CALCIUM CONDUCTANCE IN AN IDENTIFIED CHOLINERGIC SYNAPTIC TERMINAL IN THE CENTRAL NERVOUS SYSTEM OF THE COCKROACH

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SUMMARY

Intracellular microelectrodes were used to study a cholinergic synapse between two identified neurones: the lateral filiform hair sensory neurone (LFHSN) and giant interneurone 3 (G13) in the terminal ganglion of the first-instar cockroach Periplaneta americana. The presynaptic cell, LFHSN, was impaled in a region of the axon which forms large numbers of output synapses.

The sign and magnitude of the LFHSN spike afterpotential were shown to depend on \([Ca^{2+}]_o\). 1 \(\mu\)mol\(^{-1}\) tetrodotoxin (TTX) abolished LFHSN spikes but the addition of 0.1 mmol\(^{-1}\) 4-aminopyridine (4-AP) enabled regenerative depolarizations to be evoked which were followed by large EPSPs in G13. Addition of 20 mmol\(^{-1}\) tetraethylammonium ions (TEA\(^+\)) abolished the cholinergic EPSPs but resulted in long-duration LFHSN spikes. Intracellular injection of caesium ions (Cs\(^+\)) into LFHSN enabled long-duration spikes to be evoked and had no effect on synaptic transmission. Long-duration LFHSN spikes were (1) increased in amplitude by increased \([Ca^{2+}]_o\); (2) accompanied by an increase in conductance; (3) not abolished by replacement of external Na\(^+\) with Tris\(^+\) or choline\(^+\); (4) blocked by 1 mmol\(^{-1}\) Cd\(^{2+}\) and 10 mmol\(^{-1}\) Co\(^{2+}\); (5) not supported by substitution of Mg\(^{2+}\) for Ca\(^{2+}\); and (6) supported by Ba\(^{2+}\) substitution. They are thus considered to be Ca\(^{2+}\) spikes. The Ca\(^{2+}\) spikes were blocked by organic Ca\(^{2+}\) channel blockers at 0.5–1 mmol\(^{-1}\).

The putative Ca\(^{2+}\) spike was followed by a hyperpolarizing afterpotential (HAP), the duration of which was proportional to the amplitude and duration of the Ca\(^{2+}\) spike. The HAP was (1) accompanied by a conductance increase; (2) reversed at potentials 30 mV more negative than resting potential; (3) not supported by substituting Ba\(^{2+}\) for Ca\(^{2+}\); and (4) partially blocked by 150 mmol\(^{-1}\) TEA\(^+\). The HAP is considered to result from an increase in Ca\(^{2+}\)-dependent K\(^+\) conductance.

It is concluded that, in addition to Na\(^+\) channels and delayed rectifying K\(^+\) channels, Ca\(^{2+}\) channels and Ca\(^{2+}\)-dependent K\(^+\) channels are present in the axonal membrane of LFHSN, in a region which forms many output synapses.

Key words: calcium spike, calcium-activated potassium conductance, synaptic terminal, identified insect neurone.
INTRODUCTION

Many neurobiological preparations have been developed in which intracellular recording from identified pre- and postsynaptic elements of a chemical synapse is possible, but only a few allow direct access to the junctional site, particularly the presynaptic region. Examples include: the squid giant axon synapse (Bullock & Hagiwara, 1957; Llinás, Steinberg & Walton, 1981a, b), the chick ciliary ganglion (Martin & Pilar, 1963), the Mauthner cell–giant fibre synapse of the hatchetfish (Auerbach & Bennett, 1969), the lamprey Muller neurone–interneurone synapse (Martin & Ringham, 1975), the crab muscle receptor–motoneurone synapse (Blight & Llinás, 1980), the crayfish neuromuscular junction (Wojtowicz & Atwood, 1984) and the lobula giant movement detector (LGMD)–descending contralateral movement detector (LCMD) synapse in the locust (Rind, 1984).

For pharmacological studies of synaptic transmission in insect CNS the cockroach *Periplaneta americana* is particularly suitable, since the blood–brain barrier can be removed without impairing synaptic function, allowing access to pre- and postsynaptic elements of identifiable pathways. The cerci of orthopterous insects such as the cockroach are a pair of conical appendages on the posterior end of the abdomen. Each cercus bears several types of sensillum, one of which, the filiform hair, responds to air movements (Dagan & Camhi, 1979; Tobias & Murphey, 1979). Sensory axons within the cerci join to form the cercal nerves which enter the terminal abdominal ganglion. The largest neurones in this ganglion are the giant interneurones, so called because they send large-diameter axons up the nerve cord towards the head. The giant interneurones receive sensory input from the cercal sensory neurones (Callec, Guillet, Pichon & Boistel, 1971; Matsumoto & Murphey, 1977).

In the cockroach *Periplaneta americana* cercal afferent–giant interneurone synapses have been shown to be cholinergic (Callec, 1974; Sattelle, 1980) and the nicotinic antagonist α-bungarotoxin blocks transmission (Sattelle et al. 1983). The giant interneurones of *Periplaneta americana* mediate the escape response to air movements (Westin, Langberg & Camhi, 1977; Camhi & Tom, 1978; Camhi, Tom & Volman, 1978: Ritzmann & Camhi, 1978).

