The removal of acetylcholine by diffusion at nicotinic synapses in the rat otic ganglion

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1. We have examined the clearance of synaptically released acetylcholine in the otic ganglion when acetylcholinesterase was blocked with eserine.

2. Intracellular recordings were made from otic ganglion neurones, *in vitro*. The decay of the excitatory postsynaptic potential (EPSP), in response to stimulation of afferent fibres, was greatly prolonged in the presence of eserine. Low frequency (0·05–4 Hz) repetitive synaptic stimulation led to a slow depolarization of the postsynaptic cell that persisted throughout the period of stimulation. This slow depolarization was blocked by the nicotinic antagonists mecamylamine (100 μM) or (+) tubocurarine (100 μM), but was unaffected by atropine (1 μM), indicating that the response was due to the activation of nicotinic receptors.

3. Following 2 Hz synaptic stimulation (30 s), the rate of rise of the slow depolarization had a time constant of $3{\cdot}1 \pm 0{\cdot}4$ s and a peak amplitude of 12 ± 1 mV. Upon cessation of stimulation, the depolarization decayed to resting levels with a time constant of $18{\cdot}3 \pm 1{\cdot}5$ s ($n = 23$). At increasing stimulation frequencies the rate of rise of the depolarization increased. Lowering the probability of release, by adding cadmium to the perfusing solution or by lowering extracellular calcium, slowed the rise time of the response.

4. Both the onset and decay kinetics of the slow depolarization had a low temperature sensitivity, indicating that they reflect diffusional processes.

5. Repetitive stimulation (2 Hz) of the afferent nerve supplying the ganglion, in the presence of eserine, also caused a slow depolarization in cells in which we could not demonstrate a synaptic input. This indicates that synaptically released acetylcholine can spill over onto nearby neurones.

6. We conclude that at parasympathetic synapses, under physiological conditions, transmitter action is terminated by the enzymatic degradation of acetylcholine. When acetylcholinesterase is blocked, acetylcholine accumulates because its removal by diffusion is slow.

When an action potential invades the presynaptic terminal at a communication between two nerve cells, the resulting calcium influx triggers the release of neurotransmitter at specialized release sites. Transmitter then diffuses across the synaptic cleft, activates postsynaptic receptors and results in the generation of a postsynaptic current. The amplitude and time course of the postsynaptic current is determined by three factors: the time course of transmitter in the cleft, the ligand binding properties of the postsynaptic receptors, and the kinetics of the activated ion channels (Magleby & Stevens, 1972; Katz & Miledi, 1973; Gage, 1976; Jonas & Spruston, 1994; Clements, 1996).

At the vertebrate neuromuscular junction, acetylcholine released from motor nerve endings binds to nicotinic receptors and opens a cation-selective channel in the postsynaptic membrane. Acetylcholine is rapidly hydrolysed by a cholinesterase that is concentrated in the synaptic cleft and the time course of the postsynaptic current, which decays exponentially with a single time constant, is governed primarily by the deactivation rate of channels activated by acetylcholine (Magleby & Stevens, 1972; Anderson & Stevens, 1973; Gage, 1976). When acetylcholine hydrolysis is prevented by blocking the cholinesterase, the time course of the end-plate current is

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markedly slowed. This slowing is thought to result from rebinding of acetylcholine to postsynaptic receptors and re-opening of the channels as acetylcholine diffuses out of the synaptic cleft (Katz & Miledi, 1973; Magleby & Terrar, 1975).

In the parasympathetic nervous system, neurones are organized into ganglia that lie close to the organ they innervate. Generally, these cells consist of a large soma with, at most, a few short dendritic processes (Gabella, 1976), which usually receive a single suprathreshold cholinergic input (Adams & Harper, 1995). Ultrastructurally, these synapses are simple, with the innervating axon providing a number of small boutons that contact the somata of the postsynaptic cell (McMahan & Kuffler, 1971; Lichtman, 1977). As at the neuromuscular junction, released acetylcholine is rapidly hydrolysed by an acetylcholinesterase concentrated at synaptic junctions (Burnstock, 1979). At these synapses the excitatory postsynaptic current (EPSC) generated upon stimulation of the presynaptic axon(s) decays with two time constants (Rang, 1981; Callister & Walmsley, 1996). When the cholinesterase is blocked, both components are slowed (Rang, 1981). However, unlike the neuromuscular junction, it has been proposed that this slowing is unlikely to be due to persistence of acetylcholine in the synaptic cleft, but may be due to a change in channel kinetics (Rang, 1981). Slow diffusion of acetylcholine out of the synaptic cleft is not thought to be significant because (1) geometric considerations suggest that removal of transmitter at these simple junctions by diffusion alone should be fast (Eccles & Jaeger, 1958; Wahl, Pouzat & Stratford, 1996), and (2) as the fraction of released acetylcholine bound to postsynaptic receptors is small, slowing of diffusion due to rebinding of acetylcholine to postsynaptic receptors should be insignificant (Magleby & Terrar, 1975; Rang, 1981).

The otic ganglion provides parasympathetic innervation to several important structures in the head, including the parotid salivary gland and cerebral blood vessels. These cells form a homogeneous population, are morphologically simple, and always receive at least one suprathreshold synaptic input (Callister, Keast & Sah, 1997). While studying the properties of nicotinic synapses in this ganglion, we unexpectedly found that when cholinesterase was inhibited, an additional very slow component could be detected in the excitatory synaptic potential (Callister & Sah, 1996). In this study, we have re-examined the removal of acetylcholine in parasympathetic neurones under conditions where its active degradation is blocked. We find that when the acetylcholinesterase is blocked, clearance of acetylcholine is slow and accumulates during low-frequency stimulation. These results suggest that under physiological conditions, transmitter action at parasympathetic synapses is terminated by the enzymatic degradation of acetylcholine. When acetylcholinesterase is blocked, acetylcholine accumulates because its removal by diffusion alone is slow.

