Pre- and postsynaptic glutamate receptors at a giant excitatory synapse in rat auditory brainstem slices

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1. Whole-cell patch recordings were used to examine the EPSC generated by the calyx of Held in neurones of the medial nucleus of the trapezoid body (MNTB). Each neurone receives a somatic input from a single calyx (giant synapse).

2. A slow NMDA receptor-mediated EPSC peaked in 10 ms and decayed as a double exponential with time constants of 44 and 147 ms. A fast EPSC had a mean rise time of 356 μs (at 25 °C), while the decay was described by a double exponential with time constants of 0·70 and 3·43 ms.

3. Cyclothiazide slowed the decay of the fast EPSC, indicating that it is mediated by AMPA receptors. The slower time constant was slowed to a greater extent than the faster time constant. Cyclothiazide potentiated EPSC amplitude, partly by a presynaptic mechanism.

4. The metabotropic glutamate receptor (mGluR) agonists, 1*S*,3*S*-ACPD, 1*S*,3*R*-ACPD and L-2-amino-4-phosphonobutyrate (L-AP4) reversibly depressed EPSC amplitude. A dose–response curve for 1*S*,3*S*-ACPD gave an EC_{50} of 7 μM and a Hill coefficient of 1·2.

5. Analysis of the coefficient of variation ratio showed that the above mGluR agonists acted presynaptically to reduce the probability of transmitter release. Adenosine and baclofen also depressed transmission by a presynaptic mechanism.

6. α-Methyl-4-carboxyphenylglycine (MCPG; 0·5–1 mM) did not antagonize the effects of 1*S*,3*S*-ACPD, while high concentrations of L-2-amino-3-phosphonopropionic acid (L-AP3; 1 mM) and 4-carboxy-3-hydroxyphenylglycine (4C3HPG; 500 μM) depressed transmission.

7. There was a power relationship between $[Ca^{2+}]_o$ and EPSC amplitude with co-operativity values ranging from 1·5 to 3·4.

8. The mechanism by which mGluRs modulate transmitter release appeared to be independent of presynaptic Ca^{2+} or K^+ currents, since ACPD caused no change in the level of paired-pulse facilitation or the duration of the presynaptic action potential (observed by direct recording from the terminal), indicating that the presynaptic mGluR transduction mechanism may be coupled to part of the exocytotic machinery.

9. Our data are not consistent with the presence at the calyx of Held of any one known mGluR subtype. Comparison of the time course and pharmacology of the fast EPSC with data from cloned AMPA receptors is consistent with the idea that GluR-D_o subunits dominate the postsynaptic channels.

The theme of this paper is the examination of glutamate receptors underlying excitatory synaptic transmission in the CNS. Interpretation of synaptic studies in the CNS is often limited by the distortion of the synaptic response caused by propagation of the current from a remote location along the dendritic tree and the associated problems of voltage clamping long dendrites. In addition, since most neurones receive multiple inputs, it is often difficult to restrict a stimulus to any one presynaptic axon. Our aim has therefore been to develop the calyx of Held as a synaptic preparation which overcomes many of these limitations and can serve as a general model for glutamatergic transmission in the CNS. Here we examine the time course of the excitatory postsynaptic current (EPSC), establish the presence of presynaptic receptors and provide some indication of possible mechanisms for autoreceptor modulation of transmitter release in the CNS.

We have used a slice preparation of the auditory brainstem containing the medial nucleus of the trapezoid body (MNTB), whose principal neurones each receive a single

giant somatic synapse called the calyx of Held (Forsythe & Barnes-Davies, 1993*a*,*b*; Forsythe, 1994). This nucleus forms part of the binaural auditory pathway responsible for sound localization. Globular bushy cells of the anterior ventral cochlear nucleus (aVCN) give rise to axons which traverse the mid-line of the brainstem in the trapezoid body and terminate in calyceal synapses which cover up to 60% of the somata of the MNTB cells in young rats. This preparation enables synaptic transmission from a single presynaptic axon to be studied and avoids the problems associated with distally located dendritic synapses.

Previously, we have shown that transmission at the calyx of Held is mediated by glutamate receptors. A slow EPSC is generated by *N*-methyl-D-aspartate (NMDA) receptors while a fast EPSC is mediated by kainate or α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors (Forsythe & Barnes-Davies, 1993*b*). A fast excitatory synapse also exists in the aVCN (Isaacson & Walmsley, 1995). Evidence from a similar synapse in the chick nucleus magnocellularis (the avian equivalent of the aVCN) indicates that the kinetics of the AMPA receptors underlying the fast EPSC in the auditory pathway are unusually rapid (Raman, Zhang & Trussell, 1994; Zhang & Trussell, 1994) and have led to the suggestion that the auditory pathway expresses glutamate receptor channels with fast kinetics. Examination of cloned non-NMDA glutamate receptor subunits has shown that four (GluR-A to GluR-D) can be categorized as AMPA receptors (see Hollmann & Heinemann, 1994). Studies of the EPSC time course and the use of drugs such as cyclothiazide, which blocks desensitization of AMPA-type receptors (Partin, Patneau, Winters, Mayer & Buonanno, 1993) can indicate which subunits dominate the subsynaptic receptor population and show whether desensitization plays a role in the EPSC time course.

In addition to glutamate's direct action via 'ionotropic' receptors, it is also involved in a number of modulatory effects through G-protein-coupled receptors – the metabotropic glutamate receptors (mGluRs). Seven mGluRs have now been cloned and expressed in various cell lines, where their properties have been studied (Tanabe, Masu, Ishii, Shigemoto & Nakanishi, 1992; Tanabe, Nomura, Masu, Shigemoto, Mizuno & Nakanishi, 1993; Saugstad, Kinzie, Mulvihill, Segerson & Westbrook, 1994; for reviews see Nakanishi, 1994; Saugstad, Segerson & Westbrook, 1995). mGluRs 1 and 5 stimulate phosphatidyl inositol hydrolysis, whilst mGluRs 2, 3, 4, 6 and 7 are negatively coupled to adenyl cyclase. The latter group includes those presynaptic receptors (mGluRs 4, 6 and 7) which are activated by L-2-amino-4-phosphonobutyrate (L-AP4).

In a number of preparations, short-term suppression of transmitter release at excitatory synapses has been observed during application of glutamate, its phosphonic derivative L-AP4, or *trans*-1-aminocyclopentane-1,3-dicarboxylic acid (*t*-ACPD) (Forsythe & Clements, 1990; Baskys & Malenka, 1991; Lovinger, Tyler, Fidler & Merritt, 1993; Sladeczek, Momiyama & Takahashi, 1993). We confirm here that the calyx of Held possesses presynaptic mGluRs and examine aspects of their pharmacology. Beyond the question of their precise identity, there are important questions concerning the mechanism by which these presynaptic receptors may modulate exocytosis. There is strong evidence that mGluRs can modulate N- and L-type calcium channels in postsynaptic neurones (Sayer, Schwindt & Crill, 1992; Sahara & Westbrook, 1993) while other evidence suggests that presynaptic mGluRs act via potentiation of presynaptic potassium currents (Sladeczek, Momiyama & Takahashi, 1993). A third possibility is that depression of transmitter release is achieved by a mechanism independent of ion channels, through the direct modulation of part of the exocytotic machinery. Using direct and indirect recording techniques, our data are not consistent with a mechanism involving modulation of presynaptic calcium or potassium channels, implying that mGluRs act via modulation of exocytosis itself. Preliminary reports concerning aspects of this work have been published in abstract form (Forsythe & Barnes-Davies, 1993*c*; Barnes-Davies & Forsythe, 1994).

METHODS

Preparation of slices

Lister hooded rats (6–12 days old) were killed by decapitation and the brainstem removed into iced low-Na^+, high-sucrose artificial cerebrospinal fluid (ACSF) containing (mM): 250 sucrose, 2·5 KCl, 26 $NaHCO_3$, 10 glucose, 1·25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$; saturated with 95% O_2–5% CO_2, giving a pH of 7·4. The brainstem was mounted (caudal end uppermost) in the chamber of a Campden vibratome and maintained at 0–4 °C. Transverse slices (200 μm thick) were cut from the level of the 7th nerve to include the nuclei of the superior olivary complex as described previously (Forsythe & Barnes-Davies, 1993*a*; Forsythe, Barnes-Davies & Brew, 1995). Slices were incubated for 1 h at 37 °C in normal ACSF containing (mM):125 NaCl, 2·5 KCl, 26 $NaHCO_3$, 10 glucose, 1·25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 3 *myo*-inositol, 2 sodium pyruvate; and bubbled with 95% O_2–5% CO_2, giving a pH of 7·4.

Microscopy and slice perfusion

For recording, one slice was transferred to a Peltier controlled environmental chamber and maintained at 25 °C on the stage of an M2A microscope (MicroInstruments) fitted with differential interference contrast (DIC) optics. Individual cells were visualized with a ×40 water-immersion objective (NA 0·75, Zeiss). The recording chamber (300–500 μl volume) was continually perfused with ACSF at a rate of 0·75–1·0 ml min^{-1}. Changes in the Ca^{2+} and Mg^{2+} levels of the ACSF are noted in the respective Results section. Drugs were applied by switching between one of four perfusion lines, all of which entered directly into the recording chamber so as to minimize dead space. (±)-2-Amino-5-phosphonopentanoic acid (AP5), 7-Cl-kynurenic acid, 5,7-diCl-kynurenic acid, (+)-MK-801 hydrogen maleate and aniracetam were supplied by RBI. (±)-1-Aminocyclopentane-*trans*-1,3-dicarboxylic acid (*t*-ACPD), (1*S*,3*R*)-1-aminocyclopentane-1,3-dicarboxylic acid (1*S*,3*R*-ACPD), 1*S*,3*S*-ACPD, (+)-2-amino-4-phosphonobutyric

acid (L-AP4), (+)-2-amino-3-phosphonopropionic acid (L-AP3), (*RS*)-α-methyl-4-carboxy-phenylglycine (MCPG) and (*RS*)-4-carboxy-3-hydroxyphenyl-glycine (4C3HPG) were supplied by Tocris. QX314 was supplied by Alomone Laboratories (Jerusalem), cyclothiazide was a gift from Eli Lilly and adenosine and baclofen were supplied by Sigma.

Electrophysiology

Whole-cell recordings were made using Sylgard-coated (Dow Corning) patch pipettes fabricated from thin-walled borosilicate glass (Clarke Electromedical; GC150TF-15). Unless otherwise stated, pipettes contained (mM): 97·5 potassium gluconate, 32·5 KCl, 10 Hepes, 5 EGTA and 1 $MgCl_2$; pH 7·3. Pipettes had resistances of around 5 MΩ. Recordings were made (without prior cleaning) from visually identified principal neurones of the MNTB by approaching the cells with high positive pressure applied to the patch pipette. Neurones were voltage clamped using an Axopatch 200 amplifier (Axon Instruments). Recordings from the presynaptic terminal were made with standard walled glass pipettes. The methods have been described previously (Forsythe, 1994; Forsythe, Barnes-Davies & Brew, 1995). The synaptic terminal patch solution was as above, plus 2 mM ATP and 0·5 mM GTP. Series resistances were in the range 6–20 MΩ and were compensated by at least 70%. Data were filtered at 2–5 kHz and digitized at 5–20 kHz using a CED 1401 (Cambridge Electronic Design, Cambridge, UK) interface with Patch version 6 acquisition and analysis software, running on a Pericom 486 computer.

