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THE RELEASE OF ACETYLCHOLINE FROM POST-GANGLIONIC CELL BODIES IN RESPONSE TO DEPOLARIZATION

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SUMMARY

1. Acetylcholine (Ach) release from parasympathetic ganglia cell somata was investigated in denervated avian ciliary ganglia. Three days after the input to the ganglion (the oculomotor nerve) was sectioned, all presynaptic nerve terminals had degenerated.

2. Denervated ganglia were shown to contain endogenous ACh and to be capable of synthesizing [^{3}H]ACh from [^{3}H]choline added to the incubation medium.

3. In response to depolarization induced by incubation in 50 mM-$[K^+]_o$, denervated ganglia released [^{3}H]ACh into bath effluents in amounts approximately 15% of the non-denervated contralateral control. This release was shown to be Ca^{2+} dependent in both intact and denervated ganglia.

4. Antidromic electrical stimulation of ciliary nerves also elicited [^{3}H]ACh release. Nicotine (1 μg/μl.) depolarized denervated ciliary ganglion cells and evoked release of the transmitter and this release was antagonized by curare.

5. It is concluded that the ganglionic cell bodies synthesized ACh and released the transmitter in response to K^+ depolarization, antidromic stimulation and cholinergic agonists, despite the lack of morphological specializations usually associated with stimulus-induced release of neurotransmitter. The evidence suggests the existence of a mechanism of transmitter release which is Ca^{2+} dependent, probably from a cytoplasmic pool and therefore distinct from the usual vesicular release at the nerve terminal.

INTRODUCTION

It is universally accepted that acetylcholine (ACh) is released from presynaptic cholinergic nerve endings during synaptic transmission. Depolarization of the terminal results in an influx of Ca^{2+} which causes release of transmitter and the experimental evidence thus far accumulated strongly indicates that stimulus-induced release is due to the emptying of synaptic vesicles from the presynaptic nerve into the synaptic cleft (Katz, 1969). Many studies have been aimed at characterizing aspects of ACh synthesis in terminals and its release by nerve endings in neuromuscular junctions and autonomic ganglia (see review by Collier, 1977).

In the parasympathetic ganglia ACh was identified and localized not only preganglionically but also in the ganglion cell bodies (Pilar, Jenden & Campbell, 1973).

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It is not surprising that ACh is synthesized in the cell soma. Acetyl-CoA-choline D-acetyltransferase (E.C. 2. 3. 1. 6, CAT), the enzyme catalysing the ACh synthetic reaction, is produced in the cell body and transported by slow axonal flow to the terminal region (Fonnum, Frizell & Sjöstrand, 1973; Giacobini, Pilar, Suszkiw & Uchimura, 1979). The precursors of ACh are also found in the cell body. Suszkiw, Beach & Pilar (1976) demonstrated the presence of a low affinity choline uptake system in somata of the chick ciliary ganglion, in contrast to the axon terminals where a Na^+-dependent high affinity choline uptake system is coupled to ACh synthesis (Vaca & Pilar, 1979). Since acetyl coenzyme A is of mitochondrial origin, it is also present in the soma.

It is possible that the presence of ACh in the soma is merely a reflexion of the presence of the necessary synthetic machinery. The suggestion has been made that this somal ACh is transported to nerve terminals where it is incorporated into the releasable transmitter pool (Triestman & Schwartz, 1974).

On the other hand, the ability to release transmitter may not be a property of the nerve terminal alone. It was thus of interest to determine if the ACh present in the somata of ciliary ganglion cells was releasable and, if that were the case, to characterize the release process. This report confirms that ACh synthesis from labelled choline does take place in the neuronal cell body. Further experiments demonstrate that this ACh can be released in response to several stimuli, each of which depolarizes the cell, and that the release of transmitter is Ca^{2+}-dependent. A preliminary report of this work has appeared elsewhere in abstract form (Johnson & Pilar, 1978).

METHODS

Ciliary ganglia were isolated from white Leghorn chicks aged 4–7 days and dissected in oxygenated Tyrode solution containing (mM): NaCl, 150; KCl, 3; $CaCl_2$, 3; $MgCl_2$, 1; Tris, 10; glucose, 12; pH = 7·4 at room temperature. Denervation of the ganglia was accomplished by sectioning the right oculomotor nerves of 1–4 day old chicks. Birds were anaesthetized with methoxyflurane and the nerves were severed at their entrance into the orbit, 1 mm proximal to the ciliary ganglion. Completeness of denervation was assessed by pupillary dilation on the operated side as well as by visual examination of the nerve stumps at the time of dissection. In all experiments the contralateral unoperated ganglion served as the control.

Acetylcholine measurements

Ganglia were equilibrated to 37 °C for 7 min in Tyrode and then incubated for 3 min in 50 mM-K^+ Tyrode before the labelling period. Osmolarity was maintained in high [K^+] saline by equimolar substitution for Na^+. This depolarization preceding the exposure to [^{3}H]Ch stimulates high affinity choline uptake (Murrin & Kuhar, 1976; Vaca & Pilar, 1979). While the denervated cell bodies apparently do not possess a mechanism for high affinity choline uptake (Suszkiw *et al.* 1976) and, therefore, probably do not undergo increased ACh synthesis in response to this treatment, control ganglia do exhibit a high affinity system. Thus, this optimization of choline uptake and ACh synthesis via K^+ depolarization was used as a standard procedure. Following the conditioning depolarization, ganglia were incubated for 45 min at 37 °C in Tyrode containing 1–2 μm-[^{3}H]choline (8·4 Ci/m-mole, New England Nuclear, Boston, Mass.).

Extracellular labelled choline was removed by four changes of Tyrode solution (minus choline) over a 30 min period. All experimental solutions contained 35 μM-neostigmine bromide.

Ganglia were incubated for 3 min periods in 1·2 ml. volumes containing solutions as noted in the text. A 100 μl. sample was taken from each bath to determine total radioactivity in the effluent and 1·0 ml. was immediately frozen for subsequent [^{3}H]ACh determination.

