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The release of acetylcholine by acetylcholine in the cat's superior cervical ganglion

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Summary

1. The experiments described in this paper tested the effect of acetylcholine (ACh), carbachol or preganglionic nerve stimulation on the release of ACh from the cat's perfused superior cervical ganglion; radioactive tracer methods were used.

2. When the ganglion's transmitter store of ACh had been labelled, radioactive ACh was released by nerve stimulation (5 Hz for 2 min), but there was no release by ACh (0·15–15 μg) or by carbachol (1–10 μg) when these drugs were injected close to the ganglion. Perfusion with low or moderate concentrations of ACh (0·15–5 μg/ml) also failed to release ACh, but high concentrations (15–50 μg/ml) released a small amount of labelled material. There was no correlation between ganglion stimulation by ACh and release of radioactivity.

3. Ganglion-blocking concentrations of ACh did not reduce the release of ACh during continuous nerve stimulation.

4. When resting (unstimulated) ganglia were perfused with ^{3}H-choline and eserine, the extra ACh synthesized and stored by such ganglia (surplus ACh) was labelled. Preganglionic nerve stimulation (5 Hz for 2 min) did not release surplus ACh, but perfusion with ACh (0·5–15 μg/ml), or injection of carbachol (0·5–2·5 μg) did.

5. Surplus ACh released by ACh or by carbachol did not contribute to the ganglion stimulating effect of either drug.

6. It is concluded that the presynaptic effects of ACh are not of physiological importance.

Introduction

The concept that the arrival of an action potential in a presynaptic nerve terminal results in the release of enough transmitter substance to effect synaptic transmission has been challenged by Koelle (1961, 1962). Koelle's hypothesis is that, at cholinergic synapses, a nerve impulse releases too little acetylcholine (ACh) to stimulate the postganglionic cell directly, but enough to prolong the state of depolarization of the presynaptic nerve terminals so that they discharge additional ACh which does effect transmission.

This suggestion that there is a positive feed-back mechanism for release implies that exogenously applied ACh, or ACh-like agent, should release transmitter from presynaptic nerve endings, and some evidence has accumulated that this might be

true for the cat's superior cervical ganglion. However, a part of this evidence is indirect (Volle & Koelle, 1961), and the more direct support (McKinstry, Koenig, Koelle & Koelle, 1963 ; McKinstry & Koelle, 1967) is not unequivocal. The experiments of McKinstry and her colleagues showed that carbachol released ACh from ganglia perfused with an anticholinesterase agent ; however, such ganglia synthesize and store ACh additional to their normal complement of transmitter (Birks & MacIntosh, 1961), and it was not clear how much of the carbachol-induced release of ACh was from this " surplus " store, and how much was from the transmitter depot.

Procedures have now been devised whereby either of these two ACh stores can be labelled separately with radioactive ACh (Collier & MacIntosh, 1969 ; Katz & Collier, in preparation). The experiments described in this paper compared the release of ACh from the ganglion during preganglionic nerve stimulation with its release by exogenous ACh or carbachol. The results indicate that the presynaptic effects of ACh are of no physiological importance, though they might be of pharmacological interest.

A preliminary report of some of these results has been presented (Collier, Vickerson & Varma, 1969).

Methods

The techniques were similar to those described previously (Collier & Lang, 1969 ; Collier & MacIntosh, 1969).

Ganglion perfusion. Cats (1·8–2·9 kg) were used ; anaesthesia was induced with ethyl chloride followed by ether and was maintained by intravenous chloralose (80 mg/kg). The right superior cervical ganglion was perfused by Feldberg & Gaddum's (1934) modification of Kibjakow's (1933) technique. Perfusion was with Locke solution (NaCl 140 mM, KCl 5·6 mM, $CaCl_2$ 2·2 mM, $NaHCO_3$ 16 mM, glucose 5·5 mM) which contained choline chloride (Ch, $1{\cdot}07 \times 10^{-5}$M), either unlabelled or ^{3}H-labelled (63 mCi/mmol) ; the perfusion fluid was equilibrated with 3·5% carbon dioxide in oxygen throughout the experiment ; it was warmed to 37° C, and its pH was 7·4.

Perfusion was switched from one solution to another as described by Birks & MacIntosh (1961), but before testing the effect of perfused ACh on the efflux of radioactivity it was necessary to wash out the tube connecting the ACh solution to the arterial cannula, because a trace of labelled Ch diffused into its tip during the loading perfusion.