The great advantage of the first-instar nymphal cockroach as an experimental system compared to the adult, or to nymphs and adults of other orthopterous insects, lies in the small number of cercal sensory axons. There are only two filiform hairs on each cercus compared to approximately 220 in the adult, and each filiform hair sensory neurone (FHSN) is identifiable (Blagburn & Beadle, 1982; Dagan & Volman, 1982). The small size of the terminal ganglion allows it to be viewed with Nomarski optics. The FHSN axons and GI 2 and GI 3 can be located visually in the living ganglion, which greatly facilitates impalement with intracellular microelectrodes (Blagburn, Beadle & Sattelle, 1986). The first-instar FHSN–GI pathway is particularly suitable for developmental studies of, for example, synapse formation (Blagburn et al. 1985a) and chemosensitivity (Blagburn et al. 1985b).

It has been shown by electron microscopy (Blagburn et al. 1984) that the site of impalement of the LFHSN axon is also the site of large numbers of output synapses...
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(approximately $8 \mu m^{-2}$: Blagburn et al. 1985a). This gives us the opportunity to investigate the physiology of a large (6–10 $\mu m$ diameter) presynaptic terminal of an identified insect sensory neurone, while monitoring postsynaptic potential changes.

MATERIALS AND METHODS

Cockroaches (Periplaneta americana) were reared at 27°C with food and water freely available. Newly hatched first-instar nymphs were placed in a saline solution of the following composition (in mmol$^{-1}$): NaCl, 150·0; KCl, 3·1; CaCl$_2$, 5·4; MgCl$_2$, 1·0; Hepes buffer, 5·0; sucrose, 50·0; pH 7·4 (based on Callec & Sattelle, 1973).

The legs and antennae were removed with fine dissecting scissors and a ventral strip of cuticle was excised, bearing the head, nerve cord and cerci. The rectum was removed and all but the most posterior abdominal cuticle segments were pulled away from the nerve cord. The isolated CNS and cerci were then transferred to a rubber-walled chamber constructed on a glass microscope slide and anchored, ventral side up, using petroleum jelly. The connective tissue sheath around the terminal ganglion was softened by brief (5 s) exposure to saline containing 1·0 mg ml$^{-1}$ protease (Type XIV, Sigma, UK) then removed using fine forceps. All four filiform hairs were immobilized by covering the cerci with petroleum jelly.

Isolated preparations were viewed with Nomarski optics, using a 40× water-immersion objective lens (Zeiss, FDR), electrically isolated from the body of the microscope with a Perspex insert. The cell body, axon and primary dendrite of GI 3 could be identified reliably using the criteria of size, morphology, position and appearance (Fig. 1B). The LFHSN axon was located visually near the ventral margin of the neuropile, following the lateral neuropile boundary (Fig. 1B). The axon was characteristically large in this region (6–10 $\mu m$ diameter) and its many mitochondria gave it a granular appearance.

For intracellular recording, glass capillary microelectrodes were made using 'Kwik-fil' 1 mm diameter, standard-bore capillary tubing (Clark Electromedical Instruments, UK) in a vertical electrode puller (Narishige, Japan) and filled with 1·0 mol$^{-1}$ KCl and 5·0 mmol$^{-1}$ Hepes, adjusted to pH 7·2 with KOH. Alternatively, electrodes were filled with 1·0 mol$^{-1}$ potassium acetate, pH-adjusted with acetic acid. For intracellular ionophoresis of caesium ions, microelectrodes were filled with the following solution: KCl, 0·75 mol$^{-1}$; CsCl$_2$, 0·25 mol$^{-1}$; Hepes, 3·8 mmol$^{-1}$, adjusted to pH 7·2 with HCl. The microelectrodes were 30–60 MΩ in resistance, and exhibited some degree of Type II nonlinearity (Purves, 1981) when passing current of more than 2 nA. Current flow in the bath was monitored using a virtual earth circuit (Fig. 1C). Oscilloscope traces were recorded on a Racal Store 4 DS tape recorder and permanent records were made using a Medelec storage oscilloscope.
Fig. 1. Microelectrode recording from pre- and postsynaptic elements of an identified central synapse in an insect. (A) Dorsal view of first-instar cockroach (Periplaneta americana) showing position of abdominal ganglia. (B) Camera lucida tracing of the terminal abdominal ganglion seen under Nomarski optics. One lateral filiform hair sensory neurone (LFHSN) and one giant interneurone 3 (GI 3) are shown impaled with microelectrodes; each neurone has a homologous contralateral partner which is not shown here. Stippling indicates the characteristic granular appearance of the cytoplasm. (C) Experimental system.

