Synaptically Released Neurotransmitter Fails to Desensitize Postsynaptic GABA<sub>A</sub> Receptors in Cerebellar Cultures

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Mellor, J. R. and A. D. Randall. Synaptically released neurotransmitter fails to desensitize postsynaptic GABA<sub>A</sub> receptors in cerebellar cultures. J Neurophysiol 85: 1847–1857, 2001. GABA concentration jump experiments performed on membrane patches predict that postsynaptic GABA<sub>A</sub> receptors will become desensitized following the release of the contents of a single GABA-containing synaptic vesicle. To examine this we used a single synaptic bouton stimulation technique to directly examine whether postsynaptic GABA<sub>A</sub> receptors in cultured cerebellar granule cells exhibit transmitter-induced desensitization. In a large number of recordings, no evidence was found for desensitization of postsynaptic GABA<sub>A</sub> receptors by vesicularly released transmitter. This was the case even when as many as 40 vesicles were released from a single bouton within 1.5 s. In addition, postsynaptic depolarization and application of the benzodiazepine flunitrazepam, manipulations previously shown to enhance desensitization, failed to unmask transmitter-induced desensitization. In contrast, a single 2- to 3-s application of a high concentration of exogenous GABA was able to depress synaptic responsiveness for up to 70 s. Furthermore, pharmacological depletion of GABA eliminated inhibitory synaptic communication, suggesting that GABA is the transmitter and the desensitization-resistant inhibitory postsynaptic currents are not mediated by a “nondesensitizing” ligand such as β-alanine. Overall our data indicate that a specific desensitization-resistant population of GABA<sub>A</sub> receptors are present at postsynaptic sites on cultured cerebellar granule cells.

INTRODUCTION

In addition to causing channel opening, agonists of ligand-gated channels often produce receptor desensitization. This phenomenon was first described and characterized for the nicotinic acetylcholine receptors of the motor end-plate (Feltz and Trautmann 1982; Katz and Thesleff 1957; Sakmann et al. 1980) but is also expressed by most other ligand-gated ion channels (Dudel et al. 1992; Jones and Westbrook 1996). Desensitization reflects the entry of the receptor-channel complex into long-lived ligand-bound closed states (Dudel et al. 1992; Jones and Westbrook 1996; Sakmann et al. 1980). Considerable interest has been expressed in the physiological relevance of the desensitization process, particularly with regard to its role in the modulation and plasticity of synaptic communication (Jones and Westbrook 1996; Magleby and Pallotta 1981; Overstreet et al. 2000).

The GABA<sub>A</sub> receptors are ligand-gated ion channels central to fast inhibitory synaptic signaling in the vertebrate brain (Macdonald and Olsen 1994; Sieghart 1995). They are believed to be heteromeric structures with five subunits surrounding a central Cl<sup>–</sup>-selective pore. Each receptor is furnished with a number of modulatory sites, whose occupancy can significantly alter the properties of agonist-gated responses (Macdonald and Olsen 1994; Sieghart 1995). GABA<sub>A</sub> receptors on neurons and glia exhibit substantial agonist-induced desensitization (Akaie et al. 1986; Cash and Subbarao 1987; Celantano and Wong 1994; Dreifuss et al. 1969; Froesch et al. 1992; Jones and Westbrook 1995; Mellor and Randall 1998; Puia et al. 1994; Thalmann and Hershkowitz 1985; Weiss et al. 1988; Williamson et al. 1998; Wong and Watkins 1982; Zhu and Vicini 1997). In many systems, given a sufficiently prolonged exposure to a sufficiently high concentration of agonist, the large majority of GABA<sub>A</sub> receptors are found resident in desensitized states.

Although a role for GABA<sub>A</sub> receptor desensitization in inhibitory synaptic transmission has been considered by a number of workers (Ben-Ari et al. 1979; Jones and Westbrook 1996; Krnjevic 1981; Numann and Wong 1984; Thompson and Gahwiler 1989c), direct investigation of this possibility has been limited by the experimental inaccessibility of GABAergic synapses. Although this remains a problem, recent technological advances make it possible to generate exogenous agonist applications that approximate the brief agonist challenge experienced by postsynaptic neurotransmitter receptors (Clements 1996; Dudel et al. 1992). Such experiments have indicated that the processes of desensitization and deactivation (i.e., the decline in current observed following agonist removal) of GABA<sub>A</sub> receptors are closely intertwined (Jones and Westbrook 1995; Mellor and Randall 1998). Indeed, it has been suggested that the time course of the IPSC may in part reflect the entry into, and recovery from, desensitized states of the GABA<sub>A</sub> receptor (Jones and Westbrook 1995, 1996). Furthermore, experiments using multiple sequential agonist applications indicate that GABA<sub>A</sub> receptor desensitization may also contribute to short-term plasticity at GABAergic synapses (Jones and Westbrook 1995; Mellor and Randall 1997, 1998; Tia et al. 1996a,b; Zhu and Vicini 1997).