Injection of ACh or carbachol was directly into the arterial cannula ; the drugs were dissolved in Locke solution and injected in a volume of 0·1–0·2 ml.

In all experiments the first 15 min perfusion was with Ch-Locke, and this was followed by a 60 min loading perfusion designed to label one of the ganglion's ACh pools. (*A*) When the ganglion's transmitter ACh store was to be labelled (see Collier & MacIntosh, 1969) perfusion was with ^{3}H-Ch-Locke (no anticholinesterase) and the preganglionic sympathetic trunk was stimulated continuously (20 Hz, 0·5 ms, 4–8 V). (*B*) When the surplus ACh of a ganglion was to be labelled perfusion was with labelled Ch-Locke containing eserine (1–10 μg/ml) or diisopropylphosphofluoridate (DFP, 1–10 μg/ml) and the ganglion was not stimulated (preganglionic trunk cut). In either type of experiment, the loading perfusion was followed by a

washout perfusion with unlabelled Ch-Locke containing an anticholinesterase (eserine or DFP). During the first 15 min of this washout perfusion, the effluent from the ganglion was discarded ; the perfusate was then collected every 2 min and the effect of nerve stimulation or applied drug upon the efflux of radioactive material was tested.

Ganglion extraction. At the end of the perfusion, ganglia were removed and extracted with 10% trichloroacetic acid (TCA). After removing the TCA with ether, aliquots of the extract were used for liquid scintillation counting, for bioassay of ACh, or for separation of labelled ACh from other labelled material.

Measurement of radioactivity. Radioactivity in solutions was determined by liquid scintillation spectrometry in the solvent systems reported earlier (Collier, 1969) ; quench correction was made using an external standard, channels-ratio method.

Bioassay. ACh content of ganglion extracts was measured by their effect on cat's blood pressure (MacIntosh & Perry, 1950). When necessary, arterial pressure was maintained at 120 mmHg by infusing noradrenaline through the arterial cannula.

Separation of labelled materials. Radioactive ACh in ganglion extracts or effluents was separated from other labelled constituents by selective precipitation (Collier & Lang, 1969).

Nictitating membrane responses. Isometric contractions of the cat's nictitating membrane were recorded on a pen recorder to assess ganglion stimulation.

Materials. Compounds used were: methyl-^{3}H-choline chloride (New England Nuclear), unlabelled choline chloride (Nutritional Biochemicals Ltd.), acetylcholine chloride (Roche Ltd.), eserine sulphate (Brinkman Ltd.), diisopropylphosphofluoridate (DFP, K & K Ltd.), carbachol chloride (K & K Ltd.). Values are expressed as mean $\pm$ S.E. when appropriate.

Results

Release of transmitter ACh by nerve stimulation or by cholinergic drugs

In these experiments, the ganglion's normal ACh store was replaced with labelled ACh by perfusing with ^{3}H-Ch (no anticholinesterase) during continuous preganglionic nerve stimulation. At the end of this loading perfusion, ganglia have a normal total content of ACh, but about 85% of it is labelled ; a 20 min wash by perfusion with unlabelled Ch-Locke removes extracellular or loosely-bound radioactivity, but little of the labelled ACh (Collier & MacIntosh, 1969). Following this washout perfusion, nerve stimulation and injected or perfused drugs were tested for their ability to release labelled ACh.

Figure 1 shows the results of a typical experiment ; preganglionic nerve stimulation released radioactive material that was easily detected, but injected ACh (1 or 10 μg) failed to do so, although both doses of ACh stimulated the ganglion. Similar results were obtained in two other experiments with doses of ACh from 0·5–15 μg.

In two experiments the release of labelled ACh by injected carbachol (1 or 10 μg) was tested ; both doses of the drug stimulated the ganglion, but neither released a detectable amount of radioactivity. In both the experiments, nerve stimulation released labelled ACh before and after the drug injections.