EPSCs were evoked using a Digitimer isolated stimulator (model DS2) connected to a bipolar platinum stimulating electrode positioned over the trapezoid body, half-way between the mid-line and the MNTB. Although there was a low success rate (around 1 in 20) for evoking EPSCs by this means, it was favoured because once a synaptic connection had been achieved it could be maintained for up to 2 h with very little change in stimulus parameters. The criteria for establishing that an EPSC originated from a calyceal synapse were that it had an amplitude > 300 pA at −60 mV (Forsythe & Barnes-Davies, 1993*b*) and displayed all-or-nothing characteristics with changing stimulus intensity (see Fig. 1*C*).

Data analysis

EPSC averaging, measurement and exponential fitting were conducted using CED Patch software. Data from unitary EPSCs were collected at holding potentials of −60 or −70 mV and evoked at a stimulus rate of 0·5 or 1 Hz. Single traces were written to disk for later analysis. Kaleidagraph software (Abelbeck Software) was used to estimate 10–90% rise times and half-widths of the fast EPSCs by interpolation between 20 kHz data points. Exponential and other curve fitting employed the method of least squared errors. In the figures shown here, all traces are the averages of between 20 and 60 evoked responses, unless otherwise indicated. Data are quoted as means ± S.D., unless otherwise stated. Dose–response data were expressed as the ratio of the EPSP amplitudes: test/[(control + recovery)/2]. When multiple doses were applied to the same terminal, control periods sandwiched each test dose. The dose–response curve was fitted to an equation of the form:

$$r = [L^n/(1 + L^n)]M, \quad (1)$$

where n is the Hill coefficient, $L = [\text{agonist}]/EC_{50}$ and M is the maximal depression. Free parameters were n, EC_{50} and M.

The method used to assess the site of action of the agonists is based on the variance method (see Hubbard, Llinás & Quastel, 1969) and assumes quantal transmitter release which saturates the postsynaptic receptors such that channel openings do not make a significant contribution to the EPSC variance. In terms of the statistics of transmitter release, the calyx of Held is similar to the neuromuscular junction and has a number of advantages over other central synapses. It has a large number of release sites (which by lowering $[Ca^{2+}]_o$ can be adjusted to have a low probability of release) and a non-distributed synaptic input onto the soma, and experiments can be conducted under voltage clamp. A change in the apparent quantal size on application of a test drug would be interpreted as reflecting a postsynaptic change, i.e. the drug is acting at the postsynaptic receptors. A change in the total quantal content indicates a change in the probability of transmitter release (or number of release sites), which is a presynaptic phenomenon. The variance method allows a qualitative distinction to be made between these two alternatives by testing for a change in variance relative to the mean amplitude using the coefficient of variation (c.v.). The c.v., including a correction for background noise, is defined as:

$$\text{c.v.} = \sqrt{[\text{var}(a) - \text{var}(n)]}/\text{mean}(a), \quad (2)$$

where $\text{var}(a)$ is the variance in the evoked EPSC amplitude, $\text{var}(n)$ is the variance of the noise and $\text{mean}(a)$ is the mean EPSC amplitude. We have expressed these results as the ratio of $\text{c.v.}_{\text{test}}$ to $\text{c.v.}_{\text{control}}$ (i.e. the coefficient of variation ratio, c.v.r.).

For analysis of the c.v., the peak amplitude and noise measurements from between 200 and 500 records were collected under control conditions and then during perfusion of the agonist (test). In all these experiments $[Ca^{2+}]_o$ was lowered to 1 mM (or less in the case of cyclothiazide) so that the release probability would more closely approximate a Poisson distribution. Amplitude distributions of unitary EPSCs were analysed on a Macintosh Quadra computer using 'c.v.r.' software kindly supplied by Dr J. D. Clements. This software has been described previously (Clements, 1990; Forsythe & Clements, 1990). A change in the c.v.r. which significantly deviates from 1·0 indicates that the quantal content has changed and since we assume that the number of release sites is constant, this implies that the test agonist has changed the probability of transmitter release. The statistical significance of the measured c.v.r. values were determined on a cell-by-cell basis using a Monte Carlo simulation in the c.v.r. program, which tested the null hypothesis that the change in amplitude was due to a postsynaptic effect.

RESULTS

The calyx of Held generates a dual-component EPSC

Voltage-clamp recordings were made from the somata of visualized MNTB neurones in brain slices using the whole-cell patch technique (see Fig. 1*A*). Large glutamatergic EPSCs could be evoked in the principal neurones of the MNTB by stimulating the presynaptic trapezoid body fibres which give rise to the calyces of Held. These EPSCs were comprised of both slow and fast components (Fig. 1*B*), which, as previously demonstrated, can be distinguished on the basis of their time course, pharmacology and current–voltage relationship. The slow EPSC is blocked by AP5, indicating that it is mediated by NMDA receptors, while the fast EPSC is blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and therefore mediated by AMPA/kainate receptors (Forsythe & Barnes-Davies, 1993*b*). Anatomical evidence suggests that only a single

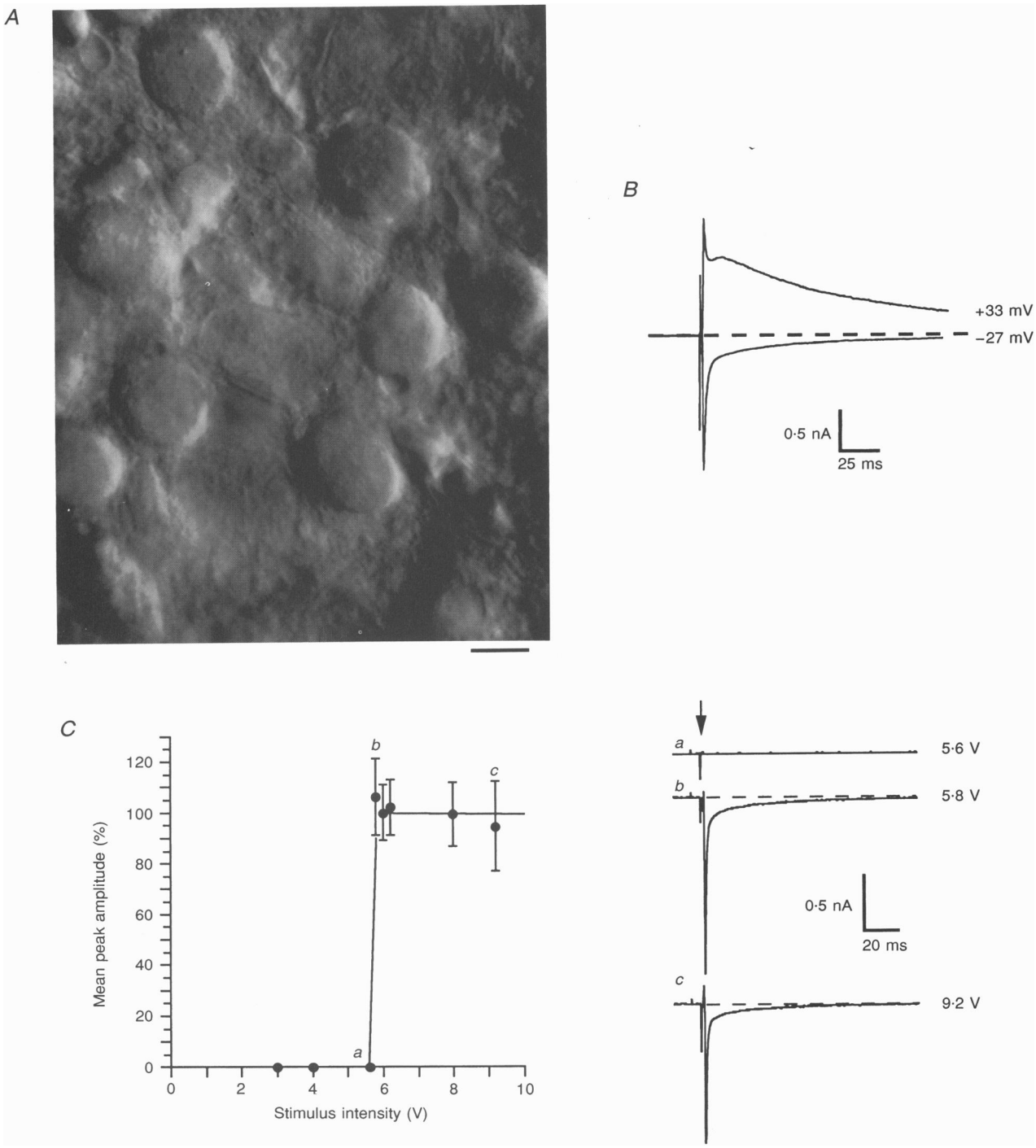

Figure 1. The calyx of Held evokes a dual-component EPSC

A, Nomarski image of the MNTB using the experimental microscope, showing the spherical somata of the principal neurones. Scale bar is 10 μm. *B*, the dual-component EPSC in an MNTB neurone evoked on stimulation of the trapezoid body. Pipette solution contained 100 mM potassium gluconate, 30 mM CsCl, 10 mM Hepes, 5 mM EGTA, 1 mM $MgCl_2$ and 5 mM QX314 to minimize outward currents at positive holding potentials. The slow, voltage-dependent NMDA receptor-mediated component can be clearly distinguished from the fast, non-NMDA receptor-mediated EPSC. *C*, plot of a stimulus–response curve shows an all-or-nothing EPSC, indicating a single giant synaptic input. Each point is the mean peak amplitude of 20–30 evoked EPSCs expressed as a percentage, with the error bars indicating the S.D. Averaged records for points *a*, *b* and *c* of the plot are shown to the right, with the arrow marking the stimulus artifact.

calyceal synapse forms around each MNTB neurone (Forsythe, 1994). This was confirmed here by examining the stimulus–response curve for evoked EPSCs, which displayed a definite threshold above which a large EPSC was evoked. Further increases in the stimulus intensity caused no change in the response amplitude, as shown in Fig. 1*C*.

Time course of fast and slow EPSCs

Our recordings of the time course of this synaptic response should be subject to minimal distortion, since the calyx of Held is a somatic synapse. The time to peak and the rate of decay of the slow EPSC were measured from averaged data recorded at between +40 and +60 mV holding potentials, where it can be distinguished from the fast EPSC and is not subject to voltage-dependent block by $[Mg^{2+}]_o$ (Fig. 2*A*). The mean time to peak measured from eight cells was 10·0 ± 1·7 ms, while the decay could be fitted by a double exponential with time constants of 44 ± 9·4 and 147 ± 26·3 ms, with the fast component contributing 65 ± 15% of the total amplitude.

