Heterogeneity and Specificity of Presynaptic Ca\(^{2+}\) Current Modulation by mGluRs at Individual Hippocampal Synapses

GABA release from axonal boutons formed by cortical interneurons shows target cell-dependent sensitivity to group III metabotropic glutamate receptor (mGluR) agonists, as well as variable dependence on presynaptic Ca\(^{2+}\) influx via N- and P-type channels. How Ca\(^{2+}\) channels interact with heterogeneous mGluR modulation to determine information flow in the synaptic circuitry is not known. Here we combine electrophysiology with two-photon microscopy to analyze Ca\(^{2+}\) influx at individual axonal varicosities of hippocampal interneurons. Action potentials triggered Ca\(^{2+}\) influx at individual varicosities, principally (>80%) via N- and P-type channels. Although Ca\(^{2+}\) influx at some varicosities was almost entirely mediated by N-type channels, P-type channels only contributed up to 60% of the action potential-evoked Ca\(^{2+}\) transient. At a subset of synapses activation of group III mGluRs depressed GABA release, and decreased Ca\(^{2+}\) influx via N-type channels (in contrast to an action on P-type channels reported at auditory brainstem calyceal synapses). The identity of the dominant channel subtype mediating Ca\(^{2+}\) influx tended to be conserved at varicosities supplied by the same axon. In contrast, neighboring varicosities often showed heterogeneous sensitivity to group III mGluR activation. Glutamatergic modulation of GABA release from individual synapses thus depends on the co-occurrence of presynaptic N-type Ca\(^{2+}\) channels and the target cell-dependent expression of group III mGluRs.

Keywords: hippocampus, interneuron, metabotropic glutamate, presynaptic calcium, two-photon microscopy

Introduction

GABA release from the terminals of interneurons is modulated by presynaptic receptors including metabotropic glutamate receptors (mGluRs) (Semyanov and Kullmann, 2000, 2001; Belan and Kostyuk, 2002). This modulation exhibits certain rules: in the hippocampus, the group III mGluR agonist L(+)2-amino-4-phosphonobutyric acid (L-AP4) relatively selectively depresses interneuron-interneuron synapses (Kogo et al., 1999; Semyanov and Kullmann, 2000), possibly reflecting the adaptive significance of disinhibiting interneurons and enhancing inhibition of principal cells when extracellular glutamate is elevated. Heterogeneity of modulation of GABA release has also been reported for kainate (Cossart et al., 2001) and GABA\(_B\) receptors (Lambert and Wilson, 1993; Pearce et al., 1995).

What mechanisms underlie the specificity of modulation of GABA release? Several metabotropic receptors are thought to reduce presynaptic Ca\(^{2+}\) influx (see, for instance, Doze et al., 1995; Huston et al., 1995). Presynaptic heterogeneity could therefore involve differential targeting of presynaptic receptors, Ca\(^{2+}\) channels, and/or linkage of Ca\(^{2+}\) channels and receptors to exocytosis.

Among group III mGluRs, mGluR7 is selectively targeted to axonal varicosities presynaptic to interneurons (Shigemoto et al., 1996, 1997), and recent work has shown that this principle applies not only to glutamatergic axons but also to GABAergic boutons (Somogyi et al., 2003). However, whether these receptors are functional is unclear. Moreover, mGluR7 is relatively insensitive to glutamate and L-AP4 (Okamoto et al., 1994), and less is known about the distribution of mGluR4 and mGluR8.

Differential targeting of Ca\(^{2+}\) channels may also underlie heterogeneity in modulation of GABA release. Unitary inhibitory post-synaptic currents (IPSCs) recorded in pyramidal neurons show a variable dependence on presynaptic Ca\(^{2+}\) influx via either N- or P-type channels, which in turn at least partly depends on the location and identity of the presynaptic interneuron (Poncer et al., 1997, 2000; Wilson et al., 2001). However, some interneurons synapse with both pyramidal neurons and other interneurons (Shigemoto et al., 1996; Cobb et al., 1997), and it is unclear whether all their terminals are supplied with the same Ca\(^{2+}\) channels. Differential sensitivity of Ca\(^{2+}\) channels to G proteins (Zhang et al., 1996) could, nevertheless, potentially underlie heterogeneous modulation of GABA release by group III mGluRs (see also Reid et al., 2003).

The conventional approach to argue that metabotropic receptors act via presynaptic Ca\(^{2+}\) channels relies on demonstrating occlusion between the effects of blocking Ca\(^{2+}\) channel subtypes and activating presynaptic receptors. When either receptors or Ca\(^{2+}\) channels are distributed heterogeneously this argument is flawed: if the receptors were co-localized with a Ca\(^{2+}\) channel subtype, abolishing transmitter release by blocking these channels would occlude the effect of the receptors even if they acted downstream of Ca\(^{2+}\) influx. Indeed, metabotropic receptors can affect spontaneous transmitter release independently of Ca\(^{2+}\) influx (Scanziani et al., 1995), although the relevance of these findings to activity-dependent transmission is unclear. Demonstrating an effect of metabotropic receptor activation on presynaptic Ca\(^{2+}\) influx (Wu and Saggau, 1994) potentially overcomes this pitfall. However, where there is heterogeneity in the identity of the presynaptic Ca\(^{2+}\) channels and/or receptors, this must be done at the level of individual synapses. Such an approach has shown that presynaptic group III mGluRs depress evoked P-type Ca\(^{2+}\) currents at synapses in the auditory brain stem (Takahashi et al., 1996). The present study extends this approach to presynaptic boutons at small cortical synapses, which are too small to be patch-clamped.