Following the release experiments, ganglia were quickly homogenized in acetic acid–ethanol and ganglionic [^{3}H]ACh was determined by high voltage electrophoresis as described previously

(Vaca & Pilar, 1979). The sum of the ganglionic [^{3}H]ACh and that collected in effluents equals the total labelled ACh synthesized by the ganglion. In addition to determining total labelled ACh, the above procedure served as a control for ganglia which may have been damaged during dissection. No preparations in this study had total [^{3}H]ACh levels so low as to suggest tissue deterioration.

Assay of labelled ACh in ganglionic effluents

[^{3}H]ACh was assayed in the superfusate after separation from [^{3}H]choline. The method of Shea & Aprison (1973) was used with some minor modifications (Johnson, Beach, Alanis & Pilar, 1977). The following is a brief summary. Choline was converted to phosphorylcholine by adding 100 μl. of a reaction mixture to the thawed 1·0 ml. sample collected during the experimental procedure. The resulting final concentrations were 10 mM-ATP, 10 mM-$MgCl_2$, 1 mM-dithiothreitol, 5 mM unlabelled choline and ACh and 0·01 u. choline kinase (Sigma, St Louis, Mo.). The enzyme-substrate solution was made in Tris base such that, after addition, the pH of the reaction solution was raised from 7·4 to 8·7, a more optimal range for the choline conversion reaction (Haubrich, 1973). Reaction mixtures were incubated at 37 °C for 10 min. The reaction was stopped by the addition of 4·0 ml. ice cold Tris HCl (0·1 M), which lowered the pH of the reaction mixture out of optimum for the conversion and into a pH range more suited for the subsequent extraction (Fonnum, 1969). ACh was extracted by the addition of 2·0 ml. Na tetraphenylboron in acetonitrile (5 g/l.). Ten ml. toluene fluor was added directly to the vial and radioactivity was determined with a Beckman LS-8000 liquid scintillation spectrometer. Cross-over from the aqueous phase containing [^{3}H]phosphorylcholine was 1·5%. Conversion of [^{3}H]choline to [^{3}H]phosphorylcholine was 96%. Greater than 97% of the labelled ACh was extracted into the organic phase. Control experiments were performed in the presence of acetylcholinesterase for 10 min at 37 °C in these assays. Following the tetraphenylboron/acetonitrile extraction only 1–2% of the total radioactivity present was detected by scintillation counting.

Endogenous acetylcholine

Levels of endogenous ACh in both normal and denervated ganglia were determined using the method of McCaman & Stetzler (1977). The method involves the conversion of ACh to choline-$^{32}PO_4$ in the presence of [^{32}P]ATP and the enzymes acetycholinesterase and choline kinase. This method is sensitive in the sub-picomole range.

Electrophysiology

Extracellular recordings from the ciliary nerves were used to determine appropriate concentrations of pharmacological agents necessary to induce action potentials in denervated cells. The ciliary nerves were dissected free of connective tissue and followed forward to the iris. A small piece of sclera surrounding the ciliary nerves' entrance into the eye was left intact. The ciliary nerve branches were dissected into fine filaments containing only a small number of axons, and the electrical activity in these filaments was recorded using suction electrodes with interchangeable polyethylene tips. The recording chamber used was similar to that previously described (Martin & Pilar, 1963) except that a small notch served to secure the piece of scleral tissue and separate the ciliary nerves from the central perfusion chamber containing the ganglion. Solutions were superfused at the rate of 0·7–1·0 ml./min. Nicotine (1 μg/μl.) was applied 2·5 mm upstream from the ganglion in a 10 μl. drop from a Hamilton microsyringe. Responses were differentially amplified (MPA-6, Transidyne General Co., Ann Arbor, Mich.), taped on a Honeywell 7600 Magnetic Tape Recorder and analysed with a Hewlett-Packard 5325A Universal Counter. A Hewlett-Packard Digital-Analog Converter allowed the generation of a frequency histogram on a Gould 220 Brush chart recorder.

Electron microscopy

Ganglia were initially fixed after preganglionic nerve section by aortic perfusion of the anaesthetized chick with a warm solution of 2% glutaraldehyde for 15 min. Later ganglia were dissected out and further fixed in 2% glutaraldehyde in phosphate buffer overnight and post-fixed with 1% osmium tetroxide for 1 hr, dehydrated and embedded in Epon. Thin sections cut on a LKB ultramicrotome were stained with uranyl acetate and lead citrate and examined with a Philips 300 electron microscope at 80 kV. Quantitation of degenerating terminals was performed on sections of ciliary cells in which nucleoli were visible.

RESULTS

Ultrastructural changes after denervation

Since both pre- and post-synaptic elements in parasympathetic ganglia are cholinergic, the preganglionic contribution was eliminated by surgical denervation. Ganglion cells receive synaptic connexions only from the oculomotor nerve whose origin is the accessory oculomotor nucleus. Intrinsic neurones and SIF cells are absent. (Cantino & Mugnaini, 1975). Therefore, sectioning the oculomotor nerve eliminated all synaptic input to the ganglion cells. Ganglia were examined with the electron microscope at 2 and 3 days after nerve section. By 2 days there was considerable axonal degeneration with many axon profiles exhibiting a break-down of compact myelin.

Preganglionic axons end in calyciform terminals extending over a large area of the ciliary cells. In favourable sections, calyces envelop almost the entire cell (De Lorenzo, 1960). Following nerve section the calyces rapidly degenerated and junctions containing vesicles and synaptic densities disappeared. The time course of degeneration of the synaptic contacts in the axon hillock region was similar to that reported in a previous paper (Giacobini *et al.* 1979) and will not be repeated here. This report will illustrate responses to denervation occurring in another area of synaptic contact between preganglionic endings and ganglion cells which is formed by the presynaptic cell interdigitating with short dendrite-like processes of the post-synaptic cell. This forms a tuft upon which synaptic specializations occur (Pl. 1*A*). These tuft-like endings are usually found near the nuclear pole of the cell opposite the axon hillock (for a description of synaptic contacts in the hilar region, see Cantino & Mugnaini, 1975). Two days after denervation the tufted areas were still visible; however, no structures resembling terminal endings were seen (Pl. 1*B*). In some sections empty spaces overlying the tufted areas were observed, presumably indicating the previous position of now degenerated presynaptic endings (Pl. 1*C*). In this example, the dendrite-like processes are clearly distinguished and contain membrane densities where synapses may have previously been present. Ciliary cell perikarya are myelinated and beneath this thin myelin layer were either degenerating terminals or spaces which could have contained synaptic endings. Using the nucleolus as a marker, fifty cells were examined for presynaptic endings. No intact terminals were seen. Degenerating debris from terminals was present at a frequency of 2–3 per cell. In contrast, several synapses were found on each of the control cells.