Like their counterparts in native tissues, recombinant GABA<sub>A</sub> receptors expressed in vitro desensitize on agonist exposure. The kinetics of this process is clearly dependent on the specific subunit composition expressed (Gingrich et al. 1995; Saxena and Macdonald 1994; Tia et al. 1996a,b; Verdoorn et al. 1990). Considerable variation in the kinetics of
desensitization of GABA$_A$ receptors can also be observed when native cells from different brain regions, developmental stages, or culture conditions are compared (Puia et al. 1994; Tia et al. 1996a; unpublished observations). Presumably, this variability in GABA$_A$ receptor desensitization kinetics in vivo reflects well-characterized cell-to-cell differences in the expression of the numerous members of the GABA$_A$ receptor subunit family (Wisden et al. 1992), although other mechanisms such as cell-type–specific receptor phosphorylation may also play a role (Gyenes et al. 1994; Jones and Westbrook 1997).

The desensitization kinetics of GABA$_A$ receptors are also dependent on the agonist employed. For example, the $\beta$-amino acids taurine and $\beta$-alanine produce much less desensitization than concentrations of GABA that produce similar levels of receptor activation (Jones and Westbrook 1995; Zhu and Vicini 1997). Activation of benzodiazepine (BDZ) and neurosteroid receptor activation (Jones and Westbrook 1995; Zhu and Vicini 1997). Kinetic and pharmacological evidence (e.g., Jones and Westbrook 1995; Mellor and Randall 1997; Zhu and Vicini 1997). Depolarization of the membrane potential exerts very similar kinetic effects to BDZs (Mellor and Randall 1998).

Although the results of rapid agonist application experiments suggest that activity-dependent transmitter-induced desensitization of GABA$_A$ receptors may contribute to short-term synaptic plasticity (Jones and Westbrook 1996), a significant caveat exists in extrapolation of data from such studies to the physiological function of GABAergic synapses. This arises from the fact that all such experiments, instead of being carried out on true postsynaptic receptors, are performed on either recombinant GABA$_A$ receptors expressed in suitable host cells (e.g., Gingrich et al. 1995; Lavoie et al. 1997; Tia et al. 1996b) or on GABA$_A$ receptors isolated from neuronal cell bodies (e.g., Jones and Westbrook 1995; Mellor and Randall 1997; Zhu and Vicini 1997). Kinetic and pharmacological evidence from this laboratory and other laboratories indicate that there are substantial functional differences between somatic and synaptic GABA$_A$ receptors (Brickley et al. 1999; Mellor et al. 2000). Furthermore, the precise subunit composition of postsynaptic GABA$_A$ receptors has not been conclusively determined in any experimental system.

In this manuscript we describe experiments that utilize single synapse stimulation (Liu and Tsien 1995) to test directly whether synaptically released GABA can desensitize postsynaptic GABA$_A$ receptors in cultured cerebellar granule cells. Our results indicate that postsynaptic GABA$_A$ receptors in granule cells are highly resistant to desensitization by synaptically released transmitter but will enter an inactivated state following prolonged application of a high concentration of exogenous GABA.

METHODS

Cell culture

Cultures of neonatal cerebellum on glass coverslips were prepared from postnatal day 5 mice as previously described (Mellor and Randall 1997). All animal sacrifice was carried out using UK Home Office Schedule 1 procedures. The culture medium contained 5 mM K$^+$ to promote the formation of active GABAergic synapses. Synaptic glomeruli resembling those formed by granule cells in vivo were not observed in any of our cultures (see also Leao et al. 2000). For the GABA depletion studies shown in Fig. 6, cultures continuously exposed to mercaptopropionic acid (100 $\mu$M), GABAase (Sigma, 4 mg/ml), and NADP (2 mg/ml) were maintained in parallel with untreated control cultures. The treated cultures appeared healthy, although they tended to exhibit a morphology similar to that seen in cultures grown in 25 mM K$^+$ medium.

Solutions and preparation of cells for recordings

Cells were only used for experiments after 10 days or more in culture. Single coverslip-containing culture dishes were removed from the incubator, and the culture medium was exchanged for a standard HEPES-buffered salt solution (HBSS) consisting of (in mM) 130 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 30 glucose, and 25 HEPES-NaOH, pH 7.3, at room temperature. The coverslip was broken into numerous shards that were individually transferred to a recording chamber mounted on the stage of an epifluorescence-equipped inverted microscope (Nikon Diaphot 200).

