DUAL INTRACELLULAR RECORDINGS AND COMPUTATIONAL MODELS OF SLOW INHIBITORY POSTSYNAPTIC POTENTIALS IN RAT NEOCORTICAL AND HIPPOCAMPAL SLICES

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Abstract—Dual intracellular recordings in slices of adult rat neocortex and hippocampus investigated slow, putative GABA$\beta$ receptor-mediated inhibitory postsynaptic potentials. In most pairs tested in which the interneuron elicited a fast inhibitory postsynaptic potential in the pyramid, this GABA$\alpha$ receptor mediated inhibitory postsynaptic potential was entirely blocked by bicuculline or picrotoxin (3:3 in neocortex, 6:8 in CA1, all CA1 basket cells), even when high-frequency presynaptic spike trains were elicited. However, in three of 85 neocortical paired recordings involving an interneuron, although no discernible response was elicited by single presynaptic interneuronal spikes, a long latency (≥20 ms) inhibitory postsynaptic potential was elicited by a train of ≥3 spikes at frequencies ≥50–100 Hz. This slow inhibitory postsynaptic potential was insensitive to bicuculline (one pair tested). In neocortex, slow inhibitory postsynaptic potential duration reached a maximum of 200 ms even with prolonged presynaptic spike trains. In contrast, summing fast, GABA$\alpha$ inhibitory postsynaptic potentials, elicited by spike trains, lasted as long as the train. Between four and 10 presynaptic spikes, mean peak slow inhibitory postsynaptic potential amplitude increased sharply to 0.38, 2.6 and 2.9 mV, respectively, in the three neocortical pairs (membrane potential −60 to −65 mV). Thereafter increases in spike number had little additional effect on amplitude. In two of eight pairs in CA1, one involving a presynaptic basket cell and the other a putative bistratified interneuron, the fast inhibitory postsynaptic potential was blocked by bicuculline revealing a slow inhibitory postsynaptic potential that was greatly reduced by 100 µM CGP 35348 (basket cell pair). The sensitivity of this slow inhibitory postsynaptic potential to spike number was similar to that of neocortical ‘pure’ slow inhibitory postsynaptic potentials, but was of longer duration, its plateau phase outlasting 200 ms spike trains and its maximum duration exceeding 400 ms. Computational models of GABA release, diffusion and uptake suggested that extracellular accumulation of GABA cannot alone account for the non-linear relationship between spike number and inhibitory postsynaptic potential amplitude. However, cooperativity in the kinetics of GABA$\beta$ transduction mechanisms provided non-linear relations similar to experimental data. Different kinetic models were considered for how G-proteins activate K$^+$ channels, including allosteric models. For all models, the best fit to experimental data was obtained with four G-protein binding sites on the K$^+$ channels, consistent with a tetrameric structure for the K$^+$ channels associated with GABA$\beta$ receptors.

Thus some inhibitory connections in neocortex and hippocampus appear mediated solely by fast GABA$\alpha$ receptors, while others appear mediated solely by slow, non-ionotropic, possibly GABA$\beta$ receptors. In addition, some inhibitory postsynaptic potentials arising in proximal portions of CA1 pyramidal cells are mediated by both GABA$\alpha$ and GABA$\beta$ receptors. Our data indicate that the GABA released by a single interneuron can saturate the GABA$\beta$ receptor mechanism(s) accessible to it and that ‘spillover’ to extrasynaptic sites need not necessarily be proposed to explain these slow inhibitory postsynaptic potential properties. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: GABA$\beta$, interneuron, hippocampus, cerebral cortex, G-protein, spillover.

Since the characterization of the GABA$\beta$ receptor and the development of the first selective antagonists many studies have described late, slow, bicuculline-insensitive inhibitory postsynaptic potentials (IPSPs) elicited by electrical stimulation, in a number of brain regions, e.g., 19 that were sensitive to Saclofen or 2-OH-Saclofen (see Ref. 23 for review). These events are mediated by G-protein-activated K$^+$ channels. Typically, a higher stimulus threshold was required to elicit GABA$\beta$ IPSPs than GABA$\alpha$ IPSPs. Moreover, spontaneous GABA$\beta$ inhibitory postsynaptic currents did not occur unless 4-aminopyridine was added to the bathing medium, presumably inducing repetitive firing in the interneuron(s) activating the GABA$\beta$ receptors.

Two hypotheses have been proposed to explain
the apparent need for intense interneuronal activation. First the ‘spillover’ hypothesis\textsuperscript{12} proposes that multiple GABA release would result in extracellular accumulation of GABA. This GABA would reach levels sufficient to activate extrasynaptically-located GAB\textsubscript{A} receptors only with sufficiently high interneuronal activity, or when GABA uptake was blocked.\textsuperscript{17,34} Second the ‘G-protein’ hypothesis\textsuperscript{9} postulated that the nonlinear activation properties arise from intracellular mechanisms involving G-protein cascades that are responsible for K\textsuperscript{+} channel activation. In this case, the GAB\textsubscript{A} response was predicted to be sensitive to the number of presynaptic spikes and a sufficiently high concentration of G-proteins was required for detectable IPSPs to be activated. A need for a build up of activated G-proteins would also explain the minimal 10–20 ms onset latency, sigmoidal rise and multi-exponential decay of GAB\textsubscript{A} IPSPs (as described by Ref. 28).

Whether mixed GAB\textsubscript{A}/GAB\textsubscript{B} IPSPs generated by single interneurons occur in cortical regions, or whether they are always separate has also been a controversial issue for some time (for discussion see Ref. 26). That the two types of IPSPs originate from quite separate interneurons has been proposed by several studies using indirect techniques, e.g.,\textsuperscript{4,32} Only two previous studies have, however, used paired recordings to study slow IPSPs. In hippocampus\textsuperscript{31} an apparently pure slow hyperpolarization was induced in a pyramidal cell by a train of spikes in a single stratum lacunosum molecular (SLM) interneuron. Conversely, in thalamus, a single GABA-ergic reticularis nucleus interneuron that elicited a fast GAB\textsubscript{A} IPSP with single spikes could also elicit a bicuculline-insensitive, CGP 35348-sensitive slow IPSP in a simultaneously recorded relay cell, provided prolonged, high-frequency firing was elicited in the interneuron.\textsuperscript{20} It is less clear whether mixed IPSPs occur in cortical regions. In some paired recordings in neocortex, prolonged high-frequency firing in a single interneuron could elicit a slowly developing and decaying event in addition to the summing fast GAB\textsubscript{A} receptor-mediated IPSPs in a simultaneously recorded postsynaptic pyramidal cell.\textsuperscript{36} This event was not apparent with spike trains briefer than 10–20 spikes. However, these events were not isolated pharmacologically and their dependence upon spike number and frequency was not studied in any detail. GAB\textsubscript{B} IPSP amplitude appears to be compromised by the concurrent activation of GAB\textsubscript{A} IPSPs.\textsuperscript{8} This could be a postsynaptic phenomenon, blockade by raised intracellular Cl\textsuperscript{−} of the G-protein activation of K\textsuperscript{+} channels,\textsuperscript{22} or Cl\textsuperscript{−} channel shunting of lower conductance events. The more effective activation of GAB\textsubscript{A} IPSPs in the presence of GAB\textsubscript{A} antagonists could equally result from the more intense activation of ‘disinhibited’ interneurons where electrical stimulation has been used to elicit the IPSPs. It was therefore also of interest to determine whether GAB\textsubscript{A} IPSPs elicited by a single interneuron in which presynaptic firing can be controlled, are masked by GAB\textsubscript{A} events, but revealed by application of GAB\textsubscript{A} antagonists.

In this paper therefore, paired intracellular recordings in slices of adult rat neocortex and hippocampus were used to determine whether slow, putative GAB\textsubscript{A} receptor-mediated IPSPs can arise from activity in a single cortical interneuron, whether they always, or conversely, never occur in the absence of coincident GAB\textsubscript{A} receptor activation. To characterize the exact relationship between the pattern of presynaptic firing and the amplitude of GAB\textsubscript{A} IPSPs and to test whether repetitive presynaptic firing is indeed required, paired recordings were tested with a range of presynaptic firing patterns. These results were then used in computational models of GAB\textsubscript{A} responses to investigate the plausibility of extracellular and intracellular hypotheses to explain any observed presynaptic/postsynaptic relations.