In five experiments the release of labelled ACh by ACh added to the perfusion fluid was tested; Fig. 2 shows the results of one typical experiment. ACh (0·15–5 μg/ml) perfused through the ganglion for 2–8 min never released detectable radioactivity, but always stimulated the ganglion; the lowest concentration of ACh was just above threshold for stimulation, and the higher concentrations produced a maximal response. Perfusion with high concentrations of ACh (15–50 μg/ml)

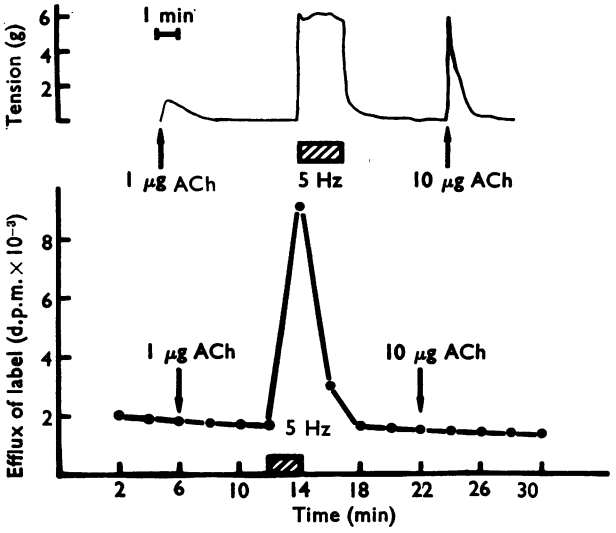

FIG. 1. Lower: Effect of nerve stimulation and injected ACh on release of radioactivity from a cat's superior cervical ganglion perfused with eserine-Ch-Locke; the ganglion's *transmitter* ACh had been labelled by perfusing the active ganglion with ^{3}H-Ch (no anticholinesterase). Upper: Contraction of the ipsilateral nictitating membrane. Preganglionic nerve stimulation (5 Hz, 0·5 ms, 5 V) during hatched bar; ACh (1 or 10 μg) injected into the perfusion stream at the arrows.

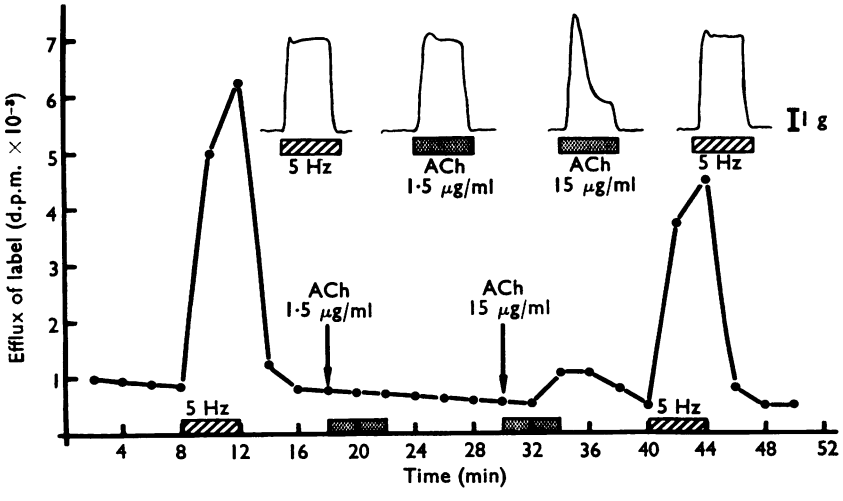

FIG. 2. Lower: Effect of nerve stimulation and perfusion with ACh on the release of radioactivity from a cat's superior cervical ganglion perfused with eserine-Ch-Locke; the ganglion's *transmitter* ACh had been labelled by perfusing the active ganglion with ^{3}H-Ch (no anticholinesterase). Upper right inset: Contraction of the ipsilateral nictitating membrane. Preganglionic nerve stimulation (5 Hz, 0·5 ms, 4 V) during hatched bars; perfusion switched to ACh (1·5 or 15 μg/ml) at the arrows and maintained for the duration of the stippled bars.

consistently released a small amount of labelled material. This release by ACh was always less than 10% of the release of ACh by nerve stimulation: in eight tests nerve stimulation (5 Hz for 2 min) released 8,150 ± 705 d.p.m., which represents about 11 ng of labelled ACh; in four tests 15 μg/ml ACh released only 337 ± 35 d.p.m., or less than 0·5 ng. The release of radioactivity by ACh was not coincident with the initial stimulant effect of ACh on the ganglion, but appeared to be associated with the subsequent phase of desensitization (see Fig. 2).

The amount of labelled material released by ACh was too small to allow its identification as labelled ACh, but that released by nerve stimulation was identified as ACh by the reineckate precipitation test.