For concentration jump experiments the bathing solution was HBSS. In contrast, the standard bathing solution for experiments in which release was elicited from FM1–43–labeled presynaptic terminals was a modified HBSS (LCHBSS) that contained only 0.2 mM Ca$^{2+}$ (to limit spontaneous release), 1 $\mu$M TTX (to block action potentials), and 5 $\mu$M 6-cyano–7-nitroquinoxaline–2,3-dione [CNQX; to block glutamatergic excitatory postsynaptic currents (EPSCs)]. As demonstrated by others, the loading of presynaptic terminals with FM1–43 requires vesicle cycling to occur. Thus to facilitate FM1–43 loading, LCHBSS was substituted for a solution (HKHBSS) that contained higher levels of both Ca$^{2+}$ and K$. HKHBSS consisted of (in mM) 50 NaCl, 85 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 30 glucose, and 25 HEPES-NaOH, pH 7.3, and for loading of terminals was supplemented with 10 $\mu$M FM1–43. Dye loading was permitted to continue for 1 min before the preparation was washed with LCHBSS for at least 5 min. For electrophysiological experiments, visualization of FM1–43 fluorescence was achieved using a Xe light source, a Nikon B–2A filter set (450–to 490-nm excitation filter, 510-nm dichroic mirror, >520-nm emission filter), and either a x40 or a x20 fluorescence objective. In addition, images of FM1–43–labeled terminals were taken with a Nd–YLF laser–based 2-photon imaging system (see Leao et al. 2000).

Data recording and analysis

All recordings were made at room temperature using an Axopatch 200A patch-clamp amplifier (Axon Instruments). The pipette solution consisted of (in mM) 110 CsCl, 10 NaCl, 5 MgCl$_2$, 5 EGTA, 2 ATP, 0.2 GTP, and 35 HEPES, pH 7.3. Concentration–jump experiments on nucleated macroplates were carried out as described previously (Mellor and Randall 1997).

Stimulation of single FM1–43–labeled synapses was carried out using minor modifications of the methods of Liu and Tsien (1995). Postsynaptic membrane current was monitored using conventional whole cell recording from individual cerebellar granule cells. A fine–tipped pressure ejection pipette was placed in the neuron’s dendritic field within 2 $\mu$m of a single FM1–43–labeled spot. Quantal release was stimulated by briefly pressure ejecting a bolus of HKHBSS solution. In all experiments FM1–43–labeled synapses were activated at 0.1 Hz using a stimulus lasting 1 s. Similar responses were also produced by pressure ejecting a hyperosmotic solution (data not shown). Recordings from cells exhibiting a high background spontaneous miniature inhibitory postsynaptic current (mIPSC) frequency were not included in the analysis shown. In most experiments drug application was through whole bath perfusion; however, for the experiments illustrated in Fig. 5E, an additional GABA–containing local perfusion barrel was placed adjacent to the synapse under study.

A number of measures were taken to ensure that only single FM1–43–labeled spots were stimulated. First, care was taken to avoid stimulating sites where two FM1–43–labeled spots appeared very
close to each other. Second, the ejection pressure was increased from 1 kPa steadily upward until just beyond the level at which release was triggered, typically 15–30 kPa. Third, it was confirmed that small lateral movements (\(~1 \mu m\)) of the pressure ejection pipette completely eliminated evoked release (Liu and Tsien 1995). As an additional control a number of imaging experiments were performed using a cooled digital charge-coupled device camera equipped with a EEV37 frame transfer chip (Astrocam, Cambridge, UK). These revealed that, under our standard stimulation conditions, destaining of stimulated boutons was more than sevenfold faster than their nearest unstimulated neighbors. This ability to selectively destain single FM1-43–labeled boutons in a stimulus-dependent fashion is in agreement with the observations of those of Liu and Tsien (1995) and others.

The response to each 1-s stimulus was filtered (2 kHz, 8-pole Bessel characteristic filter), digitized (5 kHz), and stored straight to computer disk under control of the pClamp6 software suite (Axon Instruments). In addition, continuous records of membrane current filtered at 50 kHz (4-pole Bessel characteristic filter) were stored on Digital Audio Tape. Analysis of data files was performed with pClamp6 or with programs custom written within the AxoBasic programming environment (Axon Instruments). Unless otherwise stated, data are presented as means ± SE.

**RESULTS**

**Desensitization of somatic GABA_A receptors on cerebellar granule neurons**

We have previously reported that desensitization is a prominent feature of GABA_A receptor–mediated responses in cultured cerebellar granule cells (Mellor and Randall 1997, 1998). An example of the macroscopic desensitization of a GABA response in a somatic macropatch isolated from such a cell is shown in Fig. 1A. Here 1 mM GABA was applied for 1,500 ms and caused approximately 75% macroscopic desensitization.

