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STUDIES UPON THE MECHANISM BY WHICH ACETYLCHOLINE RELEASES SURPLUS ACETYLCHOLINE IN A SYMPATHETIC GANGLION

B. COLLIER & H.S. KATZ

Department of Pharmacology and Therapeutics, McGill University, Montreal, Canada

1 Acetylcholine (ACh) releases surplus ACh from the superior cervical ganglion of the cat and the experiments described in this paper tested whether this results from exchange of endogenous ACh with exogenous ACh; the experiments also attempted to characterize pharmacologically the mechanism of this action of ACh.

2 The surplus ACh in the ganglion was radioactively labelled by perfusion of the ganglion with [^{3}H]-choline-Krebs solution containing diisopropylphosphofluoridate, and the release of surplus [^{3}H]-ACh by [^{14}C]-ACh injected close arterially to the ganglion measured. The amount of [^{3}H]-ACh released by [^{14}C]-ACh was 33 ± 5 times greater than was the amount of [^{14}C]-ACh accumulated by ganglia. The amount of exogenous ACh accumulated by ganglia that had first formed surplus ACh was not different from exogenous ACh accumulation by ganglia that had not formed surplus ACh. Thus, it is concluded that surplus ACh release by ACh is not the result of ACh exchange.

3 In other experiments, surplus [^{3}H]-ACh was accumulated in ganglia exposed to physostigmine. Nicotine, pilocarpine or ACh released surplus ACh; the effect of both nicotine and ACh was blocked by hexamethonium; atropine blocked the effect of ACh but not that of nicotine. It is concluded that both nicotinic and muscarinic receptors can be involved in the release of surplus ACh by cholinomimetic agonists.

Introduction

Acetylcholine (ACh) and ACh-like agents such as carbachol and tetramethylammonium can release ACh from the superior cervical ganglion of the cat (McKinstry & Koelle, 1967a; Fellman, 1969; Collier & Katz, 1970). The ACh that is released by ACh is not from the ganglion's normal store of transmitter but is surplus ACh (Brown, Jones, Halliwell & Quilliam, 1970; Collier & Katz, 1970). Surplus ACh is the extra ACh that is synthesized and stored by a ganglion that is exposed to an anticholinesterase agent (Birks & MacIntosh, 1961); this ACh is not immediately available for release by nerve impulses (Birks & MacIntosh, 1961; Collier & Katz, 1971)

The mechanism by which ACh releases surplus ACh is not known. It has been suggested that carbachol (McKinstry & Koelle, 1967a) and tetramethylammonium (Fellman, 1969) release ACh from ganglia by a process of cation exchange; a similar proposal was made much earlier (Renshaw, Green & Ziff, 1938) in an attempt to explain the pharmacological actions of certain choline derivatives, and it was also inferred by Collier & Katz (1970) to explain the release of surplus ACh by ACh. The present experiments were designed to test whether exogenous ACh exchanges with endogenous surplus ACh in ganglia. This was determined directly by labelling ganglionic ACh from [^{3}H]-choline and then measuring the amount of surplus [^{3}H]-ACh released by [^{14}C]-ACh and the amount of [^{14}C]-ACh accumulated by the ganglion. If exogenous ACh exchanged with endogenous ACh, a molecule for molecule exchange of the radioisotopes was expected.

Other experiments attempted to characterize the pharmacology of the process by which ACh releases surplus ACh by testing whether the effect of ACh could be mimicked by muscarinic or nicotinic agonists, and whether ACh-blocking agents block the effect.

Methods

The techniques were similar to those described before (Collier & Lang, 1969; Collier & Katz, 1970).