METHODS

All experiments were performed on neurones in the rat otic ganglion. Female animals (Wistar strain; 4–10 weeks old) were anaesthetized with halothane and decapitated according to procedures approved by the Animal Care and Ethics Committee at the University of Newcastle. An incision was made along the ventral midline of the neck and the preparation (always the left) was removed and pinned out in a small Sylgard-lined recording chamber. The preparation was perfused with a modified Ringer solution (exchange rate: four bath volumes per minute; bath volume: 1·5 ml) and viewed under a stereomicroscope at a final magnification of ×40. The perfusing solution consisted of (mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 2 $MgCl_2$, 1 NaH_2PO_4, 2·5 $CaCl_2$ and 11 glucose; pH 7·3 when bubbled with 95% O_2 and 5% CO_2. All experiments were carried out at room temperature (22–25 °C), except where indicated.

Intracellular recordings were made with sharp microelectrodes (glass diameter 1·5 mm, Clarke Instruments) using an Axoclamp-2A amplifier (Axon Instruments). Electrodes had resistances of 50–140 MΩ when filled with 0·5 M potassium chloride. During voltage-clamp experiments, the headstage was continually monitored to ensure complete settling of voltage transients between samples. Switching frequencies of between 2 and 4 kHz could be obtained providing the fluid level in the bath was kept to a minimum. The minor petrosal nerve was drawn into a close-fitting suction electrode, for stimulation of preganglionic axons, and activated by suprathreshold pulses (0·2 ms, 2–20 V) delivered from a Master-8 stimulus isolation unit (AMPI, Jerusalem, Israel). For examination of the EPSP time course, the action potential afterhyperpolarization, normally present in otic ganglion neurones (Callister *et al.* 1997), was blocked either by the addition of apamin (200 nM; $n = 3$) or by holding the cell at the potassium reversal potential (~−80 mV; $n = 3$). Signals were sampled at 1–10 kHz, and stored on an IBM compatible computer and also on videotape using a four-channel video cassette recorder (A. R. Vetter Co., Rebersburg, PA, USA) for later analysis. For responses that persisted for several seconds, data were acquired at a sampling frequency of 5 Hz. All data were analysed using software kindly provided by J. Dempster (Strathclyde Electrophysiology Software, Glasgow, UK).

All drugs were purchased from Sigma, dissolved in water as stock solutions and added to the perfusate to give the required final concentration. The low-calcium Ringer solution contained 0·5 mM $CaCl_2$ and 4 mM $MgCl_2$. Comparisons between data were made using a Student's unpaired t test or repeated measures ANOVA, and the results were considered to be statistically significant when $P < 0·05$. Data are presented as means ± S.E.M.

RESULTS

Neurones had a resting potential of −53 ± 0·7 mV ($n = 41$) and were innervated by at least one single strong cholinergic input, which on stimulation led to a suprathreshold excitatory synaptic potential (EPSP) (Callister *et al.* 1997). Under control conditions, stimulating synaptic inputs at a range of frequencies (0·5 Hz, $n = 3$; 1 Hz, $n = 4$; 2 Hz, $n = 12$; 4 Hz, $n = 3$) had no cumulative effect on the membrane potential (Fig. 1*A*). Application of the acetylcholinesterase inhibitor eserine (20 μM) had no effect on the membrane potential or input resistance; however,

repetitive low-frequency stimulation in the presence of eserine led to a slow depolarization of the postsynaptic cell (Fig. 1*A*). The amplitude of this depolarization increased as the eserine concentration rose in the bath, finally saturated after about 10 min (Fig. 1*A*) and was then stable for the duration of the experiment (0·5–2 h). Upon cessation of stimulation, the membrane potential slowly returned to control levels, often undershooting the baseline.

The inset in Fig. 1*A* compares the synaptic response at the peak of the cumulative depolarization under control conditions and in the presence of eserine. The EPSP is reduced in amplitude in the presence of eserine because of the decreased membrane resistance at the peak of the cumulative depolarization (see Fig. 1*B*).

The amplitude and kinetics of this slow depolarization varied with the stimulus frequency (see below). However, a robust response was always obtained with 2 Hz stimulation