Materials and Methods

Hippocampal slices (350 µm thick) from 3- to 4-week-old guinea pigs were placed in a submersion-type recording chamber and perfused...
with a solution containing (in mM) NaCl (119), KCl (2.5), MgSO4 (1.3), CaCl2 (2.5), NaHCO3 (26.2), NaH2PO4 (1), and glucose (11), gassed with 95% O2/5% CO2 (23–25°C). Whole cell recordings were made from CA1 stratum radiatum interneurons under infrared differential interference contrast imaging. No consistent differences were observed between interneurons with either bi- or multi-polar morphology. Displaced pyramidal cells were avoided. The following drugs were used to block α-aminoo-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate, N-methylaspartate (NMDA), GABA? reseptors and group III mGluRs, respectively: 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide disodium (NBQX, 10 μM), t-2-amino-5-phosphonovalerate (APV, 50 μM), CGP52432 (5 μM) and α-methylserine-O-phosphate (MOSP, 100 μM). N- and P-type Ca2+ channels were blocked with ω-Conotoxin GVIA (CgTx, 0.25 μM) and ω-Agatoxin IVA (AgTx, 0.1 μM), respectively.

Whole cell pipettes used to record GABAergic IPSCs contained CsCl (120), NaCl (8), HEPES (10), EGTA (2), MgCl2 (0.2), MgATP (2), GTP (0.3), QX314 Br (5) (pH 7.2, osmolarity 295 mOsm). IPSCs were evoked using 100 μs electric stimuli applied every 10 s through bipolar stimulating electrodes placed 100–500 μm away from the recorded cell. Currents were acquired with an Axopatch 1D amplifier (Axon Instruments), filtered at 1 kHz and digitized at 2–5 kHz. The access resistance was <20 MΩ, and results were discarded if it changed by more than 20%.

Fast Ca2+ Imaging

Interneurons 30–50 μm beneath the slice surface were held in whole cell mode using a pipette filled with (in mM): K-methanesulfonate (135), HEPES (10), Na-phosphocreatine (10), MgCl2 (0.4), Na-ATP (4), Na-GTP (0.4), the morphological tracer Alexa Fluor 594 (0.04), and the Ca2+ indicator Fluo-4 (0.2). Alexa Fluor 594 and Fluo-4 were excited at 810 nm using a femtosecond pulse laser (MaiTai, Spectra-Physics) optically linked to a laser scanning system (Radiance 2000; BioRad). Epifluorescence signals were chromatically separated (λm = 560 nm for Fluo-4 and 560 nm < 700 nm for Alexa 594) as described earlier (Rusakov and Fine, 2003). Cells had three to eight tortuous trajectory; (iii) specific branching pattern including T-junctions and varicosities was their size, which was 2–4 times larger than the axon diameter; (iv) presence of distinct varicosities and absence of dendritic spines. These criteria allow a clear distinction from dendrites and were validated by synaptophysin immunolabeling (Rusakov and Fine, 2003). Cells had three to eight visible primary neurites (mean 3.99 ± 0.15, n = 50), but there was no correlation between this and the effects of t-AP4 or Ca2+ channel blockers.

We first identified and traced axons using the Alexa Fluor emission channel according to the following morphological criteria: (i) small diameter of the shaft (0.2–0.4 μm compared to 0.8–1.6 μm in dendrites), without attenuation with distance from soma; (ii) often tortuous trajectory; (iii) specific branching pattern including T-junctions; and (iv) presence of distinct varicosities and absence of dendritic spines. These criteria allow a clear distinction from dendrites and were validated by synaptophysin immunolabeling (Rusakov and Fine, 2003). A routine criterion for selecting individual varicosities was their size, which was 2–4 times larger than the axon diameter; in the Z direction, this size was assessed by adjusting the focus plane and also from an increased volume-integrated brightness compared to 0.8–1.6 μm.

Because Ca2+ equilibration across axonal boutons is rapid (~2 ms) and because the recorded signal normally represents integrated fluorescence across the bouton, a one-compartmental model of Ca2+ entry and buffering is commonly applied to interpret this signal (Neher and Augustine, 1992). This model simply implies that removal of Ca2+ from the area of interest, be it by pumping, diffusion or sequestration, can be represented by a single first-order reaction, with mass loss proportional to the concentration difference/gradient. Furthermore, in the presence of high-affinity (~1 μM) endogenous buffers and Ca2+ indicators, the fluorescence signal during slow fluctuations of Ca2+ can be safely considered quasi-steady-state. For each rh buffer Bi, the kinetic equations then predict a simple relationship:

\[ \frac{[Ca]_\text{bound}}{[Ca]_{\text{total}}} = \frac{[Ca]_{2+}^2[B_i]_{\text{bound}}}{([Ca]_{2+}^2 + K_i)} \]  

where \( K_i \) is the dissociation constant of the buffer/indicator. In many cases, the first partial derivative of expression (1) with respect to \([Ca]_{2+}\) gives a useful parameter \( \kappa \), which estimates the ‘newly bound versus free Ca2+ ratio’ (Helmechen et al., 1996; Maravall et al., 2000).

