduce vesicle docking and/or repriming. Although this could conceivably affect short-term synaptic plasticity, G-protein-coupled receptor activation should, if anything, enhance short-term depression. Instead we found experimentally that baclofen had just the opposite effect, shifting short-term plasticity toward facilitation. Furthermore, the involvement of rab proteins could in no way explain the differential effects on short-term responses produced by blockade of N- versus P/Q-type channels. Hence, it seems unlikely that changes in rab protein cycling underlie the baclofen-induced changes in synaptic plasticity.

The form of short-term synaptic plasticity described here could have important implications for neuronal information processing. G-protein-mediated presynaptic inhibition may not cause an absolute quieting of the synapse but rather a selective damping of low-frequency activity. Information carried in high-frequency bursts of action potentials (Connors and Gutnick, 1990; Gray and McCormick, 1996; Lisman, 1997) may still be transferred reliably, because such bursts could partially reverse the inhibition. This could be a widespread phenomenon, because autaptic and heterosynaptic inhibition by a variety of neurotransmitters, as well as tonic adenosine receptor activation, act via G-protein-mediated inhibition of presynaptic calcium channels (Anwyl, 1991; Jones and Elmslie, 1997). For example, the diffusion of GABA from its site of release to presynaptic terminals on glutamate-releasing synapses in CNS slices has been shown to heterosynaptically inhibit excitatory transmission via activation of GABA_B receptors (Isaacson et al., 1993; Dittman and Regehr, 1997). Such inhibition could significantly alter short-term plasticity at the affected excitatory synapses, at least in part due to relief

of G-protein inhibition. Likewise, presynaptic G-protein-coupled autoreceptors can be activated by transmitter released from the same synapse, inhibiting further transmitter release (Deisz and Prince 1989; Scanziani et al., 1997). Relief of G-protein inhibition could limit the magnitude of this sort of negative feedback during high-frequency firing. Additionally, tonic G-protein inhibition may occur because of the presence of extracellular adenosine in slice preparations (Wu and Saggau, 1994; Masino and Dunwiddie, 1999). Therefore, relief of G-protein inhibition may underlie a portion of the baseline short-term plasticity at some synapses. Thus, this frequency-selective mechanism may affect many aspects of synaptic function throughout the nervous system. Inhibition by adenosine has been implicated in the regulation of arousal and wakefulness *in vivo* (Porkka-Heiskanen et al., 1997), and it will be interesting to investigate the role of possible concomitant changes in short-term synaptic plasticity in more intact systems.

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