**EXPERIMENTAL PROCEDURES**

Young adult male Sprague–Dawley rats (bred in-house), 120–200 g in body weight were anaesthetized with Sagatal (pentobarbitone sodium, Rhône Mérieux, 60 mg/kg i.p., substantially higher than the necessary anaesthetic dose for surgery), or with Fluothane followed by Sagatal and perfused transcardially with an ice-cold artificial cerebrospinal fluid (ACSF) in which the NaCl had been replaced with 248 mM sucrose and to which Sagatal (60 mg/l) was added, and then decapitated. All procedures were performed on deeply-anæsthetised animals to prevent any suffering, experiments were performed in vitro and the minimum number of animals used. Coronal slices of neocortex or hippocampus, 450–500-μm-thick were cut (Vibrisols, Camden Instruments). During slice preparation, in ice-cold ACSF, and for the first hour of incubation in the interface recording chamber (34–36°C), the slices were maintained in the sucrose containing medium (without Sagatal) equilibrated with 95%O\textsubscript{2}/5% CO\textsubscript{2}. This was then replaced with standard ACSF containing 124 mM NaCl, 25.5 mM NaHCO\textsubscript{3}, 3.3 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1 mM MgSO\textsubscript{4}, 2.5 mM CaCl\textsubscript{2} and 15 mM d-glucose in which all recordings were performed. Recordings commenced after a further hour in this medium.

**Electrophysiological recordings**

Intracellular recordings were made from pairs of neurons with conventional sharp micro-electrodes filled with 2 M KMeSO\textsubscript{4} (90–140 MΩ) and, in the majority of experiments, 2% w/v Biocytin, using an Axoprobe. Typically, a stable intracellular recording, i.e., one in which the neuron exhibited a stable membrane potential, input resistance and responses to injected current, was first obtained from one neuron in a fresh region of the slice. A second electrode was then introduced and sequential recordings made from a small number of neighbouring cells until a connection was found, or an interneuron located. If that interneuron was not synaptically connected to the first cell, the first electrode was withdrawn and a small number of other cells impaled and tested sequentially. The procedure was repeated in fresh regions until a connection was apparent. Single spikes, pairs of spikes and spike trains were elicited in the presynaptic neuron by injection of ramp and/or square wave current pulses, delivered typically once every 3 s.
amplitude and duration of the pulse and its repetition rate were modified to elicit different patterns of presynaptic firing, interleaving single spikes and brief trains with long trains of spikes and/or, low with high firing frequencies. Continuous analogue recordings from both neurons for the entire duration of the paired recording were made on magnetic tape (Racal Store 4). Postsynaptic membrane potential (MP) was maintained within 2 mV of a preset value. If sufficient data had been collected at one potential, could be changed. During continuous current injection, electrode balance was monitored by observing voltage responses to small, brief current pulses injected before, or after responses to presynaptic spikes.

In pairs in which a short latency IPSP was elicited by single presynaptic spikes, bicuculline or picrotoxin was applied after the fast IPSP had been characterized and trains of presynaptic spikes were then elicited to determine whether a slow, bicuculline-insensitive IPSP remained. Where no fast IPSP was apparent in control conditions, trains of spikes were then elicited to determine if averaged postsynaptic responses, presynaptic spike artifacts were observed or reduced graphically from averaged IPSPs. Averages of some slow IPSPs were smoothed by three or five point averaging.

Computational models

A previous model of the diffusion of GABA in the extracellular space and of GABA<sub>R</sub> receptor activation was used here with some modification. Release, diffusion and uptake of GABA were simulated using a two-dimensional grid of 0.5×0.5 μm to approximate the thin extracellular space surrounding neurons. Release was simulated by a sudden increase in the concentration of transmitter in one of the compartments, reaching a peak of 3 mM. Extracellular diffusion of GABA was simulated with a diffusion coefficient of D = 8 × 10<sup>-6</sup> cm<sup>2</sup> s<sup>-1</sup> based on values of compounds of similar molecular weight. Tortuosity of the extracellular space may reduce the effectiveness of extracellular diffusion significantly. A lower diffusion coefficient of D = 3 × 10<sup>-9</sup> cm<sup>2</sup> s<sup>-1</sup> was therefore also used (corresponding to a tortuosity factor of about 1.6). GABA uptake was simulated according to the following scheme:

\[ \text{GABA} + \text{U} \rightleftharpoons \text{B} \]

\[ \text{B} \rightarrow \text{U} + \text{GABA}_0 \]

The first step describes the reversible binding of GABA to the unbound transporter (U), leading to bound transporter (B). The second step represents the irreversible translocation of GABA into the intracellular space (GABA<sub>0</sub>). Thus, the concentration of GABA in the extracellular space is described by the following equations:

\[ \frac{dT(\dot{x},\dot{t})}{dt} = -k_1 T(\dot{x},\dot{t})(B_{in} - B(\dot{x},\dot{t})) + k_{-1} B(\dot{x},\dot{t}) + DR^2 T(\dot{x},\dot{t}) \]

\[ \frac{dB(\dot{x},\dot{t})}{dt} = -k_2 T(\dot{x},\dot{t})(B_{in} - B(\dot{x},\dot{t})) + (k_{-2} + k_2) B(\dot{x},\dot{t}) \]

where T(\dot{x},\dot{t}) and B(\dot{x},\dot{t}) are the concentration of GABA and of bound transporter, respectively at point \( \dot{x} \) and time \( \dot{t} \). \( k_1 \) and \( k_{-1} \) are the forward and backward binding rate of GABA to transporter, respectively, \( k_2 \) is the rate constant for the translocation of GABA, and \( B_0 \) is the total extracellular concentration of available transporter. A value of \((k_{-1} + k_2)/k_1 = 4 \mu M\) was estimated experimentally, showing a high affinity of the transporter for GABA. For glutamate, binding to the transporter is very fast, while the translocation step is slower. Assuming the same translocation rate as estimated for glutamate leads to the following set of parameters: \( k_1 = 30 \text{mM}^{-1} \text{ms}^{-1} \), \( k_{-1} = 0.1 \text{ms}^{-1} \) and \( k_2 = 0.02 \text{ms}^{-1} \). The total concentration of transporter molecules is difficult to estimate. It was assumed constant through the extracellular space and was tested in the range of \( B_0 = 0 \) to 1 mM.

Equations of GABA release, diffusion and uptake were simulated using a first-order implicit integration method implemented in NEURON<sup>14</sup> (see details in Ref. 9).

Activation of GABA<sub>R</sub> responses by GABA was simulated using the following kinetic equations:

\[ R_0 + \text{GABA} \rightleftharpoons R^* \]  
\[ R^* + G_0 \rightleftharpoons RG \rightleftharpoons R^* + G^* \]  
\[ G^* \rightarrow G_0 \]  
\[ K_0 + nG^* \rightleftharpoons K^n* \]

where GABA binds to the inactive form of the GABA<sub>R</sub>.
receptor (R₀), leading to the activated form of the receptor (R*; reaction 1). R* catalyses the formation of active G-proteins (G*) from their inactive form (G₀) according to a Michaelis–Menten scheme (reaction 2). Activated G-protein (G*) is hydrolysed to its inactive form (G₀; reaction 3). Finally, n activated G-proteins bind to the closed form of the K⁺ channel (K₀), leading to opening of K⁺ channels (open form K*; reaction 4).