In addition to the macroscopic desensitization produced by long applications of GABA (e.g., Fig. 1A), very brief applications (e.g., 1–5 ms) of relatively high GABA concentrations (1–10 mM) have also been shown to cause significant desensitization of GABA_A receptors. The desensitization produced by such brief applications of GABA has been characterized in experiments in which two successive GABA applications are made within a brief temporal window (Jones and Westbrook 1995; Mellor and Randall 1997, 1998; Tia et al. 1996a,b; Zhu and Vicini 1997). An extension of such an experiment is shown in Fig. 1B. Here, four successive 5-ms applications of 1 mM GABA were made with an inter-stimulus interval of 150 ms. The event-by-event decline in response amplitude reflects the increasing development of desensitization. The observed decline in response also indicates that complete recovery from desensitization must take much longer than the 150-ms inter-stimulus interval employed in these experiments; indeed complete recovery from desensitization can take as long as 30 s to occur in these cells (Mellor and Randall 1998). If the same is true of synaptic responses, we might expect that desensitization of GABA_A receptors might contribute to short-term activity-dependent plasticity such as that seen at GABAergic synapses in the hippocampus and neocortex (Davies et al. 1990; Deisz and Prince 1989; McCarren and Alger 1985; Thompson and Gahwiler 1989a). To directly test this, we employed an experimental protocol that allowed us to study postsynaptic responses at individual synaptic boutons.

**Basic properties of responses at single boutons identified with FM1-43**

Using the method of Liu and Tsien (1995), we investigated the properties of quantal IPSCs evoked at single inhibitory synapses on cultured cerebellar granule cells. Release was stimulated by pressure ejecting high K^+ HEPES-buffered salt solution (HKHBSS, see Methods section) onto individual boutons labeled with FM1-43 (Fig. 2). This caused a short intense burst of vesicle release, as detected by postsynaptic current measurements. Typical examples are shown in Fig. 3, A and B. As reported by Liu and Tsien, the first release event followed...
the start of HKHBSS application with some considerable latency, typically 300–500 ms. The overall poststimulus latency distribution for all events from a typical cell is shown in Fig. 3C.

The kinetic properties and amplitudes of IPSCs evoked by single synapse stimulation were not different from those of spontaneous mIPSCs recorded in this preparation under identical conditions (Mellor and Randall 1997, 1998). IPSCs rose to peak with a mean 20–80% growth time of about 0.6 ms, reached an average maximum current of around 30 pA, and

![Graph showing latency distribution](image1)

![Graph showing mean amplitude distribution](image2)

FIG. 4. A lack of stimulus-dependent desensitization at single inhibitory synapses. A: a graph plotting the amplitude of every synaptic event elicited at a single inhibitory synapse by 40 successive 1-s stimuli. The x-axis plots whether the IPSC was the 1st, 2nd, 3rd, or nth response elicited by the stimulus, whereas the y-axis plots the event’s amplitude. The regression line through the data indicates that on average there was no significant difference in amplitude between the 1st and last event elicited in response to each stimulus (R = 0.024, P > 0.5). B: the data in A simplified to show the average amplitude of the 1st through to the 20th event in each sweep. Note that even though the contents of 19 vesicles have been released in the previous few hundred milliseconds, the 20th event was no smaller than the 1st. C: a graph plotting pooled data from 27 single synapse recordings like that shown in B. Before averaging across cells, the mean amplitude for the 1st, 2nd, 3rd, to the 20th IPSC in each sweep was normalized to the mean amplitude of the 1st IPSC elicited by each stimulus. The lack of decline in response amplitude indicates a complete lack of postsynaptic desensitization. D: a graph plotting the average IPSC amplitude recorded in response to each of 40 consecutive 1-s stimuli. The interstimulus interval was 10 s, data from 7 recordings.

![Graph showing number of events per stimulus](image3)

![Graph showing amplitude distribution](image4)