Drugs used in the experiments were: tetrodotoxin (TTX, Sankyo Co., Japan, distributed by Koch-Light Laboratories, UK), 4-aminopyridine (4-AP, Sigma, UK), tetraethylammonium bromide (TEABr, Kodak, UK). The highly purified bromide salt of TEA was used to avoid possible contamination by triethylamine (Zucker, 1981). The organic calcium channel blockers diltiazem, verapamil (Sigma, UK), D600 and nifedipine (Bayer, FDR) were also used. Saline solution was perfused continuously at a rate of 1 ml/min⁻¹, into a bath of 0.3 ml volume. Experiments were performed at a temperature of 20–22°C.
RESULTS

Electrical properties of the lateral filiform hair sensory neurone

The resting potential recorded from the LFHSN axon was $-54 \pm 1$ mV (mean $\pm$ S.E.M., $N = 48$), and the resting potential recorded from the cell body of GI 3 was $-78 \pm 1$ mV ($N = 41$). The input resistance of LFHSN ($R_{in}$) was $20 \pm 2$ MΩ ($N = 20$) for a negative current pulse of 1 nA, and the axon exhibited pronounced delayed rectification (Fig. 2A). With the filiform hair immobilized, LFHSN spontaneously produced action potentials at a frequency of 40–60 Hz. In the majority of preparations the overall amplitude of the spikes was between 30 and 70 mV ($51 \pm 1$ mV, $N = 46$), peaking at a potential of $-4 \pm 2$ mV ($N = 46$). Preparations with a resting potential more positive than $-40$ mV, an action potential amplitude of less than 30 mV, or $R_{in}$ of less than 10 MΩ were discarded.

LFHSN spikes had a duration of approximately 1 ms, measured at a potential 10 mV positive to resting potential. Measurement of the spike duration was complicated by the variable nature of the spike afterpotential (AP), which ranged from a hyperpolarizing afterpotential (HAP) of up to $-10$ mV from resting potential, to a depolarizing afterpotential (DAP) of up to $+12$ mV from resting potential. The reversal potential for the afterpotential ($E_{AP}$) varied from $-45$ to $-70$ mV ($-58 \pm 1$, $N = 43$).

Fig. 2. (A) Averaged current–voltage relationship of nine LFHSN axons. The amplitude of the depolarization was measured 20 ms after the onset of the pulse. Delayed rectification is present in the depolarizing direction. Vertical bars represent ±S.E.M. (B) Calcium-dependence of afterpotentials (APs) following spikes recorded from the lateral filiform hair sensory neurone (LFHSN). Dependence on $[Ca^{2+}]_o$ of the size and polarity of the AP. The APs are shown at (Bi) resting, (Bii) depolarized and (Biii) hyperpolarized membrane potentials, with superimposition of those recordings obtained in (1) $20\text{mmol}^{-1}\text{L}^{-1}\text{Ca}^{2+}$, (2) $5.4\text{mmol}^{-1}\text{L}^{-1}\text{Ca}^{2+}$, and (3) $0\text{mmol}^{-1}\text{L}^{-1}\text{Ca}^{2+}$ plus $20\text{mmol}^{-1}\text{L}^{-1}\text{Mg}^{2+}$. (Ci) Calcium-dependent voltage change [$\Delta V(Ca^{2+})$] calculated by subtracting the records of spikes in zero external $Ca^{2+}$ from those in normal and high $Ca^{2+}$. (Cii) The GI 3 EPSP in (1) high $[Ca^{2+}]_o$ and (2) normal $[Ca^{2+}]_o$ is shown.
Earlier electron microscope and physiological studies have demonstrated that the LFHSN–GI3 synapse is a monosynaptic connection (Blagburn et al. 1984; Blagburn & Sattelle, 1987).

Synchronous recording from LFHSN and the contralateral GI3 cell body showed that spikes in the sensory axon give rise to depolarizing postsynaptic potentials in the interneurone. At high frequency these potentials may summate to give rise to interneurone spikes; they are therefore termed excitatory postsynaptic potentials (EPSPs). These EPSPs had an amplitude of 6.4 ± 0.3 mV (N = 91), a latency of 1.37 ± 0.06 ms (N = 21), measured from 10 mV positive to resting potential on the spike rising phase to the start of the EPSP rise, a time to peak of 2.05 ± 0.08 ms (N = 24) and a decay time constant of 4.55 ± 0.13 ms (N = 24). These values were obtained from cell body recordings and, although it has been shown that depolarizations are attenuated to 75% of their original value during electrotonic conduction from the primary dendrite to the cell body (J. M. Blagburn & D. B. Sattelle, unpublished observation), the degree to which the EPSPs were attenuated during conduction from their points of origin in the fine dendritic branches is not known.

**Evidence for a calcium component of the afterpotential following sensory axon spikes**

It was noted that the sign of the spike afterpotential (hyperpolarizing or depolarizing) varied according to the level of the resting membrane potential. The effects of changing \([\text{Ca}^{2+}]_o\) on the AP were then investigated (Fig. 2B). With a \([\text{Ca}^{2+}]_o\) of 5.4 mmol l\(^{-1}\) the EAP was 9 mV more positive than with a \([\text{Ca}^{2+}]_o\) of 0 mmol l\(^{-1}\) (with the addition of 20 mmol l\(^{-1}\) Mg\(^{2+}\) to produce complete block of Ca\(^{2+}\) channels). With a \([\text{Ca}^{2+}]_o\) of 20 mmol l\(^{-1}\) the EAP was 14 mV more positive than in 0 mmol l\(^{-1}\) Ca\(^{2+}\). The spike amplitude was not affected by changes in \([\text{Ca}^{2+}]_o\). Changes in \([\text{Ca}^{2+}]_o\) took effect in 5 min and were reversible on perfusion of normal saline.