However, the system is far from steady-state during the brief period of fast Ca2+ entry driven by an action potential. To assess the magnitude of fast Ca2+ transients during this period, it would be important to consider rapid buffering explicitly at the sub-millisecond timescale. Similar to all other one-compartment models, the remaining assumption is that the recorded space-integrated fluorescence signal simply reflects the ‘average kinetics’ across the volume of small terminals; see DiGregorio et al. (1999) for a multi-compartmental approach.

Given that Ca2+ binding to the fluorescent indicator Fi and the endogenous buffer EB follow the reactions:

\[ Ca^{2+} + Fi \overset{k_1}{\rightleftharpoons} CaFi \]

and

\[ Ca^{2+} + EB \overset{k_2}{\rightleftharpoons} CaEB \]  

Ca2+ kinetics can be described by the following simple equations:

\[ \frac{\partial [Ca]_{2+}}{\partial t} = I_{Ca} + k_{i1}[CaFi] + k_{i2}[CaEB] - I_{\text{ump}} - k_{i3}[Ca]_{2+}[Fi] - k_{i4}[Ca]_{2+}[EB] - k_{i5}[Ca]_{2+}[Fi] + k_{i6}[CaFi] + k_{i7}[CaEB] + k_{i8}[Ca]_{2+}[Fi] + k_{i9}[Ca]_{2+}[EB] - k_{i10}[Ca]_{2+}[Fi] \]

where \( I_{Ca} \) is the Ca2+ influx rate and \( I_{\text{ump}} \) is the Ca2+ removal rate (due to pumping out, diffusion and/or sequestration), \( I_{Ca} \), which is driven by an action potential, is commonly approximated with an e-function.
Because in the case of repetitive spikes this analytical approximation is not readily applicable (for arithmetical reasons), we used the Gaussian

\[ G(t) = \frac{A}{\sigma \sqrt{2\pi}} \exp\left(\frac{-(t-t_0)^2}{2\sigma^2}\right) \]  

where \( A \) (equal to the integral of \( G \) over \( -\infty < t < +\infty \)) reflects total \( \mathrm{Ca}^{2+} \) influx (in M), \( t_0 \) is the peak current time point and \( \sigma = 0.64 \) ms is set so that the effective pulse width (full-width-at-half-maximum, FWHM = 2.35\( \sigma \)) matches the average half-duration of the action potential (1.5 ms, observed range 1.2–3 ms). \( \mathrm{Ca}^{2+} \) influx during two successive spikes \( \mathcal{I}_{\mathrm{Ca}} \) could thus be represented by

\[ \mathcal{I}_{\mathrm{Ca}} = A_1 G(t-t_1) + A_2 G(t-t_2) \]  

The removal rate of \( \mathrm{Ca}^{2+} \) was represented by the first-order reaction

\[ \mathcal{I}_{\mathrm{pump}} = P([\mathrm{Ca}^{2+}] - [\mathrm{Ca}^{2+}]_{\text{rest}}) \]  

where \( P \) is an unknown parameter. The steady-state level of \( \mathrm{Ca}^{2+} \) could be estimated from a simple relationship (Tsien, 1989)

\[ \frac{[\mathrm{Ca}^{2+}]}{K_d} = \frac{F-F_{\text{min}}}{F_{\text{max}}-F} \]  

where \( F \) is the baseline fluorescence, \( F_{\text{max}} \) is the maximum fluorescence (saturated indicator), and \( F_{\text{min}} \) is the residual fluorescence of free indicator. In the case of Fluo-4, \( K_d \sim 0.4 \) \( \mu \)M and \( F >> F_{\text{min}} \), which allows estimation of the baseline \([\mathrm{Ca}^{2+}]\) by directly comparing \( F \) and \( F_{\text{max}} \) (the latter was obtained by evoking a long train of action potentials) (Maravall et al., 2000).

Equations (3–6), combined with the estimated initial/steady-state parameters, were applied to compile an explicit finite-difference model of \( \mathrm{Ca}^{2+} \) kinetics (2 \( \times \) 10⁶ steps in a routine run), as described in the text.