Fast EPSCs were studied at holding potentials of −60 or −70 mV in the presence of NMDA receptor antagonists (20 μM AP5, 5 μM 5,7-diCl-kynurenate and 4 μM MK801) and 2 mM Mg_o^{2+} to ensure block of the slow NMDA receptor-mediated component. In order to minimize series resistance errors associated with the large size of the fast EPSCs, $[Ca^{2+}]_o$ was reduced to 1 mM with 2 mM Mg_o^{2+}. This reduced the magnitude of the responses to below 1 nA at holding potentials around −60 mV. In addition, 70–90% series resistance compensation was used. The rate of decay of the fast EPSC was examined in ten cells and could be fitted by the sum of two exponentials with time constants of 0·78 ± 0·16 and 3·67 ± 0·67 ms, as shown for one response in Fig. 2*B*. The faster time constant contributed 81·3 ± 7·4% of the peak amplitude. In seven neurones, the fast EPSC rise times and half-widths were measured from single evoked responses rather than from averaged data. Care was taken to exclude responses showing asynchronous release. The mean 10–90% rise time ranged between 273 and 440 μs and mean half-width ranged between 0·95 and

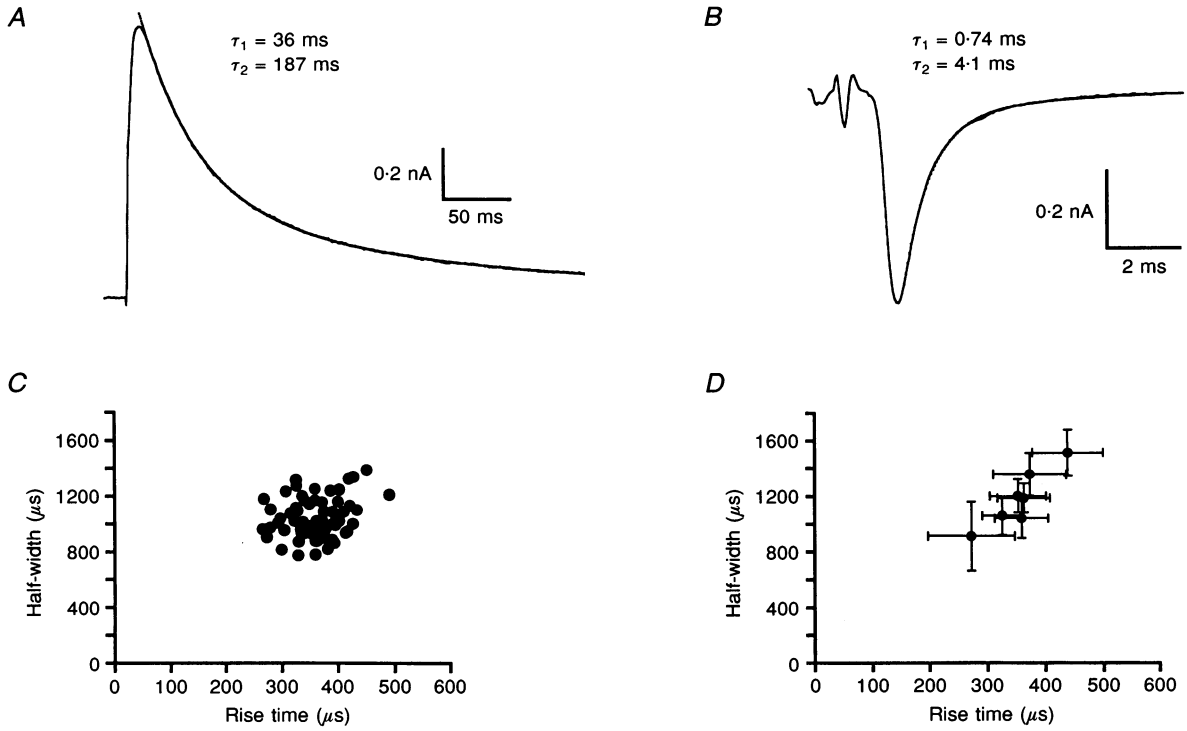

Figure 2. Time course of the dual-component EPSC

A, the decay of the slow EPSC is shown in an MNTB neurone held at +53 mV. The dotted line indicates a double exponential fit with time constants of 36 (τ_1) and 187 (τ_2) ms. The time-to-peak of the slow EPSC was 9·5 ms in this cell. *B*, the fast EPSC was examined at a holding potential of −67 mV, in the presence of NMDA receptor antagonists (see text). The decay follows a double exponential with time constants of 0·74 and 4·1 ms. *C*, plot of half-width (10–90%) *vs.* rise time for unitary fast EPSCs from a single MNTB neurone. No correlation between rise time and half-width was seen for any response. *D*, plot of the mean half-width *vs.* mean 10–90% rise time (± S.D.) for the fast EPSC in 7 MNTB neurones shows some correlation across the population of responses ($r = 0·92$; see text).

1·51 ms (Fig. 2*D*). Within any individual neurone we found no correlation between rise time and half-width (the mean correlation coefficient, *r*, for seven cells was 0·276 ± 0·180) as shown for one neurone in Fig. 2*C*. The mean 10–90% rise time for this cell was 360 ± 46 μs and the mean half-width was 1·04 ± 0·14 ms. The lack of correlation between rise time and half-width within a single neurone is consistent with a synapse whose release sites are not spatially distributed along a dendritic tree, but are close to the soma, as one would expect for the calyx of Held. When the mean values for EPSC rise time and half-width were plotted, there appeared to be some relationship across the population as a whole ($r = 0{\cdot}92$; see Fig. 2*D*). This was not correlated with animal age, series resistance or EPSC amplitude. Since all these synapses are somatic, it may suggest some differences between the expression or modulation of AMPA receptor subunits in individual MNTB neurones.

Cyclothiazide slows the rate of decay of the fast EPSC

Cyclothiazide and aniracetam have been shown to block desensitization of AMPA receptors (Isaacson & Nicoll, 1991; Vyklicky, Patneau & Mayer, 1991; Trussell, Zhang & Raman, 1993). Application of 100 μM cyclothiazide in the presence of NMDA receptor antagonists (as above) increased the amplitude and duration of the fast EPSC, as shown for one response in Fig. 3. In 0·6 mM Ca_o^{2+}, cyclothiazide increased the mean EPSC amplitude by 72 ± 26% ($n = 6$). Cyclothiazide slowed the decay time constants to 3·2 ± 0·5 (71·7 ± 2·9%) and 20·6 ± 5·6 ms ($n = 3$) as compared with 0·74 ± 0·03 (77·7 ± 14·4%) and 3·19 ± 1·29 ms in the same three responses prior to application of cyclothiazide (the value in brackets indicates the percentage of the peak amplitude contributed by the fastest time constant). Similar changes were observed in a further three cells, but the sample period was insufficient to give a reliable fit to the slowest decay time constant. In two cells, application of 2 mM aniracetam gave similar results (Table 1). An interesting feature of these data is that cyclothiazide had a differential effect on the two decay time constants of the fast EPSC, slowing the decay of the faster component to a lesser extent than the slower ($\tau_{test}/\tau_{control}$ ratios of 4·3 and 6·8, respectively), without significantly changing the contribution of each component to the peak amplitude (see Discussion).

Analysis of the coefficient of variation of the evoked EPSC amplitudes also indicated a significant presynaptic component to the potentiation in three of six experiments (see Table 1). Our data are consistent with an action of cyclothiazide at a postsynaptic site on the AMPA receptors, but may also indicate that it has a separate presynaptic action, increasing the probability of transmitter release by an unknown mechanism.

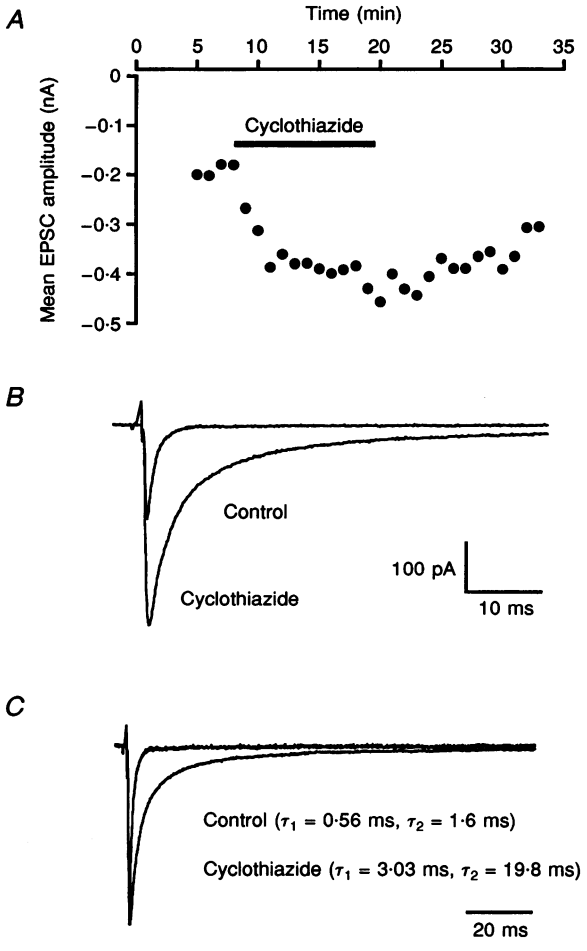

Figure 3. Cyclothiazide increases amplitude and slows the decay of the fast EPSC

A, time course for effect of 100 μM cyclothiazide on EPSC amplitude. Each point is the mean of 60 EPSCs evoked at a stimulus rate of 1 Hz. *B*, averaged traces for control (0·6 mM Ca_o^{2+}) and during perfusion of 100 μM cyclothiazide are superimposed. *C* shows the same traces normalized so as to highlight the slower decay time course in the presence of cyclothiazide. The exponential fits to these EPSC decays are indicated in parentheses alongside.

Table 1. Cyclothiazide and aniracetam potentiate EPSC amplitude

Cell no.	Agonist	Control amplitude (pA)	Test amplitude (% control)	c.v.r.
1	100 μM cyclothiazide	80	193	0·76*
2	100 μM cyclothiazide	210	157	0·93
3	100 μM cyclothiazide	190	189	0·69*
4	100 μM cyclothiazide	120	137	0·94
5	100 μM cyclothiazide	190	201	0·82*
6	100 μM cyclothiazide	260	157	0·92
7	2 mM aniracetam †	206	187	1·02
8	2 mM aniracetam †	101	197	0·81*

Data collected with 0·6 mM Ca_o^{2+} and 2 mM Mg_o^{2+} In every case, both cyclothiazide and aniracetam potentiated EPSC amplitude. † These data were collected with 1·0 mM Ca_o^{2+} and 2 mM Mg_o^{2+}. * c.v.r. values indicate a presynaptic site of action with $P < 0·01$.

Metabotropic glutamate receptor agonists depress EPSC amplitude

A number of glutamatergic synapses are known to be modulated by autoreceptors of the mGluR class. These presynaptic receptors appear to be specifically activated by the phosphonic derivative of glutamate, L-AP4, and ACPD. Similar receptors are present at the calyx of Held. Application of the metabotropic glutamate receptor agonist 1*S*,3*S*-ACPD reversibly depressed the EPSC amplitude in every case examined. In eight cells, 20 μM 1*S*,3*S*-ACPD depressed EPSC amplitude by 64 ± 14%, as shown for one cell in Fig. 4*A*, where the mean amplitude of the EPSC is plotted against time before, during and after application of ACPD. The averaged EPSCs for these data are plotted below. The effect of 1*S*,3*S*-ACPD was dose dependent; the dose–response curve is plotted in Fig. 4*B* with each point being the mean ± S.E.M. The continuous line is the best fit to all the individual data points, having an EC_{50} of 7 μM, a Hill coefficient of 1·2 and a maximal depression of 81%.