Subtraction of the action potential record obtained for 0 mmol l\(^{-1}\) Ca\(^{2+}\) from those for 5.4 and 20 mmol l\(^{-1}\) Ca\(^{2+}\) enabled examination of the effect of \([\text{Ca}^{2+}]_o\) on the shape of the spike (Fig. 2C). The calcium-dependent potential changes thus obtained were termed \(\Delta V(\text{Ca}^{2+})\). It is not known why the time course and amplitude of the \(\Delta V(\text{Ca}^{2+})\) and the EPSP are similar. The above results suggest that there may be a significant involvement of calcium ions in producing a depolarization which, superimposed on the normal HAP due to K\(^+\) efflux, reduces or reverses it. Further evidence was sought with the use of pharmacological agents which block Na\(^+\) and K\(^+\) channels.

**Effects on the nerve terminal action potential of sodium and potassium channel-blocking agents**

Tetrodotoxin (TTX) has been shown to block insect sodium channels at micromolar concentrations (Sattelle, Pelhate & Hue, 1979). After 5 min exposure to
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1 μmol l⁻¹ TTX, LFHSN spikes were reduced in amplitude to 3–10 mV; these spike remnants persisted for 5–10 min. Exposure to 1 μmol l⁻¹ TTX completely abolished LFHSN spikes and GI3 EPSPs in 10–15 min. The small spikes may have been remnants of electrotonically conducted spikes originating in distal regions of the cercal nerve in which the blood–brain barrier remained intact.

As the LFHSN spikes were blocked, EPSPs recorded from GI3 progressively decreased in size (Fig. 3), with the transmission threshold being reached when the spike amplitude was approximately 30 mV (33 ± 3 mV, N = 6). Some depolarizing slow PSPs remained in both LFHSN and GI3 long after complete spike block. These may have resulted from incomplete penetration of the ganglionic neuropile by the toxin. Alternatively, they may represent synaptic inputs from neurones with TTX-insensitive spikes or inputs from non-spiking neurones.

Addition of 0.1 mmol l⁻¹ 4-AP alone caused a doubling of LFHSN spike duration and a 30% increase in GI3 EPSP amplitude (as measured in the cell body). LFHSN delayed rectification was also reduced. 4-AP blocks K⁺ channels at membrane potentials of up to −20 mV (Pelhate & Pichon, 1974; Pelhate & Sattelle, 1982).

Addition of 0.1 mmol l⁻¹ 4-AP to a preparation in which spikes had previously been blocked with 1 μmol l⁻¹ TTX often resulted in a small decrease in resting potential (approximately +4 mV), and allowed regenerative depolarizations to be elicited by 20-ms positive current pulses (Fig. 4). These regenerative spike-like depolarizations took place when the membrane was depolarized beyond a threshold of 12 mV above resting potential (threshold potential −38 ± 1 mV, N = 18), were 3.1 ± 0.1 ms (N = 18) in duration, and reached a peak of 40 ± 2 mV (N = 18) positive to resting potential.

LFHSN depolarizations were followed, at a latency of 2.5–3.0 ms, by large (up to 35 mV) EPSPs in GI3 (Figs 4, 5). Addition of 1 mmol l⁻¹ Cd²⁺, 10 mmol l⁻¹

![Fig. 3. Transfer function for the synapse between the lateral filiform hair sensory neurone (LFHSN) and giant interneurone 3 (GI3). Amplitude of GI3 EPSPs plotted as a function of LFHSN spike amplitude during progressive spike block by 1 μmol l⁻¹ tetrodotoxin. Resting potential of LFHSN was −60 mV.](image-url)
Co$^{2+}$ or substitution of Ca$^{2+}$ with Mg$^{2+}$ abolished the regenerative depolarization, suggesting that it is dependent upon the entry of Ca$^{2+}$ through voltage-dependent channels (Hagiwara, 1973; Kostyuk, 1980). Injection of a 2.5 ms positive current pulse of approximately 3 nA elicited a rapid depolarization in LFHSN which was

![Diagram](image)

Fig. 4. Putative calcium spikes recorded in the presence of sodium and potassium channel blockers. Regenerative potentials elicited in the lateral filiform hair sensory neurone (LFHSN) in the presence of 1 μmol l$^{-1}$ tetrodotoxin (TTX) and 0.1 mmol l$^{-1}$ 4-aminopyridine (4-AP). Upper trace, current and zero potential; second trace, LFHSN potential; third trace, GI3 potential; fourth trace, LFHSN potential in the presence of 0 mmol l$^{-1}$ Ca$^{2+}$ plus 5-4 mmol l$^{-1}$ Mg$^{2+}$. 20 ms current pulses of increasing intensity were followed by a 3 nA, 3 ms pulse.