At the end of all experiments, the perfused ganglia retained appreciable radioactive ACh equivalent to 226 ± 11 ng; the sum of released and residual labelled ACh of the perfused ganglia, when compared with the total ACh content of unperfused, control ganglia (measured by bioassay to be 290 ± 15 ng), showed that the loading procedure had labelled at least 80% of the ganglion's transmitter store.

In all the experiments described above, the perfusion fluid contained eserine throughout the test period. In two other experiments without an anticholinesterase, and in one other experiment with DFP, the release of labelled material by nerve stimulation, by injected ACh (1 or 10 μg) or by perfused ACh (2 μg/ml) was the same as with eserinized ganglia.

Effect of ACh on transmitter release by nerve stimulation

In three experiments the ganglion was loaded with ^{3}H-ACh in the same way as above, and in the test period the preganglionic nerve was stimulated continuously (5 Hz). The effect of ganglion-blocking concentrations of ACh (15–50 μg/ml) on the release of radioactive ACh by nerve stimulation was tested. Figure 3 shows the results of a typical experiment in which ACh (15 μg/ml) perfused for 6 min blocked impulse transmission completely, but did not reduce the release of radioactive ACh;

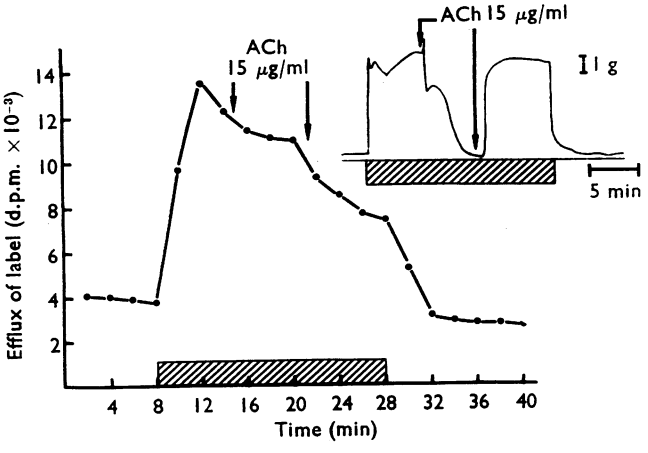

FIG. 3. Lower: Effect of nerve stimulation and simultaneous perfusion with ACh on release of radioactivity from a cat's superior cervical ganglion perfused with eserine-Ch-Locke; the ganglion's *transmitter* ACh had been labelled by perfusing the active ganglion with ^{3}H-Ch (no anticholinesterase). Upper right inset: Contraction of the ipsilateral nictitating membrane. Preganglionic nerve stimulation (5 Hz, 0·5 ms, 6 V) throughout the hatched bar; simultaneous perfusion with ACh 15 μg/ml between arrows.

indeed it appeared to cause a small increase in the release of radioactivity just as it did when applied to the resting ganglion. In two other experiments higher ACh concentrations (25 and 50 μg/ml) were tested, and in both the ACh caused only a small additional efflux of labelled material.

Release of surplus ACh by nerve stimulation or by cholinergic drugs

In these experiments, the ganglion's surplus ACh store was labelled by perfusing the resting ganglion with ^{3}H-Ch-Locke containing eserine; this procedure labels about 50% of the surplus ACh formed (Katz & Collier, unpublished). At the end of the loading perfusion the ganglia were washed out for 20 min by perfusion with unlabelled Ch-Locke (with eserine), and the release of labelled ACh during nerve stimulation or application of a cholinergic drug was then tested.

Figure 4 shows the results of a typical experiment. Perfusion with ACh (1·5 or 15 μg/ml) in the presence of eserine released easily detected radioactivity, but nerve stimulation (5 Hz) failed to do so; the release of labelled material by ACh was dose-dependent. Similar results were obtained in six other experiments in which eserine was used and in four similar experiments in which DFP was used; ACh concentrations between 0·5 and 15 μg/ml were tested. There was no obvious difference between the results with the two anticholinesterases. In one experiment, surplus ACh was labelled in the presence of DFP and the release of label by ACh (5 μg/ml) measured. The perfusion was then switched to Locke solution containing eserine and ACh was re-tested. The release of radioactivity in the presence of DFP (5,900 d.p.m.) was the same as the release obtained in the presence of eserine (5,700 d.p.m.).