FIG. 3. GABAergic inhibitory postsynaptic currents (IPSCs) evoked at single synapses. A: sweeps from an example recording in which activity was elicited at a single FM1-43–labeled synapse. The postsynaptic cell was voltage clamped at −70 mV, and transmitter release was stimulated every 10 s by pressure ejecting HKHBSS onto the FM1-43–labeled spot for 1 s (pressure ejection period indicated by arrows). Of 40 consecutive stimulation sweeps, the 1st, 10th, and 40th are shown. Note that the number of events elicited per stimulus decreases with increasing stimulus number. B: example sweeps from a different cell in which the number of events produced per stimulus did not decrease with increasing stimulus number. As in A, the 1st, 10th, and 40th are shown. C: a graph plotting the latency with which events arose after the initiation of a 1-s HKHBSS stimulus. No release occurred in the 1st 250 ms, and practically all release is complete 0.5 s after the termination of the stimulus. Data are from a different cell from those shown in A and B. D: the amplitude distribution of 964 IPSCs evoked at a single synapse of a typical cell. The holding potential was −70 mV, and an HKHBSS stimulus was applied for 1 s every 10 s (data from the same cell as C). E: cumulative amplitude distributions from 27 individual synapses (−) and their mean (−).
decayed back to baseline over a few tens of milliseconds. The IPSC amplitude distribution for one typical bouton is shown in Fig. 3D. It is clear that even at this single synapse there is a large coefficient of variation in the response amplitude, an observation that was made at all single synapses examined. This is similar to the situation reported for hippocampal synapses and has important consequences for quantal analysis (Liu and Tsien 1995). The individual cumulative amplitude distributions from 27 cells and their mean are shown in Fig. 3E.

Like spontaneously arising mIPSCs, inhibitory synaptic currents evoked at single synapses reversed polarity close to the Cl\(^-\) equilibrium potential. Furthermore, they exhibited voltage-dependent kinetics with longer lasting IPSCs being observed at positive potentials (see Fig. 5 for example). Analysis of responses evoked at synapses on, close to, and as much as served at positive potentials (see Fig. 5 for example). Analysis of responses evoked at synapses on, close to, and as much as served at positive potentials (see Fig. 5 for example). Analysis of responses evoked at synapses on, close to, and as much as served at positive potentials (see Fig. 5 for example).

We performed similar analysis on 27 different synaptic boutons. Despite as many as 40 vesicles being released within a short period of time. On average, at a stimulus-naive synapse, 19 ± 4 vesicles were released per 1-s stimulus. If synaptically released transmitter was able to induce significant desensitization of postsynaptic GABA\(_A\) receptors, this considerable barrage of vesicular release within each sweep would be expected to produce an event-by-event decline in IPSC amplitude.

A possible barrier to directly observing an activity-dependent decline in postsynaptic responsivity is the considerable inherent variability in the amplitude of IPSCs that is seen even at the level of a single bouton (Fig. 3, D and E). An illustration of this point is shown in Fig. 4A. Here, for each of 40 consecutive stimulus sweeps, we have plotted the amplitude of all the stimulus-evoked IPSCs. A regression line fit through the data indicates there was no significant decline in average IPSC amplitude between the 1st and 35th synaptic response evoked in each sweep. This is confirmed by the graph in Fig. 4B, which plots for the same cell the mean amplitudes of the 1st to the 20th IPSCs evoked per stimulus.

We performed similar analysis on 27 different synaptic boutons. Despite as many as 40 vesicles being released within around 1.5 s, in no case was any evidence found for substantial transmitter-induced desensitization. Data pooled from all 27 recordings are shown in Fig. 4C. The graph plots the normalized average of data sets like that shown in Fig. 4B and was produced by normalizing the mean amplitude of the first event in each sweep, before averaging across recordings. This confirms that the release of the contents of 20 vesicles within a brief temporal window was consistently unable to produce any measurable transmitter-induced desensitization. Furthermore, analysis of experiments in which at least 40 consecutive stimuli were applied revealed that there was no change in the mean IPSC amplitude per stimulus over the entire duration of the experiment (Fig. 4D). Thus neither the contents of 40 vesicles being released in 1 s nor the repetition of this stimulus 40 times at 0.1 Hz seems capable of desensitizing postsynaptic GABA\(_A\) receptors in cerebellar granule cells.

**Transmitter released at inhibitory synapses fails to desensitize postsynaptic GABA\(_A\) receptors**

It is clear from Fig. 3, A and B, that a 1-s stimulation of an individual synaptic bouton typically caused the release of a considerable number of quanta within a short period of time. On average, at a stimulus-naive synapse, 19 ± 4 vesicles were released per 1-s stimulus. If synaptically released transmitter was able to induce significant desensitization of postsynaptic GABA\(_A\) receptors, this considerable barrage of vesicular release within each sweep would be expected to produce an event-by-event decline in IPSC amplitude.

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**Neither postsynaptic depolarization nor BDZs unmask transmitter-induced GABA\(_A\) receptor desensitization**

In cerebellar granule cells, GABA\(_A\) receptor desensitization is enhanced by BDZ receptor agonists and membrane depolarization (Mellor and Randall 1997, 1998; Mierlak and Farb 1988). These effects are seen irrespective of whether desensitization is induced by a long-lasting (e.g., 1,500 ms, 1 mM, Fig. 4A) or a brief (e.g., 5 ms, 1 mM, Fig. 4B) GABA exposure. Given this, we tested whether postsynaptic depolarization or application of a BDZ could induce synaptically released transmitter to desensitize the postsynaptic GABA\(_A\) receptors.

