Direct patch recording from identified presynaptic terminals mediating glutamatergic EPSCs in the rat CNS, *in vitro*

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1. An *in vitro* brainstem slice preparation of the superior olivary complex has been developed permitting patch recording from a presynaptic terminal (calyx of Held) and from its postsynaptic target – the principal neurone of the medial nucleus of the trapezoid body (MNTB).

2. The fluorescent stain DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate) was used in fixed tissue and Lucifer Yellow in living slices, to identify calices enclosing single MNTB neuronal somata.

3. Whole-cell recording from the MNTB neurone shows evoked EPSCs preceded by a prespike, corresponding to the presynaptic action potential (AP). In some cases one patch pipette recorded from both pre- and postsynaptic elements, but confirmation of exclusively presynaptic recording was obtained using pipettes containing Lucifer Yellow in a further eleven cases.

4. Under current clamp, the pre- and postsynaptic sites could be distinguished by their response to step depolarizations; presynaptic terminals generated a train of APs at frequencies up to 200 Hz , while MNTB neurones gave a single AP. Each presynaptic AP had an after-hyperpolarization lasting less than 2 ms.

5. Under voltage clamp, step depolarizations of presynaptic terminals generated a tetrodotoxin-sensitive inward current followed by rapidly activating outward potassium currents at potentials more positive than −60 mV. The outward current exhibited little inactivation over the 150 ms steps and 4-aminopyridine (200 μM) blocked 63·0 ± 14·5% (mean ± S.D., $n = 3$) of the sustained current at 0 mV. Like the squid giant synapse, mammalian terminals express rapidly activating 'delayed rectifier'-type potassium currents.

This paper concerns preliminary observations using a method for direct patch recording from synaptic terminals in the mammalian CNS. The general principles of quantal synaptic transmission were established over 30 years ago by pioneering experiments at the neuromuscular junction (Katz, 1969). Much of our current knowledge of presynaptic voltage-dependent currents and their relationship to transmitter release comes from work on invertebrate models, especially the squid giant synapse (Llinas, Sugimori & Simon, 1982; Augustine 1990). Although direct examination of mammalian synapses in the CNS has been precluded by their small size, indirect methods based on quantal analysis of the postsynaptic responses have given important insights into factors affecting the probability of transmitter release. Both short-term and long-term changes in transmitter release have been identified using statistical methods based on the quantal hypothesis. Such techniques cannot distinguish between the possible mechanisms of these changes, such as modulation of presynaptic potassium and calcium currents or modulation of the exocytotic machinery itself. These questions can be addressed by direct recording. Besides the technical difficulty in conducting studies of mammalian synaptic terminals, it is important to confirm the identity of the recording site, particularly to establish the identity of the synapse under study, since most nuclei receive many inputs from several heterogeneous sources.

For this study a specialized synapse in the binaural auditory pathway called the calyx of Held has been used. The calices arise from the globular cells of the anterior ventral cochlear nucleus (aVCN) and form on the principal neurones of the contralateral medial nucleus of the trapezoid body (MNTB) by around 5 days after birth (Kandler & Friauf, 1993). This part of the brainstem auditory pathway is involved in sound localization and is adapted for rapid, high-fidelity transmission. Stimulation