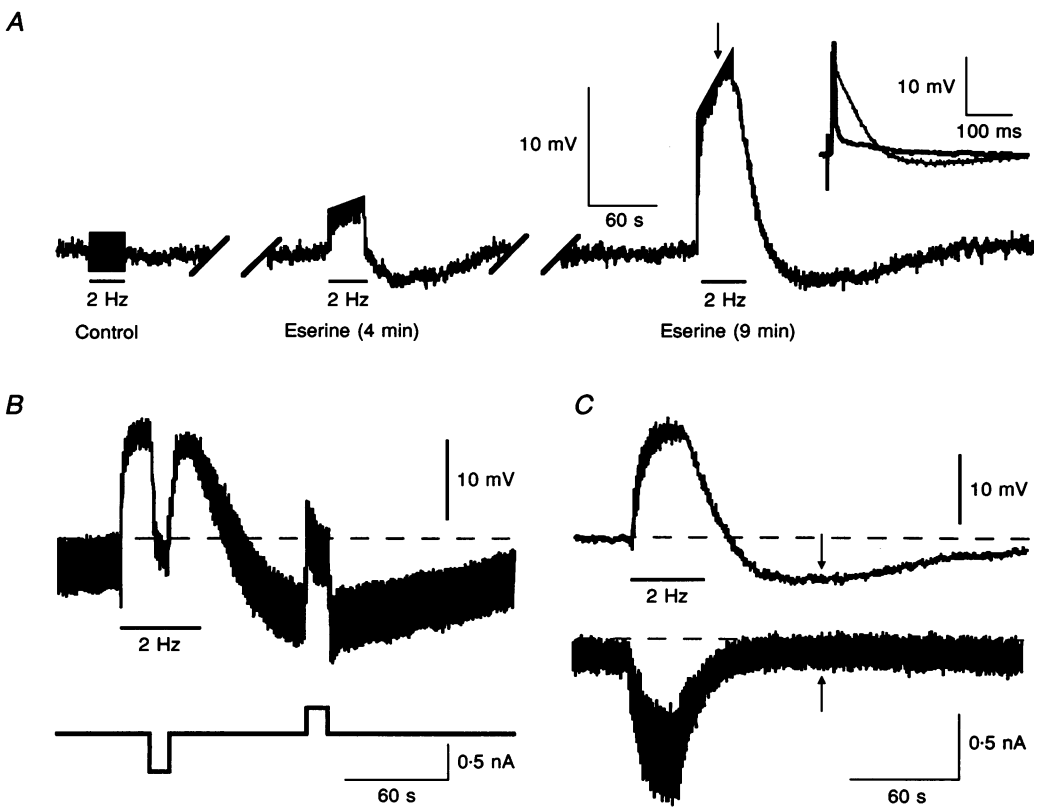

Figure 1. Effect of eserine on membrane potential during low-frequency synaptic stimulation

A, continuous record of membrane potential as eserine (20 μM) is applied to the bath (resting membrane potential, −53 mV). Afferent fibres were stimulated at 2 Hz during the period indicated by the bars. Action potentials and stimulus artifacts have been truncated to improve clarity. Traces (left to right) show responses under control conditions, and after 4 and 9 min exposure to eserine, respectively (sample frequency, 5 Hz). The inset on the right shows averaged synaptic responses under control conditions (thin trace) and in the presence of eserine (thick trace). Each trace is the average of ten consecutive sweeps sampled at the peak (arrow) of the cumulative depolarization (sample frequency, 2 kHz). The early upward deflection in both control and eserine records represents cropped action potentials. Note that the synaptic response is still suprathreshold at the peak of the depolarization. *B*, voltage recording during low-frequency stimulation (resting membrane potential, −61 mV). A hyperpolarizing pulse (0·05 nA, 250 ms) is continually passed into the cell to monitor changes in input resistance (sample frequency, 5 Hz). At the peak of the depolarization and during the slow afterhyperpolarization, the membrane potential is returned to control levels by passing constant hyperpolarizing current as indicated by the current trace. Note the reduction in input resistance at the peak of the response. In contrast, there is little change in input resistance during the slow hyperpolarization. *C*, voltage and current responses in the presence of eserine (20 μM). The top trace shows the voltage response to low-frequency stimulation (resting membrane potential, −61 mV). The corresponding membrane current, recorded in voltage clamp, is shown in the bottom trace (holding potential, −60 mV; sample frequency, 5 Hz). The cell was repetitively hyperpolarized (20 mV, 250 ms pulse) to monitor input resistance. Note the hyperpolarization observed in the voltage trace is not reflected as outward current in the voltage-clamp records (arrows).

and therefore we chose this frequency for more detailed analysis. For a stimulation frequency of 2 Hz, the average amplitude of this depolarization measured at 10 s from the onset of stimulation was 12·0 ± 1·0 mV (range 4–19 mV; $n = 23$). The depolarization was maintained as long as the stimulus continued (up to 4 min) and was always associated with a reduction in the input resistance of the cell (Fig. 1*B*). In contrast, no change in membrane conductance could be detected during the undershooting hyperpolarization observed at the cessation of stimulation. Slow afterhyperpolarizations following the activation of nicotinic receptors on autonomic ganglion cells have been attributed to several mechanisms, which include activation/deactivation of potassium currents (Hartzell, Kuffler, Stickgold & Yoshikami, 1977; Tokimasa & North, 1984) and stimulation of sodium pumps (Brown, Brownstein & Scholfield, 1972). Application of ouabain (100 μM; $n = 5$) did not affect the slow hyperpolarization, suggesting that it is not due to the action of the sodium pump. When cells were voltage clamped, a robust inward current was observed during the depolarizing phase of the response, but no membrane current was recorded during the undershoot (Fig. 1*C*; $n = 4$). This result indicates that membrane depolarization is a necessary step in generating the slow afterhyperpolarization. We do not currently understand the mechanism underlying this undershoot, and it will not be further considered in this paper.

The slow depolarization was reduced to less than 10% of its original amplitude when synaptic transmission was attenuated with 100 μM cadmium (Fig. 2*A*; $n = 3$). It was unaffected by atropine (1 μM, Fig. 2*B*; $n = 3$) but was blocked by addition of the nicotinic antagonists mecamylamine (100 μM, Fig. 2*C*; $n = 2$) or (+)tubocurarine (100 μM, Fig. 2*D*; $n = 4$). These results indicate that when acetylcholine hydrolysis is prevented by eserine, repetitive

