Two-photon imaging of spontaneous vesicular release in acute brain slices and its modulation by presynaptic GABA<sub>A</sub> receptors

Nikolai Axmacher, Jochen Winterer, Patric K. Stanton, Andreas Draguhn, and Wolfgang Müller

Introduction

γ-Aminobutyric acid (GABA) is the main inhibitory transmitter of the CNS. The precision of its synaptic transmission is a key regulator of synchronous activity of neuronal ensembles during both cognitive tasks and in pathological conditions, especially during epileptic discharges. The enhancement of GABA-mediated synaptic transmission by pharmacotherapy is of central relevance for the treatment of various neurological and psychiatric disorders (Rudolph et al., 2001). On the cellular level, spontaneous GABAergic activity is necessary to maintain the balance of excitation and inhibition in many neural networks (Miles, 2000; Stell and Mody, 2002). The presynaptic release of GABA is tightly regulated by a variety of mechanisms, most prominently by metabotropic GABA<sub>B</sub> receptors. Investigations of vesicular release at high temporal resolution are usually performed using patch-clamp recordings of spontaneous (action potential independent) postsynaptic currents (miniature IPSCs or EPSCs). One principal disadvantage of this technique, however, is that vesicular release can only be monitored indirectly by its postsynaptic effects. Here, we have taken advantage of recent developments in fluorescent recordings of vesicular release in acute brain slices to investigate a new mechanism of modulation of vesicular GABA release mediated by presynaptic ionotropic GABA<sub>A</sub> receptors.

A relationship between intracellular presynaptic GABA concentration and vesicular release probability has been heavily investigated. Depending on experimental conditions, GABA exerts either a positive (Engel et al., 2001; Golan and Grossman, 1996) or a negative (Overstreet and Westbrook, 2001) feedback effect on its own release. Most of these experiments have been performed in the presence of GABA<sub>B</sub> receptor antagonists, suggesting a different mechanism, e.g., activation of presynaptic GABA<sub>A</sub> receptors. In a recent study, we have shown by electrophysiological means that GABA also suppresses its subsequent release by activation of presynaptic ionotropic GABA<sub>A</sub> receptors (Axmacher and Draguhn, in press). This activation opens channels that conduct mainly chloride ions; dependent on the membrane potential and the Cl<sup>-</sup> concentration gradient, this can induce either a hyperpolarizing influx or a depolarizing efflux of Cl<sup>-</sup> (Misgeld et al., 1986; Müller et al., 1989; Chavas and Marty, 2003): If the reversal potential of chloride, E<sub>Cl</sub>, is more negative than the resting membrane potential, V<sub>rn</sub>, activation of GABA<sub>A</sub> receptors causes a hyperpolarization; if E<sub>Cl</sub> is more positive than V<sub>rn</sub>, it causes a depolarization. Therefore, it

Keywords: Two-photon imaging; Spontaneous vesicular release; GABA<sub>A</sub> receptors

Rapid Communication

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Available online on ScienceDirect (www.sciencedirect.com.)
is conceivable that the direction of the Cl⁻ flux and resulting feedback of GABA on its release differs between experimental situations.

Here, we characterize the effect of GABAₐ receptor activation on GABA release in acute rat hippocampal brain slices. Whole-cell, patch-clamp recordings of CA3 pyramidal cells revealed that application of muscimol induced a surprisingly long-lasting decrease of mIPSCs in conditions of high [K⁺]₀, where the baseline frequency of vesicular release is increased. To avoid the limitations of whole-cell recording of mIPSCs as a measure of presynaptic release, we directly measured spontaneous vesicular release after FM1-43 staining of vesicles. Using dual-photon laser scan microscopy, we succeeded in directly monitoring spontaneous release of vesicles from the readily releasable pool (RRP) of presynaptic vesicles. To our knowledge, this has not been done before, but will be of broader interest, as it allows to direct investigation the probability of vesicular release in slices, avoiding limitations of whole-cell recordings of miniature inhibitory postsynaptic currents (mIPSCs) from postsynaptic neurons. We found that application of muscimol to hippocampal slices slowed the fluorescence decay in proximal perisomatic boutons synapsing on CA3 pyramidal cell dendrites, believed to be mainly GABAergic (Papp et al., 2001; Soltesz et al., 1995), in a manner independent of external [Ca²⁺]. In contrast, fluorescence decay rates in distal boutons in stratum radiatum of CA3, where excitatory commissural fibers terminate, were significantly lower and not influenced by muscimol.