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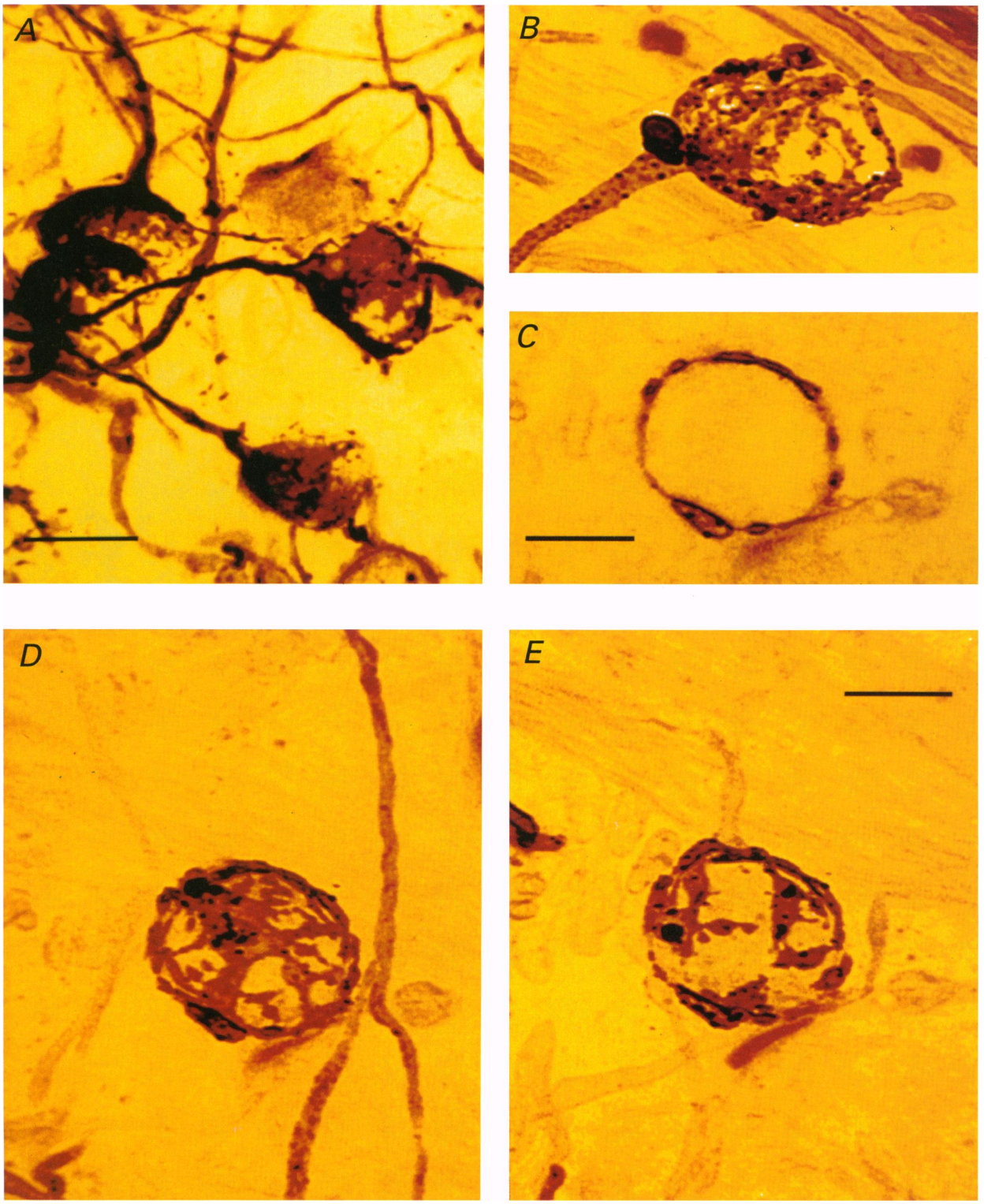

Figure 1. Structure of the calyx of Held using DiI fluorescence
A, this wide aperture confocal image of the MNTB shows several axons coursing into the nucleus and terminating in calyx-like endings. *B*, all the optical sections from a single calyx have been projected to show the axon (lower left) and terminal field around one neurone. *C*, a single optical section (0·7 μm thick) from the centre of the calyx shown in *D* and *E*. *D*, summed image of the lower half of a calyx shows 5 or 6 processes enveloping a single MNTB neurone. The axon projects down into the section from the centre of the calyx but is obscured here. *E*, summed image of upper half of the same calyx, showing the terminal processes and boutons closing round the top of the neurone. Scale in *A*, 20 μm; in *B–E*, 10 μm.

of the presynaptic axon produces a large dual component excitatory postsynaptic current (EPSC) in the MNTB neurone. A fast EPSC is mediated by α-amino-3-hydroxy-5-methylisoxazole-4-proprionate (AMPA) receptors, while a slow component is mediated by *N*-methyl-D-aspartate (NMDA) receptors (Forsythe & Barnes-Davies, 1993*b*, *c*). This study demonstrates that the size and location of this synapse permits recordings to be made from both the pre- and postsynaptic elements, giving direct observation of the presynaptic currents at a glutamatergic synapse.