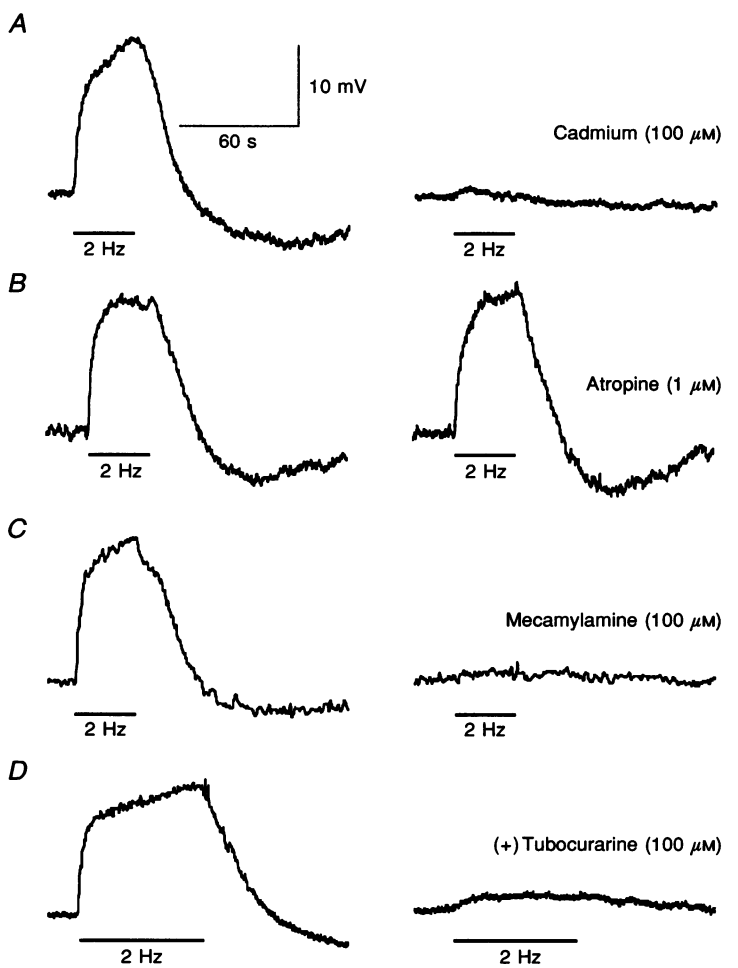

Figure 2. Pharmacology of the cumulative depolarization following low-frequency stimulation

All records show membrane potential recorded in eserine (20 μM). Control records are shown on the left and the response, in the presence of various drugs, is shown on the right (records sampled at 5 Hz, then digitally filtered (low pass) at 0·5 Hz). The response is almost abolished by cadmium (100 μM) and the nicotinic antagonists mecamylamine (100 μM) and (+)tubocurarine (100 μM), but is unaffected by the muscarinic antagonist atropine (1 μM). Resting membrane potentials were −60, −51, −50 and −52 mV for *A*, *B*, *C* and *D*, respectively.

low-frequency stimulation causes a progressive depolarization that is due to activation of nicotinic acetylcholine receptors.

The onset of depolarization was quite slow (Fig. 3*A* and *B*). In most cases, this onset could be well described by a single exponential function; however, in some cases a second slower phase was also apparent. The time constant of the initial rapid phase of the depolarization for a 2 Hz stimulus was 3·1 ± 0·4 s (range 0·9–10·4 s; $n = 22$). In those cases where a second slower component was present, its time constant was 226 ± 105 s (range 41–730 s; $n = 6$). When stimulation was stopped, the depolarization returned to baseline with a time constant of 18·3 ± 1·5 s (Fig. 3*C*; $n = 23$). This slow return of the membrane potential was mirrored by the slow decay of the inward current seen in voltage clamp (Fig. 1*C*).

Each time afferent fibres are stimulated, a given amount of acetylcholine is released from the nerve terminals. If the cumulative depolarization is due to a slow build-up of this released acetylcholine, then one would expect that stimulation at higher frequencies would speed up its accumulation, whereas reducing the amount of transmitter released per pulse should slow its accumulation. The effect of changing the frequency of stimulation is shown in Fig. 4*A*. As stimulus frequency is increased, the rate of rise of the depolarization increases. These observations are consistent with a faster accumulation of transmitter. The effect of reducing the amount of transmitter released per stimulus is shown in Fig. 4*B*. Transmitter release was lowered either by reducing extracellular calcium (0·5 mM; $n = 2$) or by the addition of cadmium (50 μM; $n = 2$). When transmitter release was reduced, such that the peak depolarization following 30 s of 2 Hz stimulation was halved, the rising phase was slowed from 2·7 ± 0·7 to 19·9 ± 2·0 s (Fig. 4*B*). In contrast, a similar reduction in the amplitude of the depolarization achieved by partially antagonizing the postsynaptic receptors with (+)tubocurarine, had no effect on the rising phase (Fig. 4*C*).

What is the origin of the slow decay of the depolarization observed upon cessation of repetitive stimulation? It could simply reflect the slow diffusion of accumulated acetylcholine, or it could be due to some other process such as an effect of eserine on the kinetic properties of acetylcholine receptors (Rang, 1981). In solution, the rate of processes that require the reactant(s) to pass through an activated state have Q_{10} values of two or more (Laidler, 1959). For example, reported Q_{10} values for the time constant of decay of the EPSC at other ganglionic synapses range from 3·7 to 6·0 (Kuba & Nishi, 1979; Derkach, Selyanko & Skok, 1983). Thus if the slow decay of the