Several approximations in this scheme, such as considering that intermediate enzymatic forms are at steady-state, that G-proteins are in excess and that open K⁺ channels are at steady state due to fast binding of G* to K₀, lead to the following simplified equations:

\[
\frac{dr}{dt} = K_1 T (1 - r) - K_2 r
\]

\[
\frac{dg}{dT} = K_3 r - K_4 g
\]

\[
I_{GABAB} = g_{GABAB} \times \left[ g' \left( g/g'_0 \right) \right] \times (V_{post} - E_K)
\]

where r is the fraction of receptors in the active form, T is the GABA concentration in the synaptic cleft, g is the normalized concentration of G-proteins in the active state, \(K_1 = 0.18 \text{ ms}^{-1} \text{ mM}^{-1}\), \(K_2 = 0.0096 \text{ ms}^{-1}\), \(K_3 = 0.19 \text{ ms}^{-1}\), \(K_4 = 0.060 \text{ ms}^{-1}\) are kinetic rate constants for reactions 1–4 above and \(K_{d} = 17.83\) is the dissociation constant for activated G-proteins bound to K⁺ channels. \(I_{GABAB}\) is the current activated by GABA and \(g_{GABAB}\) is the maximal conductance of the K⁺ channel, \(V_{post}\) is the postsynaptic membrane potential and \(E_K\) the potassium equilibrium potential. These equations were discussed in detail in previous papers.9,10

Fitting of the model to experimental data was performed using a simplex procedure. The model was run at each interaction of the simplex procedure and the parameters optimized so as to minimize the mean square error between model and data. Several initial conditions were tested to ensure that the optimal fit was unique. The behaviour was always robust within some domain of parameter values around the optimal fit. The corresponding range of parameter values are given in the text.

In the model shown above, n G-proteins bind simultaneously to act on K⁺ channels. In addition to this simple model, several other models were considered to simulate the binding of activated G-proteins to open K⁺ channels.

**Hodgkin–Huxley-like model**

A possible model for K⁺ channel activation is analogous to the model introduced by Hodgkin and Huxley:15

\[
I_{GABAB} = g_{GABAB} m^n(V - E_K)
\]

\[
\frac{dm}{dt} = \alpha g(1 - m) - \beta m
\]

where K⁺ channels open according to n independent gates, m is the fraction of gates in the open state, \(\alpha\) and \(\beta\) are rate constants and m is given by:

\[
m = g g'_{K} K_d^{1/n}
\]

where \(K_d = \beta/\alpha\) is the dissociation constant of G-protein binding on each gate.

**Allosteric model**

Another possible model is derived from the allosteric scheme introduced by Monod, Wyman and Changeux:24

\[
C \equiv O
\]

\[
O + G \equiv O_1
\]

\[
O_1 + G \equiv O_2, \ldots
\]

\[
O_{n-1} + G \equiv O_n
\]

where the closed (C) and open (O) form of the channel are at equilibrium, and activated G-proteins bind only to the open form of the receptor.
state. In this case, the total concentration of all open forms at steady-state is given by:

\[ Y = \frac{L}{1 + L(1 + g/K_2)^{t}} \]

where \( L \) is the allosteric constant (the equilibrium constant of \( C = O \) transition) and \( K_2 \) is the dissociation constant of G-protein binding. The current is then given by:

\[ I_{GABA} = \beta_{GABA} Y(V - E_K) \]

Histological processing

Following the paired recording, cells were filled with biocytin by passing 0.5 nA depolarizing current pulses in a 50% duty cycle at 1 Hz for 5–15 min. In earlier neocortical experiments, slices were incubated for at least a further hour after leaving the cell before fixation. Eventually, however, it became apparent that while this could result in good axonal filling, it typically resulted in a loss of biocytin from the soma, dendrites and the most proximal portion of the axon of interneurons (but not of pyramids), making unambiguous identification and reconstruction impossible. Probably for this reason, only one of the presynaptic neocortical interneurons discussed here was adequately recovered for reconstruction. In more recent hippocampal experiments therefore, slices were incubated for no more than a further 5–15 min after leaving the cells. Provided recordings had exceeded 45–60 min, good axonal filling resulted. Slices were then fixed overnight in 0.1 M phosphate buffer containing 1.25% glutaraldehyde and 3% paraformaldehyde. After embedding in gelatin, the slices were sectioned at 60 μm on a Vibratome. Injected biocytin was localized using the Vector ABC kit (Elite), incubated with 0.1% Triton overnight. The injected biocytin was then visualized using 3,3′-diaminobenzidine (Sigma) and the sections dehydrated and embedded in Durcupan resin (Fluka) on slides. Well-filled and characterized interneurons were fully reconstructed using a drawing tube and a ×100 objective.

RESULTS

During recordings, cells were identified as interneurons by two criteria; firstly by the fast spiking behaviour that typifies several classes of neocortical and hippocampal interneurons. Secondly, some of the interneurons elicited in a simultaneously recorded cell an IPSP with a brief and constant latency. Some regular spiking interneurons that were not recorded simultaneously with a postsynaptic partner are likely to have been missed. In addition a number of the recorded interneurons were subsequently recovered histologically (see below). In 24 experiments in neocortex (96 slices) and 17 experiments in the CA1 region of the hippocampus (68 slices) a cell that was identifiable at the time of recording as an interneuron was recorded simultaneously with a pyramidal cell in 85 neocortical paired recordings and 179 hippocampal paired recordings. This constitutes approximately 10% of all paired recordings made in these regions, the majority of which involved two pyramidal cells. Fourteen of the neocortical (14:85, or 1:6) and 39 of the hippocampal (39:179, or 1:4.5) paired recordings yielded a monosynaptic fast IPSP. The majority of neocortical interneurons in this study were in layer V (including all those eliciting slow IPSPs) and the somata of all reported CA1 interneurons were located in, or close to stratum pyramidale (SP). Fast IPSPs recorded in neocortex have been reported in some detail previously, the fast IPSPs in hippocampus form part of another study, and only those tested with bicuculline will be discussed further here.

Inhibitory postsynaptic potentials apparently mediated only by GABA A receptors

Three paired recordings in neocortex (two in layer V, one in layer III) and seven in hippocampus in which a single spike in the presynaptic interneuron elicited a short latency IPSP, were challenged with bicuculline. An additional hippocampal pair was challenged with picrotoxin. In control conditions, these IPSPs had constant latencies around 1 ms, and durations between 20 and 100 ms. In response to high-frequency spike trains, GABA A IPSPs summed (see Fig. 2, Fig. 5, Fig. 10). The second and subsequent IPSPs were usually smaller than the first (measured as the difference between the peak of that IPSP and the falling phase of the preceding IPSP, see Fig. 2, Fig. 5, Fig. 10A) and plateau potentials reached during prolonged activity were between 50% and 150% of the average amplitude of the first IPSP. These summating plateau events were maintained throughout long spike trains and then decayed at least as rapidly as the single spike IPSP. In all pairs tested, single spike responses were blocked with a low dose (1–5 μM) of bicuculline within 3–15 min. However, with these low doses, when brief, high-frequency presynaptic spike trains were elicited, a small, longer latency response was elicited in some pairs. This response, which was typically <15% of the amplitude of the control single spike IPSP appeared however to be mediated by GABA A receptors. Its occurrence was accurately time-locked to the second or third presynaptic spike and its latency, from that spike, was very constant and brief (analysis using the rising phase of the second or third spike as the trigger was essential for this procedure). When its shape was compared with the shape of the control IPSP, scaled to match in amplitude, no difference was apparent. Moreover, increasing the bicuculline concentration blocked this response entirely and even long trains of presynaptic spikes now elicited no detectable response in the three neocortical pairs or in five of the seven hippocampal pairs tested (the bicuculline resistant, slow IPSPs in the remaining two hippocampal pairs are described below). Picrotoxin blocked the IPSP totally in the one CA1 pair tested. These then were pure fast GABA A receptor-mediated responses.

One of the neocortical interneurons eliciting a ‘pure’ fast IPSP was a fast spiking cell which was fully recovered histologically and was a small, smooth layer III interneuron with a dense local axonal arbour. The other two neocortical
interneurons that elicited demonstrably pure fast IPSPs, were not fully recovered. One was a regular spiking and one a fast spiking cell (see Ref. 36 for firing pattern descriptions). The six CA1 interneurons that elicited pure fast IPSPs were fully recovered histologically and all had extensive axon ramifying throughout the depth of SP and extending little beyond its borders, i.e. a basket cell-like axonal arbour (see Fig. 3. for an example). Three of these were fast spiking cells with a rounded spike after-hyperpolarization, one a regular spiking cell (RS), and two were burst firing cells (BF, see Ref. 1 for description of these firing patterns in CA1 basket and bistratified interneurons).