**Results**

**Effects L-AP4 and CgTx on GABA Release Occlude**

We recorded monosynaptic IPSCs from stratum radiatum interneurons to address how group III mGluRs depress GABA release at synapses among interneurons. IPSCs were evoked via an extracellular electrode in stratum radiatum (in the presence of NBQX and APV, see Methods). Perfusion of the P-type \( \mathrm{Ca}^{2+} \) channel blocker \( \omega \)-Agatoxin IVA (AgTx, 0.1 \( \mu \)M) resulted in a 57 \( \pm \) 5% (mean \( \pm \) SEM) decrease in IPSC amplitude recorded in interneurons (\( n = 11 \); \( P < 0.001 \); paired \( t \)-test; Fig. 1A, C). L-AP4

![Figure 1](http://cercor.oxfordjournals.org/)

**Figure 1.** Blockade of N-type \( \mathrm{Ca}^{2+} \) channels partially occludes the depression of IPSCs by group III mGluRs. (A) Single cell example: the IPSC amplitude (normalized to baseline) was reduced in 0.1 \( \mu \)M AgTx. L-AP4 (50 \( \mu \)M) further reduced the IPSC from the new baseline (dotted line, estimated from the average of 20 trials prior to L-AP4 application). The traces shown above were averaged from 10 to 20 successive IPSCs in the following recording conditions (from left to right): control, AgTx, AgTx + L-AP4, AgTx + MSOP and AgTx + CgTx. (B) Single cell example: the IPSC amplitude was depressed to a new level by 0.25 \( \mu \)M CgTx (dotted line), but subsequent application of L-AP4 had no effect. Traces: control, CgTx, CgTx + L-AP4, CgTx + L-AP4 washout, and CgTx + AgTx; the other notations are as in B. (C) Summary of effects of \( \mathrm{Ca}^{2+} \) channel blockers and L-AP4 on IPSC amplitudes. All effects were significant at \( P < 0.001 \). (D) When P-type channels were blocked by AgTx, L-AP4 still reduced IPSCs to 52 \( \pm \) 7% of baseline (\( n = 9 \); \( P < 0.001 \)); in contrast, when N-type channels were blocked by CgTx, the IPSC reduction in L-AP4 was fourfold smaller (88 \( \pm \) 4% of baseline; \( n = 5 \); \( P < 0.04 \); the difference between the effects was significant at \( P < 0.005 \)).
(50 µM) applied after AgTx induced a further depression of 48 ± 7% (measured from a new baseline after AgTx; \( n = 9; P < 0.001 \)), not significantly different from that seen when l-AP4 was applied without Ca\(^{2+}\) channel blockers (decrease: 43 ± 7%; \( n = 4 \); Fig. 1C; see also Kogo et al., 1999; Semyanov and Kullmann, 2000).

In a separate set of experiments, the N-type blocker \( \text{ω-Conotoxin GVIA} \) (CgTx, 0.25 µM) produced a 51 ± 7% decrease in IPSC amplitude (\( n = 5; P < 0.001 \); Fig. 1B,C). In contrast to the effect of AgTx, subsequent application of 50 µM l-AP4 resulted in only a small depression (12 ± 4%; \( n = 5; P < 0.04 \)), a fourfold smaller change than when l-AP4 was applied after AgTx (\( P < 0.005 \) for difference, unpaired t-test; Fig. 1D).

Finally, when AgTx and CgTx were applied together, the IPSC was further reduced to 80 ± 2.0% of the original baseline amplitude (\( n = 8 \)), implying that non-P-, non-N-type channels alone do not sustain appreciable exocytosis.

These results are consistent with a preferential action of l-AP4 at synapses where exocytosis is triggered by N-type Ca\(^{2+}\) channels. However, P-type channel blockade did not enhance the effect of l-AP4 (Fig. 1C,D). A possible explanation of this paradox is that (i) some synapses may have both channel types (‘mixed synapses’) and (ii) synapses that have predominantly N-type channels (‘N-synapses’) are not always equipped with mGluRs. Blockade of P-type channels might therefore suppress GABA release at most mixed synapses (the sum of individual effects of CaTx and AgTx exceeds 100%, Fig. 1C) leaving an increased proportion of N-synapses that are insensitive to l-AP4. This hypothesis is consistent with data obtained in individual presynaptic varicosities (see below).

**Fast Ca\(^{2+}\) Entry in Axonal Varicosities of Interneurons: Two-photon Microscopy**

The above experiments say little about the relative roles of N- and P-type channels in triggering exocytosis at individual synapses. They do not rule out the hypothesis that both types of channels are always present, but with only one or other coupled to exocytosis. Nor do they distinguish between an action of group III mGluRs on Ca\(^{2+}\) channels and a role downstream in the exocytosis cascade.

We therefore applied two-photon excitation microscopy to image fast Ca\(^{2+}\) transients in individual axonal varicosities of stratum radiatum interneurons (Fig. 2A–G). Using the intense emission from Alexa Fluor 594, we identified the axon as a fine process (diameter \(-0.2–0.4 \) µm) with distinct varicosities (Fig. 2B,C), while dendrites always had a larger diameter (0.8–1.5 µm), and were either uniformly smooth or heavily populated with 0.5–1 µm wide ‘stubby’ spines (see Materials and Methods for details).