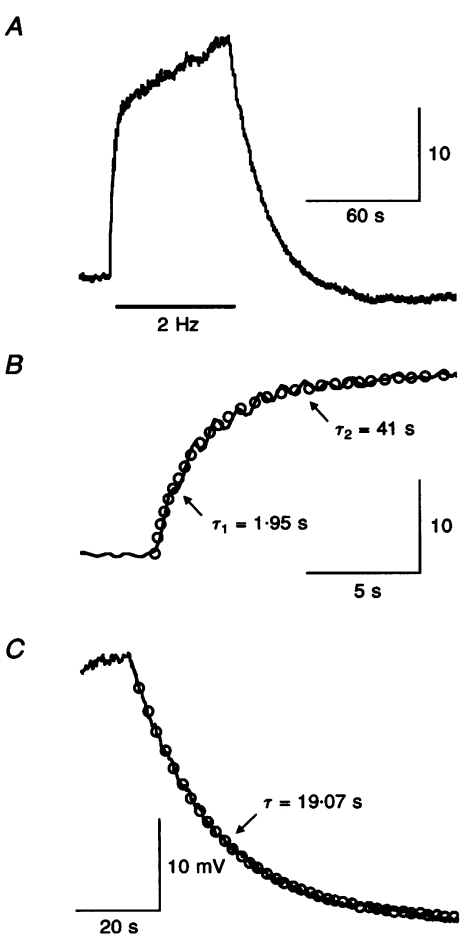

Figure 3. Kinetics of the rise and decay phase of the cumulative depolarization following low-frequency stimulation

A, membrane potential changes recorded (resting membrane potential, −60 mV) in the presence of eserine (20 μM). *B*, rising phase of the response in *A* on an expanded time scale. In this particular cell, the rise time had a fast (τ_1) and slow (τ_2) component. *C*, decay phase of the response shown in *A* on an expanded time scale. The decay phase could be described by a single exponential (all records digitized at 5 Hz, then digitally filtered (low pass) at 0·5 Hz).

response, in the presence of eserine, is due to a change in channel kinetics, we expect it to be temperature sensitive. Raising the ambient temperature from 23 to 34 °C had little effect on the kinetics of the slow depolarization (Fig. 5). In six cells recorded at 34 °C, the peak amplitude obtained for a 2 Hz, 30 s stimulation was 13·0 ± 1·8 mV, the rising phase of the response had a time constant of 2·4 ± 0·3 s and the decay had a time constant of 20·3 ± 1·8 s (Fig. 5*B*; $n = 6$). These measurements were not statistically different from those obtained at a temperature of 23 °C (Student's unpaired t test). We therefore conclude that both the rising phase and decay of the slow depolarization we observed in otic ganglion neurones, in the presence of eserine, are likely to represent processes that are rate limited by simple diffusion (Hille, 1992).

If accumulated acetylcholine diffuses away slowly, one prediction is that when acetylcholinesterase is only partially blocked, the decay of depolarization would be faster because acetylcholine removal would be faster due to its hydrolysis.

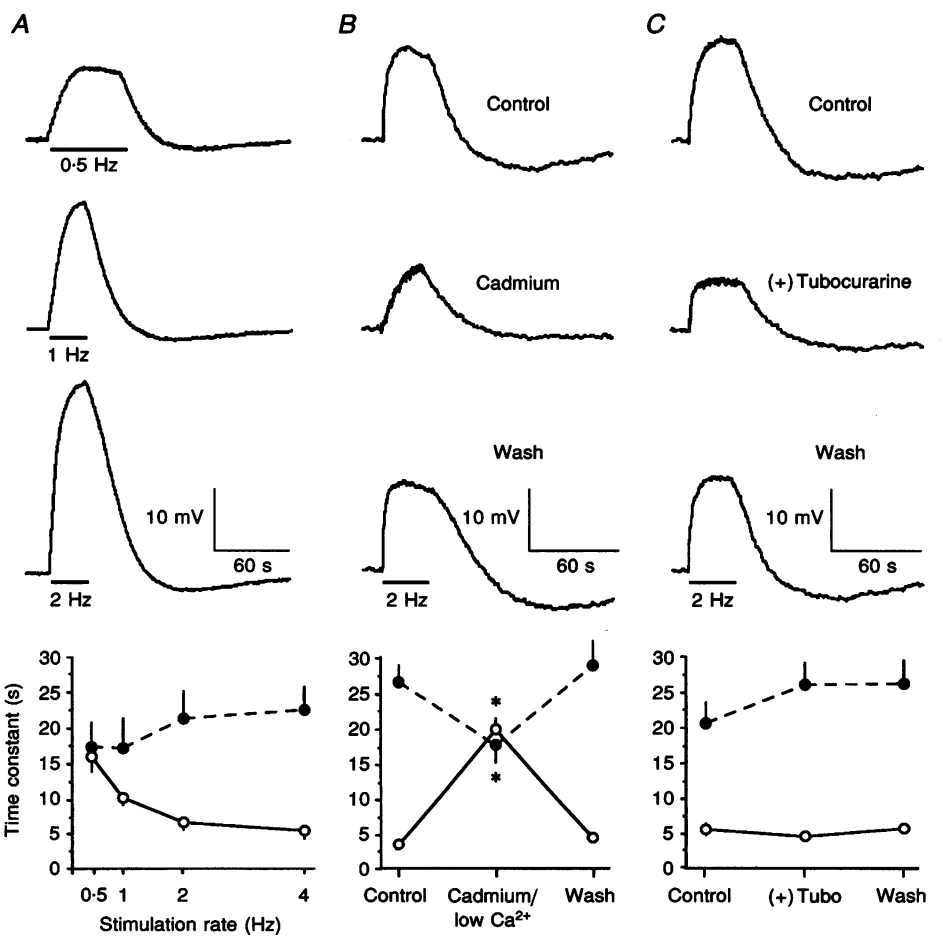

Figure 4. The cumulative depolarization is due to a slow accumulation of acetylcholine