Choroid cells comprise 60% of the ganglion cell population and are morphologically distinct from the ciliary cells. Presynaptic axons end upon choroid cells in distributed boutons similar to those of other vertebrate parasympathetic neurones (Cantino & Mugnaini, 1975). The degeneration of these boutons followed a time course similar to that of the ciliary cell synapses.

No further differences were found in the appearance of the ganglion at 3 days after nerve section. No intact terminals were observed in fifty cells whose entire perimeter was examined (Pl. 2). Evidence of degenerating terminals was still present at a frequency of 2–3 per cell and spaces were found where synaptic endings may have once been. It should be emphasized that the somal contents in all the denervated preparations were normal in all other respects: the mitochondria, rough endoplasmic

reticulum, Golgi apparatus and both free ribosomes and ribosomal rosettes appeared similar to those in the unoperated control ganglion.

The short period of degeneration reported in this paper contrasts with the longer time course of denervation changes occurring at the frog neuromuscular junction (Birks, Katz & Miledi, 1960). However, the time course of nerve terminal degeneration is largely dependent upon the length of the residual nerve stump and temperature (Birks *et al.* 1960). In this study both factors favour a rapid disappearance of nerve endings. The oculomotor nerve was sectioned at a point 1–2 mm proximal to the ganglion leaving a very short nerve stump and the core temperature of the chick is 40 °C. Consistent with these observations, the rate of presynaptic degeneration in this study was similar to that found in denervated pigeon ciliary ganglia (Giacobini *et al.* 1979) and in the decentralized major pelvic ganglion of the rat (Dail & Evan, 1978).

An estimate was made of the number of functional terminals necessary to account for the ACh release measured in this report (see section on ACh release). The ciliary ganglion contains roughly 3000 cells; each ciliary cell is innervated by a single preganglionic axon, each choroid cell is contacted by two or three afferent axons. Since the denervated ganglion released approximately one sixth of the ACh released from an intact ganglion, one would expect to find about 500 functional terminals remaining. Another possibility is that all the terminals remained functional but each released only a portion of its normal amount. This large a population could not be easily overlooked microscopically. In fact, no normal presynaptic elements were observed. These observations also ruled out the possibility that the ganglion cells, after denervation, made synaptic connexions among themselves, as is the case for parasympathetic cells of the denervated frog heart (Sargent & Dennis, 1977).

ACh levels in intact and denervated ganglia

Endogenous ACh was assayed in both denervated and control ganglia, as was the labelled ACh pool which was determined by summing the amount of [^{3}H]ACh collected in effluents with that remaining in the ganglion after an experiment.

Table 1 presents values of both the endogenous levels and the total ACh labelled during incubation with [^{3}H]Ch. Approximately equal proportions of the ACh pool were labelled in both denervated and control situations. Two sham-operated controls had values of endogenous ACh of 38·3 and 42·9 p-mole, within the range of control values (control values $46{\cdot}7 \pm 3{\cdot}3$, mean $\pm$ S.E., $n = 13$). Since an anticholinesterase was used in these experiments, it was important to determine whether the ACh synthesized by the denervated ganglia was surplus ACh (Birks & McIntosh, 1961; Collier & Katz, 1971). Surplus ACh accumulates when precursor incubation is carried out in the presence of an anticholinesterase and this surplus pool can be released by high $[K^+]_0$. In our study neostigmine was chosen because it would not penetrate the terminal membrane in sufficient quantities during the incubation time to effect a surplus pool, and because quaternary amines are less effective than tertiary amines (e.g. eserine) in causing surplus ACh (Collier & Katz, 1971). Furthermore, in all experiments the drug was added to the bathing solutions after the [^{3}H]Ch incubation period had ended and while excess [^{3}H]Ch was being washed out of the ganglion. Finally, synthesis of [^{3}H]ACh was not increased by the presence of neostigmine. In denervated and control ganglia total radioactivity in bath effluents and [^{3}H]ACh in ganglia was measured following the incubation schedule outlined in Methods, except

that no anticholinesterase was added during the washout and collection periods and these values were compared to those obtained when neostigmine was used.

In the presence of neostigmine the total radioactivity in bath effluents of denervated ganglia was 20,469 ± 4895 d.p.m. (n = 7) and ganglionic [^{3}H]ACh was 0·7 ± 0·09 p-mole (n = 29); in its absence total radioactivity was 17,632 (n = 2) and ganglionic [^{3}H]ACh level was 0·5 p-mole (n = 2). The difference between the two

TABLE 1. Acetylcholine levels in normal and denervated ganglia

	Total ACh	Labelled ACh	% Labelled
Denervated	17·3 ± 1·7 (n = 11)	0·94 ± 0·11 (n = 29)	5·4 ± 0·8
Control	46·7 ± 3·3 (n = 13)	3·17 ± 0·63 (n = 7)	6·8 ± 1·4

All values are in p-mole and expressed as mean ± S.E.
The % labelled is the ratio of the means ± S.E.

groups was not statistically significant. Similar results were also found in control ganglia. Total radioactivity and [^{3}H]ACh in the presence of neostigmine was 38,946 ± 6207 d.p.m. (n = 7) and 1·91 ± 0·53 p-mole (n = 7), respectively, and when drug was omitted, the total radioactivity was 31,424 d.p.m. (n = 2) and ganglionic [^{3}H]ACh was 1·0 p-mole (n = 2). The difference between both groups was not statistically significant. Thus, with the incubation schedule used in the present experiments, anticholinesterase did not alter the amount of [^{3}H]ACh in the ganglia, and it is reasonable to conclude that the ACh release induced by a variety of treatments shown below is not surplus ACh.