![Diagram](image)

Fig. 5. Relationship between size of presynaptic depolarization and postsynaptic EPSP amplitude for the lateral filiform hair sensory neurone (LFHSN) – giant interneurone 3 (GI3) synapse. (A) Postsynaptic depolarization plotted against presynaptic depolarization recorded in the presence of 1 μmol l$^{-1}$ tetrodotoxin (TTX) and 0.1 mmol l$^{-1}$ 4-aminopyridine. (B) Postsynaptic depolarization plotted against presynaptic depolarization in the presence of 1 μmol l$^{-1}$ TTX and with Cs$^{+}$ injected into LFHSN.
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terminated before a full regenerative potential was elicited. This depolarization gave rise to an EPSP which was similar in shape to those produced by normal LFHSN spikes (Fig. 4).

Subsequent addition of 20 mmol l\(^{-1}\) TEA\(^+\) greatly increased the amplitude and duration of the regenerative depolarization, or putative Ca\(^{2+}\) spike. External TEA\(^+\) blocks K\(^+\) channels in Helix neurones with weak voltage-dependence (Hermann & Gorman, 1981), when compared to the strongly voltage-dependent blocker 4-AP, and external TEA\(^+\) and 4-AP block pharmacologically distinct K\(^+\) channels in Tritonia (Thompson, 1977). In saline containing 5·4 mmol l\(^{-1}\) Ca\(^{2+}\) the putative Ca\(^{2+}\) spike depolarized the membrane to near 0 mV potential (−2 ± 2 mV, \(N = 13\)) and lasted for 15–20 ms (18 ± 2 ms, \(N = 7\)). The spike was followed by an HAP approximately 400 ms in duration and 9–23 mV peak hyperpolarization from resting potential (15 ± 3 mV, \(N = 5\)). A second spike, elicited 700 ms after the first, was reduced in duration, as was the HAP (Fig. 6A,C). Hyperpolarization of LFHSN showed that the HAP was reversed at membrane potentials approximately 30 mV more negative than resting potential (HAP reversal potential −78 ± 3 mV, \(N = 5\)) (Fig. 7A).

Increasing the TEA\(^+\) concentration from 5 to 50 mmol l\(^{-1}\) did not result in an increase in duration of the putative Ca\(^{2+}\) spikes. However, increasing the concentration to 150 mmol l\(^{-1}\) resulted in a partial block of the HAP and an increase in spike duration.

Fig. 6. Calcium spike elicited in the presence of 1 \(\mu\)mol l\(^{-1}\) tetrodotoxin (TTX), 0·1 mmol l\(^{-1}\) 4-aminopyridine (4-AP) and 20 mmol l\(^{-1}\) tetraethylammonium (TEA\(^+\)). The first and second spikes in a series are superimposed, the first being of longer duration. (A,C) Spikes in 5·4 mmol l\(^{-1}\) Ca\(^{2+}\). (B,D) Larger-amplitude and longer-duration spikes recorded in 10 mmol l\(^{-1}\) Ca\(^{2+}\). C and D are on a slower timebase to show the hyperpolarizing afterpotentials. Upper trace: current and zero potential; lower trace: lateral filiform hair sensory neurone (LFHSN) potential.
Increasing the [Ca\textsuperscript{2+}]\textsubscript{o} to 10 mmol\textsuperscript{-1} increased the amplitude and duration of the putative Ca\textsuperscript{2+} spike (Fig. 6B). The first spike in a series peaked at about +5 mV and lasted for 100 ms, with an HAP reaching −18 mV from resting potential and lasting for 800 ms. Putative Ca\textsuperscript{2+} spikes could not be elicited during the HAP but the next spike peaked at 0 mV and lasted for 30 ms, with a proportionate reduction in the duration of the HAP (Fig. 6D). In both 5·4 and 10 mmol\textsuperscript{-1} Ca\textsuperscript{2+} there was little further change in spike duration after the second spike.

20 mmol\textsuperscript{-1} TEA\textsuperscript{+} also had the effect of enhancing the size and duration of EPSPs in LFHSN (Fig. 6C,D) to the extent that putative Ca\textsuperscript{2+} spikes could be elicited by these EPSPs. TEA\textsuperscript{+} also enhanced some PSPs in GI 3 but the EPSPs elicited by the cholinergic LFHSN were blocked. This reversible blocking of cholinergic synaptic inputs (see Twarog & Roeder, 1957) by the action of TEA\textsuperscript{+} as a quaternary ammonium ion (Adler \textit{et al.} 1979) means that extracellular TEA\textsuperscript{+} cannot be used in studies of both the pre- and postsynaptic sides of the synapse.

![Fig. 7.](image-url)
In order to circumvent the cholinergic receptor-blocking action of external TEA⁺ and the synapse-blocking action of internal TEA⁺ (Blagburn & Sattelle, 1987a), LFHSN was impaled with microelectrodes containing caesium ions. Internal Cs⁺ blocks outward K⁺ currents at depolarized potentials (Hille, 1984). This was followed by total block of action potentials with 1 µmol l⁻¹ TTX, and subsequent injection of positive current pulses into the presynaptic terminal for 2–5 min (1 nA amplitude, 1 Hz frequency, 500 ms duration). Injection of K⁺ alone had no significant effect.