All records were obtained in 20 μM eserine, sampled at 5 Hz, then digitally filtered (low pass) at 0·5 Hz. *A*, response to increasing stimulation rates (resting membrane potential, −56 mV). At higher stimulation rates, the amplitude of the depolarization is larger and its rate of rise is faster. The decay phase of the response was not significantly affected. *B*, response under conditions of reduced transmitter release (resting membrane potential, −57 mV). The addition of cadmium (50 μM) to the bath reduced the amplitude, rate of rise, and decay time constant of the response. The characteristics of the response returned to control values when the cadmium was washed off. *C*, response when postsynaptic receptors were partially antagonized by (+)tubocurarine ((+)Tubo; 50 μM). The addition of (+)tubocurarine decreased the amplitude of the response but had no effect on the rising phase (resting membrane potential, −56 mV). The characteristics of the response returned to control values when the cadmium was washed off. The graphs at the bottom show mean responses for six cells (*A*) and four cells (*B* and *C*). The centre points in *B* represent pooled results from responses in cadmium ($n = 2$) and low calcium ($n = 2$). The open and filled circles represent the time constants of the rising and falling phases of the response, respectively (as described in Fig. 3). The asterisks in *B* indicate significant differences for responses *vs.* control and wash (repeated measures ANOVA, with bath conditions (control, drug, wash) as within factor).

When the eserine concentration was lowered, so that there was incomplete block of the esterase, the decay of the depolarization was considerably faster (Fig. 6). This was also apparent as the eserine was either washed on or off; the time constant of the response was always faster when the eserine concentration was lower. These data indicate that the removal of released transmitter at synapses in the otic ganglion by diffusion alone must be slow. Indeed, when we examined the effect of eserine on the response to a single stimulus, a slow tail to the EPSP was apparent (Fig. 7*A*). Under control conditions, the EPSP decayed with a time constant of 33·3 ± 7·6 ms. In the presence of eserine (20 μM), this time constant increased to 74·3 ± 20·3 ms ($n = 6$; Fig. 7*A*, inset). In addition, a very slow tail was apparent, which lasted several tens of seconds (Fig. 7*A*). It is the summation of this slow tail that produces the cumulative depolarization during repetitive stimulation (Fig. 7*B*).

In three cases we recorded from cells in which we could not demonstrate a synaptic input; presumably the afferent axon had been damaged during dissection of the nerve from surrounding connective tissue. These cells had membrane properties indistinguishable from those of the innervated cells. Repetitive stimulation (2 Hz, 30 s), in the presence of eserine, still produced a slow depolarization (Fig. 7*C*), which was characteristically slower in onset (rate of rise, 11·7 ± 3 s) and smaller in amplitude (3·8 ± 0·7 mV) than the response observed in cells with synaptic inputs.

DISCUSSION

In this paper we have shown that when the endogenous acetylcholinesterase was blocked in parasympathetic neurones, the decay of the EPSP was slowed dramatically. Repetitive low-frequency synaptic stimulation led to a summation of these prolonged EPSPs and produced a cumulative depolarization. When stimulation was stopped, the depolarization dissipated slowly, with a time constant of many seconds. This depolarization was blocked by nicotinic antagonists and was insensitive to the muscarinic antagonist atropine. In neurones where the innervating axon could not be directly stimulated, repetitive stimulation of afferents to surrounding cells also produced a similar response. Together, these results indicate that the removal

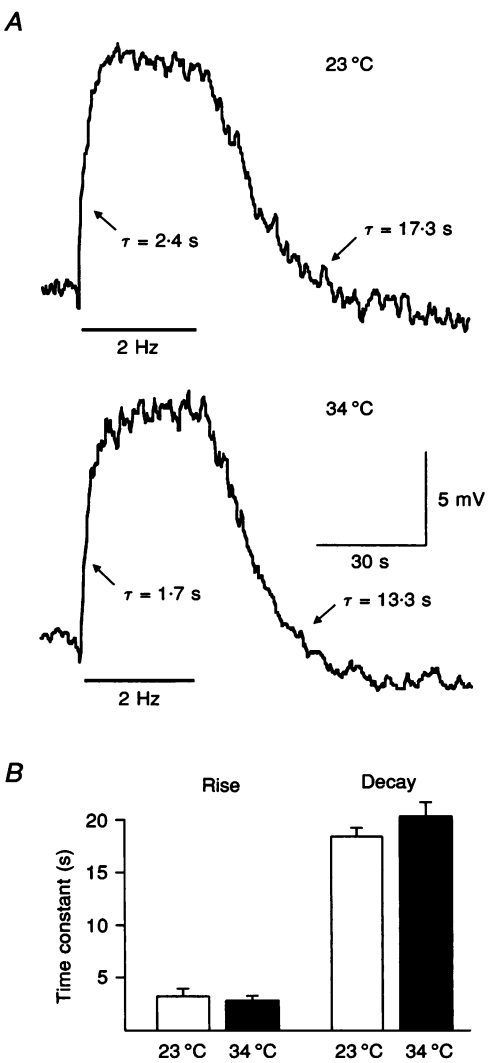

Figure 5. Effect of temperature on the kinetics of the cumulative depolarization

A, response to low-frequency stimulation in the presence of eserine (20 μM) at room temperature (23 °C) and when the temperature was raised to 34 °C. Records were digitized at 5 Hz and digitally filtered (low pass) at 0·5 Hz (resting membrane potential, −56 mV).
B, bar graphs compare means for the rate of rise and decay times for experiments carried out at 23 °C ($n = 23$) and 34 °C ($n = 6$) in different cells. Neither the rate of rise nor the decay of the response were markedly affected by changing the temperature.

of synaptically released acetylcholine by diffusion alone is slow, and it can spill over onto adjacent neurones.