Two experiments (three tests) showed that injected carbachol (0·5–2·5 μg) also released surplus ACh, and the results of a typical experiment are shown in Fig. 5.

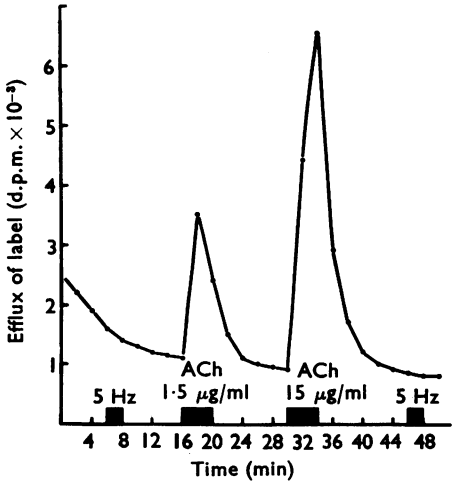

FIG. 4. Effect of nerve stimulation and perfusion with ACh on the release of radioactivity from a cat's superior cervical ganglion perfused with eserine-Ch-Locke; ganglion's *surplus* ACh had been labelled by perfusing the resting ganglion with ^{3}H-Ch-Locke in the presence of eserine. Preganglionic nerve stimulation (5 Hz, 0·5 ms, 5 V) during the hatched bars; perfusion switched to ACh (1·5 or 15 μg/ml) during the stippled bars.

In most of the experiments that demonstrated the release of radioactivity by exogenously-applied cholinergic drugs, the released labelled material was identified by differential precipitation tests ; in all cases the extra radioactivity released by the drugs was labelled ACh.

Ganglion stimulation by ACh or carbachol in the presence or absence of surplus ACh

In four experiments the contribution of ACh released by cholinergic drugs to the ganglion stimulation that they produced was tested.

(*a*) *Acetylcholine.* The results of a typical experiment are shown in Fig. 6. A ganglion was perfused with eserine-Locke (no Ch) and every 10 min perfusion was

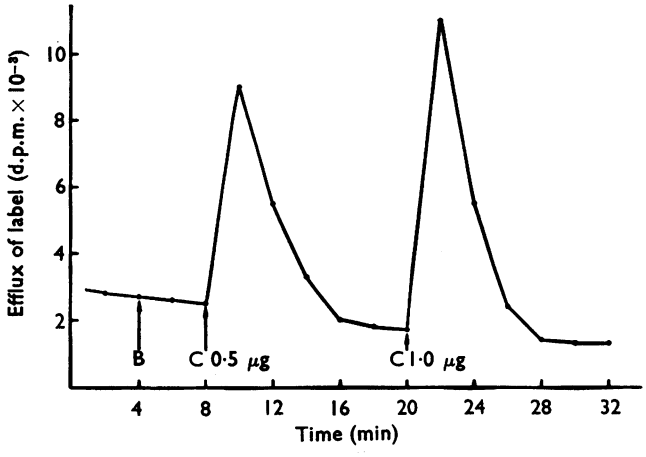

FIG. 5. Effect of injected carbachol on release of radioactivity from a cat's superior cervical ganglion perfused with eserine-Ch-Locke ; the ganglion's *surplus* ACh had been labelled by perfusing the resting ganglion with ^{3}H-Ch in the presence of eserine. Carbachol (C, 0·5 or 1 μg) injected into the perfusion stream at the arrows marked C ; control injection of 0·2 ml of Locke at arrow marked B.

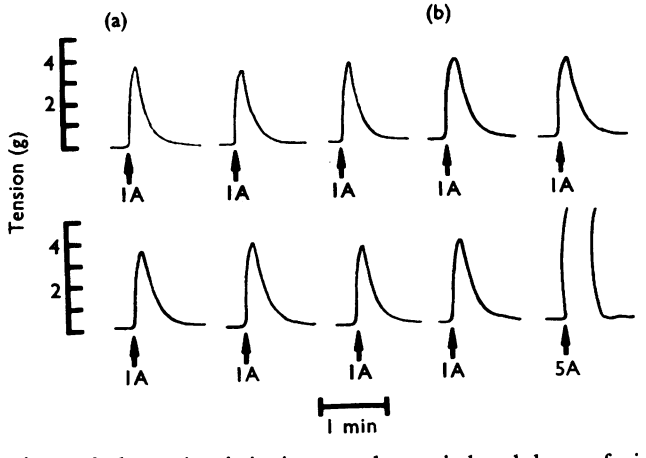

FIG. 6. Contractions of the cat's nictitating membrane induced by perfusing the superior cervical ganglion with ACh ; (a) during perfusion with eserine-Locke (no Ch) and (b) during perfusion with eserine-Ch-Locke. Perfusion switched to medium containing ACh (1 μg/ml) for 25 s every 10 min as indicated by arrows marked 1A ; at arrow marked 5A perfusion switched to ACh (5 μg/ml) to demonstrate maximal response (off recording paper).