Ionophoretic injection of Cs⁺ into LFHSN allowed putative Ca²⁺ spikes to be evoked (Fig. 7B). These were similar in amplitude to those evoked in the presence of external TEA⁺ and were 10 ms in duration. The putative Ca²⁺ spikes were followed by HAPs of proportionate duration, and elicited EPSPs in GI 3, similar to those produced by the shorter-duration putative Ca²⁺ spikes seen in the presence of TTX and 4-AP. Using internal Cs⁺ it was possible to determine the relationship between the amplitude of the presynaptic putative Ca²⁺ spike and that of the GI 3 EPSP in the absence of any possible effects on the membrane properties of GI 3 resulting from 4-AP (Fig. 5B).

Fast hyperpolarizing current pulses into LFHSN were used to monitor Rₘ during putative Ca²⁺ spikes evoked by enlarged non-cholinergic EPSPs (Fig. 8A). Rₘ was reduced, i.e. there was an increase in membrane conductance, during the initial EPSP, then Rₘ dropped to near zero during the spike. A conductance increase persisted during the HAP.

Because TTX-resistant spikes have been reported in vertebrate sensory cells (Heyer & MacDonald, 1982), an increase in Na⁺ conductance was ruled out by replacing all the Na⁺ in the saline with either Tris⁺ or choline⁺. The putative Ca²⁺ spikes were not affected by removal of Na⁺ (Fig. 8B), suggesting that the spike is not the result of an increased TTX-insensitive Na⁺ conductance. Zero-Na⁺ saline abolished LFHSN action potentials within 5 min so it is unlikely that sodium ions remained in the intercellular spaces surrounding the axon. Lengthy treatment with zero-Na⁺ saline (over 20 min) resulted in a loss of membrane resistance.

Barium ions carry current through Ca²⁺ channels (Hagiwara & Byerly, 1981). Substitution of Ba²⁺ for Ca²⁺ had little effect upon the amplitude or duration of normal LFHSN action potentials, but abolished the EPSPs elicited in GI 3 by LFHSN spikes. In contrast, other background synaptic activity in both neurones was enhanced.

Substitution of Ba²⁺ for Ca²⁺ had little effect upon the amplitude of the putative Ca²⁺ spike, but prolonged it to 1·5 s (Fig. 8C). No HAP was observed. A conductance increase took place during the first half of the extended spike, and Rₘ returned to its original value before the termination of the spike. Ba²⁺ spikes were blocked with 1 mmol l⁻¹ Cd²⁺ or 10 mmol l⁻¹ Co²⁺.

Effects of calcium channel-blocking compounds

The phenylalkylamine Ca²⁺ channel blockers D600 and verapamil had little effect on Ca²⁺ spikes at 0·1 mmol l⁻¹ but blocked spikes reversibly at 1 mmol l⁻¹. Similarly,
Fig. 8. Calcium spikes in the lateral filiform hair sensory neurone (LFHSN) axon recorded in the presence of 1 μmol l⁻¹ tetrodotoxin (TTX), 0·1 mmol l⁻¹ 4-amino-pyridine (4-AP) and 20 mmol l⁻¹ tetraethylammonium (TEA⁺). TEA⁺ blocks the cholinergic EPSPs in GI 3. (A) Changes in the membrane input resistance of the LFHSN axon were monitored by injection of 1 nA hyperpolarizing pulses. There is a conductance increase during the spike-initiating EPSP and during the hyperpolarizing afterpotential (HAP), while the input resistance drops to near zero during the spike. (B) Ca²⁺ spike elicited in the absence of external sodium (replaced by Tris⁺). (C) Barium substituted for calcium supports long-duration spike with no HAP. Upper traces, current and zero potential; centre traces, LFHSN potential; lower traces, GI 3 potential.

the benzodiazepine diltiazem blocked the Ca²⁺ spikes reversibly at 1 mmol l⁻¹, but not at 0·1 mmol l⁻¹. The dihydropyridine nifedipine blocked the Ca²⁺ spikes irreversibly at 0·5 mmol l⁻¹ but not at 10 μmol l⁻¹.

**DISCUSSION**

The combination of Nomarski optics with conventional electrophysiological techniques has enabled us to record intracellularly from an identified insect synaptic terminal while monitoring the postsynaptic potential changes. The lateral filiform hair sensory neurone (LFHSN) can be impaled in a region of the axon which forms hundreds of output synapses with giant interneurone 3 (GI 3). The closest synapses to the recording site are less than 5 μm from the microelectrode, while the furthest are less than 100 μm; to our knowledge this is considerably closer than in other preparations, even the squid giant synapse.