Voltage-clamp studies of the synaptic current generated at similar synapses in the submandibular ganglion have shown that the decay of the excitatory postsynaptic current (EPSC) is best described by two components with time constants of approximately 7 and 34 ms, respectively (Rang, 1981; Callister & Walmsley, 1996). When acetylcholinesterase is blocked, both phases of the decay are slowed, with the second component increasing to approximately 85 ms (Rang, 1981). We have not voltage clamped synaptic inputs in the otic ganglion; however, the change in the decay rate of the EPSP, in the presence of eserine (from 33 to 74 ms), is consistent with these data. In this study we have described a third, much slower component to the EPSP (Fig. 7*A*), which was not described by Rang (1981) in submandibular ganglion neurones. The cumulative depolarization during repetitive stimulation is most likely to be due to summation of this additional slow component of the EPSP (Fig. 7*B*). The amplitude of the slow component, measured at 500 ms from the stimulus artifact, was $1{\cdot}9 \pm 0{\cdot}5$ mV ($n = 5$). At 2 Hz stimulation, the peak amplitude of the summated depolarization was approximately 12 mV. Assuming linear summation, this amplitude would be attained after the summation of six stimuli, and is consistent with our observations.

It is generally believed that the removal of synaptically released neurotransmitter by free diffusion is fast. Except for synapses where the geometry is complex, and the area of synaptic contact large (Rossi, Alford, Mugnaini & Slater, 1995; Otis, Wu & Trussell, 1996), diffusion of transmitter out of the synaptic cleft can be described by multiple time constants, the slowest being less than about 10 ms (Eccles & Jaeger, 1958; Clements, 1996; Holmes, 1996). While the ultrastructure of synapses on otic ganglion cells has not been investigated, such information is available for other parasympathetic ganglia. For example, in the submandibular ganglion where neurones receive similar strong inputs, a single fibre provides a number of small boutons onto the soma of the neuron (Lichtman, 1977; Callister, Keast & Walmsley, 1995). The organization of the synapses in the otic ganglion is also similar (R. J. Callister, J. Keast &

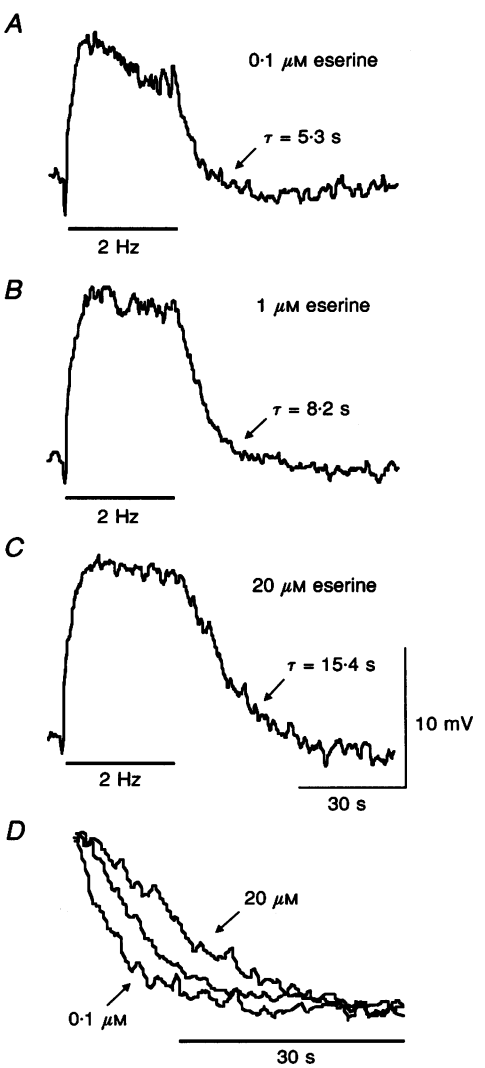

Figure 6. Responses to low-frequency stimulation in increasing concentrations of eserine

A, *B* and *C*, the rate of rise of the response was not markedly affected by increasing the concentration of eserine; however, the time constant of the decay phase of the response increased as the concentration of eserine was elevated in the bath (resting membrane potential, −50 mV). *D*, the decay phase of the response generated in the three different eserine concentrations shown in *A*, *B* and *C* has been normalized and superimposed on an expanded time scale. Note the rate of decay is slowest in 20 μM eserine. All records were digitized at 5 Hz and digitally filtered (low pass) at 0·5 Hz.

P. Sah, unpublished observations). Our data indicate that, at this simple synapse, diffusion of acetylcholine must be slow. In the absence of the esterase, synaptically released acetylcholine must persist in regions close to the neuronal membrane for several seconds.

A slow depolarization, similar to that described here, has been previously reported at the neuromuscular junction (Katz & Miledi, 1975) and at nicotinic synapses in the superior cervical ganglion (Bennett & McLachlan, 1972). However, in both these cases, much higher stimulation frequencies (10–50 Hz) were used to evoke the depolarization; the effect of lower stimulation frequencies were not reported. For the neuromuscular junction, the explanation for the slow clearance of transmitter was thought to be retention of a small amount of transmitter in membrane infoldings such as T-tubules and caveolae (Katz & Miledi, 1975). Such anatomical equivalents are not present in mammalian parasympathetic ganglia, although complicated membrane foldings do exist in the frog cardiac ganglion where synapses are tightly covered with a glial sheath, which is lamellated in places (McMahan & Kuffler, 1971). Such glial sheaths might form a barrier that slows the escape of acetylcholine out of the ganglion. It should be noted that the slow diffusion of acetylcholine is unlikely to be due to immobilization of transmitter by binding to acetylcholine receptors, because (i) the fraction of released acetylcholine bound by postsynaptic receptors is small (Rang, 1981) and (ii) the decay of the depolarization was not significantly faster when postsynaptic receptors were blocked with (+)tubocurarine (Fig. 4*C*) (Katz & Miledi, 1973; Magleby & Terrar, 1975).