The average caliper (maximum projection) area of individual varicosities was \( S = 2.44 ± 0.18 \) µm\(^2 \) (\( n = 86 \)). We positioned a scanning line across the varicosity and acquired scans at 500 Hz for 600 ms. Two successive spikes (Fig. 2D) reliably evoked two Fluo-4 fluorescence transients in the varicosity (Fig. 2E–G). The average \( \Delta F/F \) response to the first spike measured 23 ± 3% (\( n = 95 \) varicosities; see Methods) and the time constant of decay was 265 ± 8 ms (single exponent; \( n = 75 \)). In control experiments, a train of 100 action potentials 20 ms apart resulted in an average saturated versus baseline Ca\(^{2+}\) fluorescence ratio \( F_\text{max} / F_1 = 9.1 ± 2.4 \) (\( n = 6 \); Fig. 2H,I; the entire sweep duration in these experiments was 2.5 s). Substituting these data into equations (7) and then (1) suggests that the average baseline [Ca\(^{2+}\)] was \(-50 \) nM and Fluo-4 bound Ca\(^{2+}\) (CaFl) was \(-22 \) µM. The average post-spike fluorescence level (quasi-steady-state, integrated over 50 ms post-spike) \( F_1 \) thus corresponded to the ratio \( F_\text{max} / F_1 = 9.1/1.25 = 7.4 \), implying \(-62 \) nM of quasi-steady-state [Ca\(^{2+}\)], or \(-27 \) µM CaFl, following a single action potential. These data suggested little impact of saturation in a typical case. In addition, we measured the second-to-first \( \Delta F/F \) ratios in fluorescence responses where two successive transients were clearly distinguishable (e.g. Fig. 2F,G); the average ratio was 1.98 ± 0.07 (\( n = 19 \)) thus confirming that \( \Delta F/F \) signals were not saturated to any significant degree. In these varicosities, we saw no Ca\(^{2+}\) transient failures in >1000 trials, suggesting that action potentials propagated reliably (>99.9%), at least at a low spiking frequency.

Is it possible to estimate the kinetics of total and free Ca\(^{2+}\) inside the varicosity from the average kinetics (amplitude, decay, paired-pulse ratio) of the fluorescent signal? The large dynamic range of Fluo-4 (50–100) implies that the recorded fluorescence simply reflects the kinetics of CaFl. This, however, involves the two unknowns: the amount of an endogenous Ca\(^{2+}\) buffer (assuming \( K_a = 0.5 \) µM, as estimated in mossy fiber synaptic boutons (Jackson and Redman, 2003)) and the Ca\(^{2+}\) removal rate \( P \). Because these determinants could vary among cells/varicosities, we simply explored two likely limiting cases of the buffer concentration: 50 µM (2–3 times lower than estimated by Jackson and Redman (2003) for mossy fibers) and 400 µM (twice the concentration of Fluo-4). Once the buffer concentration was ‘fixed’, one unknown parameter, total Ca\(^{2+}\) influx, was adjusted to reproduce the observed 23% \( \Delta F/F \) increment (corresponding to a CaFl concentration step from 22 µM to an average of \(-27 \) µM within a 50 ms window); the other parameter, Ca\(^{2+}\) removal rate, was adjusted to reproduce the \( \Delta F/F \) decay; within the explored range these two variables were effectively orthogonal (independent). The results predict 9 µM and 20 µM Ca\(^{2+}\) influx, and 2.5 ms\(^{-1}\) and 5 ms\(^{-1}\) Ca\(^{2+}\) removal rate, in conditions of low and high buffer, respectively (Fig. 2J,K; black dotted lines correspond to the experimental average for fluorescence kinetics). Interestingly, in both cases the computed free [Ca\(^{2+}\)] transient peaked at 400–450 nM suggesting that \( \Delta F/F \) reflects this variable almost independently of the assumptions about the endogenous buffer.

Given that one Ca\(^{2+}\) channel carries, on average, \( Q = 0.2 \) µC \( × \) 2 ms = \( 4 × 10^{-17} \) coulomb, or \( Q/2e = 125 \) Ca\(^{2+}\) ions, during an action potential (Koester and Samkoon, 2000), and that the average varicosity volume was \( 1.33πr^2h^2 = 2.86 \) µm\(^3\), an increase of 9–20 µM implies an influx of 1000–3000 Ca\(^{2+}\) ions or the opening of 8–25 Ca\(^{2+}\) channels.

**l-AP4 Suppresses Ca\(^{2+}\) Entry in Axonal Varicosities Enriched in N-type Channels**

We next examined the relative contribution of N- and P-type channels to the Ca\(^{2+}\) transients and the possible modulating role of group III mGluRs at individual varicosities. We applied l-AP4 prior to the channel blockers (otherwise, irreversible blockade of one channel type could suppress the Ca\(^{2+}\) influx completely and thus prevent any further investigation of that varicosity). Two experiments were performed: (i) the blockers were applied without prior application of l-AP4; (ii) the blockers were applied after MSOP reversed the effect of l-AP4 (Fig. 3A–D). Because MSOP reversed the effects of l-AP4 (see...
average data in Fig. 3E, and also Semyanov and Kullmann, 2000), we grouped together data from experiments (i) and (ii) to yield information on the effect of Ca\textsuperscript{2+} channel blockers alone. Experiment (ii) yielded information on the relationship between the effects of L-AP4 and Ca\textsuperscript{2+} channel blockers within individual varicosities. L-AP4 (50 µM) on its own, bath-applied after a 15–20 min baseline, produced a highly variable decrease in the fluorescence transient \( \Delta F/F \). The average decrease, including all varicosities, was 21 ± 3% (Fig. 3E, n = 50). In 42 experiments, the group III mGluR antagonist MSOP was subsequently added. This reversed the effect of L-AP4 (∆F/F = 94 ± 9% of baseline, Fig. 3E).