First we analyzed the effects of a BDZ receptor agonist Flunitrazepam (FLU) on IPSCs evoked at single inhibitory boutons. To do this, single synapse stimulation experiments were performed in the presence and absence of FLU (1 μM). As we have previously reported in this system, this BDZ produced a substantial slowing of the decay of the IPSC (Mellor and Randall 1997) while only slightly increasing mean IPSC amplitude. Analysis similar to that shown in Fig. 4 revealed that FLU was completely ineffective in unmasking transmitter-induced desensitization (Fig. 5, A and B).

In the next series of experiments, we investigated how the postsynaptic membrane potential affected synaptic GABA\(_A\) receptor desensitization. Single synapse stimulation experiments were performed with the postsynaptic cell voltage clamped at +50 rather than −70 mV. An example recording is shown in Fig. 5C and pooled data from seven such experiments in Fig. 5D. It is clear from this graph that, even at this positive postsynaptic potential, the release of the contents of as many as 20 synaptic vesicles in around 1.5 s fails to induce any postsynaptic GABA\(_A\) receptor desensitization.

**Prolonged applications of exogenous GABA depress mIPSC amplitude**

Having consistently failed to produce desensitization through the synaptic liberation of transmitter at the GABAergic
inputs to cerebellar granule cells, we examined whether prolonged applications of exogenous GABA to synaptic sites could produce any effects commensurate with GABA_A receptor desensitization (Overstreet et al. 2000). To do this we again used the single synapse stimulation approach but placed an additional local perfusion pipe to allow exogenous GABA to be applied in the vicinity of the stimulated synapse.

With this experimental configuration in place, we stimulated single boutons as described above. After a baseline period of single synapse stimulation, we locally applied 1 mM exogenous GABA for 2–4 s. This produced an inward current and increase in membrane noise demonstrating a successful activation of postsynaptic GABA_A receptors. Furthermore, following the application of GABA, a large decrease (≈70%) in the mIPSC amplitude evoked by single synapse stimulation was observed (Fig. 5E). This decrease in mean single synapse mIPSC amplitude was slow to recover following the removal of exogenous GABA (and the accompanying loss of the inward current it evoked). Indeed, on average it was not until around 70 s after the removal of exogenous GABA that the mIPSC amplitude recovered to values not significantly different to those seen in the control period (Fig. 5E). These data indicate that GABA_A receptors at postsynaptic sites in cultured cerebellar granule cells are capable of desensitizing given a sufficiently sustained agonist challenge.

Is GABA the transmitter at the inhibitory synaptic inputs to cerebellar granule cells?

One possible explanation for the lack of desensitization at GABAergic synapses is that a molecule other than GABA acts as the transmitter. Other possible transmitter candidates are the β-amino acids taurine and β-alanine (DeFeudis and Martin del Rio 1977; Fykse and Fonnum 1996; Höslí and Höslí 1980; Sandberg and Jacobson 1981; Saransaari and Oja 1993). Both compounds are taken up into neurons and have been reported to be released in a Ca<sup>2+</sup>-dependent manner (Fykse and Fonnum 1996; Holopainen et al. 1989; Saransaari and Oja 1993). Furthermore, brief applications (e.g., 1–5 ms) of suitable doses (10–20 mM) of both taurine and β-alanine can produce IPSC-like currents in voltage-clamped membrane patches (Jones and Westbrook 1995; Zhu and Vicini 1997). However, contrary to results obtained using GABA as an agonist, paired agonist application protocols have revealed that brief exposures to taurine and β-alanine do not produce significant GABA_A receptor desensitization (Jones and Westbrook 1995; Zhu and Vicini 1997).

In macropatches isolated from cultured granule cell bodies, taurine and β-alanine both functioned as agonists, consistently producing robust inward currents. Application of the inhibitory amino acid glycine produced no current responses whatsoever, thus indicating that the actions of β-alanine and taurine did not arise from activation of the glycine receptor. Examples of the responses elicited by GABA (1 mM), β-alanine (10 mM), taurine (10 mM), and glycine (1 mM) are shown in Fig. 6A. Although consistently producing responses, 10 mM taurine was a relatively ineffective agonist. In contrast, 10 mM β-alanine produced responses of a similar size to those elicited by 1 mM GABA, a maximally effective agonist concentration in granule cells. By comparing the relative sizes of the first and second responses in paired application protocols, it was clear that, consistent with earlier reports, neither of the β-amino acids produced the same degree of desensitization as GABA (Jones and Westbrook 1995; Zhu and Vicini 1997). Indeed as a result of its submaximal receptor occupancy at 10 mM, taurine responses tended toward paired pulse facilitation, although this did not reach statistical significance (see also Jones and Westbrook 1995). Pooled data from four paired agonist application experiments are shown in Fig. 6B. Given the desensitization-resistant nature of granule cell IPSCs, we wondered whether β-alanine or taurine, rather than GABA, is the inhibitory neurotransmitter at the GABAergic synapses of cultured cerebellar granule cells.