Release of [^{3}H]ACh in response to $[K^+]_o$ depolarization

Exposure for a 3 min period to Tyrode containing 50 mM-K^+ resulted in a marked increase in released ^{3}H-labelled ACh from control ganglia (Fig. 1). In Fig. 1 the first depolarization yielded a release of approximately 520 ± 101 f-mole/3 min (mean ± S.E., n = 7), a nine or tenfold increase above base line levels, and the second depolarization, 9 min later, resulted in a release of 244 ± 36 f-mole (mean ± S.E., n = 7) approximately 6 times that of the base line.

Denervated ganglia subjected to two similar exposures to high [K^+] saline also showed an increase in transmitter release. In Fig. 1 it is seen that during both stimulation periods, ACh release increased fourfold over the base line amount. Levels of [^{3}H]ACh in bath effluents were 16·5 ± 3·9 % (mean ± S.E., n = 7) that of the contralateral unoperated control during the first release period and 22·7 ± 5·5 % during the second. Also, the resting efflux of [^{3}H]ACh in denervated ganglia was approximately 25–30 % that of contralateral intact control.

Ca^{2+}-dependence of $[K^+]_o$-induced [^{3}H]ACh release

Exposure of ganglia to 50 mM-K^+ in Tyrode lacking Ca^{2+} (0·1 mM-EGTA added) resulted in no increase in the [^{3}H]ACh in ganglionic effluents. However, addition of 3 mM-Ca^{2+} to the bath yielded marked release in response to elevated $[K^+]_o$ (Fig. 2). Each experiment in this series included a period of exposure to high [K^+] in the presence of Ca^{2+} between two trials in the absence of Ca^{2+}. This insured that the

release process was intact and operable. Thus, the release of labelled ACh from denervated ganglia is by a Ca^{2+}-dependent process. It is also shown in Fig. 2 that immediately after Ca^{2+} was added to the incubation solutions, the resting leakage of transmitter was slightly elevated.

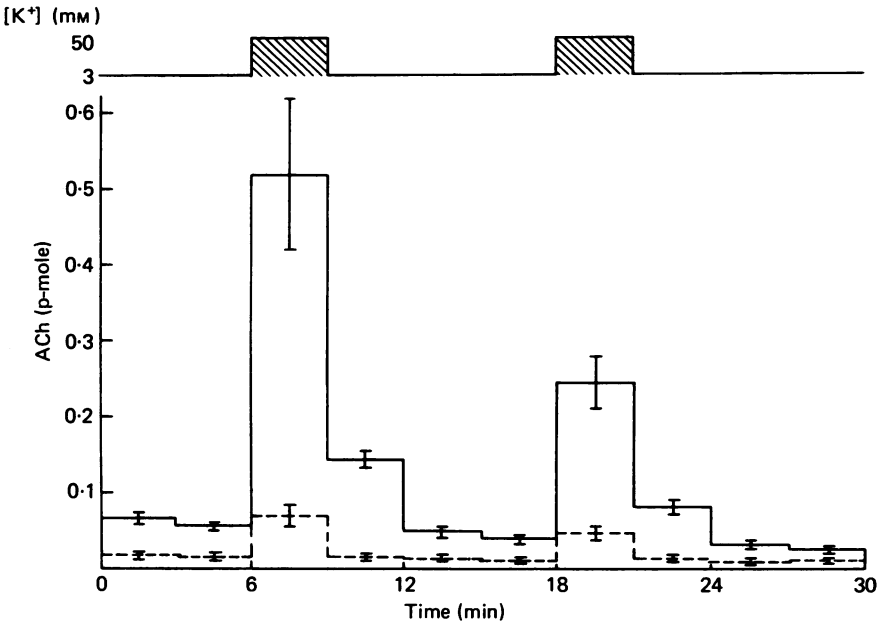

Fig. 1. Release of labelled ACh from intact (continuous lines) and 3-day denervated ganglia (dashed lines) in response to high $[K^+]_o$ stimulation. [^{3}H]ACh was collected during 3 min periods. Similar collection times were used in Figs. 2 and 3. Stippled areas indicate the time of exposure to 50 mM-K^+. In each experiment similar $[K^+]_o$ challenges were applied to a denervated ganglion and its contralateral control. Note that [^{3}H]-ACh is released from the denervated ganglia during $[K^+]_o$ stimulation. In this and Figs. 2, 3 and 6 vertical lines indicate mean ± S.E. (n = 7).

Release of [^{3}H]ACh by antidromic electrical stimulation

Ciliary nerves of ganglia denervated 3 days earlier were stimulated for 3 min at 20 Hz by supramaximal pulses via a suction electrode. Under these conditions the concentration of [^{3}H]ACh in the bath effluents increased threefold. Elevated levels of release also occurred in the period subsequent to stimulation (Fig. 3). This prolonged release after stimulation was not observed when [^{3}H]ACh was collected after $[K^+]_o$ challenges. This difference may be due to a slightly modified procedure in handling the preparation. In the previous experiments, the ganglia were transferred at 3 min intervals to different vials, each with the same amount of saline and a sample was taken from each container. In the electrical stimulation experiment, the same amount of saline was removed every 3 min and replaced with fresh saline. [^{3}H]ACh was measured from aliquots of each period. The wash-out of labelled ACh was probably not complete after the saline replacement and therefore, some of the labelled ACh contaminated the subsequent collection periods.

Antidromic stimulation of an intact ganglion caused a similar elevation in ACh release. The amount collected in this effluent was greater than that of denervated ganglia probably due to the activation of presynaptic elements via electrical junctions (see Martin & Pilar, 1963).