On average, CgTx (0.25 µM) reduced ∆F/F by 42 ± 6% (n = 33), while AgTx (0.1 µM) reduced ∆F/F by 31 ± 5% (n = 26; Fig. 3E). When applied together or sequentially, the two blockers reduced ∆F/F by 78 ± 5% (n = 17), implying that these channels account for the majority of the Ca\textsuperscript{2+} transient. Finally, perfusing 100 µM Cd\textsuperscript{2+} completely abolished the fluorescence transient (residual fluorescence 2 ± 1%, n = 11; Fig. 3E).
The effect of CgTx among varicosities was bimodal (Fig. 3F): in 22 varicosities CgTx had a small effect (group I), and in 11 varicosities it produced a much larger reduction (group II). We then compared the reduction in ∆F/F by L-AP4 in varicosities from group I with that in varicosities from group II. L-AP4 had a threefold larger effect on varicosities that were sensitive to CgTx (∆F/F reduction: 14 ± 4% in group I and 42 ± 8% in group II, n = 19 and n = 8, respectively, P < 0.001; Fig. 3G; in six varicosities, L-AP4 was not applied). This implies that varicosities that show L-AP4-sensitive Ca2+ transients also tend to express N-type Ca2+ channels; pooled together, the corresponding changes in ∆F/F showed a significant positive correlation (r = +0.51; n = 27, P < 0.008).

To dissect the contribution of P-type channels in the Ca2+ influx, we conducted similar experiments with AgTx (Fig. 4A–C). The effect of AgTx alone again fell into two distinct categories (Fig. 4D). In this case, however, varicosities that were sensitive to AgTx (group II in Fig. 4D) exhibited a significantly smaller reduction in ∆F/F by L-AP4 than varicosities that were less sensitive to AgTx (∆F/F reduction: 16 ± 5% and 42 ± 10%, n = 6 and n = 7, respectively; P < 0.04; Fig. 4E; in 13 varicosities, L-AP4 was not applied). Pooled together, these effects of L-AP4 and AgTx showed a negative correlation: r = −0.56, n = 13, P < 0.045).

Thus, L-AP4 predominantly affects varicosities where N-type channels mediate most of the action potential-dependent Ca2+ influx. These findings imply that group III mGluRs negatively modulate N-type channels, and that N- and P-type channels play complementary roles. Further supporting this, when we compared the contributions of N- and P-type channels to the Ca2+ transients in individual varicosities by sequential application of the selective toxins, their effects on ∆F/F were negatively correlated (r = −0.83, P < 0.001, n = 17; Fig. 4F).
Neighboring Varicosities Tend to Have Similar Ca$^{2+}$ Channels but Show Variable Sensitivities to L-AP4

These results argue for considerable heterogeneity in the roles of N- and P-type Ca$^{2+}$ channels, and of group III mGlus, at different varicosities. It is not known whether the identity of the presynaptic neuron, that of the postsynaptic cell, or a combination of both, determine the distribution and function of presynaptic Ca$^{2+}$ channels and G protein-coupled receptors. We therefore asked whether distinct varicosities supplied by a single axon showed similar sensitivities to L-AP4, CgTx or AgTx. We explored this by testing the hypothesis that the difference in the effects of the drugs between neighboring varicosities (‘neighbor-pair difference’) was smaller than the difference in randomly selected pairs of varicosities (‘random-pair difference’). We first calculated the absolute average difference in the reduction in \(\Delta F/F\) between neighbors (usually 5–10 \(\mu\)m apart, Fig. 5A). The example in Figure 5A–D shows fluorescence signals recorded simultaneously from two neighboring axonal varicosities (1 and 2) in which application of LAP4 produced contrasting effects. We then reshuffled the data collected from all such recordings and computed the average difference between randomly selected pairs of varicosities. This reshuffle was repeated 10 times to generate a predicted cumulative distribution of ‘random-pair’ differences. This distribution was then compared to the observed cumulative distribution of ‘neighbor-pair’ differences: whenever these distributions differ significantly, this test indicates similarity between neighbors. The result of this Monte Carlo test is shown in Figure 5E: neighboring varicosities showed similar sensitivities to CgTx (\(P < 0.005, n = 23\)) and AgTx (\(P < 0.01, n = 32\)), but not to L-AP4 (\(n = 34\)).