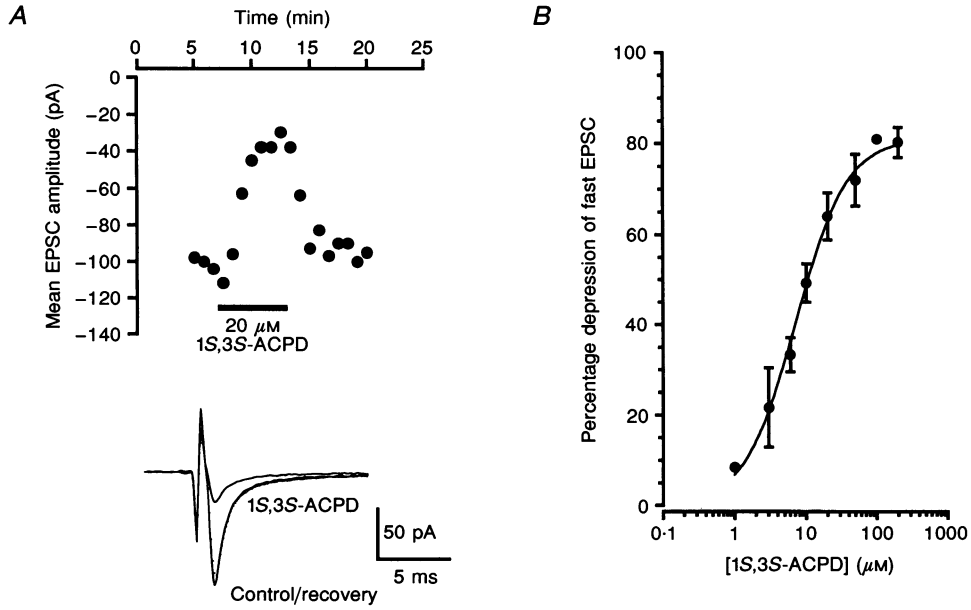

Figure 4. Depression of EPSC amplitude by 1*S*,3*S*-ACPD

A, plot of fast EPSC amplitude against time from an MNTB neurone held at −60 mV in 1 mM Ca_o^{2+} and 2 mM Mg_o^{2+}. Each point is the mean of 50 EPSCs evoked at a stimulus rate of 1 Hz. Application of 20 μM 1*S*,3*S*-ACPD (indicated by the filled bar) reduced EPSC amplitude by about 70% in this cell. Averaged EPSCs in control and after application of 1*S*,3*S*-ACPD are shown below, with the dotted line showing recovery. *B*, dose–response curve for the depression of the fast EPSC by 1*S*,3*S*-ACPD. Each point is the mean depression (± S.E.M.) in 3–7 cells with the exception of 1 ($n = 2$) and 100 μM ($n = 1$). The curve is the fit of eqn (1) to all the individual data points, giving an EC_{50} value of 7·2 μM, a Hill coefficient of 1·2 and a maximal depression of 81%.

In two cells to which both 1*S*,3*R*- and 1*S*,3*S*-ACPD (50 μM) were applied sequentially, little difference was seen in the level of depression produced, as shown for one case in Fig. 5*A* where the depression was 58 and 60%, respectively. The mGluR agonist L-AP4, which is particularly potent at presynaptic mGluRs, also reduced EPSC amplitude in every case tested, as shown for one response in Fig. 5*B*. The mean depression produced by 20 μM L-AP4 was 80 $\pm$ 7% ($n = 5$).

The site of action of mGluR agonists

The above data confirm that mGluR agonists depress transmission, but in order to determine whether the site of action is pre- or postsynaptic, it is necessary to examine the amplitude distributions of the evoked or miniature EPSCs. Spontaneous or miniature EPSCs are very rare at the calyx of Held ($\ll$ 1 Hz; authors' unpublished observations). In addition, we have shown previously that the MNTB may receive smaller but significant inputs from high-threshold afferents (Forsythe & Barnes-Davies, 1993*b*), so data collected using spontaneous activity would not reflect the same homogeneous population of release sites as examined with the evoked response. Unfortunately we have not been able to resolve the quantal amplitude at the calyx of Held from distributions of EPSC amplitudes, either because the quantal amplitude is too small or because it has a large variance, which combined with a large number of release sites makes it difficult to distinguish discrete peaks in the amplitude distribution. Under these circumstances, examination of the coefficient of variance ratio (c.v.r.; see Methods) can be used to give an assessment of the site of action of a putative agonist (Forsythe & Clements, 1990).

The basis of the c.v.r. method is illustrated in Fig. 6, where superimposed traces are shown to illustrate the underlying variance in EPSC amplitude. Under control conditions, stimulation of the presynaptic axon generates a large-amplitude EPSC (Fig. 6*A*) which shows relatively little variation from trial to trial. Exchange of the normal ACSF for one containing 1 mM Ca_o^{2+} decreases the probability of transmitter release and results here in a decrease in the mean amplitude of the unitary evoked responses from 2400 to 680 pA, while the c.v. increases from 0·039 to 0·243 (the latter change in the c.v. can be seen in the raw data as an increased range of EPSC amplitudes, relative to the mean amplitude). Subsequent application of the mGluR agonist 1*S*,3*S*-ACPD (20 μM) causes a further reduction in the mean EPSC amplitude from 680 to 160 pA with an additional increase in c.v. from 0·243 to 0·389 (Fig. 6*A c*). The peak amplitudes of each of the evoked responses have been plotted (after equilibration with 1 mM Ca^{2+} and 2 mM Mg_o^{2+}), showing the onset and recovery from addition of 20 μM ACPD.

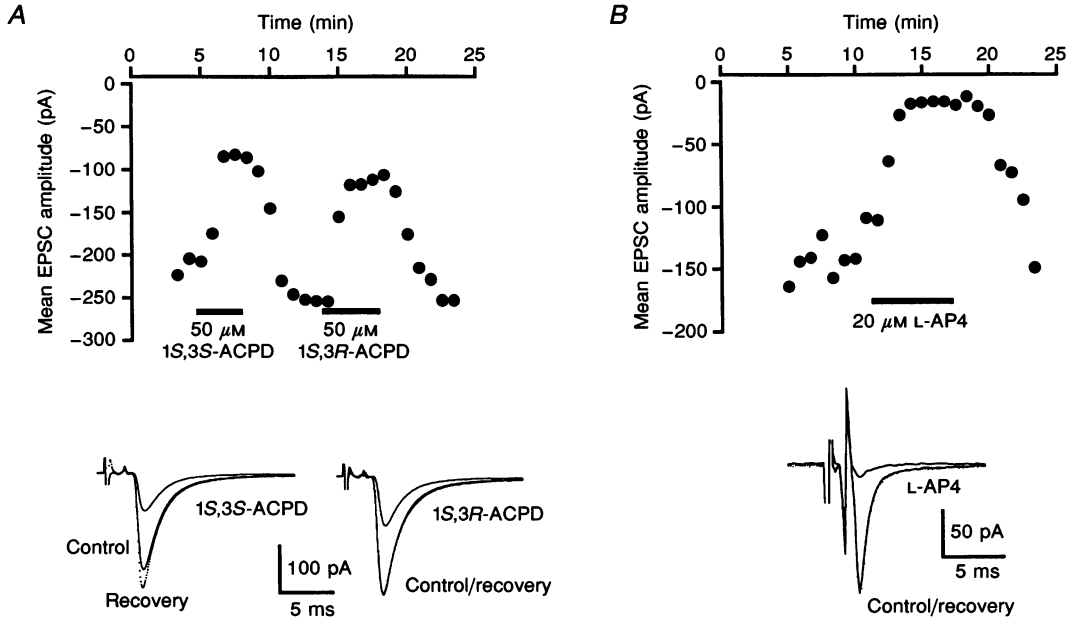

Figure 5. Depression of EPSC amplitude by ACPD isomers and L-AP4

A and *B*, plot of fast EPSC amplitude against time from an MNTB neurone held at −60 mV in 1 mM Ca_o^{2+} and 2 mM Mg_o^{2+}. Each point is the mean of 50 EPSCs evoked at a stimulus rate of 1 Hz. The averaged EPSCs are shown below for control and test, with the dotted line indicating recovery. *A*, 1*S*,3*S*-ACPD and 1*S*,3*R*-ACPD depressed the EPSC amplitude to a similar extent when applied to the same cell. In this cell, 1*S*,3*S*-ACPD depressed EPSC amplitude by 60% and 1*S*,3*R*-ACPD depressed EPSC amplitude by 58%. *B*, application of 20 μM L-AP4 reduced the EPSC amplitude by 88%.

In order to quantify the increased variability shown above and determine whether the depression in evoked EPSC amplitude by mGluR agonists was due to a pre- or postsynaptic effect, an analysis of the c.v. was carried out (see Methods). This involved collecting measurements of noise and EPSC amplitudes in control and test conditions for > 200 evoked events. In every cell tested, the depression in EPSC amplitude by 50 or 20 μM 1*S*,3*S*-ACPD ($n = 6$), and 20 μM L-AP4 ($n = 5$) resulted in a c.v.r. value greater than 1. Assessment of the statistical significance of these data was carried out on a cell-by-cell basis and in every case was shown to be highly significant with $P < 0{\cdot}01$, as shown in Table 2.

The amplitude distributions and c.v.r. values for two such experiments with ACPD and L-AP4 are shown in Fig. 7. Each plot is presented as a probability density distribution. Under control conditions (with 1 mM Ca^{2+} and 2 mM Mg^{2+}) the amplitude distributions show a high probability of large-amplitude events, whilst in the presence of ACPD or L-AP4, there is a reduction in the probability of large-amplitude events and an increase in the probability of smaller EPSCs and failures. The c.v.r. values for these data were 2·09 and 1·96, respectively, indicating that ACPD and L-AP4 are acting presynaptically. The increased number of failures is also consistent with a presynaptic site of action.

Presynaptic adenosine and baclofen receptors

There is also evidence for the presence of presynaptic $GABA_B$ and adenosine receptors at the calyx of Held. Application of 5 μM baclofen caused a reduction in mean EPSC amplitude of 89 ± 6·5% in three cells (Fig. 8*A*). This reduction was probably due to activation of presynaptic $GABA_B$ receptors, which have been reported at many central excitatory synapses (Forsythe & Clements, 1990; Scanziani, Capogna, Gähwiler & Thompson, 1992), since analysis of the c.v.r. values indicated a statistically significant reduction in transmitter release (see Table 2). The c.v.r. values for the effect of baclofen on all three cells were greater than 1, with a significance level of $P < 0{\cdot}01$, indicating a presynaptic effect. Application of 50 μM

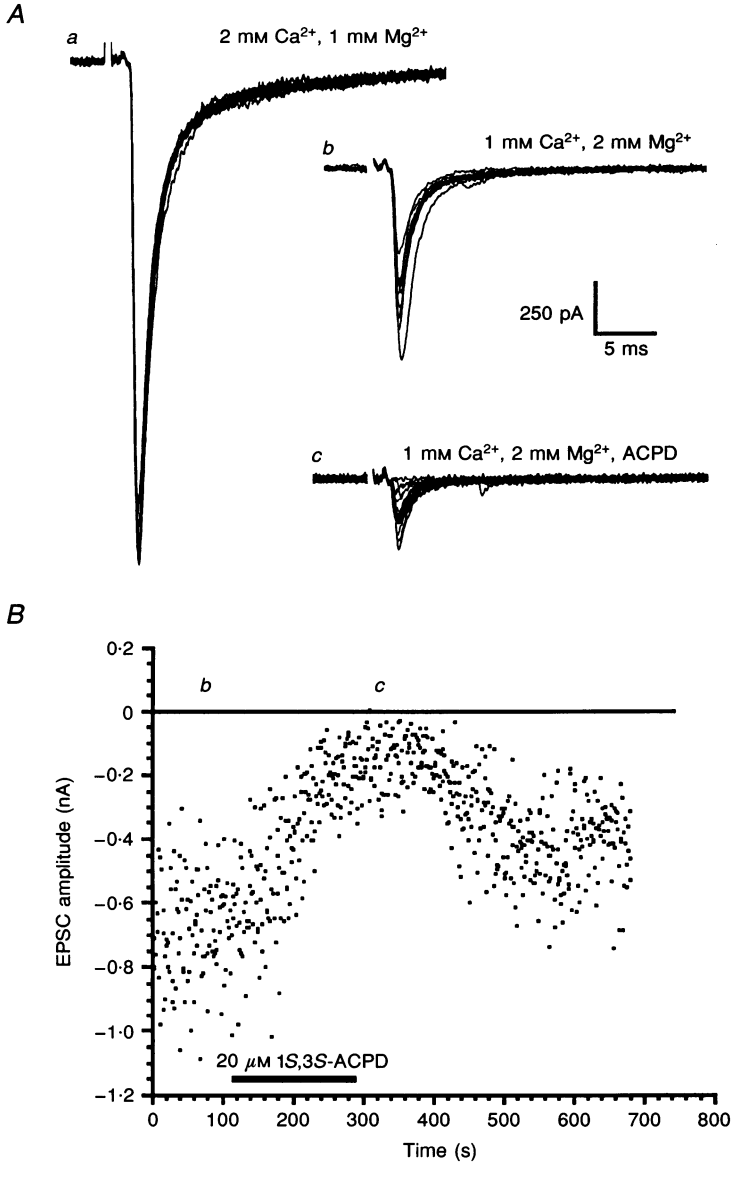

Figure 6. Effect of reducing $[Ca^{2+}]_o$ and application of ACPD on raw EPSC amplitudes