Methods

Slice electrophysiology

Transverse hippocampal slices (200 μm thick; 300 μm thick for imaging experiments) were obtained from 9- to 14-day-old Wistar rats as described previously (Frahm et al., 2001; procedures approved by state Government authorities). Whole-cell patch-clamp recordings were performed from visually identified hippocampal CA3 pyramidal neurons at room temperature (22–25 °C) using an EPC-7 amplifier (List Medical, Darmstadt, Germany) and an upright microscope (Zeiss Axioskop) equipped with DIC. Input and series resistances were regularly controlled throughout the experiment and cells were excluded if they showed significant (>20%) resealing or parallel decreases in synaptic current amplitude and series conductance. Intracellular solution contained (in mM): CsCl 140, MgCl₂ 2 and HEPES 10; pH is adjusted to 7.3 with CsOH. If not indicated differently, extracellular solution contained (in mM): NaCl 129, KCl 3, MgSO₄ 1.8, CaCl₂ 1.6, glucose 10, NaH₂PO₄ 1.25, NaHCO₃ 21, pH 7.4, when gassed with 95% O₂ + 5% CO₂. Spontaneous (miniature) GABAₐ-receptor-mediated inhibitory postsynaptic currents (mIPSCs) were isolated by recording in the presence of the glutamatergic receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 30 μM) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 30 μM) and tetrodotoxin (TTX, 1 μM). All chemicals were provided by Sigma-Aldrich, Taufkirchen, Germany. Miniature IPSCs were analyzed using TIDA software (HEKA, Lamprecht, Germany) and the Strathclyde Electrophysiology Packet written by J. Dempster (University of Glasgow, Scotland) for successive intervals of 1 min.
postsynaptic effects of the released transmitter. Attenuation and filtering along the dendritic tree, as well as receptor desensitization, can disturb the detection of vesicles fusing with the presynaptic membrane. This situation is even more complicated when drugs are used that affect both pre- and postsynaptic receptors, as in our experimental paradigm. Therefore, we directly measured the effect of muscimol on vesicular release using two-photon excitation of the fluorescent dye FM1-43. Though this has not been previously reported, we hypothesized that imaging spontaneous release should be possible, using the increased resolution yielded by two-photon methods and specific staining of the RRP with the protocol of Stanton et al. (2003). With this approach, we were able to measure spontaneous vesicular release from the RRP in acute brain slices in the presence of 1 μM TTX.

We compared the decay of FM1-43 fluorescence (corresponding to vesicular release) during control conditions and in slices exposed to 1 μM muscimol for 10 min. While in electrophysiological recordings, this prolonged application induces substantial desensitization of postsynaptic receptors (data not shown) and hence confounds detection of release events, whereas desensitization of postsynaptic receptors did not affect our direct presynaptic measurements.

In a first series of experiments, we restricted our analysis to perisomatic synaptic boutons within 50 μm of the pyramidal cell layer, where inhibitory synapses have been shown to terminate (Fig. 3A; Soltesz et al., 1995). Mossy fiber boutons in the dendritic part of this region were easily identifiable as threedimensional clusters of four to five closely associated bright puncta (representing several active release sites) and were systematically excluded from analysis. Only terminals with single release sites (isolated puncta) were analyzed. Fig. 3B displays in magnification the marked region of Fig. 3A during the time course of a typical experiment. Arrows indicate examples of putative inhibitory synaptic boutons. Muscimol was applied after the second image (9′). Subsequently, the fluorescence decay was reduced, so that even after >30 min, some boutons remain visible. In control experiments where no muscimol was applied, fluorescence decayed much further, until virtually all labeled vesicles had disappeared at the end of the experiment (Fig. 3C). Eventually (>40 min), fluorescence decayed to plateau backgrounds of approximately 50% of start-

**Results**

First, we were interested in whether activation of presynaptic GABA<sub>A</sub> receptors directly reduces vesicular release of FM1-43 from the RRP. To make spontaneous release signals more easy to detect with optical imaging, we increased release frequency by increasing [K<sup>+</sup>]<sub>out</sub> to 15 mM (normally 3 mM in standard ACSF; in exchange with NaCl), which increased spontaneous destaining rates from −2.55±1.8% for the first 10 min in normal ACSF, to −4.64±1.0% per 10 min in 15 mM [K<sup>+</sup>]<sub>out</sub> (n = 6, P < 0.05, Student’s t-test). This changes in [K<sup>+</sup>]<sub>0</sub> will affect the resting membrane potential as well as the intracellular Cl<sup>−</sup> concentration (see Discussion).