It was observed that LFHSN spikes exhibited a variable afterpotential, which, in some cases, reversed at membrane potentials more positive than resting potential.
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The size and sign of the spike afterpotential (AP) were dependent upon the membrane potential and the external calcium ion concentration. It is thought that this component of the spike, which is similar in time course to the GI 3 EPSP, represents the sum of the HAP due to $K^+$ efflux and the depolarization due to $Ca^{2+}$ influx. The balance of these will be depolarizing or hyperpolarizing, depending on the resting membrane potential. A depolarizing afterpotential (DAP), which was independent of $[Ca^{2+}]_o$, has been seen after spikes in the crayfish claw-opener excitatory motor axon and has been considered to be a passive membrane response following activation of a $K^+$ channel (Wojtowicz & Atwood, 1984). However, in the case of the crayfish central giant axons it has been suggested that the DAP is caused by an increase in $Ca^{2+}$ conductance (Yamagishi & Grundfest, 1971).

Progressive spike block by TTX allows the input/output relationship of the LFHSN–GI 3 synapse to be determined. EPSPs in GI 3 decrease in amplitude as the height of the LFHSN spike decreases, with a threshold spike height of 33 ± 3 mV from resting potential, i.e. $-22 ± 3$ mV. Clearly the attenuation undergone by the EPSP may render this estimate inaccurate.

The synaptic transfer curve is similar in shape to those obtained for other synaptic preparations in which phasic transmission normally takes place. The threshold value for the presynaptic depolarization of -22 mV (33 mV from resting potential) for the LFHSN–GI 3 synapse compares with values of -30 to -40 mV (20–40 mV from resting potential) for the squid synapse (Takeuchi & Takeuchi, 1962; Katz & Miledi, 1966), -35 to -40 mV (35–40 mV from resting potential) for the lamprey (Martin & Ringham, 1975), -40 to -45 mV (25–30 mV from resting potential) for the hatchetfish (Auerbach & Bennett, 1969), -40 mV (10 mV from resting potential) for the locust LGMD (Rind, 1984) and -27 mV (48 mV from resting potential) for the crayfish neuromuscular junction (Wojtowicz & Atwood, 1984).

The tonic synapses of the non-spiking barnacle photoreceptor (Ross & Stuart, 1978) and the non-spiking crab T-fibre (Blight & Llinás, 1980) exhibit lower thresholds for transmission: -50 mV (7 mV from resting potential) for the barnacle photoreceptor and -70 mV (10 mV from resting potential) for the crab T-fibre.

The results show that LFHSN can support $Ca^{2+}$ spikes in the presence of the $K^+$ channel blockers 4-AP and TEA$^+$. The regenerative potentials satisfy the usual criteria for $Ca^{2+}$ spikes (Hagiwara & Byerly, 1981) in that (1) regenerative depolarization in the presence of $K^+$ channel blockers was accompanied by an increase in membrane conductance; (2) the spike was not blocked by TTX or by replacement of extracellular $Na^+$ with Tris$^+$ or choline$^+$; (3) the spike was blocked by replacement of extracellular $Ca^{2+}$ with $Mg^{2+}$, and by exposure to 1 mmol$^{-1}$ Cd$^{2+}$ or 10 mmol$^{-1}$ Co$^{2+}$; (4) the spike height and duration were dependent upon the external concentration of $Ca^{2+}$; and (5) $Ba^{2+}$ substituted for $Ca^{2+}$ and produced spikes of longer duration. The threshold for the $Ca^{2+}$ spikes of -38 mV is somewhat more negative than the threshold for synaptic transmission.

In the presence of TTX and 4-AP only, $Ca^{2+}$ spikes of relatively short duration can be evoked in LFHSN, and these produce large EPSPs in GI 3. It is possible that reduction of the delayed rectifier $K^+$ conductance in GI 3 will reduce the degree of
attenuation undergone by large dendritic EPSPs recorded from the cell body. EPSPs evoked by LFHSN Ca\(^{2+}\) spikes in the presence of TTX and with Cs\(^+\) inside the presynaptic terminal attain a maximum amplitude of 25 mV, 5 mV lower than those produced with external 4-AP. This suggests that delayed rectification has a small effect on the size of the EPSP recorded in the cell body of GI3.

It is possible that a regenerative Ca\(^{2+}\) current adds to the height of the GI3 EPSPs. This is unlikely because the EPSPs exhibited no threshold for regenerative depolarization as did the presynaptic Ca\(^{2+}\) spikes. In addition, no TTX-insensitive depolarizations could be evoked in GI3 in the presence of external TEA\(^+\).

The organic Ca\(^{2+}\) channel blockers D600, verapamil, nifedipine and diltiazem have been found to be potent blockers of Ca\(^{2+}\) currents in vertebrate smooth and heart muscle (Hagiwara & Byerly, 1981; Lee & Tsien, 1983) at concentrations of 1–10 \(\mu\)mol\(^{-1}\). However, in some invertebrate preparations, such as the squid giant axon (Baker, Meves & Ridgway, 1973) and snail neurones (Kostyuk, Krishtal & Shakhovalov, 1977), these compounds block Ca\(^{2+}\) currents only at high concentrations (0.1–2 mmol\(^{\text{-1}}\)). The results of the present study, in which these compounds have been tested on insect Ca\(^{2+}\) spikes for the first time, are consistent with the latter findings. Recently it has been shown that chick dorsal root ganglion neurones exhibit three types of Ca\(^{2+}\) channel, only one of which, the L-type, is sensitive to micromolar concentrations of dihydropyridine agonists (Nowycky, Fox & Tsien, 1985). It has been postulated that N-type channels may mediate dihydropyridine-insensitive synaptic transmission (McCleskey, Fox, Feldman & Tsien, 1986), and it is possible that LFHSN synaptic Ca\(^{2+}\) channels are of this type.