One potential confounding factor in these analyses is that any changes in the physiological conditions of an axon could, in principle, affect all its varicosities to a similar degree and thus induce a spurious positive correlation between the neighbors. However, the fact that the largest sample (L-AP4 experiments) showed no correlation between neighbors argues against such a spurious effect. Direct spread of Ca$^{2+}$ transients between the neighbors was also unlikely to affect the analysis: an inter-neighbor distance \(x = 5–10 \mu\)m along the axon corresponds to a characteristic diffusion time of \(t = 0.5 \times x^2 D^{-1} = 0.5–2 \mu\)s, given an intracellular Ca$^{2+}$ diffusion coefficient \(D = 0.01–0.02 \mu\)m$^2$/ms (Gabso et al., 1997) – this is beyond the measurement window for \(\Delta F/F\). Although varicosities supplied by different branches could not be compared, these results are consistent with the hypothesis that the identity of the presynaptic neuron determines whether N-type Ca$^{2+}$ channels predominate at the release sites, but not whether group III mGlus are effective in reducing Ca$^{2+}$ influx.

Discussion

Results from two complementary methods applied here converge on the conclusion that a major effector mechanism for presynaptic group III mGlus is depression of N-type Ca$^{2+}$ currents. This conclusion agrees with the evidence that N-type Ca$^{2+}$ channels are very sensitive to G protein-mediated modulation (Zhang et al., 1996), which therefore provides a direct intracellular signaling mechanism to depress evoked GABA release.

The present study argues against several alternative hypotheses relating group III mGlus to N-type channels. Thus, the hypothesis that both channel types are present in a constant proportion, but with only one or other linked to exocytosis,
can be rejected on the basis of the negative correlation between the contributions of N- and P-type channels in individual varicosities (Fig. 4). Moreover, modulation of Ca²⁺ transients by L-AP4 and the positive correlation in sensitivities to L-AP4 and CgTx make highly unlikely (although do not disprove) the hypothesis that group III mGluRs depress transmitter release independently of an action on Ca²⁺ channels.

Although the present study argues strongly for modulation of N-type Ca²⁺ channels by group III mGluRs (see also Millan et al., 2002a,b), a smaller effect on P-type channels cannot be excluded: L-AP4 produced a significant, albeit small, depression of IPSCs in the presence of CgTx. This may be due to the sensitivity of the assay used (Corti et al., 2002). An additional effect of group III mGluRs downstream of Ca²⁺ influx cannot be ruled out. Indeed, several metabotropic receptor agonists can affect transmitter release under conditions where presynaptic Ca²⁺ channels do not contribute (Scanziani et al., 1995; Capogna et al., 1996; see also Schoppa and Westbrook, 1997; Krieger et al., 1999). Whether these actions are relevant to glutamatergic modulation of GABA release remains to be determined. Group III mGluRs may have other actions elsewhere in the CNS. At least one subtype (mGluR4) is also expressed postsynaptically (Shigemoto et al., 1997; Corti et al., 2002), and is a candidate receptor for mediating a decrease in somatodendritic Ca²⁺ influx by group III agonists. At least part of this effect, in contrast to the present results, has been attributed to an action on P-type channels (Stefani et al., 1998).

Although some varicosities apparently had only N-type channels (close to 100% reduction in ΔF/F by CgTx), varicosities equipped exclusively with P-type channels did not occur (the maximal effect of AgTx was ∼60%). Some varicosities had residual Ca²⁺ transients that were resistant to both toxins (Fig. 4F), suggestive of a role for R-type channels. Whether these channels are also modulated by group III mGluRs cannot be determined from the present results. Another potential target of group III mGluRs is Ca²⁺ release from intracellular stores, which has been implicated in spontaneous transmitter release.
and short-term plasticity (Llano et al., 2000; Emptage et al., 2001). However, rapid action potential-dependent exocytosis of GABA depends on the presynaptic kinetics of free Ca$^{2+}$ inside the terminal, which also determine the size of fluorescence transients recorded with Ca$^{2+}$-sensitive dyes. Thus, indirect effects of mGluRs on Ca$^{2+}$ kinetics are unlikely to alter the main conclusions of the present study.

Interestingly, although the effect of N-type or P-type Ca$^{2+}$ channel blockade was correlated among varicosities supplied by the same axon, this was not the case for the effect of L-AP4 (moreover, not every varicosity that responded to CGTx was profoundly modulated by L-AP4; this divergence among neighbors might reflect the necessity to spatially restrict the actions of glutamate that escapes neighboring excitatory synapses; Rusakov and Lehre, 2002). This implies that N-type channels are not always co-localized with group III mGluRs, even though on average such co-localization is clearly above the random coincidence rate (see Fig. 3F). In other words, the sub-group of varicosities sensitive to CGTx (group II in Fig. 3F) should exhibit a relatively higher variability for the effects of L-AP4 (because some of these varicosities might be insensitive to L-AP4). Indeed, the inherent variances for the effects of CGTx and L-AP4 in this group were strikingly different (coefficient of variation 0.11 and 0.55, respectively, $P < 0.001$, Levene’s test; see group II in Fig. 3F and data scatter in Fig. 3G). Because a 100% reduction in $\Delta F/F$ by a single channel blocker was rarely seen, our data also suggest that a significant proportion of varicosities contain a mixture of N- and P-type channels. Taken together, these considerations support our explanation why the P-type channel blockade did not enhance the effect of L-AP4 (Fig. 1C,D, see Results): the proportion of releasing varicosities with N-type channels that are insensitive to L-AP4 could increase as a result of silencing synapses with mixed channel types by application of AgTx alone.