switched for 25 s to solution containing ACh (1 μg/ml); ACh produced a transient submaximal stimulation of the ganglion, as recorded by the nictitating membrane contraction. Perfusion was then changed to eserine-Ch-Locke and the tests were continued. With this solution the ganglion would begin to accumulate surplus ACh and its content would approximately double within an hour. The response of the ganglion to ACh when surplus ACh was being produced was the same as when it was absent.

(*b*) *Carbachol.* This drug is resistant to cholinesterase and could be tested more simply than could ACh. The effects of submaximal stimulant doses of injected carbachol (1 μg) were compared during ganglion perfusion with Ch-Locke (no anti-cholinesterase and therefore no surplus ACh) and during subsequent perfusion with DFP-Ch-Locke (surplus ACh forming). The surplus ACh that carbachol released from the ganglion did not enhance its stimulant effect: response in the presence of DFP was 95–104% of the response in its absence.

Discussion

It is now well established that ACh and ACh-like agents initiate antidromically-propagated discharges in certain nerve terminals (for example Masland & Wigton, 1940; Feng & Li, 1941; Riker, Roberts, Standaert & Fujimori, 1957; Randić & Straughan, 1964), and that ACh can depolarize non-myelinated nerve fibres (Armett & Ritchie, 1960) including preganglionic sympathetic nerve terminals (Koketsu & Nishi, 1968).

Koelle (1961, 1962) suggested that the presynaptic effect of ACh is an integral part of the physiology of synaptic transmission. This hypothesis was based, in part, on experiments by Volle & Koelle (1961) which showed that carbachol is a less effective stimulant of the chronically decentralized superior cervical ganglion of the cat than of normally innervated ganglia. This was interpreted as evidence that a part of the effect of this drug on the normal ganglion is due to release of endogenous transmitter. Although this difference between the effect of carbachol on normal and denervated ganglia has been questioned (Brimblecombe & Sutton, 1968; Brown, 1969), McKinstry *et al.* (1963) and McKinstry & Koelle (1967) clearly demonstrated that carbachol can release ACh from perfused ganglia. However, ganglia supplied with Ch in the presence of eserine (as they were in McKinstry's experiments) synthesize and store ACh in excess of their normal transmitter depot (Birks & MacIntosh, 1961), and this " surplus " ACh was probably, at least partly, the source of the ACh released by carbachol. With the techniques that she used, McKinstry could not test whether carbachol could release ACh from the ganglion's normal store.

The development of procedures that label selectively either the transmitter ACh or the surplus ACh has allowed us to test the presynaptic effects of ACh itself, and to distinguish the release of surplus ACh from the release of the normal transmitter store.

Transmitter release by ACh

Perfusing an active ganglion with ^{3}H-Ch in the absence of anticholinesterase labels the ganglion's normal ACh store, but surplus ACh is not formed. Koelle's (1961) hypothesis predicts that a small amount of exogenously applied ACh should

release a large amount of labelled transmitter. This was not so. Low concentrations of ACh stimulated the ganglion but did not release labelled ACh. It is quite clear from the present experiments that the postsynaptic membrane is much more sensitive to ACh than are the presynaptic nerve endings. The amount of labelled material released by high concentrations of ACh was much smaller than that released by nerve stimulation, and release of label by ACh was not correlated with ganglion stimulation.

The mechanism by which ACh released labelled material from ganglia whose transmitter ACh was labelled is not clear from the present experiments. The concentration of ACh required was in the range shown by Koketsu & Nishi (1968) to depolarize presynaptic terminals of the amphibian sympathetic ganglia ; if ACh depolarizes preganglionic terminals, it would be expected to release ACh, for at the neuromuscular junction transmitter release has been shown to depend on the level of presynaptic polarization (Liley, 1956). However, it is possible that the release of labelled material by ACh in the present experiments was a consequence of K^+ leaking from the postsynaptic cells that were depolarized by ACh (Fatt & Katz, 1951 ; Takeuchi & Takeuchi, 1960) ; a raised K^+ concentration effectively releases labelled ACh from loaded ganglia (Collier, 1969), as would be expected from Brown & Feldberg's (1936a) experiments.