The long duration of Ba\(^{2+}\) spikes is similar to results obtained from Helix and lamprey neurones (Kerkut & Gardner, 1967; Leonard & Wickelgren, 1985) and may be due to the inability of Ba\(^{2+}\) to inactivate the Ca\(^{2+}\) channel (Eckert & Tillotson, 1981) and/or to activate the Ca\(^{2+}\)-dependent K\(^+\) conductance (Gorman & Hermann, 1979; Meech & Thomas, 1980).

The HAP which followed the Ca\(^{2+}\) spikes in the presence of TEA\(^+\) was proportional in duration to the amplitude and duration of the Ca\(^{2+}\) spikes. It was accompanied by a conductance increase and was blocked by substitution of Ba\(^{2+}\). The HAP reversed at potentials approximately 30 mV more negative than resting potential. This evidence suggests that the HAP results from an increase in Ca\(^{2+}\)-dependent K\(^+\) [K\(^+\)(Ca\(^{2+}\))] conductance, although a possible contribution by a Cl\(^-\) conductance was not ruled out. The HAP was not blocked by 50 mmol\(^{-1}\) TEA\(^+\), but 150 mmol\(^{-1}\) TEA\(^+\) caused some block of the HAP and a 10-fold increase in duration of the Ca\(^{2+}\) spike. External TEA\(^+\) is not an efficient blocker of the K\(^+\)(Ca\(^{2+}\)) current in many vertebrate and invertebrate neurones (Stanfield, 1983) although in some molluscan neurones the K\(^+\)(Ca\(^{2+}\)) conductance is very sensitive to external TEA\(^+\). It has been suggested that there are two types of K\(^+\)(Ca\(^{2+}\)) currents which differ in their activation kinetics: fast ones, which are sensitive to TEA\(^+\) and insensitive to apamin; and slow ones, which are insensitive to TEA\(^+\) and blocked by apamin (Mallart, 1985). The K\(^+\)(Ca\(^{2+}\)) conductance in the cockroach LFHSN may
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belong to the latter class, although it appears to be insensitive to apamin (J. M. Blagburn & D. B. Sattelle, unpublished observation).

From studies of the small number of preparations in which voltage changes in the presynaptic terminal can be monitored, it appears that a $K^+$(Ca$^{2+}$) conductance is often associated with the synaptic Ca$^{2+}$ conductance. A $K^+$(Ca$^{2+}$) conductance has been investigated by intracellular recording at the squid giant synapse (Augustine & Eckert, 1982). Indirect measurement of electrical signals has been used to study synaptic Ca$^{2+}$ and $K^+$(Ca$^{2+}$) conductances in the giant barnacle photoreceptor (Stockbridge & Ross, 1984), the frog neurohypophysis (Obaid, Orkand, Gainer & Salzberg, 1985) and the mouse neuromuscular junction (Mallart, 1985).

Both Ca$^{2+}$ and $K^+$(Ca$^{2+}$) channels have been found in adult cockroach cell bodies (Pitman, 1979; Thomas, 1984), and Ca$^{2+}$ conductances have been described in embryonic grasshopper neuronal cell bodies (Goodman & Spitzer, 1981) but neither has previously been described in synaptic regions of any insect. In other arthropods, presynaptic Ca$^{2+}$ and $K^+$(Ca$^{2+}$) conductances have been described only in the barnacle photoreceptor (Ross & Stuart, 1978), lobster neuromuscular junction (Niwa & Kawai, 1982) and crab sinus gland neurosecretory terminal (Cooke, 1985).

We have no direct evidence that the LFHSN axon within the cercal nerve does not support Ca$^{2+}$ spikes. However, it was frequently observed that small Na$^+$ spike remnants, originating in the axon further down the cercal nerve, persisted after a short period of TTX application. These spike remnants were superimposed on Ca$^{2+}$ spikes, suggesting that the LFHSN axon within the nerve is not able to produce prolonged Ca$^{2+}$ spikes. This property appears to be confined to the ganglionic areas of the LFHSN axon. The presence of Ca$^{2+}$ and $K^+$(Ca$^{2+}$) channels in this region of LFHSN is presumably related to it being a site of large numbers of output synapses, in effect a large synaptic terminal (Blagburn et al. 1984, 1985a).

The LFHSN-G13 preparation is well suited to studies of integrative synaptic physiology and presynaptic Ca$^{2+}$ and $K^+$(Ca$^{2+}$) channels, avoiding the need to deduce the properties of synaptic areas from cell body membrane properties. This preparation allows unrivalled access to an identified cholinergic synaptic terminal, at a distance of less than 5 $\mu$m from the presynaptic sites.

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