A, unitary evoked EPSCs are shown superimposed, illustrating the change in amplitude and the coefficient of variation (c.v.) during an experimental protocol to determine the site of action of ACPD. The holding potential is −60 mV, stimulus rate 1 Hz, with extracellular divalent cation concentrations indicated above each trace. *a*, 10 superimposed unitary fast EPSCs from an MNTB in normal ACSF. The c.v. was 0·039. *b*, another 10 superimposed fast EPSCs from the same cell after changing to ACSF containing 1 mM Ca^{2+} and 2 mM Mg^{2+} with NMDA receptor antagonists. The c.v. was 0·243. *c*, again from the same cell, 14 superimposed EPSCs collected after application of 20 μM 1*S*,3*S*-ACPD. The c.v. was 0·389. *B*, plot of the raw EPSC amplitude against time from the same cell as in *A*. Zero time was taken from the point at which the EPSC equilibrated in the 1 mM Ca^{2+}, 2 mM Mg^{2+} ACSF.

Table 2. Presynaptic effects of mGluR agonists, adenosine and baclofen

Cell no.	Agonist	Control amplitude (pA)	Test amplitude (% control)	c.v.r.
1	20 μM L-AP4	285	20	1·97*
2	20 μM L-AP4	60	28	1·99*
3	20 μM L-AP4	198	18	2·80*
4	20 μM L-AP4	208	17	2·88*
5	20 μM L-AP4	128	10	1·96*
6	10 μM L-AP4	95	40	1·63*
7	100 μM *t*-ACPD	254	28	2·21*
8	50 μM 1*S*,3*S*-ACPD	187	33	2·09*
9	50 μM 1*S*,3*S*-ACPD	246	43	1·28*
10	50 μM 1*S*,3*S*-ACPD	561	13	2·77*
11	20 μM 1*S*,3*S*-ACPD	215	57	1·23*
12	20 μM 1*S*,3*S*-ACPD	114	32	1·62*
13	20 μM 1*S*,3*S*-ACPD	200	44	1·64*
14	5 μM baclofen †	303	16	3·20*
15	5 μM baclofen	98	3	5·87*
16	5 μM baclofen	142	13	2·63*
17	50 μM adenosine	628	35	1·69*
18	50 μM adenosine	812	67	1·18*
19	50 μM adenosine	251	69	1·22*

The test amplitude (as a percentage of the control) is shown along with the control EPSC amplitude and the c.v.r. for each result. Data collected with 1 mM Ca_o^{2+} and 2 mM Mg_o^{2+}, except for †, which were data collected with 2 mM Ca_o^{2+} and 2 mM Mg_o^{2+}. * c.v.r. values indicate a presynaptic site of action with $P < 0{\cdot}01$.

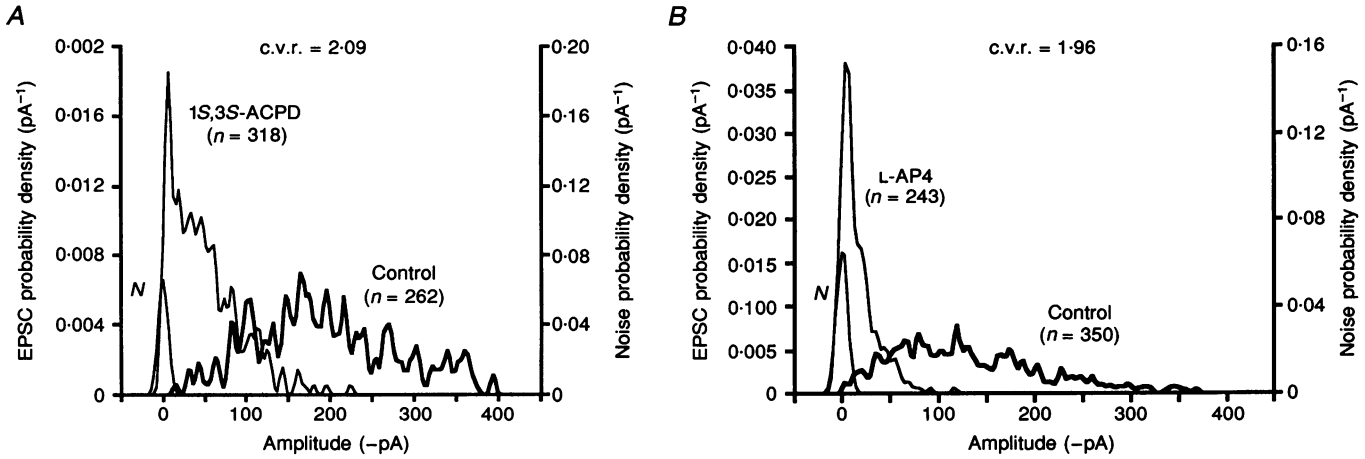

Figure 7. 1*S*,3*S*-ACPD and L-AP4 have a presynaptic site of action

Probability density distributions of unitary EPSC amplitudes collected from the same neurone, held at −60 mV with 1 mM Ca^{2+}, 2 mM Mg^{2+} ACSF. The thick line shows the distribution of EPSC amplitudes during control conditions, while the thin line shows the distribution for the same response during perfusion of the test agonist. The noise distributions are marked (*N*) and the number of events (*n*) measured in control and test conditions are indicated in parentheses above the respective curve. *A*, application of 50 μM 1*S*,3*S*-ACPD depressed the mean EPSC amplitude by 70% (thin line). *B*, application of 20 μM L-AP4 (thin line) depressed the mean EPSC amplitude by 90%. Both ACPD and L-AP4 caused a reduction in the probability of large-amplitude events, with a corresponding increase in the probability of small-amplitude events and failures. The changes in c.v.r. for the depression induced by both agonists were shown to be significant ($P < 0{\cdot}01$).

adenosine resulted in a reduction in mean EPSC amplitude by 54 ± 14·3% in three cells. This was also accompanied by an increase in the c.v.r. in all cells tested ($P < 0·01$; see Fig. 8*B*), confirming the existence of presynaptic adenosine receptors as suggested previously for other synapses (Thompson, Haas & Gähwiler, 1992).

Pharmacology of presynaptic mGluRs

Although specific antagonists for each of the mGluR classes are not yet available, pharmacological tools can shed some light on the receptor subtypes involved in the presynaptic effects seen here. As shown above, the action of L-AP4 suggests that mGluR-4 or -7 may be involved (as mGluR-6 is expressed only in the retina). MCPG is an antagonist at mGluR-1 and -2 but not at mGluR-4 (Hayashi *et al.* 1994). In order to examine the effect of MCPG on the presynaptic modulation we used an experimental protocol in which 1*S*,3*S*-ACPD was applied twice to the same cell: once on its own and then during application of MCPG. MCPG had no effect on the EPSC amplitude alone, nor did it antagonize the effect of 20 or 50 μM ACPD. The results for two different cells are shown in Fig. 9. In six experiments we applied MCPG at either 0·5 or 1 mM concentrations using 20 or 50 μM ACPD and saw no antagonistic effect. Another phenylglycine derivative, 4C3HPG, is an agonist at mGluR-2 but not -4, while acting as an antagonist at mGluR-1$_\alpha$ (Hayashi *et al.* 1994; Thomsen, Boel & Suzdak, 1994). At the calyx of Held, 500 μM 4C3HPG had an agonist effect depressing EPSC amplitude by 85% in two cells (Fig. 10*A*). L-AP3 has also been shown to antagonize some effects of ACPD, such as the depression of somatic calcium currents in hippocampal neurones (Sahara & Westbrook, 1993). We found no effect of 300 μM L-AP3 on EPSC depression by 1*S*,3*S*-ACPD (data not shown), but in three cases at higher concentrations (1 mM), L-AP3 had an agonist action, depressing EPSC amplitude when applied alone by 60·3% ± 9·8, ($n = 3$), as shown for one example in Fig. 10*B*.

Extracellular calcium and transmitter release

The relationship between extracellular calcium concentration and the fast EPSC amplitude was measured in six synapses over a range of $[Ca^{2+}]_o$ from 1·0 to 0·3 mM (with a constant Mg_o^{2+} of 2 mM). EPSCs were averaged only after stabilization

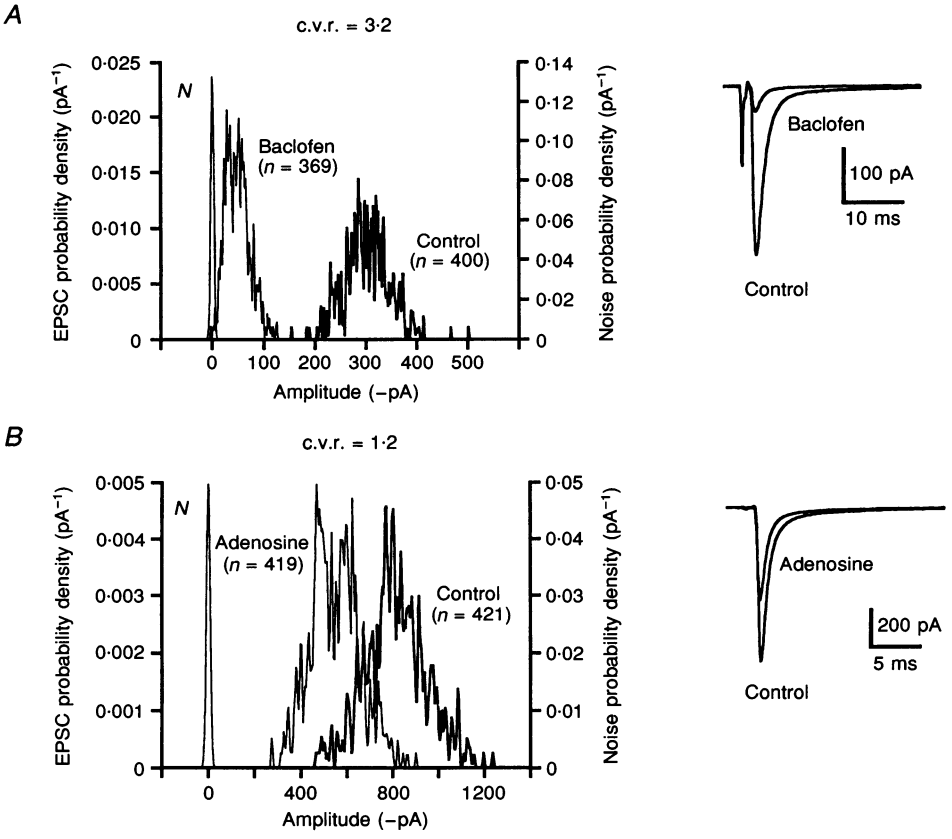

Figure 8. Baclofen and adenosine depress EPSC amplitude

A, probability density distributions of unitary EPSC amplitudes in the absence (thick line) and presence (thin line) of agonist, with the noise distribution marked (*N*) and the number of amplitude events measured (n) indicated beside the distributions. Averaged EPSCs are shown superimposed on the right of each plot . *A*, 5 μM baclofen depressed EPSC amplitude by 84%. Analysis of the c.v.r. between control and test periods shows a presynaptic site of action with a c.v.r. value of 3·2 . *B*, 50 μM adenosine depressed EPSC amplitude by 33%. The c.v.r. of 1·2 confirmed a presynaptic site of action ($P < 0·01$).

of the response amplitude at the new calcium concentration, which required about 15 min. Because of this, it was not usually possible to obtain data from any one synapse for more than two calcium concentrations. A plot of normalized amplitude against $[Ca^{2+}]_o$ for pooled data gave a good fit with a power function of 2·2, with fits to individual responses ranging from 1·5 to 3·4. The data for one cell are shown in Fig. 11, where log EPSC amplitude is plotted against log $[Ca^{2+}]_o$. The line is the best fit to a power function ($y = x^n$), showing that amplitude is proportional to the calcium concentration raised to the power of 2·01.