**Interneurons eliciting pure slow inhibitory postsynaptic potentials in neocortex**

In three of the 85 paired recordings in neocortex that involved an interneuron, a single interneuronal action potential elicited no discernible response in the simultaneously recorded pyramidal cell, even when the postsynaptic pyramidal cell was depolarized up to and beyond spike threshold and when large spontaneous fast IPSPs were apparent. However, trains of spikes elicited a long-latency IPSP. In one of these three examples the classical fast spiking (CFS) presynaptic interneuron was fully recovered histologically (960509B2, reconstructed in Fig. 7). In the other two examples, what appeared to be interneuronal axon was apparent in the recorded region and beaded aspiny interneuronal dendrites were also recovered histologically from one fast spiking presynaptic cell (950209A1). Since one of these interneurons was a CFS cell (960509B2), one a regular spiking cell (RS, 960716A1) and one a fast spiking cell (FS, not classical, 950209A1), the presynaptic firing pattern did

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**Fig. 2.** Two inhibitory connections in CA1 that appear to be mediated solely by GABA\_A receptors. In control conditions (A and C), single spikes and double spikes (C) elicit brief, short latency IPSPs. Following addition of bicuculline (B and D), all postsynaptic response, even to high-frequency spike trains, is blocked. In D the control response to a similar high-frequency spike train (normal trace) is compared with the response in bicuculline (bold trace). The dotted lines in C and D indicate the time-course of the single spike control IPSP. In each case, a typical single sweep record of the presynaptic interneuron’s firing is shown above an average (30–100 sweeps) of the postsynaptic response. A reconstruction of the interneuron that elicited the IPSPs in C and D is shown in Fig. 3 (970618B1, 970619C1.)

**Fig. 3.** The IPSPs illustrated in Fig. 2, were both elicited by CA1 interneurons with basket-like axonal arbours. One is reconstructed here in its entirety at ×100 magnification. The axon (blue) ramified throughout the depth of stratum pyramidale (SP), but extended little beyond its borders and its dendrites (red) extended through stratum oriens (SO) and through stratum radiatum (SR) and into stratum lacunosum moleculare (SLM). The dotted lines indicate the borders of these hippocampal layers. The insert indicates the position of this cell in the slice from which it was recorded. (970618C1.)
Fig. 3.
not appear to predict the ability of an interneuron to elicit a slow IPSP.

Since two of the presynaptic neurons were fast spiking cells and sufficiently recovered histologically for identification, these two slow IPSPs were demonstrably elicited by interneurons. Fast spiking behaviour has never been reported for a pyramidal cell, but is regularly described in identifiable interneurons. The third presynaptic cell was regular spiking and therefore not so easily distinguishable from a pyramidal cell by its electrophysiological characteristics and only its axon was recovered histologically. This pair (960716A1) was however tested with bicuculline to exclude the possibility of a disynaptic fast IPSP resulting from a pyramid–interneuron–pyramid circuit. The slow IPSP was not reduced by bicuculline (10 μM, Fig. 4), which blocked spontaneous fast IPSPs in the postsynaptic pyramidal cell within 3 min of application. In addition, in all other respects this slow IPSP resembled those elicited by the more easily identifiable interneurons.

Inputs to ‘pure slow’ inhibitory postsynaptic potential generating interneurons

Before the connection involving the slow IPSP was studied, two of these interneurons were found to receive inputs from other cells. One was postsynaptic to a second interneuron which elicited in it a short latency IPSP (Fig. 5) with a width at half amplitude of 9 ms, i.e. briefer than any of the IPSPs reported in pyramidal cells to date (range 10 to >100 ms). This IPSP summed on repetitive activation of the presynaptic interneuron. Another of the interneurons presynaptic to a slow IPSP was postsynaptic to a layer III pyramidal cell, which elicited in it an excitatory postsynaptic potential (EPSP) about 1 mV in amplitude. This EPSP exhibited pronounced paired pulse depression (Fig. 5). To generate the ≥3 spike bursts necessary for activation of the slow IPSP, such an excitatory input would probably therefore be inadequate alone.

Amplitude of pure slow inhibitory postsynaptic potentials and effect of presynaptic spike number

Data subsets in which the postsynaptic MP and average presynaptic firing rate were the same were selected and where at least 10 and up to 30 sweeps involved the same number of presynaptic spikes, average IPSP amplitudes were calculated. With presynaptic spike trains of ≥100 Hz, and at least 10 spikes, mean IPSP amplitudes were 0.38 ± 0.3 (S.D., 950209A1), 2.91 ± 0.8 (960509B2) and 2.6 ± 0.81 mV (960716A1), respectively, for the three pairs at postsynaptic MPs between −60 and −65 mV. In all three pairs, IPSP amplitude was strongly influenced by spike number between three to four and 10 spikes. No discernible IPSP was elicited with single or double presynaptic spikes and only a barely detectable response to three spikes, while between four and 10 spikes, IPSP amplitude increased sharply with spike number. However, beyond 10 spikes little additional increase in amplitude was apparent (Fig. 6, see Fig. 15B for the model fit to these data). The presynaptic interneuron for the pair illustrated in Fig. 6 is reconstructed in Fig. 7.

Effect on slow inhibitory postsynaptic potentials of presynaptic spike train duration

Provided spike frequency was >50–100 Hz, the
duration of the presynaptic spike train had little or no influence on the amplitude of the IPSP, when spike number was held constant. For example, trains of 10 spikes at 100 Hz lasting 90 ms and 10 spike trains at 250 Hz lasting 36 ms elicited similar amplitude IPSPs. Spike train duration also had only a small influence on IPSP duration (see Fig. 8D) and the correlation between these parameters was weak. In one pair (960509B2), spike trains briefer than 60 ms generated IPSPs with a mean duration of $117\pm38$ ms ($n=54$), while trains 100 to 160 ms in duration resulted in IPSP durations of $171\pm36$ ms ($n=38$). However, responses to more prolonged trains peaked and had almost decayed before the spike train ended (Fig. 9B). Thus, in contrast to the summing GABA_A receptor-mediated IPSPs elicited in other pairs by long spike trains, these slow IPSPs lasted no longer than 200 ms after the first spike, even with long spike trains.

**Effect of presynaptic spike frequency on slow inhibitory postsynaptic potential time-course**

Presynaptic spike frequencies below 50 Hz appeared to generate little postsynaptic response and were not studied systematically. Between 75 and 300 Hz presynaptic firing frequency had only a small, non-significant influence on IPSP amplitude, when trains with similar numbers of spikes were compared. Frequency had a small influence on latency however (see Figs 8A and 9A). In one pair (960716A1), frequencies of 170–200 Hz resulted in latencies of $41\pm5.5$ ms ($n=13$), frequencies of 200–220 Hz, latencies of $36\pm3.6$ ms ($n=19$), frequencies of 220–250 Hz latencies of $33\pm3.2$ ms ($n=13$) and frequencies of 250–300 Hz latencies of $26\pm4$ ms ($n=4$). In another pair (950509B2) frequencies of 140–180 Hz yielded latencies of $39\pm10$ ms ($n=83$) and frequencies of 200–230 Hz, latencies of $31\pm9$ ms ($n=49$). In one pair firing frequency had no significant effect on IPSP rise time, in another frequencies between 75 and 150 Hz resulted in rise times of $42\pm12$ ms ($n=58$) and rise time decreased at frequencies above 180 Hz to $30\pm13$ ms ($n=56$). Single sweep events in the third pair were too small to analyse in this way. To determine whether repetition rate influenced IPSP amplitude, slow IPSPs were elicited at two repetition rates (0.33 and 0.1 Hz) and events elicited by spike trains of similar duration and frequency compared. There was no significant difference in the amplitudes of the IPSPs at these two rates.

The largest data subset in which all parameters were constant was selected and an averaged IPSP computed. Neither the rising phase nor the decay of this averaged IPSP was well fit by a single exponential, the decay approximating to the sum of two to three exponentials and the rising phase to a sigmoidal curve (as reported previously, 28). A model fit to the time-course of this IPSP is shown in Figs 14 and 15.
Voltage relations of slow inhibitory postsynaptic potentials

In one pair (960509B2), slow IPSPs generated by trains of $\geq$10 spikes were measured at four MPs. The amplitudes of these IPSPs are plotted against MP in Fig. 8C. A linear regression through these points yielded an extrapolated reversal potential of $-62\text{mV}$.