METHODS

Brain slice preparation

Six- to twelve-day-old Lister Hooded rats were killed by decapitation and the brainstem region removed. Transverse slices (200 μm thick) were prepared as described previously (Forsythe & Barnes-Davies, 1993*a*), except that equi-osmolar concentrations of sucrose were substituted for NaCl in the artificial cerebrospinal fluid (ACSF) used for slicing. After incubation for 60 min (37 °C), one slice was mounted in a Peltier-driven environmental chamber on the stage of a M2A microscope (MicroInstruments) fitted with Nomarski optics and epifluorescence, with a Zeiss ×40 (NA 0·75) water-immersion objective. The normal ACSF consisted of (mM): NaCl, 125; KCl, 2·5; $NaHCO_3$, 26; glucose, 10; NaH_2PO_4, 1·25; sodium pyruvate, 2; *myo*-inositol, 3; $CaCl_2$, 2; $MgCl_2$, 1. It was gassed with 95% O_2–5% CO_2 and continually perfused over the preparation (0·75 ml min^{-1}, 25 °C).

Electrophysiology

Whole-cell patch recordings were made from neurones using thin-walled glass filled with (mM): potassium gluconate, 97·5; KCl, 32·5; EGTA, 5; Hepes, 10; $MgCl_2$, 1. The presynaptic axons were stimulated in the trapezoid body using a bipolar electrode. Whole-cell patch recordings were made from visually identified neurones, without prior cleaning. Series resistances were 8–20 MΩ and compensated by 80–90%. Patch recordings from presynaptic terminals were made with thick-walled glass, filled with the above patch solution plus 2 mM K_2ATP, 0·5 mM Na_2GTP and 1–3 mg ml^{-1} Lucifer Yellow (Aldrich). Voltage- and current-clamp recordings were made using an Axopatch 200, with a 1401 interface and 'Patch' software (Cambridge Electronic Design) running on a Dell 486 computer. Digitization rates were between 2 and 20 kHz with filtering of 1–5 kHz. Unless otherwise stated, chemicals/drugs were from Sigma (UK). Drugs were applied by perfusion in the ACSF. Averaged data are means ± S.D.

Confocal microscopy

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes; 2·5 mg ml^{-1}) was dissolved in absolute ethanol and injected into the trapezoid body of freshly dissected 10- to 14-day-old rat brainstems. The brainstem was immediately fixed in 4% formaldehyde–phosphate-buffered saline and kept at 36 °C for 3–6 months (see Kandler & Friauf, 1993). Transverse sections 100–200 μm thick were then cut on a vibrotome. Stained calyces were examined using a confocal microscope (MRC 600) with an argon laser and rhodamine (DiI) or fluorescein (Lucifer Yellow) filter sets. Single terminals were optically sectioned at 0·5–0·7 μm (*z*-interval).

RESULTS

Structure of the calyx of Held

Orthograde diffusion of the DiI along the axons in the trapezoid body revealed that they formed large calyceal terminals in the contralateral MNTB as shown in Fig. 1*A*. The summed image projected from one calyx (Fig. 1*B*) shows the axon and terminal field enclosing a single MNTB neurone. The projected image of the lower half of another calyx is shown in Fig. 1*D*. The axon (not visible) opens out into a palm-like apposition on contacting the MNTB neurone, which then divides into finer processes enfolding one neurone. A single optical section from the middle of the series (Fig. 1*C*) shows a cross-section of the presynaptic processes; the 'hole' in the middle indicates the position of the unstained neurone. Continuation of the projection through the upper half of the same calyx shows the synaptic processes closing round the MNTB neurone (Fig. 1*E*).

Postsynaptic response

Whole-cell patch recordings from the postsynaptic MNTB neurone, reveal that stimulation of the trapezoid body evokes a short latency, all-or-nothing current (prespike) which is rapidly followed by a large EPSC (Fig. 2*A*). In control ACSF a fast EPSC is mediated by AMPA receptors, while a slow EPSC is mediated by *N*-methyl-D-aspartate (NMDA) receptors (Forsythe & Barnes-Davies, 1993*b*, *c*).