Depression and facilitation of paired pulses

Examination of changes in the degree of paired-pulse facilitation has been used to characterize the site of action of an agonist as pre- or postsynaptic. According to the residual calcium hypothesis (Katz & Miledi, 1968), an agonist acting at a presynaptic site to modulate calcium influx (either directly or indirectly) will change the degree of facilitation experienced by a second stimulus following closely after the conditioning stimulus. Interpretation of such data in the case of glutamatergic synapses in the CNS is difficult since there are competing influences, such as postsynaptic AMPA receptor desensitization, autoreceptor activation and/or vesicle depletion, which would cause depression of the second synaptic response. Under conditions where the release probability is low, so as to minimize desensitization and endogenous autoreceptor activation, we have found no evidence for changes in paired-pulse facilitation by 1*S*,3*S*-ACPD.

With normal external divalent concentrations (2 mM Ca^{2+} and 1 mM Mg^{2+}), the calyx of Held shows paired or triple-pulse depression as shown in Fig. 12*A*. However, when the probability of transmitter release is reduced by lowering $[Ca^{2+}]_o$, the second EPSC of a train is now potentiated (Fig. 12*B*) and as $[Ca^{2+}]_o$ is reduced further, facilitation of both the second and third EPSCs in a triplet is obvious (Fig. 12*C*). So it seems that a mixture of depression and facilitation is present at this synapse; at high release probabilities depression is dominant, while lowering the probability of release unmasks paired-pulse facilitation (PPF). Similar observations have been made at a giant synapse in the chick (Trussell *et al.* 1993). PPF is defined as a greater-than-unity ratio of the peak (P) amplitudes of the second EPSC (P2) to the first EPSC (P1), i.e. P2/P1.

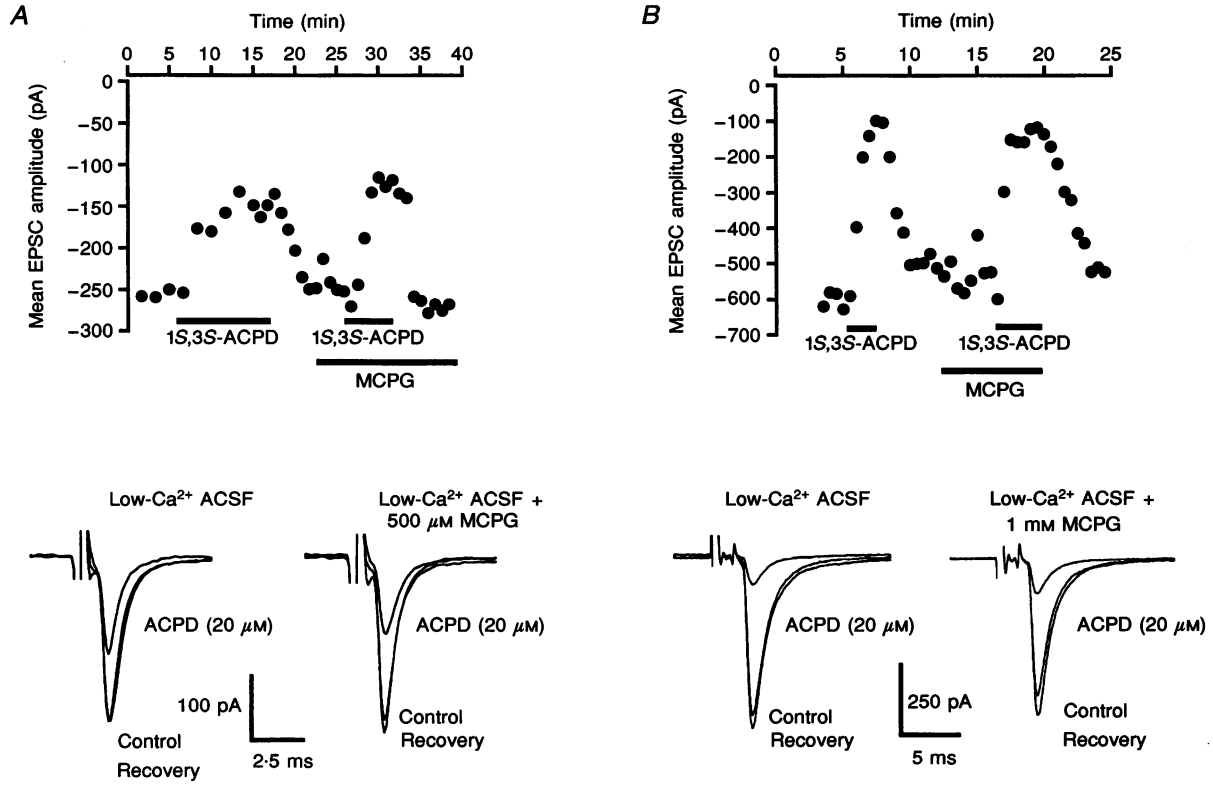

Figure 9. MCPG does not block the presynaptic action of 1*S*,3*S*-ACPD

A and *B*, plots of the EPSC amplitude against time from two MNTB neurones held at −60 mV. Each point represents the mean of 60 EPSCs evoked at a frequency of 1 Hz. The averaged EPSCs showing the effect of ACPD in the absence and then presence of MCPG are shown below. *A*, 20 μM 1*S*,3*S*-ACPD depressed EPSC amplitude by 43%, while perfusion of 500 μM MCPG had no effect, with ACPD now causing depression of 54% in this example. *B*, data from another cell in which 20 μM 1*S*,3*S*-ACPD depressed EPSC amplitude by 81%, during application of 1 mM MCPG, and a second response to ACPD still depressed EPSC amplitude by 73%.

Use of a triple-pulse regime (triplet) allows one to determine whether the factors inducing depression are significant. These factors become less significant with low probabilities of release. Thus the triple-pulse protocol in Fig. 12*B* shows a combination of facilitation and depression, indicating that the depression is still significant; these combined phenomena would not have been obvious using a paired-pulse protocol. Application of ACPD under these conditions appears to potentiate the degree of facilitation (Fig. 12*D*). When $[Ca^{2+}]_o$ is reduced to 0·6 mM (compared with the 1 mM in Fig. 12*B*), facilitation is now the dominant feature of the triplet (Fig. 12*C*). Under these conditions, ACPD still depressed the EPSC amplitude but the amplitude ratio P2/P1 was now unchanged by the presynaptic agonists (Fig. 12*C* and *E*).

Triplet responses from eight cells could be divided into two groups: Group 1, consisting of those that showed facilitation of the third EPSC over the second EPSC in a triplet, and Group 2, consisting of those that did not show facilitation of the third EPSC. In the first group (6 EPSCs, in which facilitation was the dominant feature, i.e. the facilitation was unmasked), the ratio of P2/P1 was unchanged during perfusion of ACPD (see Fig. 13). In the second group (4 responses, where no facilitation of the third EPSC was apparent, i.e. facilitation was masked) the ratio of P2/P1 amplitudes increased on application of ACPD (Fig. 13). We interpret the difference between these two groups as reflecting masking of the Group 2 facilitation by postsynaptic desensitization and presynaptic autoreceptor activation, since lowering the release probability (by reducing $[Ca^{2+}]_o$) converts the triplet response from Group 2 to Group 1 (Fig. 12*B* and *C*). In support of this interpretation, the maximum level of facilitation in both groups (i.e. in the presence of ACPD) is the same (Fig. 13). We consider that the Group 2 response is an artifact caused by superimposed facilitation and depression. Interpretation of the Group 1 data in the light of the residual calcium hypothesis indicates that ACPD does not depress Ca^{2+} entry to the presynaptic terminal either directly through blocking calcium currents, or indirectly through enhancing potassium currents.

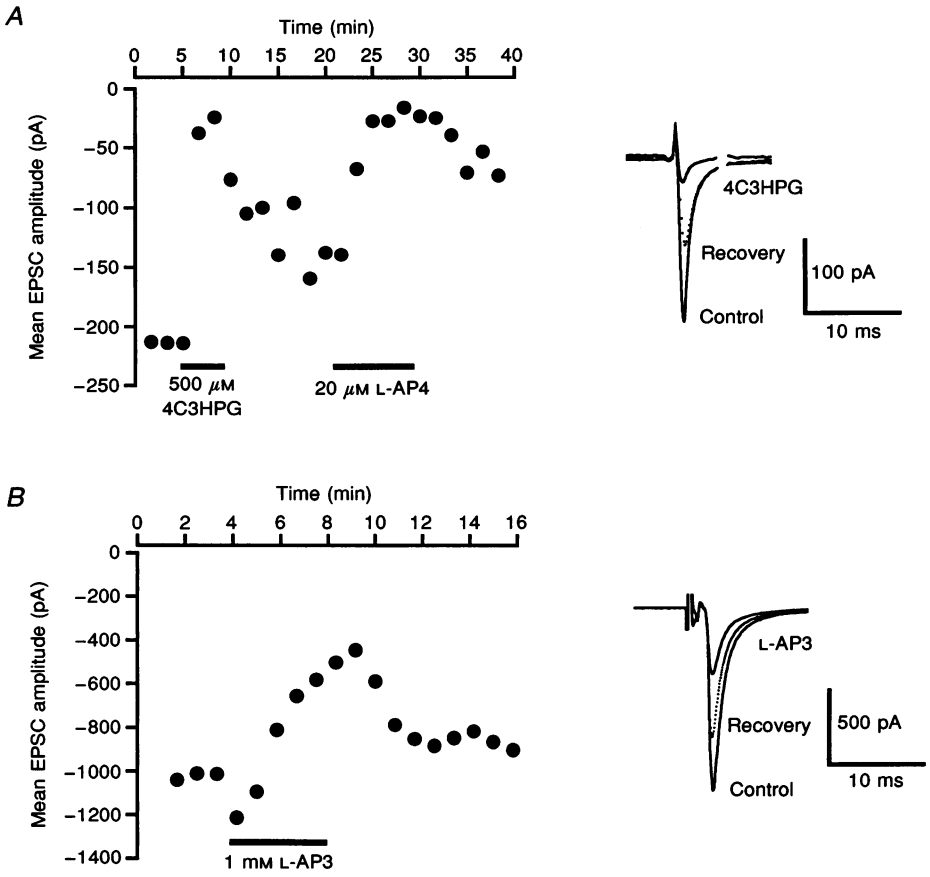

Figure 10. L-AP3 and 4C3HPG both depress the EPSC

A, plot of EPSC amplitude against time. Each point is the mean of 20 EPSCs evoked at a frequency of 0·2 Hz. 500 μM 4C3HPG depressed the EPSC amplitude by 85%; subsequent application of 20 μM L-AP4 in this cell depressed EPSC amplitude by 80%. *B*, plot of EPSC amplitude against time with each point representing the mean of 50 EPSCs evoked at a frequency of 1 Hz from an MNTB neurone held at −60 mV. 1 mM L-AP3 depressed EPSC amplitude by 60%. The averaged EPSCs are shown to the right.