Fig. 6. Slow IPSPs elicited by a single fast-spiking interneuron increase in amplitude with increasing numbers of presynaptic spikes. Spike trains and postsynaptic responses to them were collected into data subsets according to the number of spikes and the spike frequency and averaged. The number of spikes in the train that elicited each response is indicated to the left of each average and the mean frequency of the spike trains included to the right of each averaged IPSP. Single sweep examples of typical presynaptic spike trains are shown on the far left. The interneuron that was probably responsible for this IPSP is reconstructed in Fig. 7. (960509B2.)

Fig. 7. A light level ($\times\ 100$ magnification) drawing tube reconstruction of the layer V interneuron that elicited the slow IPSPs shown in Fig. 6. (960509B2). A level of ambiguity as to the identity of this interneuron exists because two interneurons were recorded in this region of the slice (see Fig. 5. for the IPSPs elicited in the slow IPSP-generating interneuron by another interneuron). However, the other interneuron was recorded only relatively briefly, not actively filled with biocytin and then incubated for $>1.5\text{h}$ in the bath before fixation. Such long post-filling incubation times typically lead to loss of biocytin from soma, dendrites and most proximal axon even of actively and well-filled interneurons (but not of pyramids). The slow IPSP generating interneuron on the other hand, was recorded for $>1\text{h}$, actively filled by current injection and fixed within 20--25 min of filling. The soma and dendrites of the reconstructed interneuron (red) were completely, but less intensely stained than pyramidal cells filled during the same experiment and the axon (blue) was intensely stained. The insert shows the position of this interneuron in the slice from which it was recorded. (960509B2.)
A similar voltage relation was apparent in the other two pairs, but was studied less systematically.

Inhibitory postsynaptic potentials mediated by both GABA_A and GABA_B receptors

The sensitivity of the neocortical slow IPSPs to current injected at the soma might suggest that these were relatively proximal events. However, the presynaptic neocortical interneurons were either not adequately recovered, or their treatment with Triton precluded confirmation of synaptic sites with correlated ultrastructural morphology. To determine therefore whether proximal inhibitory inputs could be mediated by GABA_B receptors, paired recordings were made in SP of the CA1 region of the hippocampus where the subcellular postsynaptic targets of filled interneurons can be more readily identified than in neocortex. None of the paired recordings to date involving SP interneurons has yielded a demonstrably pure slow IPSP (>100 connected pairs), but two yielded a mixed GABA_A/GABA_B IPSP. Both of these presynaptic CA1 interneurons were BF cells. One was only partially recovered. Its axon appeared to ramify in both stratum oriens (SO) and stratum radiatum (SR), but less in SP, i.e. it resembled a bistratified cell. The slow, bicuculline-resistant component of the IPSP generated by this putative bistratified interneuron was small (0.2–0.3 mV) and it was not studied in detail, but its shape and time-course were similar to those of the other mixed IPSP.

The other CA1 presynaptic interneuron was fully recovered and had a basket cell-like axonal arbour (Fig. 12). Under control conditions, single presynaptic basket cell spikes elicited an IPSP 1.07 mV in average amplitude, latency 1 ms, rise time 9 ms and width at half amplitude 33 ms in this pair (970319A). Trains of presynaptic spikes elicited summing IPSPs. The average amplitude of second and subsequent IPSPs in trains was, however, smaller than that of first IPSPs. The amplitude of the second IPSP (measured from its peak to the falling phase of the 1st IPSP) was 33% of the amplitude of the first at interspike intervals between 7 and 9 ms. Subsequent IPSPs were further depressed (Fig. 10A), but during prolonged trains a plateau was attained and maintained. At the time of recording, a mixed fast/slow IPSP was suspected, but the slow component is not

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Fig. 8. (A) Slow IPSP latency is weakly inversely correlated with the presynaptic firing frequency preceding the IPSP (average frequency of the first four spikes in each sweep). (B) IPSP duration is weakly correlated with spike train duration. Each point was obtained from a single sweep record. (C) The extrapolated reversal potential for one of the slow IPSPs in neocortex is close to −90 mV. Maximum IPSP amplitudes for sweeps with presynaptic firing rates >100 Hz and 10 or more spikes in the train are plotted against postsynaptic MP, for one pair (960509B2).

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−88 mV. A similar voltage relation was apparent in the other two pairs, but was studied less systematically.
readily apparent in averaged recordings under control conditions. Indeed, as illustrated in Fig. 10A, the slow component coincided with the rebound depolarization that followed short trains of fast IPSPs.

The single spike fast IPSP was completely blocked by 10 μM bicuculline within 5 min. Complete blockade of the summing GABA_A IPSP elicited by longer spike trains took 20 min. GABA_A receptor blockade revealed a long-latency, slow IPSP (Fig. 10A) which was greatly reduced by addition of 100 μM CGP 35348 within 20 min (Fig. 10B), at which time the recording was lost. Like the ‘pure’ slow IPSPs in neocortex, the amplitude of this IPSP was strongly influenced by the number of presynaptic spikes that elicited it (see Fig. 11). No discernible postsynaptic response was elicited by one or two presynaptic spikes. Between three to four and 10 spikes IPSP amplitude sharply increased and little additional increase in amplitude was achieved with larger numbers of spikes. The stereotypic firing pattern of this BF interneuron, whose threshold response was a high-frequency burst of at least two and more usually four or five spikes, precluded assessment of the influence of a wide range of tonic firing frequencies. This slow IPSP was, however, longer in duration than the ‘pure’ slow IPSPs in neocortex and the response to 200 ms spike trains did not decline significantly until the train had ended and it lasted ≈400 ms. Fig. 12 shows the reconstruction of the presynaptic interneuron of this pair. Its axonal arbour spans the entire depth of and is almost entirely restricted to SP indicating that this was a basket cell. The dendritic arbour is, however, unusual for a basket cell (compare Fig. 12 with Fig. 3), having a single ‘apical’ dendrite that does not branch until it has traversed approximately half the depth of SR and then branches significantly in SLM.

**Computational models**

‘Spillover’ hypothesis. Models first investigated whether the ‘spillover’ hypothesis explained the nonlinear spike number-dependencies apparent for the slow IPSPs. A model of extracellular release, diffusion and uptake of GABA (see Fig. 13A and Experimental Procedures) was used to determine whether extracellular mechanisms can lead to accumulation of GABA only when multiple high-frequency presynaptic spikes occur. Simulation of a terminal releasing at 200 Hz in the presence of uptake led to a very brief presence of GABA in the extracellular space immediately adjacent to the release site and a limited duration presence 1 μm and 2 μm away from the release site (Fig. 13B, Control). Without uptake, the time-course was still brief in the cleft but the spillover of GABA outside the cleft was enhanced (Fig. 13B, No uptake). With a smaller diffusion coefficient to account for extracellular space tortuosity, and with uptake, the peak concentration of GABA was diminished extrasynaptically, but its accumulation was enhanced (Fig. 13B, Low diffusion).

If an extracellular mechanism is to account for the observed spike number-dependency of GABA_B responses, a similar dependency on spike number should be apparent in the model at extrajunctional sites. To test this, the total integrated amount of GABA at a distance of 2 μm from the release site was computed. The relative amount is represented for different numbers of presynaptic spikes at 200 Hz in Fig. 13C. In control conditions and without uptake, the relation can be seen to be perfectly...
linear. However, a significant deviation from linearity occurred for high concentrations of GABA transporter ($B_m = 1 \text{mM}$). Variations in other parameters, such as the size of the grid, the diffusion coefficient, the uptake parameters and the amount of GABA released led to similar behaviour: the relation was linear for most parameter values. Non-linear relations occurred only with high concentrations of transporter and only at a substantial distance from the release site ($2 \text{m}$ here). Saturation of the response with larger spike numbers (as observed experimentally) was, however, not obtained with this model.