Ganglion perfusion

Cats were anaesthetized with ethyl chloride followed by ether and then by intravenous

chloralose (80 mg/kg). In the experiments in which only one ganglion was to be perfused, the right superior cervical ganglion was prepared for perfusion (Feldberg & Gaddum, 1934), and the left ganglion was removed and used as the control for ACh content. In other experiments, both ganglia were prepared for perfusion. The medium used for perfusion was Krebs solution of the following composition (mM): NaCl 120, KCl 4.6, $CaCl_2$ 2.4, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 9.9; it was equilibrated throughout the experiment with 5% CO_2 in O_2 so that the pH was 7.4 at 37° C. In all experiments, perfusion during the first 15 min was with Krebs solution containing choline (10^{-5} M). When the ganglion's surplus ACh was to be radioactively labelled, ganglia (preganglionic trunk cut, but not stimulated) were perfused for 60 min with Krebs solution containing [^{3}H]-choline (10^{-5} M) and an anticholinesterase agent which was either diisopropylphosphofluoridate (DFP, 5×10^{-5} M) or physostigmine (2×10^{-5} M); this procedure labels surplus ACh to a greater specific activity than depot ACh (Collier & Katz, 1971). In experiments in which [^{3}H]-ACh release by injected ACh or an ACh-like agent was measured, the drug was dissolved in 0.2 ml of Krebs solution and injected directly into the arterial cannula. Before injection of the drug it was necessary to wash out the tube leading to the arterial cannula because a little [^{3}H]-choline diffused into its tip during the perfusion with [^{3}H]-choline; to make sure this wash was effective, 0.2 ml of Krebs solution was injected before the drug was tested.

Nerve stimulation

The preganglionic nerve was cut low in the neck and stimulated when necessary with supramaximal rectangular pulses (10 Hz, 0.3 ms, 5-8 V). Contractions of the nictitating membrane were recorded to give some indication of the effectiveness of nerve stimulation or of ganglion stimulation by injected drug.

Preparation of ganglion extracts and bioassay

Ganglia were removed, and extracted with 2.0 ml of 10% trichloroacetic acid (TCA). The TCA was removed by shaking with ether, residual ether was removed by aeration, and separate aliquots of the aqueous phase (pH 4-5) were used for bioassay, for liquid scintillation counting and for separating radioactive ACh from other labelled material.

The ACh content of ganglion extracts and of effluent collected from the stimulated ganglia was measured by bioassay on the blood pressure of the eviscerated cat (MacIntosh & Perry, 1950).

Separation of radioactive materials

In experiments in which ganglia were perfused with [^{3}H]-choline, labelled ACh in ganglion extracts was separated from other ^{3}H-labelled material by the sequential ammonium reineckate and gold chloride precipitation method described before (Collier & Katz, 1971). In the experiments in which ganglia were perfused with [^{14}C]-ACh, labelled ACh in ganglion extracts was separated from labelled acetate by precipitation with ammonium reineckate. In this procedure, unlabelled ACh (1.0 mg) and unlabelled sodium acetate (1.0 mg) were mixed with an aliquot of the ganglion extract, and 2.0 ml of a saturated aqueous solution of ammonium reineckate was added. The reineckate precipitate was washed with saturated ammonium reineckate solution, and ACh was recovered from the precipitate by the anion-exchange resin, Dowex AG2-X8 (Collier & Katz, 1971); ammonium reineckate precipitated ACh (> 95%), but not acetate (< 1%). This procedure was also used to separate [^{14}C]-acetate from unhydrolysed [^{14}C]-ACh in the experiments that tested the hydrolysis of ACh by acetylcholinesterase and to separate [^{14}C]-acetate from [^{3}H]-choline in acetylcholinesterase-treated effluents collected from ganglia in the experiments that measured the release of [^{3}H]-ACh by [^{14}C]-ACh.

Acetylcholine hydrolysis by acetylcholinesterase

When the hydrolysis of ACh by acetylcholinesterase was tested, standard amounts of [^{14}C]-ACh were dissolved in 1.0 ml of Krebs solution without an anticholinesterase or containing DFP; acetylcholinesterase was added and the mixture was incubated at 25° C for 30 minutes. The hydrolysis of ACh was stopped by adding 0.1 ml of 1.0 N HCl, and the amount of ACh remaining was measured after separating [^{14}C]-ACh from [^{14}C]-acetate by precipitation with ammonium reineckate (see above). Aliquots of effluent collected from perfused ganglia were treated in the same way and [^{14}C]-acetate was separated from [^{3}H]-choline by reineckate precipitation.