In whole-cell recordings from CA3 pyramidal neurons, the frequency of mIPSCs was higher in high [K<sup>+</sup>]<sub>out</sub> compared to standard ACSF (188.5 ± 20.5 in 15 mM [K<sup>+</sup>]<sub>out</sub> vs. 87 ± 9.3 events/min in normal ACSF; P < 0.05, repeated measures ANOVA, post hoc Scheffe’s test). Addition of muscimol did, however, still reduce mIPSC frequency (Figs. 2A,B). This reduction was less pronounced than in standard ACSF; the normalized frequency decreased by approximately 40%, as compared to a reduction by 48% in standard ACSF (Axmacher and Draguhn, in press). The amplitude of mIPSCs was unchanged in elevated [K<sup>+</sup>]<sub>out</sub> compared to standard ACSF (54 ± 12 vs. 50 ± 11 pA; P = 0.48); after application of muscimol, it did not change significantly (P > 0.1).

Electrophysiological recordings of mIPSCs only indirectly indicate the frequency of vesicular release by measuring the percent decay of fluorescence over the first 12 min before drug addition, in comparison to the last 20 min of the experiment (Fig. 3B). To check whether the difference in the first and in the second part of the experiment was due to an exponential decay of fluorescence or a linear one, we used both linear and single exponential regression fits. Since both types of fits yielded equivalent results, linear fits were used for average data. Vertical bars depict the SEM for the averages of all normalized and corrected boutons across experiments.

**Fig. 2.** Muscimol reduces mIPSC frequency in ACSF with [K<sup>+</sup>]<sub>out</sub> = 15 mmol. (A) Example traces of mIPSCs recorded during control conditions, after application of muscimol and washout of the drug. (B) mIPSC frequency during control, after application of muscimol and during washout (approximately 35 min after application of muscimol). mIPSC frequency was reduced significantly (n = 6, washout n = 4; P < 0.05).
ing brightness, similar to RRP-evoked destaining end points observed previously at excitatory terminals (Stanton et al., 2003). Fig. 3D directly compares the mean fluorescence decay of \( n = 13 \) proximal synaptic boutons in a typical control slice, versus a slice where muscimol was applied. The summary of six experiments is depicted in Fig. 3E. During the first 12 min of the experiment, fluorescence decayed by 12.4 ± 2.4%; during the last 15 min, the decay was reduced to 5.2 ± 0.9% (\( P < 0.05 \), Wilcoxon test). In six control experiments (Fig. 3F), fluorescence decayed by 8.1 ± 2.1% in the first part and by 10.1 ± 2.5% in the second part of the experiment (\( P > 0.4 \), Wilcoxon test). We obtained qualitatively and quantitatively similar results when we applied alternative measures of fluorescence decay rate, such as the decay time constant of single exponential fits of decay time courses.

To check whether the effect of muscimol was restricted to proximal boutons (with a presumably higher contribution of GABAergic synapses), we also analyzed the effect of muscimol on synapses more remote from the pyramidal cell layer in the same slices (\( n = 12 \)), presumptive excitatory synapses. We found that, under control conditions, the fluorescence decay of distal boutons was significantly lower than in proximal boutons (5.3% vs. 10.2%; \( P < 0.05 \); \( n = 12 \); Wilcoxon test), corresponding to a wealth of data showing a lower frequency of mEPSCs compared to mIPSCs. Fig. 4A shows typical examples of the mean fluorescence decay in distal boutons in a slice where muscimol had been applied, versus
an untreated control slice. Fig. 4B displays mean results from six experiments where muscimol was applied. In contrast to proximal boutons, muscimol had no effect on the fluorescence decay of these distal boutons (P > 0.5, n = 6, Wilcoxon test). Fig. 4C displays results from six control experiments.