Approximately 30% of varicosities imaged in the present study showed a marked response to L-AP4 (residual $\Delta F/F < 50\%$ of baseline), but such varicosities were frequently near to others that showed little or no effect of L-AP4. The most parsimonious explanation is that L-AP4-sensitive varicosities correspond to interneuron–interneuron synapses, because GABA release at these synapses is sensitive to L-AP4 (Semyanov and Kullmann, 2000). This hypothesis also agrees with the target cell-dependence in the distribution of some group III mGluR subtypes on both glutamatergic and GABAergic boutons (Shigemoto et al., 1996, 1997; Corti et al., 2002; Somogyi et al., 2003). Of the group III mGluRs, the most likely candidate receptors mediating the actions of L-AP4 studied here are mGluR4 and mGluR8, because the affinity of mGluR7 for L-AP4 is relatively low (Okamoto et al., 1994), and mGluR6 does not occur in the hippocampus (Nakajima et al., 1993).

Although the account given above reconciles the physiology, imaging and immunocytochemistry data, it leads to a paradox concerning the relative numbers of synapses formed on interneurons versus pyramidal neurons. Visual inspection suggests that pyramidal cells outnumber stratum radiatum interneurons by a factor of 20–50. Other interneurons in the CA1 area (stratum pyramidale and stratum oriens) appear to follow a similar relationship (Woodson et al., 1989). Thus, there is a discrepancy between the relatively small numbers of potential postsynaptic target interneurons and the large proportion of varicosities sensitive to L-AP4. However, stratum radiatum interneurons are a mixed population, including calretinin-positive cells that project mainly to other interneurons, and calbindin- or VIP-positive cells that principally innervate pyramidal neurons (Gulyas et al., 1996). Thus, a possible resolution of the paradox concerning the expected proportion of L-AP4-sensitive varicosities is that stratum radiatum interneurons are ~10-fold more likely to project to other interneurons than predicted by the frequency of these cells.

We did not attempt to classify interneurons on morphological or immunohistochemical grounds in the present study. Although such a classification is not always straightforward (Parra et al., 1998), it has been recently suggested that cholecystokinin-positive interneurons use N-type Ca$^{2+}$ channels to release GABA, and that these channels are also the target of cannabinoid receptors that mediate depolarization-induced suppression of inhibition of pyramidal neurons (Wilson et al., 2001; see also Katona et al., 1999; Tsou et al., 1999). It remains to be determined whether cannabinoids directly depress Ca$^{2+}$ influx at these synapses, as shown for L-AP4 in the present study, but if such experiments confirm that activation of CB1 receptors leads to a decrease in Ca$^{2+}$ influx via N-type channels, this could form a striking counterpart to the mechanism of action of group III mGluRs. Thus, transmitter release from boutons that depend on N-type Ca$^{2+}$ channels could be depressed by glutaamate if the postsynaptic cell is an interneuron, or by cannabinoids if the target cell is a pyramidal neuron.

In conclusion, group III mGluRs heterogeneously expressed at presynaptic varicosities of interneurons depress Ca$^{2+}$ influx principally via an action on N-type Ca$^{2+}$ channels. This result is in striking contrast with the MNTB synapse, where group III mGluRs act via P-type channels (Takahashi et al., 1996). The mGluR-dependent depression of presynaptic Ca$^{2+}$ influx mediates a reduction of GABA release in response to elevations of extracellular glutamate relatively selectively at synapses between interneurons (Semyanov and Kullmann, 2000). This phenomenon may contribute to the disinhibition of interneurons under conditions of excessive firing of principal neurons, and therefore play an important role in regulating hippocampal excitability. A conjunction of both presynaptic and postsynaptic factors underlies the specificity of this phenomenon: the expression of presynaptic Ca$^{2+}$ channels appears to be determined mainly by the identity of the presynaptic neuron (Poncer et al., 1997; Wilson et al., 2001). The expression of mGluRs, on the other hand, is determined at least in part by the identity of the postsynaptic neuron (Shigemoto et al., 1996, 1997; Corti et al., 2002; Somogyi et al., 2003). Whether such influences are specific to mGluRs remains to be established: for instance, analyzing Ca$^{2+}$ kinetics at individual hippocampal mossy fiber boutons did not reveal heterogeneity in the actions of presynaptic GABA$_A$ receptors (Ruiz et al., 2003). Finally, we propose a testable hypothesis for the organization of metabotropic modulation of GABA release at individual synapses: notwithstanding the heterogeneity of interneuron subtypes, N-type Ca$^{2+}$ channels act as the principal effector mechanism, but the identity of the presynaptic receptor is at least in part determined by the target cell identity.
Notes

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Address correspondence to Dmitri A. Rusakov or Dimitri M. Kullmann, Department of Clinical and Experimental Epilepsy, Institute of Neurology, University College London, Queen Square, London WC1N 3BG, UK. Email: d.rusakov@ion.ucl.ac.uk; d.kullmann@ion.ucl.ac.uk.

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