Fig. 10. A mixed GABA$_A$/GABA$_B$ IPSP in the hippocampus. (A) A burst firing presynaptic CA1 interneuron with a basket cell-like axon (see Fig. 12) elicits in a pyramidal cell, a short latency, summing fast IPSP (composite average of responses to single spikes, double, triple and quadruple spikes, and brief five spike trains triggered from the 1st...5th presynaptic spikes, respectively) in control conditions (bold trace). After addition of bicuculline, the fast IPSP is blocked and a slow IPSP, generated by a similar spike train is revealed. (B) The slow IPSP revealed by blockade of GABA$_A$ receptors (normal trace, labelled bicuculline) was greatly reduced by addition of $100 \mu \text{M CGP 35348}$, a selective GABA$_B$ antagonist. Two representative presynaptic spike trains of the types elicited in the presence of CGP 35348 are shown above. (970319A1.)

The G-protein hypothesis. To investigate a possible intracellular origin of the observed non-linearity, we also investigated a kinetic model of the G-protein transduction mechanisms underlying GABA$_B$ responses (see details in Experimental Procedures and references therein). The principle of G-protein transduction is schematized in Fig. 14A: GABA binds to the receptor, leading to its activated form. The activated receptor catalyses the activation of G-proteins (GDP-GTP exchange) on the intracellular side of the membrane. Active G-proteins (G*) can either affect the gating of the channel or be degraded back to the inactive form. Simulation of this mechanism is shown in Fig. 14B, with the typical slow time course of G-protein illustrated for a single presynaptic spike as well as for a train of 10 spikes at 200 Hz. The peak G-protein concentration was proportional to the number of presynaptic spikes, according to a standard Michaelis–Menten saturation curve (Fig. 14C). This model can result in the saturation of the

Fig. 11. Spike number dependence of the slow component of the mixed GABA$_A$/GABA$_B$ IPSP. Three typical presynaptic spike trains are illustrated above and averaged responses to two (normal trace), five (bold), eight or nine (normal trace), and 10 to 13 (bold) presynaptic spikes are compared below. Two presynaptic spikes elicited almost no postsynaptic response. An almost maximal response was elicited by eight or nine spikes. The response to the longer spike trains was, however, prolonged and the IPSP elicited by the longest spike trains (10–13 spikes) hardly decayed before the end of the train. (970319A1.)

Fig. 12. The CA1 basket cell that elicited a mixed GABA$_A$/GABA$_B$ IPSP is drawn in its entirety ($\times 100$ magnification). The axon (blue) ramifies throughout the depth of stratum pyramidale (SP) and the dendrites (red) extend through stratum oriens (SO), and through stratum radiatum (SR) branching in stratum lacunosum moleculare (SLM). The dotted lines indicate the borders of these hippocampal layers. The insert shows the position of the interneuron in the slice. (970319A1.)
Fig. 12.
response with large numbers of spikes. However, if the K+ currents were simply proportional to the peak G-protein concentration, this mechanism alone would be insufficient to account for the non-linearity observed experimentally with low spike numbers.

A model with a nonlinear transduction mechanism has been proposed based on multiple binding sites for G-proteins on the K+ channels.9 Using a similar model here (see Experimental Procedures), the experimental data were used to constrain the model directly and to estimate (i) to what extent this model can account for the data and (ii) what are the optimal values for parameters such as the number of binding sites. Fitting was provided by using directly the information from the paired intracellular recordings. The train of action potentials recorded in the presynaptic interneuron was used in the presynaptic compartment of the model, and the time-course of the simulated postsynaptic response was compared with the averaged IPSP recorded in the postsynaptic pyramidal cell (largest data set available with consistent MP, spike number

Fig. 13. Simulation of the ‘Spillover hypothesis’ for GABA_B responses. (A) Extracellular space was simulated using a 12 x 12 two-dimensional grid consisting of 0.5 x 0.5 μm extracellular compartments. A release site for GABA is indicated in light grey, as well as two sites in which the transmitter concentration was analysed. (B) Time-course of [GABA] following successive releases occurring at the same site at 200 Hz, shown in the three sites indicated in A. The time-course of GABA is compared in control conditions (Control), following block of uptake (No uptake) and using a smaller diffusion coefficient (Low diffusion). (C) Transmitter presence 2 μm from the release site shown as a function of the number of presynaptic spikes. The extracellular GABA concentration was integrated and normalized to the value obtained with 20 spikes. The relation was approximately linear for control conditions (B_n = 0.1 mM), low diffusion and without uptake (B_n = 0). However, a significant deviation from linearity occurred for high concentrations of GABA transporter (B_n = 1 mM).
and frequency was used). Best fits of the model for different numbers of binding sites \((n)\) are shown in Fig. 15 (left panel). The minimal error was attained with \(n = 2\), while \(n = 4\) and \(n = 8\) showed relatively small errors. In addition, a complete spike-number/response amplitude curve, for this pair was used to constrain the model further (Fig. 15B, mean IPSP peak amplitudes for a series of data sets with similar MP and firing frequencies, but different numbers of spikes are plotted). In this case, the optimal fits were obtained with \(n = 4\) and \(n = 8\). Using both constraints therefore, the number of binding sites that was most consistent with the data was \(n = 4\).

The observation, that in neocortex (though not in CA1) slow IPSP duration reached a maximum which could not be increased by increasing spike train duration, was also tested in the model. Although the increase in simulated IPSP duration with increases in train duration was not linear, it did not saturate. Thus while the present model reproduces well the CA1 mixed IPSP data, another component is required to describe fully the neocortical slow IPSP durations.

For the pooled data plot illustrated in Fig. 16, single sweep records were first separated into subsets in which spike number and firing frequency were the same and peak amplitudes of each single sweep IPSP were measured. Mean peak IPSP amplitudes were then plotted against spike number (separately for each frequency range and for each pair) and peak IPSP amplitudes normalized using the peak amplitude achieved with a sigmoidal curve fit to the data. The optimal sigmoid for the pooled, normalized data is shown in Fig. 16A. The model fits to these pooled data show that \(n = 4\) binding sites reproduces the data from all three pairs relatively well (continuous line in Fig. 16B) while a linear model (\(n = 1\); dotted line in Fig. 16B) had a Michaelis–Menten type of saturation curve (as in Fig. 14C) and did not capture well the GABAB response curve. The optimal fit shown in Fig. 16B was refined from Fig. 15 (\(n = 4\)) by optimizing the value of \(K_d\) with same kinetic parameters (\(K_1\) ...\(K_4\), see Experimental Procedures and Fig. 15 legend). In this case, the model captured well both the dependency on the number of spikes and the 10-spike IPSP time-course (inset Fig. 16B).

Other models of activation of \(K^+\) channels with G-proteins were also tested to fit the nonlinear relationship between number of presynaptic spikes and IPSP amplitude. A Hodgkin–Huxley\(^{15}\) (1952) like model, in which each \(K^+\) channel had \(n\) independent gates and each gate was opened by G-protein binding (see Experimental Procedures), fit the data optimally when \(n = 4\) (not shown). Another model tested was the allosteric activation of the \(K^+\) channel by binding of G-proteins on the open state of the channel (see Experimental Procedures). Here again, \(n = 4\) binding sites gave the best fits to the data (Fig. 16C). For the three models tested, computing the dose–response relation between simulated IPSP amplitude and GABA concentration during a 1 s extracellular GABA application, led to Hill

\[ GABA_B \] IPSPs in neocortex and hippocampus

Fig. 14. Simulation of the G-protein transduction mechanism underlying GABA\(_B\) responses. (A) Scheme of the release of GABA, activation of the receptor by binding of GABA, and catalysis of G-proteins into activated form (\(G^*\)) by the bound receptor. Subsequently, activated G-proteins may affect the gating of ion channels as well as participating in other biochemical mechanisms, or be degraded into inactive form (indicated by the dashed arrow). (B) Typical slow time-course of G-protein concentration following one or 10 presynaptic spikes at 200 Hz. (C) Peak G-protein concentration as a function of the number of presynaptic spikes (same parameters as in Experimental Procedures).
coefficients of $1.8 \pm 0.2$ for $n = 4$ binding sites (not shown), consistent with the values of 1.4–2.1 measured experimentally. These models all indicate that $n = 4$ G-protein binding sites can optimally account for the GABA$_B$ non-linearity, and that this conclusion is not dependent on the particular kinetics assumed for the models, within the context of the present approximations.