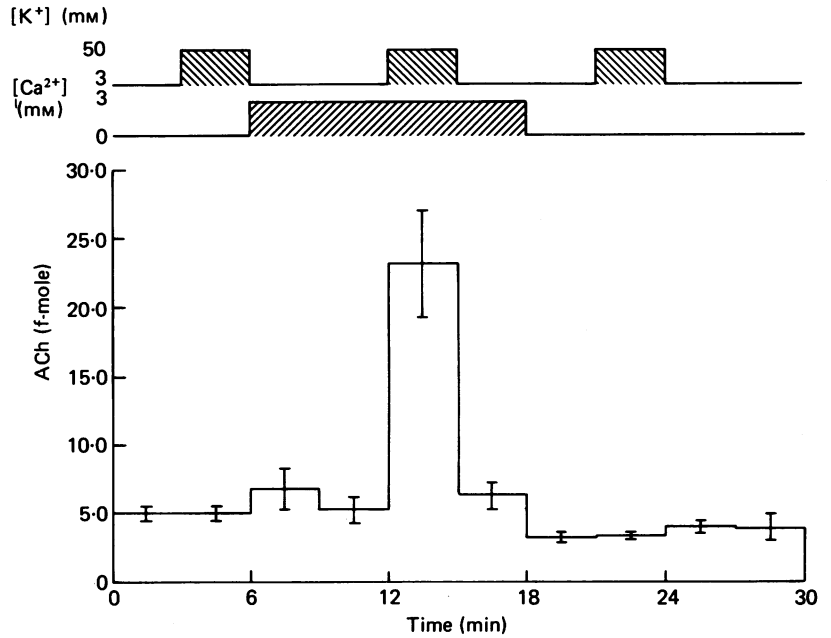

Fig. 2. Ca^{2+} dependence of $[K^+]_o$-induced release of [^{3}H]ACh from denervated ganglia. Ganglia were exposed to 3 min stimulation in high $[K^+]_o$ (upper stippled area). Ca^{2+} was added to the bathing medium following the first $[K^+]_o$ challenge and was removed prior to the third (lower stippled area). [^{3}H]ACh collected in bath effluents during $[K^+]_o$ stimulation was elevated only in the presence of Ca^{2+}. (n = 6).

Release of [^{3}H]ACh in response to nicotine

To discern whether the release of labelled ACh could be induced by receptor-activated depolarization of ganglion cells, nicotine, a cholinergic agonist known to activate ciliary ganglion neurons, was used.

Electrophysiological tests were conducted to insure that the cells in a ganglion denervated for 3 days could respond to nicotine and to determine the concentration of agonist necessary to elicit action potentials.

In the absence of nicotine there was no spontaneous activity in axons of denervated ciliary cells. Following drug application action potentials were evoked. Fig. 4*A* shows the first spikes from several units recorded 3·2 sec after applying nicotine to the bath. By 8 sec the frequency of firing had increased greatly (Fig. 4*B*) and by 180 sec action potentials were again low in frequency (Fig. 4*C*). The addition of curare (50 μg/ml.) eliminated the action potentials elicited by nicotine (Fig. 4*D*, *E*). Figs. 4*D* and *E* were taken at times similar to *A* and *B*, respectively, following a nicotine challenge in the presence of curare, and no responses were evoked. A

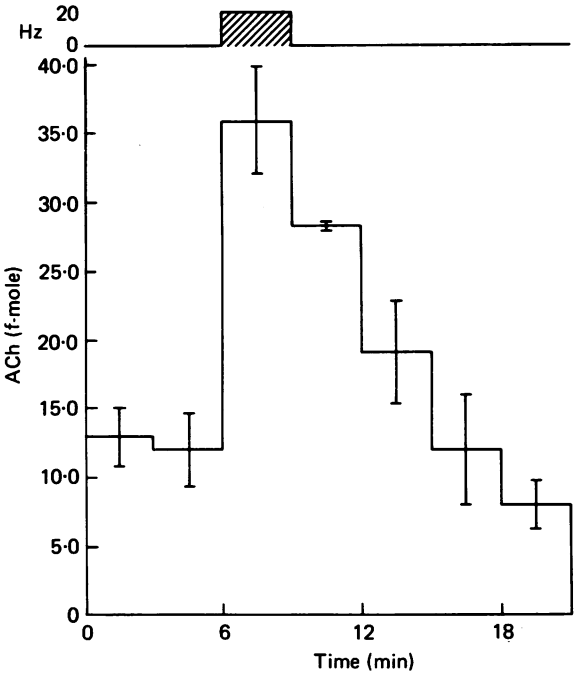

Fig. 3. Release of [^{3}H]ACh by antidromic stimulation of denervated ganglia. Ciliary nerves were electrically stimulated at 20 Hz for 3 min (stippled area). [^{3}H]ACh was released not only during the stimulation period, but persisted for several min. See text for explanation (n = 4).

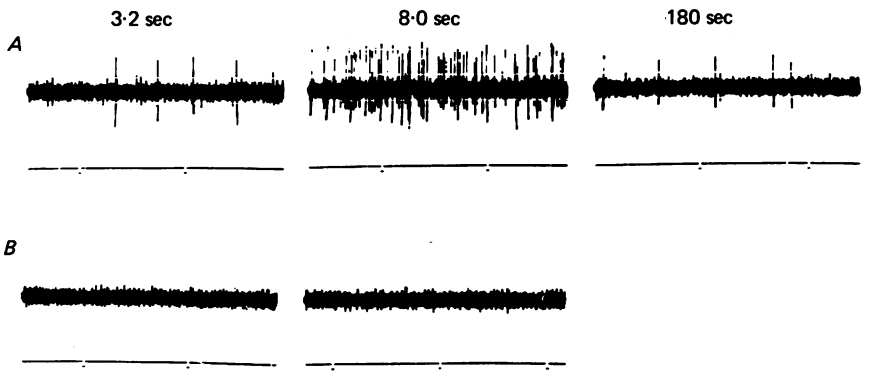

Fig. 4. Responses of denervated ciliary cells to nicotine. Spike activity recorded from ciliary nerve filaments allows the individualization of single axons and quantitation of the response. The electrical activity was recorded continuously, and the recordings illustrated are samples taken at the indicated time after drug application. *A*, control responses after a 10 μl. drop of nicotine (1 μg/μl.). The same application was repeated in *B* after curare was added to the superfusate for 30 min. Calibration bottom line, each square-pulse is separated by 100 msec.