To test this hypothesis we adopted a strategy designed to completely deplete GABA from our cerebellar cultures. Cul-
tures were maintained in standard media supplemented with a GABA synthesis inhibitor and an enzyme that metabolizes GABA. Drug-free control cultures were prepared from the same animals and were maintained in parallel under identical culture conditions. After 10 days in culture, we tested the two different groups for functional GABAergic synaptic transmission. As usual >80% of cells from control cultures exhibited frequent spontaneous IPSCs. In contrast, no IPSCs were observed in 17 of 18 cells previously exposed to the GABA depleting agents (Fig. 6C). In the remaining cell, IPSCs were present but arose at a very low frequency indeed (<0.1 Hz, compared with around 2.5 Hz in control cells). Thus blockade of GABA synthesis combined with its enzymatic breakdown once released are together able to almost completely eliminate inhibitory synaptic communication in cerebellar cultures. This indicates that GABA rather than another molecule such as β-alanine acts as the transmitter in this system.

**DISCUSSION**

This study depends crucially on the methods used to stimulate single synaptic boutons. The major possible pitfalls arise from questions regarding whether the contents of every vesicle released from a single FM1-43–positive bouton impinge on the same population of postsynaptic receptors. To fulfill this criterion it is important that stimulation applied is restricted to single boutons. This seemed to be the case as neighboring spots either did not destain at all or destained at rates far below that of the stimulated site. In addition, small lateral movements of the stimulation puffer pipette (~2 μm) completely and reliably eliminated all evoked vesicular release.

Having established with a reasonable degree of certainty that we were indeed stimulating single FM1-43–labeled boutons, the possibility still remains that each FM1-43–labeled spot contains a multitude of independent active zones, each having its own independent complement of postsynaptic receptors. To fully address this possibility, a full ultrastructural serial section reconstruction of a number of GABAergic synapses from our cultures would be required, information that is unfortunately not currently available. The most closely relevant previous study is the detailed analysis of Schikorski and Stevens (1997), who reconstructed 16 presumed excitatory synapses from rat hippocampal cultures. In this study each bouton exhibited either a single (69%) or two (31%) active zones; similar findings were also made for the excitatory synapses of the hippocampus in vivo. However, studies of neurons from outside the hippocampus have revealed that greater numbers of active zones per bouton can be found in vivo (Hamos et al. 1987; Pierce and Mendell 1993; Streichart and Sargent 1989; Yeow and Peterson 1991). For example as many as eight active zones were reported to be present on presynaptic boutons in the thalamus (Hamos et al. 1987).

Although it is difficult to draw any strong parallels between the work of Schikorski and Stevens on hippocampal cultures and our cerebellar system, it is noteworthy that 2-photon excitation imaging of FM1-43–labeled spots in our cultures reveals that they are of similar dimension (approximately 0.15 μm^3) to the boutons observed in hippocampal cultures (Leao et al. 2000) and therefore may have a broadly similar number of active zones. Additionally, if three or four completely independent active zones where present per bouton, the large numbers of quanta released by each of our high K^+ stimuli (i.e., 20–40) would still produce on the order of 6–10 release events per active zone per stimulus and therefore might be expected to produce significant desensitization. It is clear that, given the additional insights it would provide for this and other studies, there is a pressing need for a full and detailed ultrastructural analysis of GABAergic synapses in neuronal culture.

In addition to demonstrating a lack of activity-dependent desensitization of postsynaptic GABA_ A receptors, our data also indicate that shifts in postsynaptic intracellular Cl^– concentration are not induced by repetitive synaptic activity under our recording conditions where cells are preloaded with Cl^– ions. It will be interesting to examine whether activity-dependent shifts in [Cl^–]_i, can modulate inhibitory synaptic strength when a more physiological Cl^– gradient (such as that present in gramicidin-perforated patch recordings) is imposed (Thompson and Gahwiler 1989b,c). Recent data gathered with a novel chloride imaging method suggest that such shifts in [Cl^–]_i can be quite substantial (Kuner and Augustine 2000).