**DISCUSSION**

This paper reports for the first time ‘pure’ slow IPSPs elicited in neocortical pyramidal cells and the first demonstrably mixed. GABA$_A$/GABA$_B$ receptor-mediated IPSP in a cortical region using dual intracellular recordings. It also answers a number of relatively controversial issues. Firstly, the firing of a single hippocampal interneuron can release sufficient GABA to activate GABA$_B$ receptors. Simultaneous firing in several interneurons with very close terminal zones might activate extra-synaptic receptors, but simultaneous activity is not always a requirement. Secondly, confirming suggestions made in studies using less direct methods, some IPSPs in both neocortex and in the CA1 region of the hippocampus were found to be mediated solely by GABA$_A$ receptors. Even prolonged trains of high-frequency firing induced no discernible postsynaptic response when GABA$_A$ receptors were blocked. Whether the GABA released by these terminals can access extra-synaptic GABA$_B$ receptors, but activate them only when additional GABA is released by other terminals, was not determined. Thirdly, ‘pure’ slow IPSPs can be elicited in neocortex. In 85 dual recordings in which one of the neurons was a neocortical interneuron, three yielded a slow IPSP, i.e. with a random selection of interneurons and of putative pyramidal targets in layer V, a slow IPSP was elicited in one of 30 pairs. Since 14 of the 85 pairs in this series yielded a fast IPSP, ‘pure’ slow IPSPs occurred in three of 17 (or approximately one in five) monosynaptic inhibitory connections studied in layer V of the neocortex in this series. No mixed IPSPs were encountered in neocortex in this series (although only three fast IPSPs were challenged with a GABA$_A$ antagonist) and no ‘pure’ slow IPSPs have yet been seen in CA1 when the outputs of SP interneurons are studied. Finally, these experiments demonstrated that single axon CA1 IPSPs can be mixed. In two of the eight SP connections challenged with bicuculline or picrotoxin a slow IPSP, which was later blocked by CGP 35348 (in one pair), was revealed.

**Disynaptic inhibitory postsynaptic potentials?**

One obvious concern is that long latency events, particularly those elicited by repetitive presynaptic firing, might be disynaptic. Disynaptic IPSPs have been studied extensively in both regions (unpublished data), but have distinctly different properties from the slow IPSPs reported here. Although disynaptic IPSPs latencies are a few milliseconds longer and somewhat more variable (2–5 ms after the 1st, 2nd or 3rd spike) than those of monosynaptic fast IPSPs (1 ms), fast rising events can clearly be seen on single sweeps. Moreover, the disynaptic IPSPs studied to date have been readily blocked by 1–5 μM bicuculline. Unlike the present slow IPSPs, large, disynaptic IPSPs in neocortex are readily elicited by two to three spikes and in CA1 by one to two presynaptic pyramidal spikes in appropriate paired recordings. Moreover, the amplitude of these disynaptic events is not increased by increasing spike number. In addition, with disynaptic IPSP recordings the presynaptic neuron displays typical pyramidal behaviour and no filled interneuronal axons or dendrites have been apparent on subsequent histological processing.

In this study, both CA1 interneurons mediating mixed GABA$_A$/GABA$_B$ IPSPs and one of the neocortical interneurons eliciting a slow IPSP were fully recovered histologically and filled interneuronal axons and in one case dendrites, were apparent in the recorded region in the two remaining experiments. Two of the presynaptic interneurons in neocortex were fast-spiking layer V cells and unlikely therefore to be pyramidal cells and the IPSP elicited by the third (RS) neocortical interneuron was insensitive to bicuculline. The neurons eliciting slow IPSPs in the present study are therefore likely to be GABAergic interneurons. The question therefore arises as to whether an interneuron–interneuron–pyramidal disynaptic connection could have resulted in the present data.

Although a strong IPSP can result in ‘rebound’ activation of a postsynaptic cell,7,36 it is unlikely that this would occur before the presynaptic spike train and therefore the inhibition had ended. If, therefore, these slow IPSPs had resulted from a disynaptic connection involving two serially connected interneurons, latencies longer than the presynaptic spike train would have resulted (for an example of a summing IPSP recorded in an interneuron see Fig. 5). This was clearly not the case. Moreover, single spikes and very brief trains would have been just as effective as longer trains.

The final possibility explored was electrical gap junction coupling between the recorded interneuron and others nearby. Two observations suggest that this possibility can be discounted in relation to the present data; firstly the extremely tight correlation between recorded presynaptic spike number and slow IPSP amplitude even when trains with different numbers of spikes were elicited by identical current pulses and secondly the absence of any ‘dye-coupling’ between well filled interneurons and any other cells in the slice. Gap junctions could be a very effective way of recruiting interneuronal
populations, but do not appear to contribute to the present results. It seems likely therefore that the ‘pure’ slow IPSPs in neocortex and the mixed IPSPs in CA1 originated from monosynaptic connections.

A proximal location for some GABA_B receptor-mediated inhibitory postsynaptic potentials?

The relatively steep voltage sensitivity of one of the neocortical slow IPSPs and the basket-like
axonarbour of one of the interneurons mediating a mixed CA1 IPSP, demonstrate that slow IPSPs can be elicited by proximal inputs. Whether GABA<sub>B</sub> IPSPs are more likely to occur in distal dendritic regions must await a systematic study using postsynaptic dendritic recordings. The small amplitude of the bicuculline-insensitive component of the mixed IPSP elicited by a bistratified interneuron in CA1 may have resulted from its less proximal location, although its time-course cannot be entirely explained by dendritic filtering since the GABA<sub>A</sub> IPSP from this connection was no slower (width at half amplitude 42 ms) than many of the ‘fast’ IPSPs elicited by basket cells. The present estimate, that one in five of the relatively proximal inhibitory connections in layer V of the neocortex results in a pure slow IPSP and may be mediated solely by GABA<sub>B</sub> receptors, indicates that these may not be uncommon occurrences.

GABA<sub>A</sub> inhibitory postsynaptic potentials indicate a relatively high release probability

Slow IPSPs elicited in thalamic relay cells required longer presynaptic spike trains than those required in the present study in cortex and hippocampus. Several explanations could be proposed to account for this difference. Perhaps the most likely derives from the differences apparent in the frequency-dependent characteristics of fast IPSPs elicited by spike trains in the two studies. In cortical regions, both in this and in our previous studies, fast IPSPs typically displayed paired-pulse depression, indicating a relatively high probability of GABA release in response to the first spike. Moreover, failures of transmission are very rare when large fast IPSPs are studied. In contrast, in thalamus, strong facilitation of fast IPSPs was apparent, indicating that each contributory synaptic release site has a low probability of release. Clearly, if repetitive release from the same terminal is required for the extracellular GABA transient to be extended and for receptor activation to be prolonged thereby, higher frequencies of firing, or longer presynaptic spike trains would be required where release probabilities were low. That postsynaptic cortical inhibitory sites are exposed to repetitive GABA release at GABA<sub>A</sub> receptor-mediated connections was indicated by the results obtained with threshold antagonist doses of bicuculline. These doses could block all response to single spikes, but a second or third spike in quick succession appeared to release sufficient additional GABA
to compete with the antagonist and partially relieve the blockade.

**Strength and time-course of slow, putative GABA<sub>B</sub> receptor-mediated inhibitory postsynaptic potentials**

The largest single sweep slow IPSPs reported here were between 4 and 5 mV in amplitude at postsynaptic MPs of -57, or -52 mV. They can therefore be large enough to hyperpolarize the postsynaptic cell significantly and, indeed, at these MPs could delay spontaneous postsynaptic firing. In this they were, however, less effective than many proximal GABA<sub>A</sub> IPSPs. What is perhaps rather surprising is the relatively brief and very restricted duration of the neocortical ‘pure’ slow IPSPs. Not only was the entire event only twice the duration of some of the broader GABA<sub>A</sub> IPSPs recorded in these areas, but in neocortex, the slow IPSP could not be prolonged by longer spike trains.

The model that best reproduces the other characteristics of the slow IPSP does not predict this restricted time-course. An additional mechanism is required. Desensitization/inactivation of one of the events in the cascade might occur, or the pattern of neurotransmitter release at these slow neocortical connections might differ from that at other cortical GABAergic synapses. In a mixed GABA<sub>A</sub> connection, the pattern of GABA release could be assessed from the fast IPSPs elicited. Although release declined during high-frequency activity, it rapidly reached a plateau that was then maintained for long spike trains. Interestingly, at these mixed CA1 connections, the slow IPSP was not as restricted in duration as ‘pure slow’ neocortical IPSPs. Release at these ‘pure slow’ connections cannot be assessed in the same way and might have declined significantly towards the end of longer spike trains.