Determination of radioactivity

Radioactivity in solutions was measured by liquid scintillation spectrometry. In experiments in which a single isotope was to be measured, the solvent system was that described before (Collier & Lang, 1969), and in experiments in which ^{3}H- and ^{14}C-radioactivity was to be measured simultaneously, the solvent system was Aquasol

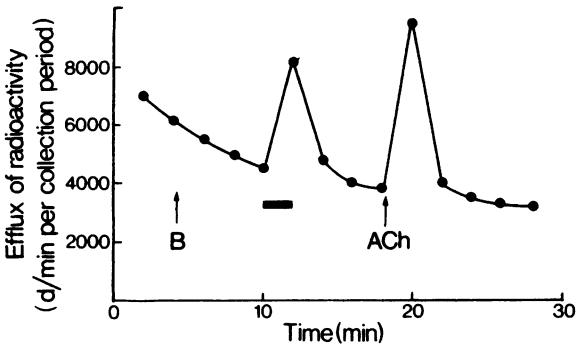

Figure 1 Effect of Krebs solution (0.2 ml indicated by B), of nerve stimulation (10 Hz, during the 2 min period indicated by the horizontal bar), and of injected acetylcholine (ACh, 55 nmol in 0.2 ml Krebs) upon the efflux of radioactivity from the superior cervical ganglion of the cat perfused with diisopropylphosphofluoridate (DFP)-choline-Krebs. The ganglion had previously accumulated labelled surplus ACh during perfusion with [^{3}H]-choline-DFP-Krebs for 60 min; it had then been washed by perfusion with DFP-choline-Krebs for 20 minutes.

(New England Nuclear). Correction for quench and for isotope spill in double-label counting was made, using an internal standard.

Drugs

Compounds used were: methyl-[^{3}H]-choline chloride (50-100 mCi/mmol, New England Nuclear), acetyl-[1-^{14}C]-choline chloride (13.7 mCi/mmol, Amersham/Searle), diisopropylphosphofluoridate (DFP, K&K), acetylcholine chloride (Lematte-Boinot), choline chloride and nicotine hydrogen tartrate (BDH), acetylcholinesterase (*Electrophorus electricus*; 150 units/mg (1 unit hydrolyses 1 μmol ACh/min at 25°C); Schwartz/Mann), physostigmine sulphate, atropine sulphate, and pilocarpine nitrate (Nutritional Biochemicals), and hexamethonium bromide (Poulenc).

Results

The release of surplus acetylcholine by acetylcholine

The previous demonstration (Collier & Katz, 1970) that ACh releases surplus ACh was qualitative, but for the present experiments it was necessary to quantify the release of surplus ACh by ACh. Two experiments in which unlabelled ACh was used to release surplus [^{3}H]-ACh demonstrated how the amount of surplus ACh released could be calculated. In these experiments, the ganglia (not stimulated) were first perfused for 60 min with Krebs solution containing DFP and [^{3}H]-choline to label surplus ACh; perfusion was then switched to DFP-Krebs containing unlabelled choline and when the efflux of radioactivity had declined to a reasonable level (after 20-25 min), the release of [^{3}H]-ACh by preganglionic nerve stimulation (10 Hz for 2 min) and then the release of [^{3}H]-ACh by injected ACh (55 nmol dissolved in 0.2 ml of Krebs solution) was measured. The results of a typical experiment are illustrated in Figure 1; nerve stimulation and injected ACh released radioactivity, but the control injection (0.2 ml of Krebs solution) did not. The other experiment gave similar results. The release of [^{3}H]-ACh by nerve stimulation in these experiments was similar to that measured under similar conditions by Collier & Katz (1971), but was greater than was measured by Collier & Katz (1970) who used slightly different conditions. At