The calyx covers half of the surface area of the MNTB neuronal soma, so it was anticipated that presynaptic recordings would be made 'accidentally'. In fact such recordings have been extremely rare, although some of the data are consistent with recording from both the pre- and postsynaptic sites (Fig. 2*B*). In whole-cell recordings from the postsynaptic neurone, a component of the presynaptic current flow associated with invasion of the AP into the surrounding calyx is detected as a positive-going current (Fig. 2*A*). In some cases a large negative-going prespike, which possessed an identical threshold to that of the EPSC, was observed instead (Fig. 2*B*). Given the veil-like apposition of parts of the calyx, we interpret this as a postsynaptic recording in which some part of a presynaptic process has been trapped and ruptured under the patch pipette, giving a high series resistance recording of the presynaptic current. On two occasions the latter configuration spontaneously changed so that the prespike increased in amplitude by more than an order of magnitude, while the EPSC diminished in magnitude, as shown for one recording in Fig. 2*C*–*E*. This presumably reflects a reduction in series resistance with the presynaptic terminal relative to the postsynaptic element.

Presynaptic recording from the calyx of Held

In order to prove that recordings could be obtained exclusively from the presynaptic terminal, Lucifer Yellow was included in the patch solution, so that the recording site could be identified. Current-clamp recording from the postsynaptic MNTB neurones show a highly characteristic single AP response (Fig. 3*A*), even with depolarizations well in excess of the threshold, as described previously (Forsythe & Barnes-Davies, 1993*a*). Inadvertent recording from the postsynaptic neurone was common during this study and was characterized by the labelling shown in Fig. 3*B*. MNTB neurones have a round soma with few dendrites; the largest process in Fig. 3*B* is the axon, orientated toward the lateral brainstem.

A total of eleven recordings were made from presynaptic terminals where some electrophysiology was also obtained (i.e. MNTB neurone soma remained unstained, but was partially enclosed by a fluorescent profile as shown in Fig. 3*D*). Presynaptic terminals could be differentiated from postsynaptic neurones by their response to injection of depolarizing current steps under current clamp and their fluorescent profile. Each of the four presynaptic terminals examined under current clamp exhibited trains of action potentials throughout the depolarizing current step (Fig. 3*C*), with the terminals sustaining firing rates of up to 200 Hz (at 25 °C). The resting or zero current potential of seven terminals was $-73{\cdot}0 \pm 5{\cdot}4$ mV, with input resistance of 388 ± 228 MΩ ($n = 6$). The presynaptic AP always overshot 0 mV and was characterized by a distinct after-hyperpolarization (AHP) more negative than −90 mV and lasting $1{\cdot}8 \pm 0{\cdot}2$ ms ($n = 3$), as illustrated in the inset of Fig. 3*C*.