Interestingly, we recorded from three cells in which a fast EPSP could not be evoked. Presumably, the axon(s) innervating these cells were damaged during the dissection. However, repetitive activation of the inputs to the ganglion, and presumably nearby cells, produced a qualitatively similar slow cumulative depolarization (see Fig. 7*C*) to that observed in innervated cells. Such 'spillover' of neurotransmitter onto adjacent neurones is well recognized for neurotransmitters mediating slow (G protein-coupled) synaptic transmission (Jan & Jan, 1983). For neurotransmitters mediating fast excitatory transmission, such spillover has been demonstrated for GABAergic synapses in the hippocampus following strong stimuli that activate multiple GABAergic afferents. This spillover is greatly potentiated by blockade of GABA uptake (Isaacson, Solis & Nicoll, 1993). Similarly in the otic ganglion, when the esterase is active, transmitter action is restricted to the

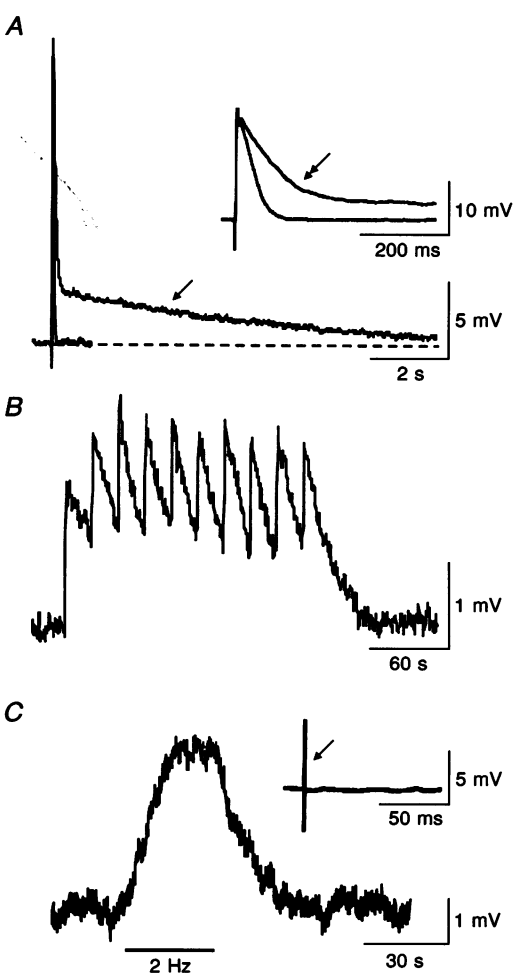

Figure 7. Mechanism underlying the cumulative depolarization and evidence for transmitter spillover onto adjacent neurones

A, suprathreshold excitatory postsynaptic potential (EPSP) generated under control conditions and in the presence of 20 μM eserine (arrows). Each trace is the average of five sweeps (stimulation rate, 0·06 Hz; sample frequency, 2 kHz). The cell was held at the reversal potential for potassium (~−80 mV) in order to abolish the contaminating effect of the large afterhyperpolarization present in otic ganglion neurones. Note the appearance of a slow tail (single arrow) on the falling phase of the EPSP, which is not present in the control EPSP. The inset shows the EPSP on an expanded time scale. Action potentials have been truncated. Note the slowing of the early part of the falling phase of the EPSP (double arrow). *B*, membrane potential recorded in the presence of 20 μM eserine (stimulation rate, 0·05 Hz; sample frequency, 2 kHz). The cumulative depolarization during repetitive stimulation is due to the summation of the slow tail on the decay phase of the EPSP. *C*, evidence for spillover of transmitter from synapses on neighbouring cells. The membrane potential was recorded in a non-innervated cell following low-frequency stimulation of synaptic inputs onto surrounding cells (resting membrane potential, − 54 mV). The inset shows the mean of the first ten responses during stimulation. Note the absence of an EPSP after the stimulus artifact (arrow).

synapse at which it is released, but spills over to nearby cells when the esterase is blocked. It is now well known that neurones in peripheral ganglia can express multiple types of acetylcholine receptors that differ in their agonist affinities, pharmacology and kinetics (Sargent, 1993; McGhee & Role, 1995). Furthermore, receptor subtypes can differ in their spatial location on the neuronal membrane (Wilson Horch & Sargent, 1995). Our findings raise the possibility that, in the presence of eserine, synaptically released acetylcholine may also be activating a population of high-affinity receptors located extrasynaptically.

Nicotinic synapses are not restricted to the periphery. In the central nervous system, a fast nicotinic synapse is formed between collaterals of motoneurone axons and Renshaw cells. Delivery of an antidromic volley to the relevant motor nerve evokes a characteristic repetitive discharge in the Renshaw interneurone that lasts tens of milliseconds. When acetylcholinesterase is blocked, by the intravenous administration of eserine, the discharge of Renshaw cells to the same antidromic volley increases to several seconds (Eccles, Fatt & Koketsu, 1954). This result is consistent with a prolonged action of acetylcholine in the presence of eserine, and suggests that removal of acetylcholine by diffusion at these synapses must be slow (Eccles, 1957). Thus slow clearance of synaptically released acetylcholine may be a feature common to all nicotinic synapses.

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