Finally, we tested the role of [Ca\(^{2+}\)]\(_{\text{out}}\) for the effect of muscimol on GABA release, by applying muscimol in nominally Ca\(^{2+}\)-free ACSF containing 100 \(\mu\)M EGTA (Fig. 5). The frequency of mIPSCs was reduced to 41 \pm 2.8 events/min, compared to 87 \pm 9.3 events/min in control conditions (n = 29, 1-min epochs in seven Ca\(^{2+}\)-free cells and n = 38 epochs in 12 control cells; P < 0.05, repeated measures ANOVA, post hoc Scheffe’s test). The direction of this change is consistent with a partial dependence on extracellular Ca\(^{2+}\) of spontaneous release of GABA. Muscimol reduced the normalized mIPSC frequency by 74% in Ca\(^{2+}\)-free ACSF (Fig. 5B, P < 0.05, n = 7). Besides, muscimol significantly reduced the mIPSC amplitude by approximately 30% (P < 0.05). To test whether the apparent reduction in frequency was caused by a decreased detectability of small events (Zhou et al., 2000), we increased the driving force by recording at \(-72\) mV, instead of \(-60\) mV. Under this condition, the amplitude decrease was less pronounced (\(\leq 10\%\), P > 0.05), whereas mIPSC frequency was still significantly reduced. From these data, we conclude that presynaptic hyperpolarization via GABA\(_A\) receptors reduces vesicular release by mechanisms other than blockade of tonic influx of extracellular [Ca\(^{2+}\)].

**Discussion**

*Action of presynaptic GABA\(_A\) receptors is largely independent of changes in the chloride reversal potential*

Presynaptic ionotropic GABA receptors at axon terminals were first described by Eccles et al. (1963), and are also present at various glycineergic (Jang et al., 2002; Turecek and Trussell, 2002), glutamatergic (Ruiz et al., 2003; Stasheff et al., 1993) and GABAergic terminals (Axmacher and Draguhn, in press; Kamermans and Werblin, 1992; Pouzat and Marty, 1999; Vautrin et al., 1994). Depending on the direction and amplitude of the presynaptic potential change induced, such presynaptic GABA\(_A\) receptors can either trigger (Stasheff et al., 1993) or suppress (Ruiz et al., 2003) antidromic action potentials, and facilitate (Kamermans and Werblin, 1992) or suppress transmitter release (Eccles et al., 1963). The exact mechanisms of presynaptic action in many systems remain to be elucidated. As GABA\(_A\) receptors are ionotropic receptors coupled to chloride channels, the effects of agonists on these receptors depend on both membrane potential

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**Fig. 4.** Application of muscimol had no effect on distal boutons. (A) Typical fluorescence decays of boutons in slices with (●) and without (○) application of muscimol. (B) Mean fluorescence decay in six slices before (first part) and after (second part) application of muscimol. (C) Mean fluorescence decay in the first and second part of six control experiments.

**Fig. 5.** Application of muscimol decreased mIPSC frequency in Ca\(^{2+}\)-free ACSF. (A) Example traces. (B) Mean ± SEM from seven cells.
and the Cl⁻ gradient (Chavas and Marty, 2003; Misgeld et al., 1986). Attempts to directly manipulate the Cl⁻ reversal potential by decreasing the extracellular [Cl⁻] may fail. In this case, the K⁺–Cl⁻ cotransporter KCC2 has an increased driving force. This counteracts the change in $E_{\text{Cl}}$. Indeed, we saw that, in ACSF with a reduced [Cl⁻]$_{\text{out}}$ of 30 mM, application of muscimol still reduced the frequency of mIPSCs ($n = 3$; data not shown). Under physiological conditions, Cl⁻ is known to be mainly depolarizing in young rats (<p7) due to a high intracellular [Cl⁻]. Later, Cl⁻ becomes hyperpolarizing in many central neurons, due to an enhanced expression of the KCC2 K⁺–Cl⁻ cotransporter (Rivera et al., 1999). The driving force for this K⁺–Cl⁻ cotransporter can be reduced by an increased extracellular [K⁺] when membrane potential is kept stable (Jarolimek et al., 1999). We were interested in whether the negative feedback of GABAA receptor activation in CA3 of the hippocampus also depends on [K⁺]$_{\text{out}}$. An increased [K⁺]$_{\text{out}}$ reduces the driving force of KCC2, but also tends to depolarize the presynaptic membrane. The net effect on the driving force for Cl⁻ is therefore expected not to reverse but to be weaker than when membrane potential is kept stable. In agreement with this scenario, reduction of mIPSC frequency by muscimol was less pronounced than in normal ACSF (Fig. 2). Interestingly, Ruiz et al. (2003) have demonstrated a role for the presynaptic Cl⁻ gradient in GABA$_A$-receptor-mediated modulation of excitability of mossy fiber terminals. Overall, we found that the effect of muscimol on the release of GABA is surprisingly stable in a variety of conditions, including ACSF with a reduced [Cl⁻]$_{\text{out}}$ or increased [K⁺]$_{\text{out}}$.