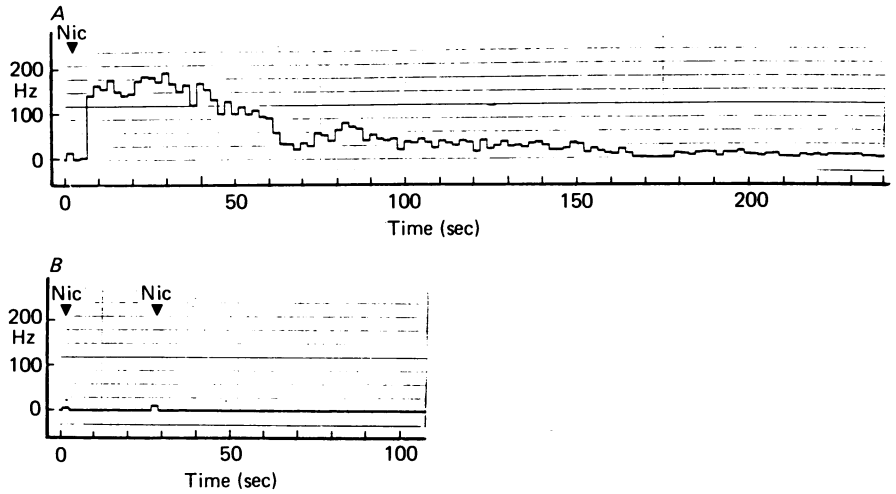

Fig. 5. The time course of electrical activity after nicotine applications (marked by arrows) is illustrated. Spike activity was similar to that in Fig. 4. *A*, following nicotine application, action potential frequency increased within 10 sec and fell to low levels by 2–3 min. *B*, no nerve activity was evoked by nicotine in the presence of curare. A second drop of nicotine, 30 sec after the first, still did not elicit any activity from the curarized ganglion.

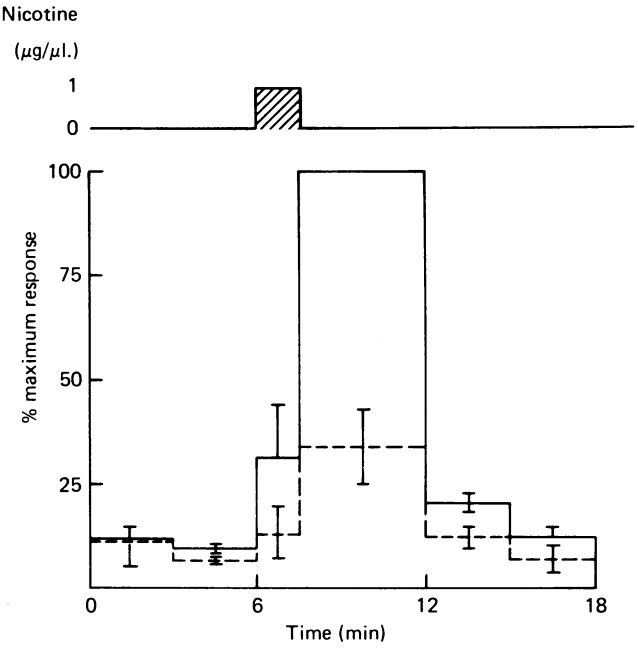

Fig. 6. Release of [^{3}H]ACh from denervated ganglia by nicotine (continuous line) and its block by curare (dashed line). Ganglia were exposed to nicotine during stippled area period. Each experiment consisted of two denervated ganglia, one exposed to nicotine in the presence of curare, the other exposed to the drug in the absence of curare. Data expressed as per cent maximum release for each pair. The collection procedure, slightly different from the one used in previous experiments, is detailed in the text (n = 4).

frequency histogram of the discharges recorded from a nerve filament shows the time course of the nicotine effect (Fig. 5*A*) and its blockade by curare (Fig. 5*B*).

Superfusion of the ganglion with nicotine solution (1 $\mu g/\mu l.$), produced rapid desensitization unlike the response seen with dropwise application. Therefore, for the purpose of ganglionic effluent collection, a slightly different release-inducing procedure was used. Each ganglion was immersed in nicotine-containing Tyrode for 30 sec, then Tyrode without nicotine for 90 sec, this being repeated 3 times. The bath samples from the three 30 second periods were then pooled, as were those from the three 90 sec periods. Ganglia subjected to similar treatments in Tyrode alone did not release ACh. Levels of [^{3}H]ACh were elevated in the ganglionic effluents following exposure to nicotine and this release was inhibited by curare (Fig. 6). The data is normalized to the maximum amount of ACh collected for each pair (experimental and control).

DISCUSSION

Denervated chick ciliary ganglion cell bodies were shown to be capable of synthesizing ACh. Release of this ACh into bath effluents was enhanced during depolarization of the denervated ganglia by ionic, pharmacologic and electrical stimuli. Several lines of evidence indicate that the transmitter was released from the ganglion cell soma.

Ciliary ganglion cell somata possess a low affinity choline uptake system and synthesize [^{3}H]ACh from [^{3}H]choline (Suszkiw *et al.* 1976). This study confirms the latter finding of that report. Following denervation, a significant amount of endogenous ACh ($17{\cdot}3 \pm 1{\cdot}7$ p-mole, mean $\pm$ S.E.) was present in the ganglion. Incubation in [^{3}H]choline labelled approximately 5·4 % of this pool. In the intact ganglion 6·8 % of the ACh pool was labelled. These figures are comparable to those of Szerb (1975) who reported that incubation for 60 min in 1 μM [^{3}H]choline labelled 3·1 % of the ACh pool in the myenteric plexus of the guinea-pig ileum.

Masserelli, Ciesielski-Treska, Ebel & Mandel (1974) demonstrated a low affinity Ch uptake system in differentiated glial cell cultures and a high affinity uptake system in undifferentiated glial cells. Perhaps in response to denervation the glial cells may assume an undifferentiated state and develop a high affinity choline uptake. The presence of either a low or high affinity uptake system could account for the Ch taken up which may be converted to ACh if the cells have the ability to synthesize ACh, but there is no evidence that glia have CAT activity. The discussion below argues against glial cells as those elements mediating the ACh release reported.

Characterization of the release process

A series of experiments was conducted to characterize the mechanism of release of [^{3}H]ACh from denervated ganglia. It was found that it was clearly Ca^{2+}-dependent; in the absence of Ca^{2+}, levels of release in response to high $[K^+]_o$ stimulation were not increased above base line. Upon re-addition of Ca^{2+} to the bathing solution, high [K+] depolarization elicited the release of substantial quantities of ACh. Release of [^{3}H]ACh from cell somata was also elicited by antidromic electrical stimulation. These experiments required the use of denervated ganglia since ciliary neurones are both chemically and electrically coupled with the preganglionic oculomotor nerve

terminals (Martin & Pilar, 1963). Because of these electrical junctions, electrical activation of post-synaptic neurons can lead to the retrograde activation of the presynaptic terminals. If the coupling potential reaches threshold, significant transmitter release from the presynaptic terminal may occur. This sort of release was observed in control experiments. However, 3 days after denervation, the release from antidromically activated presynaptic elements was no longer detectable.