Prolonged applications of a high concentration of exogenous GABA to the synaptic region are capable of producing an effect reminiscent of desensitization, namely a decrease in the size of stimulus-evoked mIPSCs. Although this depression of the mIPSC amplitude could be attributed to ongoing GABA receptor occupancy while the exogenous GABA is present, its persistence long after the exogenous GABA is removed provides evidence for a true process of desensitization. A second but less likely explanation for the depression caused by exogenous GABA is that it arises from a massive depletion of intracellular chloride, which, although likely to occur in the presence of a physiological Cl^– gradient (Kuner and Augustine 2000), is not likely to be significant in cells loaded with elevated levels of intracellular Cl^–, as in our experiments.

Our data reveal substantial differences between the desensitization behavior of cell body and GABA_ A synaptic receptors. We have previously provided evidence for a number of other differences between the GABA_ A receptors at these two locations (Mellor et al. 2000). The most likely explanation for this is that the postsynaptic GABA_ A receptors but not those on the cell body are of a subunit composition that resists desensitization. Other sources of desensitization-resistant postsynaptic responses are feasible. One possibility is that the GABA_ A receptors at synapses interact with some other protein or proteins that inhibit their ability to desensitize. To enter desensitized states the GABA_ A receptor changes its conformation in a manner that involves movement of charge within the membrane field (Mellor and Randall 1998). It is possible that the binding of some synaptic protein, for instance a cytoskeletal element, could significantly restrict the conformational change(s) required to enter desensitized states while still permitting other open-closed transitions. A phenomenon could be produced by other synapse-specific receptor modifications, for example phosphorylation or dephosphorylation by enzymes specifically compartmentalized at postsynaptic sites.

Another potential source for the desensitization-free phenotype of IPSCs in cultured granule cells is the transmitter utilized by the synapse. Rapid agonist application experiments have shown that brief applications of either taurine or β-alanine can activate GABA_ A receptors to produce IPSC-like responses without producing substantial desensitization (Jones and Westbrook 1995; Zhu and Vicini 1997). This lack of desensitization probably arises from the rapid unbinding (and therefore low affinity) exhibited by both molecules. Could
either taurine or β-alanine act as the fast neurotransmitter at inhibitory synapses on granule cells? The definitive identification of the transmitter responsible for synaptic communication at a given synapse is far from a trivial task (Apron and Werman 1968). Taurine is unlikely to act as a fast transmitter, however, because although it is transported into nerve terminals, it is seemingly not taken up into synaptic vesicles (Fykse and Fonnum 1996). For fast synaptic transmission a vesicular mechanism would seem essential. This is because nonvesicular release mechanisms cannot produce a fast enough rise to a sufficiently high transmitter concentration to explain the rapid rising phase of synaptic currents (Mody et al. 1994). β-Alanine, in contrast, is taken up into vesicles on the same transporter as GABA and glycine and can also be released in a Ca2+-dependent manner (Fykse and Fonnum 1996; Saramaa and Oja 1993). For this reason β-alanine has previously been suggested to act as a transmitter (Fykse and Fonnum 1996; Sandberg and Jacobson 1981).

An argument against a prominent role for β-alanine in synaptic communication is its low concentration in the brain compared with, for example, GABA (Fonnum and Walberg 1973; Fykse and Fonnum 1996; Martin del Rio et al. 1977). Granule cell processes in culture are heavily decorated with terminals that stain positive for glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA (Leao et al. 2000). Given that so much GAD in cultured terminals is present in terminals presynaptic to granule cells, plus the ubiquitous presence of glutamate in cytoplasm, it is clear that considerable levels of GABA will be synthesized (Apron and Werman 1968). For fast synaptic transmission a vesicular mechanism would seem essential. This is because nonvesicular release mechanisms cannot produce a fast enough rise to a sufficiently high transmitter concentration to explain the rapid rising phase of synaptic currents (Mody et al. 1994). β-Alanine, in contrast, is taken up into vesicles on the same transporter as GABA and glycine and can also be released in a Ca2+-dependent manner (Fykse and Fonnum 1996; Saramaa and Oja 1993). For this reason β-alanine has previously been suggested to act as a transmitter (Fykse and Fonnum 1996; Sandberg and Jacobson 1981).

In summary we have shown that transmitter released at GABAergic synapses fails to desensitize postsynaptic GABA_A receptors. This suggests that desensitization of postsynaptic receptors does not contribute to short-term synaptic plasticity at this synapse. The most likely source of the desensitization-resistant IPSCs is the subunit structure of the postsynaptic receptors. Because different cells express different GABA_A receptor subunits, it will be of interest to see whether transmitter-induced desensitization of postsynaptic GABA_A receptors occurs in other neurons prepared from different regions of the CNS.

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