**Effects of muscimol on spontaneous release can be measured by two-photon imaging**

All attempts to measure the probability of release by the frequency of postsynaptic currents are principally hindered by factors influencing the postsynaptic detection of vesicles. This situation is especially difficult when investigating feedback mechanisms where similar pre- and postsynaptic receptors are involved. On the other hand, direct measurements of the vesicular release machinery by fluorescence imaging was long restricted to cell culture or isolated synapse systems (Cochilla et al., 1999; Murthy and Stevens, 1999), due to excessive background fluorescence in brain tissue. Recently, however, several groups have succeeded in imaging evoked vesicular release from the total vesicle pool in acute brain slices (Kay et al., 1999; Pyle et al., 1999). In previous studies, we demonstrated outstanding suitability of dual-photon laser scan microscopy for such measurements and developed a paradigm for specifically labeling the RRP in acute brain slices (Stanton et al., 2001, 2003). We were interested in whether it was possible with these techniques to measure the spontaneous release of vesicles directly. We loaded synaptic vesicles with a hyperosmotic sucrose solution according to Stanton et al. (2003) and measured the fluorescence decay in ACSF with an increased [K⁺]$_{\text{out}}$ to enhance the baseline vesicular release frequency. We found that single proximal release sites (that are to a high percentage GABAergic, Papp et al., 2001; Soltesz et al., 1995) have a significantly higher rate of fluorescence decay than distal (presumably predominantly glutamatergic) boutons (Figs. 3 and 4). This fits with electrophysiological data showing that GABAergic terminals spontaneously release GABA with a higher frequency than glutamatergic terminals release glutamate. Upon application of muscimol, the fluorescence decay (as a measure of release probability) decreased in proximal boutons (Fig. 3), corresponding to the muscimol-induced reduction in mIPSC frequency (Fig. 2). In marked contrast, the fluorescence decay of distal, largely glutamatergic boutons, was not affected (Fig. 4), indicating a specific effect of muscimol on the release of GABA.

Recently, Ruiz et al. (2003) have shown that mossy fiber terminals possess GABA$_A$ receptors whose activation reduces mossy fiber conduction. Although mossy fibers terminate near the soma of CA3 pyramidal cells, they contain clusters of four to five release sites that are recognized in our imaging experiments with high reliability and appear to have much less spontaneous release.

A recent work by Brager et al. (2003) supports our conclusion that most perisomatic boutons were GABAergic. Using styryl dyes to measure action potential-driven release of GABA-containing vesicles in organotypic slice cultures, they concluded that boutons in the pyramidal cell layer were almost exclusively GABAergic, because release from them was not blocked by adenosine and they were selectively labeled in GAD65-eGFP-expressing mice.