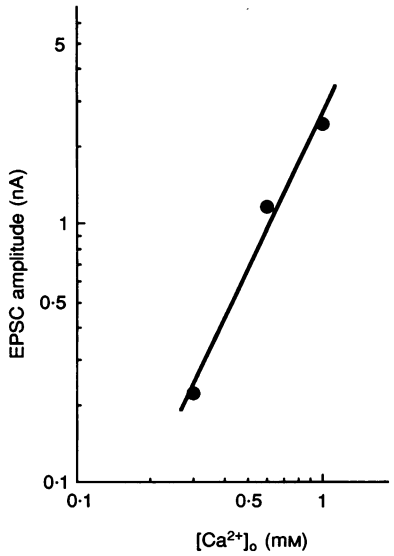

Figure 11. Calcium dependence of transmitter release at the calyx of Held

A, plot of log EPSC amplitude against log $[Ca^{2+}]_o$ for 1 neurone. The line is the fit to a power function indicating that EPSC amplitude is proportional to the $[Ca^{2+}]_o$ raised to the power of 2·01.

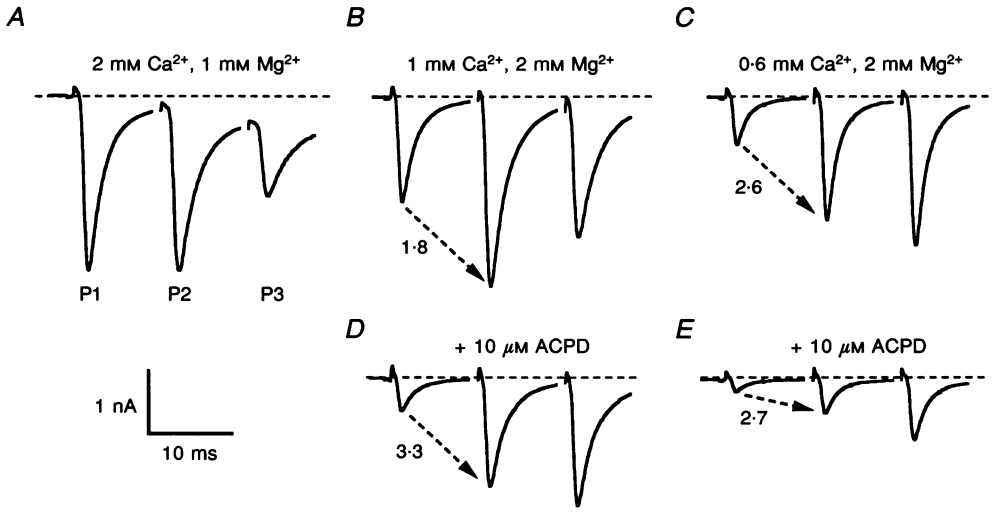

Figure 12. Paired-pulse facilitation as an indicator of $[Ca^{2+}]_i$ change

Each trace is the mean of 30 trains of 3 EPSCs (P1, P2, P3; triplet) evoked at 10 ms intervals with a frequency of 0·33 Hz. All data are from the same neurone at a holding potential of −60 mV. Divalent concentrations are indicated above the respective column, while the addition of ACPD under these conditions is indicated on the bottom row. In *A*, *B* and *C*, the top row shows that as calcium is reduced from physiological levels, the response amplitude of the first EPSC falls, while there is a relative increase in the response amplitude of the second EPSC (P2). The paired-pulse ratio, P2/P1, is indicated alongside the dashed arrow. Triplet *B* falls into Group 2 on the basis of the depression of the third pulse, while triplet *C* is in Group 1, since its third response is potentiated above P2 (see text for basis of groups). In *D* and *E*, the bottom row shows the changes on addition of 10 μM 1*S*,3*S*-ACPD (*B* is the control for *D* and *C* is the control for *E*). As expected, ACPD depressed the absolute amplitude. In Group 2 (*B* and *D*), ACPD increased the P2/P1 ratio but in Group 1 (*C* and *E*) the P2/P1 ratio did not change, suggesting that calcium influx into the terminal has not been altered by ACPD (see text).

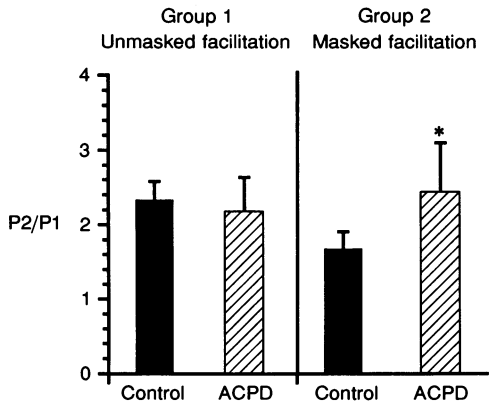

Figure 13. Paired-pulse facilitation (PPF) does not change on application of ACPD
This histogram summarizes the PPF findings for 8 responses. Those triplets in which P3 was not potentiated above P2 showed facilitation which was partially masked (Group 2), while those triplets in which P3 was potentiated above P2 showed unmasked facilitation (Group 1). Although ACPD significantly increased PPF (P2/P1) in Group 2 (* $P = 0{\cdot}07$), it had no effect on PPF in Group 1.

ACPD has no effect on the presynaptic action potential duration

Further evidence against the involvement of presynaptic potassium currents was obtained by examining the effect of ACPD on the duration of the action potential (AP) recorded from the calyx of Held. Whole-cell patch recordings were obtained from identified presynaptic terminals as described previously (Forsythe, 1994; Forsythe, Barnes-Davies & Brew, 1995). Under current-clamp conditions, depolarizing steps led to a train of APs in the presynaptic terminal. Each short-duration AP overshoots zero millivolts and is accompanied by a rapid after-hyperpolarization, as shown in Fig. 14. In order to minimize washout, ATP and GTP were included in the pipette solution and the experiments were conducted as soon as possible after going 'whole-terminal'. Further precautions to minimize delay in the experiment were taken by applying ACPD with a perfusion pipette positioned within 50 μm of the terminal rather than by perfusion of the whole chamber. Application of 200 μM 1*S*,3*S*-ACPD had no effect on the AP amplitude or duration in the three terminals examined, nor did it affect the resting membrane potential. In contrast, as shown above, 1*S*,3*S*-ACPD concentrations in excess of 3 μM depressed the EPSC in every case examined.

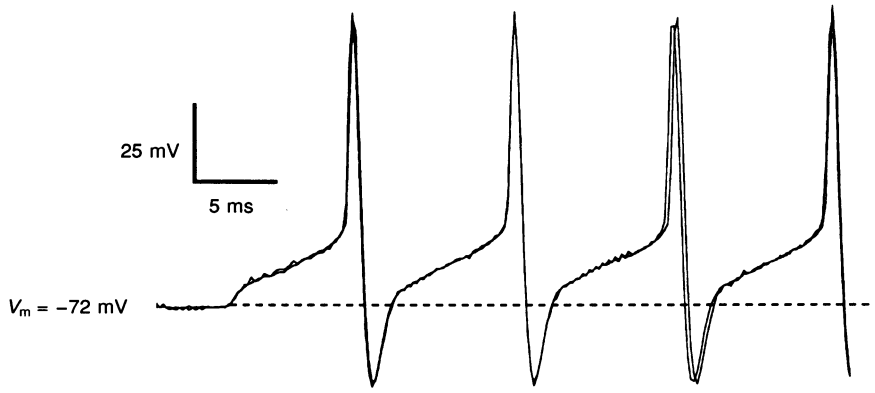

Figure 14. Presynaptic action potentials are unaffected by ACPD
Using direct whole-terminal patch recording, the terminal was depolarized to induce a low-frequency train of action potentials. (2 mM ATP, 0·5 mM GTP and 1–3 mg of Lucifer Yellow were included in the patch pipette.) Application of 200 μM ACPD from a local puffer pipette had no effect on the action potential amplitude, duration or after-hyperpolarization, as shown here by the superimposition of a control trace with a second trace recorded during the application of ACPD.

DISCUSSION

We have exploited the unusual morphology of the calyx of Held to examine the time course and presynaptic modulation of excitatory synaptic transmission in the binaural auditory pathway. Stimulation of the presynaptic axon results in a large-magnitude, dual-component EPSC which is generated at the soma of the MNTB neurone. The fast EPSC has very rapid kinetics and is mediated by AMPA receptors. It can be modulated at a presynaptic site by metabotropic glutamate receptors, which are insensitive to antagonism by MCPG. Two current hypotheses suggest that autoreceptors function by direct modulation of presynaptic calcium or potassium currents. The results of direct current-clamp recordings from the presynaptic terminal and the use of a paired-pulse protocol to test these ideas at the calyx of Held were inconsistent with the involvement of either Ca^{2+} or K^+ currents. It is possible therefore that mGluRs act through modulation of one or more components of the exocytotic pathway.

The slow, NMDA receptor-mediated EPSC

The calyx of Held shows a characteristic dual time course EPSC, with a slow voltage-dependent component mediated by NMDA receptors, preceded by a large-magnitude non-NMDA receptor-mediated component (Forsythe & Barnes-Davies, 1993*b*) similar to that observed in many areas of the CNS (Forsythe & Westbrook, 1988; Silver, Traynelis & Cull-Candy, 1992; Stern, Edwards & Sakmann, 1992; McBain & Dingledine, 1992). Estimates of the time course of the synaptic glutamate pulse suggest that it decays very rapidly with a time constant of about 1 ms (Clements, Lester, Tong, Jahr & Westbrook, 1992). In contrast, the slow EPSC can last for over 500 ms. The time course of the slow EPSC appears to be determined by the channel kinetics, since single-channel data show very long 'super-clusters' of openings (Gibb & Colquhoun, 1992) and competitive glutamate antagonists have no effect on the time course of the slow EPSC when applied during the synaptic response (Lester, Clements, Westbrook & Jahr, 1990). The latency to first opening of the NMDA receptor will also influence the slow time course of the EPSC (Edmonds & Colquhoun, 1992).

Time course and identity of receptor subunits contributing to the fast EPSC

For most of the experiments reported here, NMDA receptors were blocked by both competitive and non-competitive antagonists, so that the fast EPSC was observed in isolation. Under these conditions the fast EPSC had a rise time of between 270 and 440 μs and decayed with a double exponential time constant. The rapid rise time and restricted distribution of half-widths were consistent with a somatic location for this synapse. The kinetics of the fast EPSC are similar to those observed in other auditory synapses, such as the avian nucleus magnocellularis (Trussell, Zhang & Raman, 1993) and the endbulbs of Held in the rat cochlear nucleus (Isaacson & Walmsley, 1995).