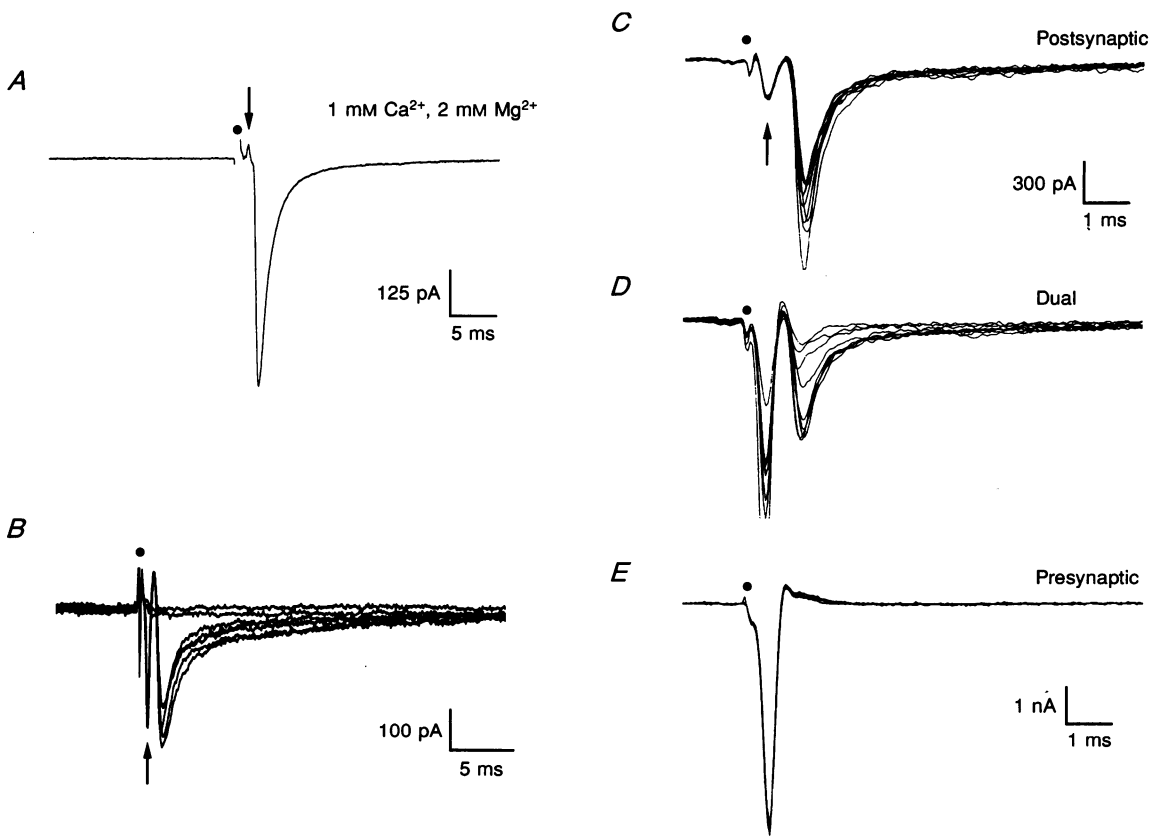

Figure 2. In voltage clamp (holding potential −60 mV) a field corresponding to the presynaptic spike is observed in postsynaptic recording configurations on stimulation of the trapezoid body

A, in averaged data a prespike precedes the evoked EPSC by about 0·5 ms (artifacts indicated by ●, and the prespike by the arrow). *B*, in the raw data from a different neurone a negative prespike is observed, possessing an identical threshold to the postsynaptic EPSC, as indicated here by the EPSC and prespike failing together. The prespike is all-or-nothing in amplitude, while the EPSC fluctuates in amplitude from trial to trial. *C*, *D* and *E*, in another recording the prespike spontaneously increases in amplitude, while the EPSC declines, such that the patch pipette is now recording invasion of an orthodromic AP. This is interpreted as a recording from the presynaptic terminal. Note the change of gain in panel *E*.

Current–voltage relation of the presynaptic terminal

Voltage-clamp recording from the calyx of Held revealed the presence of an inward sodium current, followed by rapidly activating outward currents. Orthodromic stimulation of the axon also produced a rapid inward current, which was presumably generated in unclamped regions of the axon (inset in Fig. 4*A*). Step depolarizations evoked a rapid low-threshold inward current which was blocked by 1 μM tetrodotoxin (TTX). The current–voltage (I–V) relationship of the outward currents was examined in five terminals. In the presence of 1 μM TTX (Fig. 4*B*) an outward current rapidly activated on depolarizations