The present failure to demonstrate a presynaptic effect of ACh (except in very high concentrations) on the autonomic ganglion is consistent with the observations of Ginsborg & Guerrero (1964) that ACh-like agents do not increase the frequency of miniature synaptic potentials recorded from the frog sympathetic ganglion. The inability of ACh to release transmitter confirms the more indirect pharmacological evidence against Koelle's hypothesis, at least in the autonomic ganglion: tubocurarine, which inhibits presynaptic depolarization by ACh (Koketsu & Nishi, 1968) does not reduce the amount of ACh released by nerve stimulation (Brown & Feldberg, 1936b ; Matthews, 1966), and ganglion stimulation by ACh is not reduced when the transmitter store has been depleted by hemicholinium-treatment (Collier *et al.*, 1969).

If ACh could produce sustained depolarization of presynaptic nerve terminals, it is likely that it would reduce the amount of transmitter released by nerve impulses, and such an effect has been seen at the neuromuscular junction (Ciani & Edwards, 1963 ; Hubbard, Schmidt & Yokota, 1965). The present experiments failed to show this effects at the autonomic ganglion, even when ACh completely blocked synaptic transmission. It is most likely, therefore, that ganglion block from ACh is entirely a postsynaptic phenomenon.

Release of surplus ACh by ACh

Perfusing a resting ganglion with labelled Ch and an anticholinesterase agent labels surplus ACh without significant labelling of transmitter ACh (depot ACh). ACh or carbachol released surplus ACh and this provides an explanation for the observations of McKinstry *et al.* (1963) and McKinstry & Koelle (1967) that carbachol can release ACh from perfused ganglia.

In the present experiments, short periods of nerve stimulation did not release labelled surplus ACh ; this shows that the loading procedure did not label a significant amount of releasable ACh, and confirms Birks & MacIntosh's (1961) sugges-

tion that surplus ACh is not immediately available for release by nerve impulses. The failure of neuronally released ACh to release surplus ACh implies that the concentration of the mediator at the site at which exogenous ACh acts to release surplus ACh must be less than about 0·5 μg/ml during nerve stimulation. The concentration of ACh in the synaptic cleft during nerve stimulation is almost certainly greater than 0·5 μg/ml (see, for example, Nishi, Soeda & Koketsu, 1967; Collier & MacIntosh, 1969). The precise location of surplus ACh is not yet known, but it appears to be synthesized in the preganglionic nerve endings, and it may exist free in the cytoplasm; it would then be analogous to that ACh found within the cytoplasm of synaptosomes prepared from brain tissue (Whittaker, Michaelson & Kirkland, 1964). If surplus ACh is distributed throughout the nerve-terminal cytoplasm and is not concentrated at transmitter release sites, ACh released by nerve impulses would be quickly diluted by diffusion (Ogston, 1955) to a concentration well below that necessary to release surplus ACh. Similarly, if much of the surplus ACh released by injected or perfused cholinergic drugs is liberated some distance from the postsynaptic ACh-receptors, this extra ACh would not contribute significantly to drug-induced ganglion stimulation; the present experiments showed that ganglion stimulation by ACh or by carbachol is unaltered when surplus ACh is present.

There are various possible mechanisms by which exogenous ACh might release surplus ACh. It is known that ACh can depolarize non-myelinated nerve fibres (Armett & Ritchie, 1960) and sympathetic nerve terminals (Koketsu & Nishi, 1968), but the concentrations needed are much higher than those that release surplus ACh. Moreover, ACh in concentrations that released surplus ACh did not release transmitter ACh, and so it is unlikely that the nerve terminals were appreciably depolarized. This finding also makes it unlikely that K^+ leaking from the postganglionic cell in response to exogenous ACh was responsible for the release of surplus ACh. The mechanism by which ACh is released is therefore not at all clear. The effect is reminiscent of the release of noradrenaline from adrenergic nerve terminals by certain sympathomimetic amines (Burn, 1932; reviewed by Trendelenburg, 1963), and may depend on similar mechanisms.

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