Table 1 Calculation of the amount of surplus acetylcholine (ACh) released by ACh

	Measured		*Calculated*	
	[^{3}H]-ACh (d/min)	*Total ACh (pmol)*		
Released by nerve stimulation	2,800	219	Specific radioactivity of releasable ACh	= 12.8 d min^{-1} pmol^{-1}
Control ganglion		1,648	Total releasable ACh (85%)	= 1,401 pmol
			Total radioactivity of releasable ACh	= 17,934 d/min
Test ganglion	43,900	3,186	Total surplus ACh	= 1,538 pmol
			Radioactivity of surplus ACh	= 25,966 d/min
Released by ACh	5,300		Radioactivity of surplus ACh at test	= 31,266 d/min
			Specific radioactivity of surplus ACh	= 20.3 d min^{-1} pmol^{-1}
			Amount of surplus ACh released	= 261 pmol

the end of the experiment, the perfused ganglion was removed and assayed for total ACh and for [^{3}H]-ACh. The total ACh content of the ganglion effluent collected during nerve stimulation, and the ACh content of the control (not perfused) ganglion were also measured. These measured values were then used to calculate the amount of surplus ACh released by the injected ACh using the procedures summarized in Table 1. The specific radioactivity of the ganglion's releasable ACh was calculated from the measured release of ACh by nerve stimulation; this is valid provided that the period of nerve stimulation is short (Collier, 1969). Multiplication of this value by the total amount of releasable ACh yielded the total radioactivity of releasable ACh; for this calculation, the total ACh content of the test ganglion was taken to be equal to the total ACh content of the control ganglion (Brown & Feldberg, 1936; Feldberg, 1943) and it was assumed that 85% of the total ACh was releasable (Birks & MacIntosh, 1961; Collier & MacIntosh, 1969). Subtraction of the total releasable [^{3}H]-ACh (d/min) from the amount (d/min) of [^{3}H]-ACh measured in the ganglion at the end of the experiment gave the radioactivity of surplus ACh in the ganglion at the end of the experiment, and the sum of this value and the measured release of [^{3}H]-ACh by ACh was taken to be equal to the amount (d/min) of surplus [^{3}H]-ACh in the ganglion at the time that the ACh was injected. The total amount of surplus ACh in the ganglion was calculated from the difference in ACh content of the test and the control ganglia, and this was taken to be the amount of surplus ACh at the time of the ACh injection because the ACh content changes little in the second hour of perfusion with an anticholinesterase (Birks & MacIntosh, 1961; Collier & Katz, 1971). The amount (d/min) of surplus [^{3}H]-ACh in the ganglion divided by the total amount of surplus ACh (pmol) gave the specific activity of surplus ACh, from which the amount (pmol) of ACh released by injected ACh could be calculated. In the two experiments done, the amount of ACh released was 261 pmol and 220 pmol.

The lack of exchange of acetylcholine for surplus acetylcholine

The exchange of exogenous ACh with endogenous surplus [^{3}H]-ACh was tested by experiments like those described above; surplus ACh release was evoked by injection of [^{14}C]-ACh and the amount of [^{14}C]-ACh accumulated by the ganglion at the end of the experiment was also measured. Preliminary experiments of this type showed that the [^{3}H]-ACh content of ganglion extracts could readily be measured in the presence of the accumulated [^{14}C]-ACh by standard double-isotope methods. However, the presence of an excess of ^{14}C- to ^{3}H-radioactivity in the effluent collected from the ganglion immediately following the injection of [^{14}C]-ACh precluded the use of standard double-isotope counting techniques for measurement of the released [^{3}H]-ACh. This problem was overcome by using acetyl-[1-^{14}C]-choline and hydrolysing the ACh in the effluent collected from the ganglion with acetylcholinesterase. The released [^{3}H]-ACh, which had been synthesized *in situ* from precursor choline, was N-methyl-labelled whereas the exogenous ACh was acetyl-labelled; hydrolysis yielded [^{3}H]-choline from released ACh and [^{14}C]-acetate from injected ACh and these two labelled materials were easily separated by precipitation with ammonium reineckate (see methods section). Because the irreversible anticholinesterase agent, DFP, was used in these experiments it could safely be omitted from the perfusion fluid during the 2 min period that immediately followed the injection of [^{14}C]-ACh; the concentration of DFP in fluid collected would then be $< 5 \times 10^{-5}$ M. The maximum concentration of ACh in the collected effluent was calculated to be 6×10^{-5} M. The results of *in vitro* experiments that measured the hydrolysis of ACh by acetylcholinesterase in the presence or in the absence of DFP showed that 5.4 units/ml of the enzyme hydrolysed all of the added ACh even in the presence of DFP; this concentration of acetylcholinesterase was used in the experiments that tested the release of ACh by [^{14}C]-ACh.