For significant, long-lasting inhibition, repetitive activation and summation of GABA<sub>A</sub> IPSPs may be more effective than activation of slow IPSPs. The functional relevance of a brief GABA<sub>B</sub> component of a mixed IPSP is unclear. This component was almost completely masked under control conditions by the higher conductance summing fast components in CA1 pyramids. It remains possible that the activation of GABA<sub>B</sub> receptors results in an additional postsynaptic effect that is not readily apparent in electrophysiological recordings.

**Interneurons mediating slow inhibition**

It is unclear at present whether specific interneurons elicit postsynaptic effects only via GABA<sub>B</sub> receptors. The three presynaptic neocortical cells reported here each had different firing characteristics. If these correlate with other parameters, more than one ‘class’ of neocortical interneuron could activate pure slow IPSPs. Moreover, the slow IPSPs recorded in neocortex were ‘pure’ only in the sense that no GABA<sub>A</sub> receptor-mediated component was apparent in the postsynaptic target that was recorded. Other targets, that were not recorded, may have responded via GABA<sub>A</sub> receptors or via both GABA<sub>A</sub> and GABA<sub>B</sub> receptors. It remains a possibility that some targets are rapidly and profoundly inhibited with low-frequency presynaptic firing via GABA<sub>A</sub> receptors, while others are unaffected in the absence of repetitive firing and even with long trains of interneuronal spikes inhibited only for a relatively brief time. Answers to these questions must await multiple target recordings and/or immunolocalization of the postsynaptic GABA<sub>B</sub> receptors. However, the relatively brief events reported here are not the only inhibitory events elicited by repetitive firing of a single interneuron in neocortex. Although the pharmacology of the more slowly activating and decaying events elicited by some fast spiking basket cells that also elicited fast IPSPs, was not determined, it remains possible that these events represent a more long-lasting GABA<sub>B</sub> receptor-mediated inhibition that can outlast the summed fast IPSP. Conversely, either the present relatively brief, or the previously reported more prolonged slow components, might have been mediated by peptidergic receptors.

**Computational models of GABA<sub>B</sub> receptor-mediated inhibitory postsynaptic potentials**

Slow IPSPs were never observed to follow single or double presynaptic spikes. This observation alone may explain why more intense activation of interneurons has been required in previous studies using less direct methods. It is also consistent with the absence of GABA<sub>B</sub> components in GABAergic miniature events, suggesting that a single release event is insufficient to activate detectable GABA<sub>B</sub> currents. The present observation is also similar to previous reports of the activation of slow versus fast EPSPs in the myenteric plexus, the slow events requiring at least two and the fast events only one presynaptic action potential. In addition, between three and 10 spikes, all reported slow IPSPs increased steeply in amplitude, but beyond 10 spikes little additional increase in amplitude was apparent in any. Experiments therefore reveal an unusual sigmoidal relation between spike number and response amplitude for putative GABA<sub>B</sub>-mediated IPSPs.

Two possible hypotheses were investigated to account for this spike-number dependence. Firstly, a computational model suggested that a ‘spillover’ hypothesis, GABA<sub>B</sub> receptors would be located extrasynaptically and their activation would require significant extracellular accumulation of GABA and therefore the intense activation of interneurons. Simulated diffusion of GABA in a 2-dimensional extracellular space with uptake indicated that accumulation of GABA could...
Indeed occur at extrasynaptic sites (Fig. 13B). However, the integrated amount of GABA accumulated extrasynaptically increased linearly with number of spikes for most of the parameters tested. One exception was that with high concentrations of transporter (∼1 mM) the accumulation of GABA at a distance of 2 μm from the cleft showed nonlinear summation in the micromolar range (Fig. 13C, $B_m = 1$).

Thus, if GABA$_B$ receptors are located extrasynaptically and are sensitive to the micromolar GABA concentration range and if GABA transporters are present at concentrations as high as 1 mM, then the ‘spillover’ mechanism may account for some non-linearity in the relationship between GABA$_B$ IPSP amplitude and number of presynaptic spikes. Predictions of the ‘spillover’ hypothesis are therefore that GABA$_B$ receptors should be located at a significant distance outside the cleft and that the GABA transporters should be present all through the extracellular space at concentrations in the millimolar range. It must be noted that this mechanism does not account for the saturation of GABA$_B$ IPSP amplitudes with 10–12 spikes evidenced in the present experiments. A ‘spillover’ model would need additional mechanisms to explain this observation.

Secondly, the ‘G-protein’ hypothesis postulated that the nonlinear stimulus-dependence of GABA$_B$ responses was due to the properties of the transduction mechanisms linking GABA$_B$ receptors to K$^+$ channels via G-proteins. This mechanism was tested here using different possible transduction mechanisms. All models tested, including Hodgkin–Huxley and allosteric types, predicted that, if the binding of several G-proteins is needed to activate each K$^+$ channel, an optimal number of four binding sites accounts for the present experimental data. In addition, this model also reproduced the sigmoidal rise and multi-exponential decay of GABA$_B$ IPSPs.

Thus, the ‘G-protein’ hypothesis accounts for both the shape of the GABA$_B$ IPSPs (reported both here and previously) and the present spike number/IPSP amplitude relationship, including its saturation, with no need for an extrasynaptic localization of the receptors. If there are multiple G-protein binding sites on K$^+$ channels, a sufficient G-protein concentration must be accumulated to evoke detectable K$^+$ currents. We therefore interpret the absence of GABA$_B$ components in miniature GABAergic events and the absence of any discernible GABA$_B$ response following one or two presynaptic spikes (present data) as attributable to the fact that a single release event does not produce sufficient levels of G-proteins to activate the K$^+$ currents. On the other hand, with multiple high-frequency spikes, G-proteins reach membrane concentrations that can activate K$^+$ currents.

Predictions of the ‘G-protein’ hypothesis would be that the K$^+$ channels underlying GABA$_B$ responses should express several binding sites for G-proteins and that activation of G-protein-dependent K$^+$ currents in membrane patches should reveal nonlinear dose–response relations. A Hill coefficient greater than unity has indeed been observed for GABA$_B$ responses in dissociated hippocampal neurons and is similar to the Hill coefficient of 1.8 obtained in the present model with four G-protein binding sites.

This model is also consistent with other modelling studies of the activation of K$^+$ channels. Since the work of Hodgkin and Huxley, a tetrameric structure has been demonstrated for most K$^+$ channels and shown to account for voltage-dependent or calcium-dependent properties of various K$^+$ channel subtypes. In the present model, the K$^+$ channel associated with GABA$_B$ receptors could be described as a multimer composed of four identical subunits, with one G-protein binding site on each subunit. A similar conclusion has indeed been reached by a combined patch-clamp and modelling study of the K$^+$ channels associated with muscarinic receptors. In this case, the channel was found to activate according to an allosteric model with four binding sites. Our results are in perfect agreement with this type of model, indicating perhaps a principle valid for all G-protein-activated K$^+$ channels.

**Conclusions**

We conclude that some IPSPs in neocortex and hippocampus are mediated solely by GABA$_A$ receptors, others in neocortex appear mediated solely by non-ionotropic, possibly GABA$_B$ receptors, while some in CA1 are mediated by both GABA$_A$ and GABA$_B$ receptors. The GABA released by a single interneuron, provided it fires at least three spikes at high frequency, is sufficient to activate postsynaptic GABA$_B$ receptors. The nonlinear relationship between spike number and slow IPSP amplitude is best predicted by a model in which the non-linearity is due to the transduction mechanisms underlying the putative GABA$_B$-mediated responses. The multiplicity of G-protein binding sites on K$^+$ channels is alone sufficient to account for experimental data. In this case, the precise location of the receptors; sub-or peri-synaptic, does not matter if they have high sensitivity to GABA, which does indeed seem to be the case. GABA uptake may contribute an additional non-linearity if transporters are present at high density, but an extrasynaptic location for the receptors need not be proposed to account for the present data.

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