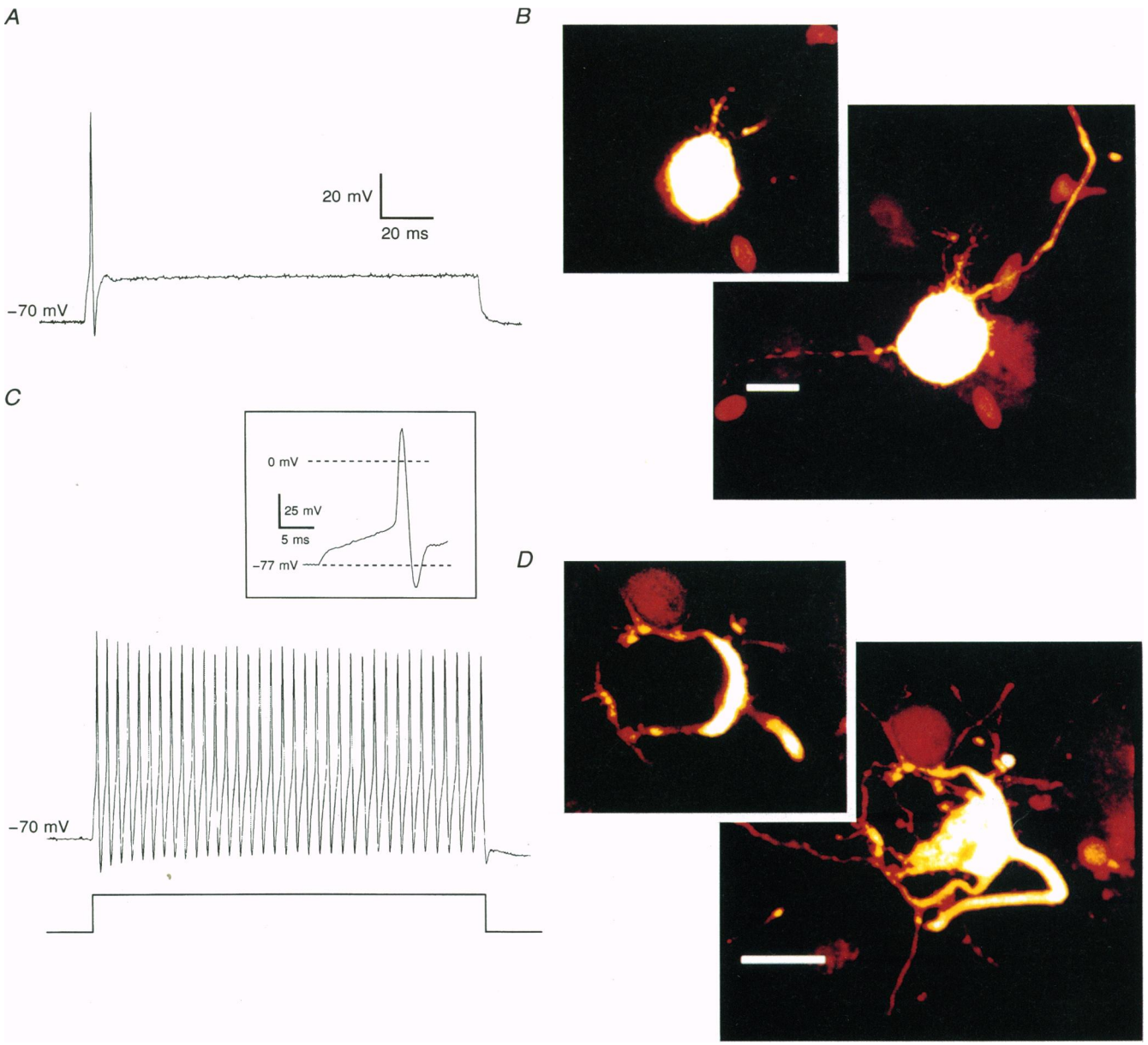

Figure 3. Combined fluorescence and current-clamp recordings from either post- or presynaptic elements
A, MNTB neurone stained with Lucifer Yellow: in response to 1 nA step depolarizations a single AP is generated. *B*, a summed confocal projection of the same neurone shows a characteristic round soma with one major process (axon) orientated to the lateral superior olive and several fine dendritic processes. Inset, a single optical section just below the centre of the cell. *C*, calyx of Held stained with Lucifer Yellow: step depolarizations induce a train of short APs, each with an after-hyperpolarization as shown in the inset. *D*, confocal projection of this calyx shows its axon on the right, with the dye restricted to a crescent-shaped profile around the circumference of a neurone, as shown by the single optical section in the inset. Scale, 10 μm.