The third method used to depolarize the denervated ciliary cells was activation of the nicotinic cholinergic receptors. Spontaneous electrical activity in denervated cells was very low. Application of nicotine evoked high frequency discharges from ciliary cells. The sensitivity of this firing to curare blockade suggests that it results from the activation of nicotinic receptors. Ganglia incubated in [^{3}H]choline released [^{3}H]ACh into the bath effluents upon exposure to nicotine. This release was also inhibited by curare. These two pharmacological experiments suggest that receptor mediated depolarization induces the ACh release.

It is conceivable that this release is from satellite or glial cells in the ganglion. Indeed ACh released by glia at the denervated frog neuromuscular junction has been demonstrated (Miledi & Slater, 1968; Bevan, Grampp & Miledi, 1976), and can be brought about by direct depolarization of the Schwann cell (Dennis & Miledi, 1974). Such could be the case in the present experiments; i.e. both high [K^+] depolarization and antidromic firing leading to a local increase in the extracellular $[K^+]_o$ could depolarize glial cells and evoke release. However, the release process described in the present report is clearly Ca^{2+}-dependent, unlike the glial ACh release which is antagonized by Ca^{2+} (Ito & Miledi, 1977).

In summary, these experiments rule out a pre-terminal origin and demonstrate a Ca^{2+}-dependent ACh release that is not attributable to glial elements. Three separate procedures (high [K^+] depolarization, antidromic electrical stimulation and ACh receptor activation), each designed to depolarize the neuronal cell bodies, clearly induced release.

Functional implications of the somatic ACh release

The release process described in this study resembles the presynaptic release of transmitter. However, in all electron microscopic sections examined, there were no clusters of vesicles similar to those present in presynaptic terminals and no membrane specializations were found which might be analogous to releasing sites. But since no neurones were examined in their entirety by serial sectioning, the possibility of vesicular clusters cannot be eliminated. Nevertheless the quantity of [^{3}H]ACh collected suggests that a large number of vesicles or vesicle clusters would be observed.

While the vesicular hypothesis of transmitter release is well established and generally accepted, recent reports have suggested the existence of a cytoplasmic ACh release mechanism (Birks, 1974; Birks & Fitch, 1974). Also, continuous leakage of ACh has been shown to occur from frog motor nerve terminals. However, it was not determined if this release was Ca^{2+}-dependent or increased by nerve impulses (Katz & Miledi, 1977).

Our results can be reconciled with these different observations if we assume that the somatic release of ACh is from a cytoplasmic pool. The Ca^{2+}-dependence of this

process is still puzzling for Ca^{2+}-dependency has usually been associated with the vesicular release at nerve endings (Heuser, Reese & Landis, 1974). Ascertaining whether the somatic release occurs in a quantal or nonquantal fashion will aid in determining whether different cellular mechanisms are involved in vesicular and cytoplasmic ACh secretion.

There remains the question of the physiological significance of this phenomenon. Does the ACh that can be released from the soma play a feed-back role, influencing further presynaptic release or acting to alter the sensitivity of post-synaptic ACh receptors? The experiments in this report were conducted in the presence of neostigmine which prevented the enzymatic hydrolysis of ACh and allowed greater diffusion of transmitter. In the normal ganglion, this hindrance to ACh diffusion may be sufficient to block any presynaptic action of the post-synaptically released ACh, but may not be so large a barrier as to prevent alterations in post-synaptic ACh receptor sensitivity. Furthermore, it needs to be determined if the release of transmitter from cell bodies is an event that occurs in the intact preparation or one which appears only following denervation. This question requires separation of presynaptic and post-synaptic events by a method other than denervation.

Finally, based on our observations, it would seem that the capacity to release ACh is not restricted to the membrane of the nerve terminal, but rather may be distributed over the membrane of the entire neurone. This situation, which may merely reflect an evolutionary imperfection, might also explain the widespread distribution of cholinesterase in cholinergic neuronal membranes, in that the esterase present could hydrolyse the extra-terminal ACh released.