The procedures described above were then combined to test the exchange of exogenous [^{14}C]-ACh for endogenous surplus [^{3}H]-ACh. Injected [^{14}C]-ACh released [^{3}H]-radioactivity and the calculations described above (Table 1) were made for these experiments to provide an estimate of the amount of surplus [^{3}H]-ACh released. The amount of [^{14}C]-ACh accumulated was calculated from the ^{14}C-radioactivity measured in the ganglion extract and the specific activity of ACh used. Table 2 summarizes the results of three such experiments; the amount of ACh released by ACh was 24-40 times greater than the amount of ACh accumulated. Thus, exogenous ACh does not release endogenous ACh by a mechanism of ACh exchange.

This conclusion that exogenous ACh does not exchange with endogenous surplus ACh was supported by other experiments that measured ACh accumulation by ganglia that had, or had not, formed surplus ACh. In these experiments, both superior cervical ganglia were perfused. One ganglion was perfused for 60 min with Krebs

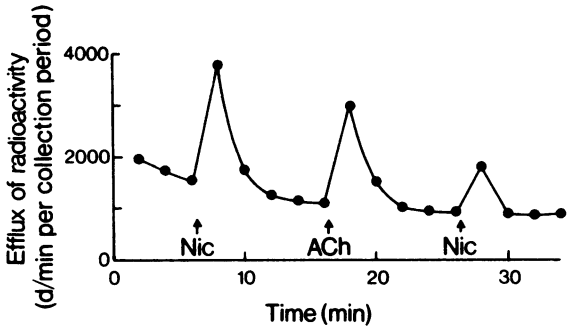

Figure 2 Effect of 50 nmol of nicotine (NIC) and of acetylcholine (ACh) on the efflux of radioactivity from the superior cervical ganglion of the cat perfused with Krebs solution containing physostigmine and choline. The ganglion had previously accumulated surplus [^{3}H]-ACh during perfusion with [^{3}H]-choline-Krebs containing physostigmine for 60 min, and had then been washed by perfusion with physostigmine-choline-Krebs for 30 minutes.

solution containing DFP and choline (surplus ACh accumulated) and the other ganglion was perfused with choline-Krebs solution (no anticholinesterase, therefore, no surplus ACh accumulated). Both ganglia were then perfused for 90 min with choline-free Krebs solution containing DFP and perfusion was switched to the same medium containing [^{14}C]-ACh (2.8×10^{-6} M) for 5 min every 10 minutes. At the end of the experiment, the ganglia were removed and their [^{14}C]-ACh content and their total ACh content was measured. The results of these experiments are summarized in Table 3. The ganglia that had been exposed to DFP and choline during the first 60 min of perfusion contained 45% more ACh, but no more [^{14}C]-ACh than did the ganglia that had not been exposed to DFP in the first 60 min of perfusion.

The release of surplus acetylcholine by acetylcholine-like drugs

To test whether the release of surplus ACh by ACh involved an action of the drug upon nicotinic or muscarinic receptors, the release of surplus ACh by nicotine or by pilocarpine was tested in five experiments. In these experiments, surplus [^{3}H]-ACh was allowed to accumulate in ganglia perfused with [^{3}H]-choline in the presence of physostigmine, perfusion was then continued with Krebs solution containing unlabelled choline and physostigmine and the effect of the drugs upon the release of [^{3}H]-ACh was measured by injecting them (50 nmol) into the arterial cannula. Both nicotine and pilocarpine, like ACh, released radioactivity (Figures 2 and 3), and the extra ACh

Table 2 Comparison of the amount of surplus [^{3}H]-acetylcholine (ACh) released by [^{14}C]-ACh to the amount of [^{14}C]-ACh accumulated by ganglia