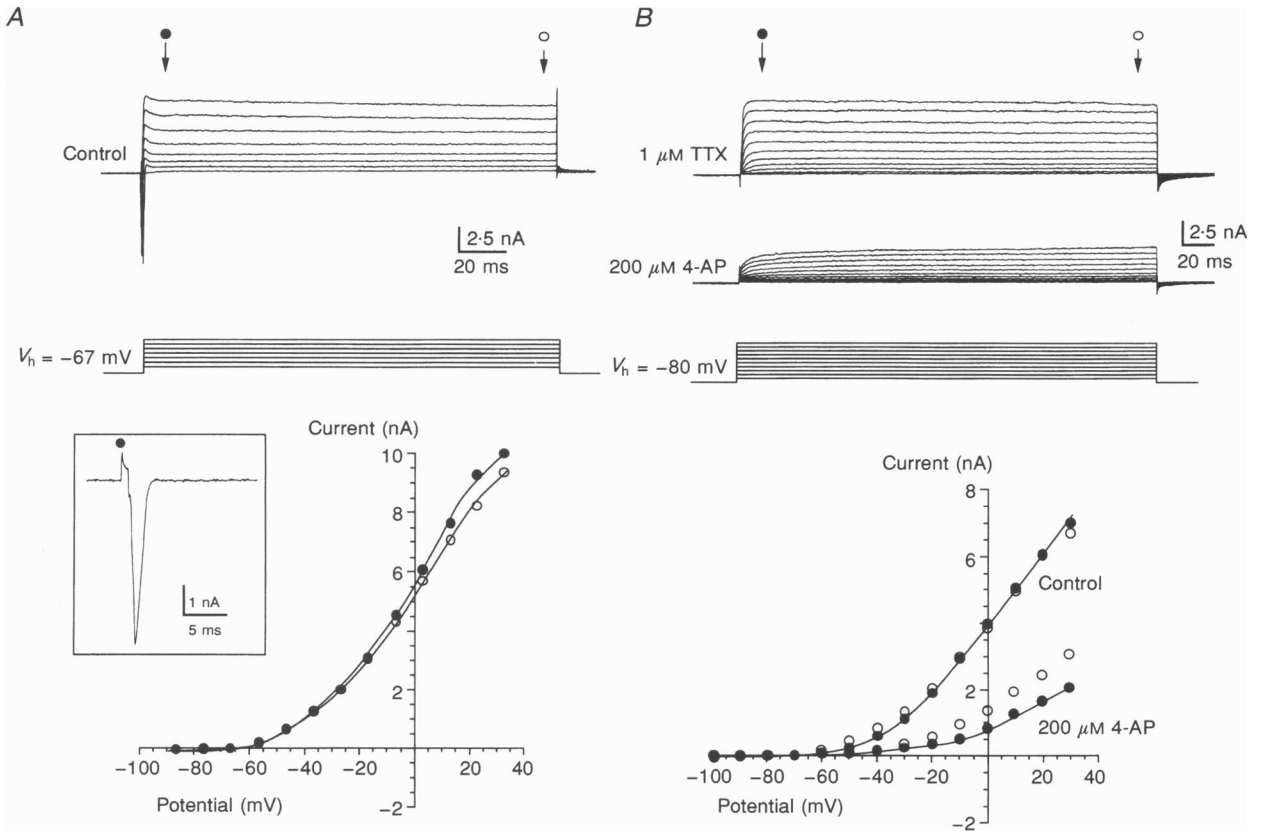

Figure 4. Voltage clamp of presynaptic currents shaping the action potential
Current traces are shown on the top with voltage protocols below and I–V relations at the bottom plotted at the times indicated by the open and filled symbols. *A*, in normal ACSF a TTX-sensitive inward current is followed by rapidly activating and sustained outward current. Inset shows the response on stimulation of the trapezoid body, with the filled circle indicating the stimulus artifact. *B*, in another calyx (in the presence of 1 μM TTX) the outward currents activated by low-threshold depolarizations are substantially blocked by 200 μM 4-AP.

more positive than −60 mV, peaking in 2·3 ± 0·8 ms ($n = 5$) on steps to positive potentials. This current showed little inactivation over the 150 ms time course of these step commands, but a voltage-dependent inactivation process is implied since steps from a holding potential of −60 mV produced smaller outward currents than from −80 mV (data not shown). 4-Aminopyridine (4-AP, 200 μM) substantially blocked the low-threshold component of the total outward current (63·0 ± 14·4% block at 0 mV, $n = 3$) as shown for one cell in Fig. 4*B*.