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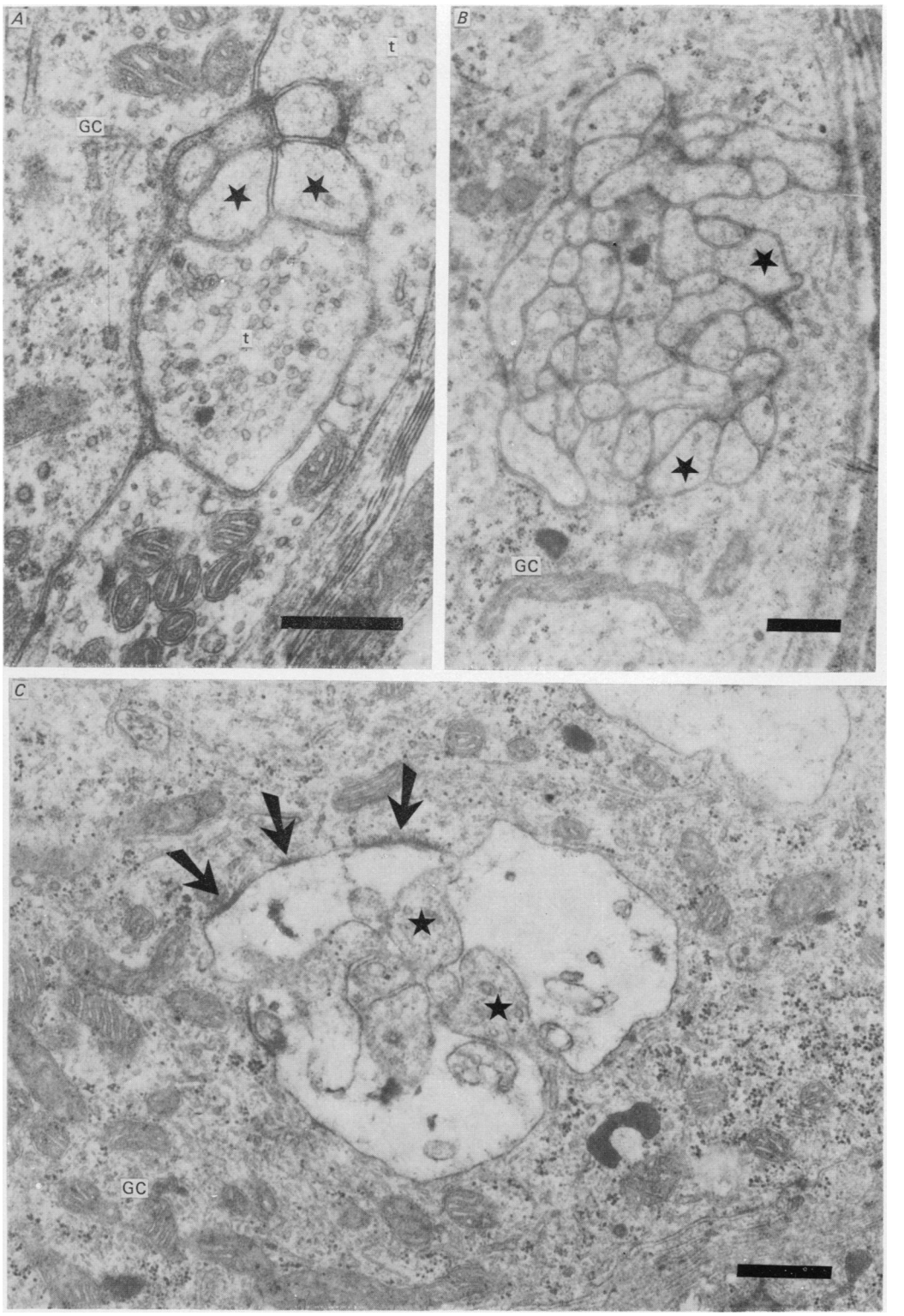
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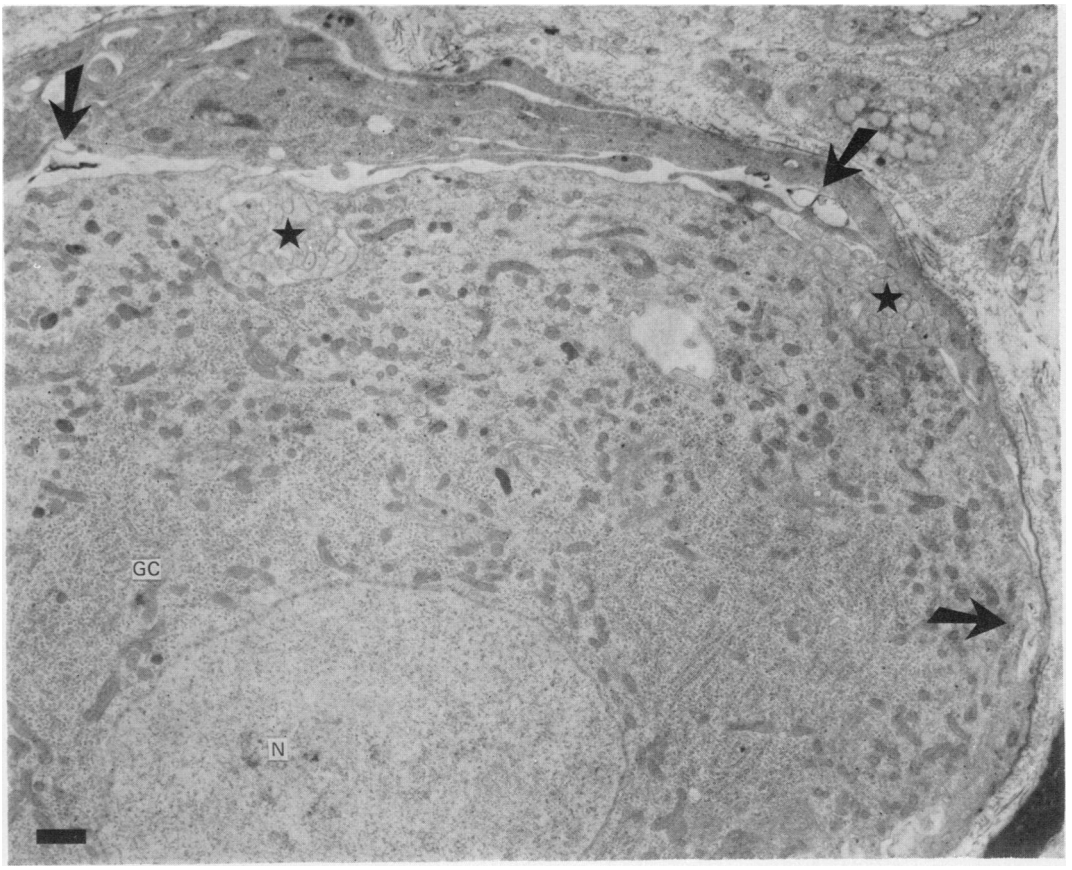
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EXPLANATION OF PLATES

PLATE 1

All electron micrographs illustrate the same area of synaptic contact on ciliary ganglion cells formed by interdigitations of terminals and post-synaptic processes.

A, presynaptic nerve terminals (t) filled with synaptic vesicles contact tuft-like endings (*) of a normal ganglion cell (GC).

B, micrograph of a tufted area in a 2 day denervated ganglion. There is no indication of presynaptic endings. It appears that the region is now occupied by only post-synaptic processes clustered together, some of which are indicated by the *.

C, section of a similar synaptic region 2 days after denervation. The empty area was presumably occupied by degenerated terminal endings, which were removed during the fixation procedures, some remnants still being visible. The dendrite-like processes of the cell body which formed the tufts are clearly seen, and membrane densities (arrows) indicated. Calibration bar, 0·5 μm for *A*, *B* and *C*.

PLATE 2

Low power micrograph of a ciliary cell 3 days after denervation. The cytoplasmic organization is similar to control cells. There are no presynaptic terminals in the periphery of the cell, where they are normally seen. Debris (arrows) partially fills the space between the satellite cells and the ganglion cells. Tufts can be recognized (*). Calibration bar, 1·0 μm.