Postsynaptic glutamate receptor desensitization can be blocked by both cyclothiazide and aniracetam, with the former selectively blocking desensitization of AMPA-type glutamate receptors (Partin *et al.* 1993). At the calyx of Held, cyclothiazide increased the amplitude and slowed the rate of decay of the fast EPSC. The potentiation in the amplitude could indicate that a proportion of the postsynaptic receptors are desensitized even with the low rates of stimulation used in these experiments (1 Hz). However, an increase in the probability of transmitter release was detected in half of the cases examined here, using the c.v.r. method. Our data do not exclude the possibility that there was a presynaptic effect at the remaining synapses, since it is superimposed on the established postsynaptic block of desensitization and is therefore difficult to detect using the c.v.r. method. A presynaptic effect of aniracetam was discounted in earlier reports using the c.v.r. method (Vyklicky *et al.* 1991), but the sensitivity of the c.v.r. technique is poor when testing for an increased probability of transmitter release under physiological release conditions (2 mM Ca_o^{2+}) when the probability of release may be already high (i.e. $P > 0{\cdot}5$). Our evidence would suggest that cyclothiazide and aniracetam can also potentiate transmitter release, in addition to blocking desensitization of postsynaptic AMPA receptors. A recent report suggests that cyclothiazide can modulate mGluR-1 and -5 (Sharp, Mayne & Burnett, 1994).

The speed of the synaptic event is an important feature for the physiological function of this synapse, which is known to maintain transmission at stimulus rates of over 600 Hz at 37 °C (Wu & Kelly, 1993). Given that the synaptic glutamate transient is rapid and that glutamate uptake does not shape the decay of the EPSC (Sarantis, Ballerini, Miller, Silver, Edwards & Attwell, 1993), the kinetics of the EPSC are likely to reflect the receptor's subunit composition and the spliced variants expressed, combined with RNA editing (see Hollmann & Heinemann, 1994). Molecular studies on cloned AMPA receptors give some important clues to the possible identity of the subunits underlying the fast EPSC at the calyx of Held. Recently it has been shown that homomeric and heteromeric channels containing GluR-C and -D subunits which include a dominant 'flop' spliced variant desensitize more quickly than those containing the alternative 'flip' cassette (Mosbacher, Schoepfer, Monyer, Burnashev, Seeburg & Ruppersberg, 1994). Flop and flip forms of the same subunit are designated GluR-D_o and GluR-D_i, respectively.

In contrast to many areas of the CNS, AMPA receptors in the chick auditory pathway have been shown to be calcium permeable (Otis, Raman & Trussell, 1995), implying that the channels probably do not contain edited GluR-B subunits (which form calcium-impermeable receptors; see Hollmann & Heinemann, 1994). It seems likely that equivalent receptors are present in the mammalian auditory system. Certainly, the reversal potential of the

fast EPSC at the calyx and endbulbs of Held are more positive than expected for a monovalent cationic permeability (+7 mV, Forsythe & Barnes-Davies, 1993*b*; +10 mV, Isaacson & Walmsley, 1995). In addition, immunocytochemical evidence suggests that GluR-D predominates over GluR-B in the auditory brainstem (Hunter, Petralia, Vu & Wenthold, 1994).

The rapid time course of the currents generated by receptors, which include the GluR-D_o subunits (the fastest of the GluR subtypes, $\tau = 0{\cdot}8$–$0{\cdot}9$ ms; Mosbacher *et al.* 1994) closely matches that observed here for the dominant sub-millisecond decay phase of the EPSC at the calyx of Held. Interestingly, the duration of glutamate application has little influence on the decay time course of the GluR-D_o-mediated current (Mosbacher *et al.* 1994) suggesting that the time course of this fast EPSC is likely to be independent of the duration of the transmitter pulse. Extrapolation of the data from cloned AMPA receptors to the slower component of the EPSC decay is more difficult ($\tau = 3{\cdot}3$ ms, which reflects 10–20% of the peak current), since several subunit combinations have decay times in this range. But an attractive hypothesis is that the slower time constant represents current flow through similar channels to those generating the fastest decay, but which instead contain a dominant flip subunit (i.e. GluR-D_i). Such a hypothesis is consistent with the decay time constants of channels containing the cloned GluR-D_i and also with the differential sensitivity that we observed for the two decay components of the fast EPSC to cyclothiazide, since the flop subunits are less sensitive to cyclothiazide than their flip variants (Partin, Patneau & Mayer, 1994).

Identity of presynaptic mGluRs

We have found that L-AP4, 1*S*,3*S*-ACPD and 1*S*,3*R*-ACPD are agonists for presynaptic receptors. Additionally, 4C3HPG and L-AP3 at high concentrations also depress transmission at the calyx of Held. MCPG did not affect synaptic transmission or antagonize the depression induced by 1*S*,3*S*-ACPD. This is in contrast to studies of field potentials generated in the ventral root on stimulation of the 1 a primary afferents, where MCPG blocks the depression induced by both ACPD and L-AP4 (Kemp *et al.* 1994), and in contrast to studies of synaptic depression in the nucleus tractus solitarii (Glaum, Sunter, Udvarhelyi, Watkins & Miller, 1993). However the presynaptic depression of transmission by ACPD in rat neocortical brain slices is insensitive to MCPG (Burke & Hablitz, 1994). Comparison of our data with the agonist–antagonist profiles for cloned mGluRs expressed in CHO or BHK cells can give some clues, but as shown below, no clear identification of the presynaptic receptor(s) can be made at present.

The L-AP4-sensitive receptors comprise mGluRs 4, 6 and 7. Although mGluR-6 may be excluded on the grounds that it is not expressed outside the retina, the pharmacological profile is very similar to that observed here in that both 1*S*,3*S*- and 1*S*,3*R*-ACPD and 4C3HPG are agonists (Thoreson & Miller, 1994). mGluR-4 is relatively insensitive to *t*-ACPD (the active isomer of which is 1*S*,3*R*-ACPD; Tanabe *et al.* 1993), but we have observed similar potencies for both 1*S*,3*S*- and 1*S*,3*R*-ACPD. The phenylglycine derivative 4C3HPG has no effect on the cloned MGluR-4 but is an antagonist at mGluR-1 and an agonist at mGluR-2 (Hayashi *et al.* 1994; Thomsen *et al.* 1994). Since 4C3HPG depresses transmission at the calyx of Held, this might imply that mGluR-2 is present; however, mRNA for GluR-2 is not detectable in cells of the VCN which give rise to the calyx of Held (Ohishi, Shigemoto, Nakanishi & Mizuno, 1993). Even if present it seems unlikely that mGluR-2 can be the only receptor involved as it is insensitive to L-AP4 (Tanabe *et al.* 1992). In addition MCPG is a competitive antagonist at mGluR-2 (and mGluR-1; Hyashi *et al.* 1994), but it does not block presynaptic depression at the calyx of Held. The other L-AP4-sensitive receptors, mGluR-4 and -7, are also insensitive to the antagonist MCPG. These results do not fit with any one of the known mGluRs, suggesting that either the presynaptic receptor has yet to be cloned, or that there are multiple mGluRs expressed (such as mGluR-2 with mGluR-4 or -7). Such a possibility is supported by recent evidence that the methyl derivative of L-AP4 (MAP4) and (2*S*,3*S*,4*S*)-α-(carboxycyclopropyl)glycine (MCCI), respectively block presynaptic L-AP4 receptors and those receptors preferentially activated by 1*S*,3*S*-ACPD in the rat spinal cord (Jane, Jones, Pook, Tse & Watkins, 1994). The best evidence for the identity of a presynaptic mGluR comes from the accessory olfactory bulb, where mGluR-2 modulates transmission at a GABAergic dendro-dendritic synapse (Hayashi *et al.* 1993).

Mechanism of autoreceptor regulation of transmitter release

We have established that ACPD acts at presynaptic receptors to depress transmitter release. There are two general mechanisms which could mediate this depression: (1) modulation of calcium channels directly, or indirectly via channels responsible for the AP duration (i.e. K^+ channels), or (2) modulatory action on components of the exocytotic machinery influencing the availability of vesicles or their probability of exocytosis. One way to distinguish between these two possibilities is to test for changes in the level of paired-pulse facilitation which, according to the residual calcium hypothesis, will increase as calcium influx declines (irrespective of whether this is a direct effect or indirect through potentiation of potassium currents).

It is important to point out that paired-pulse protocols cannot be generally applied in the CNS because of the occurrence of desensitization, autoreceptor activation or presynaptic vesicle depletion. In this study we have taken precautions to minimize these confounding influences by reducing the probability of transmitter release and

employing a triple-pulse protocol. In addition, we have two special advantages at the calyx of Held: (1) like the neuromuscular junction, all release sites arise from the same axon, so we can exclude artifacts caused by axon threshold changes, and (2) the postsynaptic neurone is voltage clamped, so that secondary activation of voltage-gated currents cannot occur.

Short-term facilitation of release is explained within the residual calcium hypothesis (Katz & Miledi, 1968) as reflecting a transient elevation in the $[Ca^{2+}]_i$ which, given the power relationship between calcium and transmitter release (Augustine & Charlton, 1986), results in a higher probability of release for the second of two closely timed stimuli. We have found that while ACPD reduces the overall amplitude of the EPSC, it did not change the P2/P1 ratio in responses with unmasked facilitation. This implies that $[Ca^{2+}]_i$ has not changed, thus excluding both the direct modulation of calcium channels and their indirect modulation via potentiation of potassium currents. An alternative explanation is that residual calcium does not account for the facilitation at this synapse, but further work would be required using paired pre- and postsynaptic recordings to test such a hypothesis. Confirmation of the lack of involvement of presynaptic potassium channels was apparent from the direct presynaptic terminal recordings, which showed no change in AP duration. Inhibition of spontaneous transmitter release at hippocampal synapses by adenosine and baclofen also occurs independently of calcium influx (Scanziani *et al.* 1992; Scholz & Miller, 1992). Direct confirmation of this mechanism will require further work using dual pre- and postsynaptic recordings and examination of exocytosis using the capacitance method.

Physiological significance of autoreceptors

A fundamental question arises concerning the functional significance of autoreceptors, especially at a synapse such as the calyx of Held which serves as a secure relay (see Forsythe, Barnes-Davies & Brew, 1995). Autoreceptors could serve both short-term and long-term modulatory functions. As suggested previously (Forsythe & Clements, 1990), given the sensitivity of the presynaptic receptors for glutamate, any changes in the efficacy of local glutamate transporters could modulate presynaptic transmitter release.

Presynaptic receptors could also serve as a means of regulating release locally at clusters of release sites on the same or adjacent axons. Such a mechanism would result in the suppression of exocytosis from and around those release sites with the highest intrinsic release probabilities. This would be physiologically significant at the calyx of Held, which supports high transmission rates (Wu & Kelly, 1993). The superior olivary complex is known to compare both binaural timing and relative loudness as part of the mechanism for estimating the source of sound in auditory space. Such temporal cues rely on a high fidelity of transmission along the auditory pathway. In order to maintain transmission, such calyceal synapses have a high safety factor (Wu & Kelly, 1993; Zhang & Trussell, 1994; Brew & Forsythe, 1995). Although autoreceptors would reduce the mean probability of exocytosis from any given release site, the number of release sites is high, and thus their activation under physiological conditions is unlikely to depress exocytosis sufficiently to induce transmission failure. Indeed, autoreceptors are likely to help preserve transmission, since reducing the probability of release from the most active release sites will give more time (on average) for recovery of the desensitized postsynaptic receptors (Trussell, Zhang & Raman, 1993) and for priming/recycling of presynaptic vesicles during high-frequency trains.

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