DISCUSSION

Here are reported direct observations of the time course and currents underlying the action potential in a mammalian synaptic terminal which utilizes glutamate as its transmitter. Although the calyx of Held may superficially be considered as a 'giant' synapse, its structure and unusual configuration are best interpreted as those of an axon collateral which forms its terminal field exclusively on the soma of one neurone. This synapse might be expected to be a general model for excitatory transmission in the CNS, since the presynaptic cell is not a primary afferent.

Previously, patch recordings have been made from neurohypophysis nerve terminals (Thorn, Wang & Lemos, 1991), the ciliary ganglion calyx (Stanley, 1991; Yawo & Momiyama, 1993) and recently preliminary reports have also been made of recordings from two excitatory synapses: mossy fibre terminals in the hippocampus (Gray, 1993) and primary afferents in the chick auditory pathway (Sivaramakrishnan & Laurent, 1993).

The method used here for making unambiguous recordings from the calyx of Held required application of both electrophysiological and fluorescence techniques. This presynaptic terminal expresses rapidly activating potassium currents, permitting short APs with high firing rates. The sensitivity of the current to micromolar concentrations of 4-AP supports the suggestion from work on synaptic transmission (Jack, Redman & Wong, 1981; I. D. Forsythe & M. Barnes-Davies, unpublished observations) that 4-AP potentiates release by blocking a presynaptic potassium current. In contrast to data from neurohypophysis nerve terminals (Thorn *et al.* 1991), there is no obvious transient outward currents contributing to AP repolarization in this glutamatergic pathway, indeed the outward currents are

remarkably similar to those of the squid giant synapse (Augustine, 1990). Although there is evidence for *Shaker*-related potassium channels in axons and particularly inhibitory terminals (McNamara, Muniz, Wilkin & Dolly, 1993; Sheng, Tsaur, Jan & Jan, 1994), the pharmacology, kinetics (Kirsch & Drewe, 1993) and localization of currents observed here are perhaps most closely matched with the *shaw*-related channel Kv3.1 (Perney, Marshall, Martin, Hockfield & Kaczmarek, 1992). Like the MNTB neurone, the globular cell, from which the calyx of Held arises, will fire only one or two APs (Manis & Marx, 1990), while in this study it is shown here that the terminal will sustain trains of APs during a depolarizing step; this suggests that there are differences in the insertion of potassium channel subunits or differences in their modulation between the two locations.

An interesting feature of the AP in the synaptic terminal is the profound after-hyperpolarization, in marked contrast to microelectrode recordings from myelinated axons, which exhibit a slow after-depolarizing potential (Gavriel, Barrett & Barrett, 1993). The after-hyperpolarization is probably generated by the rapidly activating outward potassium currents, rather than a calcium-dependent potassium current, which generally has a slower time course. Such an after-hyperpolarization could be a general feature of axons with high transmission rates, for it would serve the dual role of minimizing the refactory period by re-activating sodium channels and maximizing the driving force for calcium ions. Further work is required to determine the contribution of calcium or calcium-activated currents to the AP and to examine presynaptic calcium currents themselves.

It has been suggested that the calyx of Held guarantees transmission, by providing a massive synaptic current in the MNTB neurone. The time course of the fast EPSC is very rapid, with a major decay time constant of less than 1 ms (Forsythe & Barnes-Davies, 1993*c*), similar to the chick nucleus magnocellularis (avian equivalent of the cochlear nucleus; Raman & Trussel, 1992). This has led to the idea that the auditory pathway may specifically express rapidly acting glutamate receptors. It seems likely that the rapidly activating potassium currents observed here may also be a general feature of fast excitatory synaptic pathways.

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