How is it possible to directly compare destaining rates of styryl dyes with the frequency of mIPSCs? Although both phenomena reflect the same underlying mechanism, i.e., the fusion of vesicles with the presynaptic membrane, a direct comparison is difficult for two reasons. First, FM1-43-stained vesicles may not lose all dye when fusing with the membrane (Sara et al., 2002), especially during kiss-and-run fusion (Aravanis et al., 2003), leading to an underestimate of the real release rate. Second, the frequency of mIPSCs reflects release from an unknown number of GABAergic synapses converging on the recorded pyramidal neuron. However, estimation suggests that the respective rates are at least of the same order of magnitude. A reduction in fluorescence of approximately 20% in 40 min results in a reduction of approximately 0.5% per minute, assuming a linear decay. Given estimates of 10 docked vesicles per terminal (Stanton et al., 2003), this results in one released vesicle each 20 min. On the other hand, an IPSC frequency of 100/min results in approximately one event per 1 min (assuming 100 GABAergic synapses converging onto one pyramidal neuron soma; Morin et al., 1999). With 10 docked vesicles ready for spontaneous release, a vesicle would be released once every 10 min. This number matches well the rate of one vesicle each 8 min measured in cultured neurons (Murthy and Stevens, 1999). The lower release rate measured with fluorescence would be consistent with incomplete FM1-43 destaining of fused vesicles during kiss-and-run release (Aravanis et al., 2003).

**Reduction of GABA release via GABA$_A$ receptors is Ca$^{2+}$ independent**

Upon activation of GABA$_A$ receptors, influx of Cl⁻ hyperpolarizes the membrane and may thereby reduce the influx of Ca$^{2+}$. Influx of Ca$^{2+}$ from the extracellular space is known to be important both for action potential-dependent and -independent transmitter release. Recent data, however, suggest that release can also be directly affected by changes in membrane potential (Hochner et al., 1989; Parnas et al., 2002). Therefore, we were interested in whether the negative effect of muscimol on GABA release depends on Ca$^{2+}$. We measured the effect of muscimol on mIPSCs recorded in ACSF without Ca$^{2+}$, where residual Ca$^{2+}$ was buffered by addition of EGTA. In our preparation, the frequency of mIPSCs was reduced compared to normal ACSF. To our surprise, we found that muscimol still reduced the frequency of the remain-
ing mIPSCs in the absence of [Ca\textsuperscript{2+}]\textsubscript{out} (Fig. 5). This suggests that presynaptic GABA\textsubscript{A} receptors can inhibit both [Ca\textsuperscript{2+}]\textsubscript{dependent and [Ca\textsuperscript{2+}]-independent vesicle fusion in a Cl\textsuperscript{−}/voltage-dependent manner. Potential mechanisms include effects of swelling of the presynaptic terminal produced by GABA\textsubscript{A} receptor-mediated Cl\textsuperscript{−} and H\textsubscript{2}O influx, and/or pH-dependent modification of the vesicular release machinery.

The effect of muscimol on release is long-lasting

In previous experiments, we have shown that muscimol activates both post- and presynaptic GABA\textsubscript{A} receptors (Axmacher and Draguhn, in press). The presynaptic effect, however, was longer-lasting than the postsynaptic one. Furthermore, in several experimental conditions, we found that the effect of muscimol was only partially reversible (Figs. 2 and 5). For several reasons, we do not believe that this is due to damage from prolonged whole-cell recording. First, input and series resistance were carefully checked during the experiments and remained stable for >1 h. Second, in experiments in normal ACSF, mIPSC frequency reversed by at least 80% (Axmacher and Draguhn, in press). Third, long-lasting effects of muscimol have been reported by other groups (Hays et al., 1999). This might be explained by different affinities of pre- and postsynaptic GABA\textsubscript{A} receptors to GABA (Stell and Mody, 2002). Alternatively, capacitative effects might cause long-lasting changes in pH or intraterminal [Ca\textsuperscript{2+}]. This could even provide a link to prolonged reduction of synaptic transmission (LTD) by presynaptic effects (Chevaleyre and Castillo, 2003; Stanton et al., 2003). Further work remains to be done to address this possibility.

In conclusion, we have shown that spontaneous vesicular release from the readily releasable pool can be measured in acute brain slices using dual-photon imaging of the styryl dye FM1-43. Our data directly demonstrate presynaptic inhibition of vesicular release by activation of GABA\textsubscript{A} receptors. This method might be helpful in clarifying the hypothetical long-term plasticity of presynaptic release by activation of GABA\textsubscript{A} receptors.

Acknowledgments

This work was supported by the DFG grants TR-3/C5 to WM, TR-3/B5 to AD, GK 238 to NA and JW and NINDS grant NS44421 to PKS.

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