	[^{3}H]-ACh released		*[^{14}C]-ACh accumulated*		*[^{3}H]-ACh released/ [^{14}C]-ACh accumulated*
Expt.	*d/min*	*pmol*	*d/min*	*pmol*	
1	4132	203	170	5.7	36
2	4800	308	380	12.7	24
3	5600	292	220	7.3	40
Mean ± s.e.	4844 ± 424	268 ± 32	257 ± 63	8.6 ± 2.1	33 ± 5

Table 3 [^{14}C]acetylcholine (ACh) uptake by ganglia that had (test) or had not (control) previously accumulated surplus ACh

	[^{14}C]-ACh (d/min)		*Total ACh content (pmol)*	
Experiment	*Test ganglion*	*Control ganglion*	*Test ganglion*	*Control ganglion*
1	478	511	2198	1538
2	909	969	2747	1648
3	608	596	1813	1483
Mean ± s.e.	665 ± 128	692 ± 141	2253 ± 262	1556 ± 53

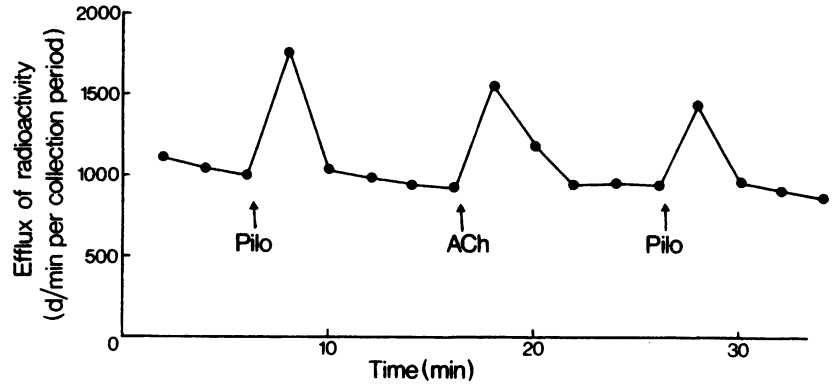

Figure 3 Effect 50 nmol of pilocarpine (Pilo) and of acetylcholine (ACh) on the efflux of radioactivity from the superior cervical ganglion of the cat. Experimental conditions were the same as for Figure 2.

released by the drugs was [^{3}H]-ACh (see methods section). The nictitating membrane contracted in response to ACh or to nicotine, but little if any contracture resulted after injecting pilocarpine.

The effect of acetylcholine-blocking agents upon the release of surplus acetylcholine induced by acetylcholine or by nicotine

In these experiments, surplus [^{3}H]-ACh was formed by ganglia in the presence of physostigmine, perfusion was continued with medium containing physostigmine and non-radioactive choline and the release of surplus [^{3}H]-ACh by the agonist was tested before, during, and after exposing the ganglion to the antagonist. Four experiments showed that atropine (5×10^{-5} M) completely blocked the release of ACh induced by ACh (Figure 4a); the injection of ACh in the presence of atropine still induced a contraction of the nictitating membrane. Two experiments showed that atropine (2×10^{-4} M) did not block the release of ACh induced by nicotine (Figure 4b). Two experiments showed that hexamethanium (2×10^{-4} M) clearly reduced the release of ACh induced by nicotine (Figure 5a). Five experiments tested whether hexamethonium (2×10^{-4} M) blocked the release of surplus ACh induced by ACh; in four of these release was almost completely abolished (Figure 5b), but in the other, release was not clearly blocked. In all experiments, hexamethonium almost completely blocked the contraction of the nictitating membrane that resulted from injecting nicotine or ACh.

Discussion

The present experiments were designed to test whether exogenous ACh releases endogenous ACh from the cat superior cervical ganglion by a process of ACh exchange. The most direct test of the mechanism was to compare the amount of exogenous [^{14}C]-ACh accumulated by ganglia with the amount of endogenous [^{3}H]-ACh released from ganglia by the [^{14}C]-ACh. If endogenous ACh is released as the result of ACh exchange, a molecule for molecule exchange of the isotopes would be expected in these experiments. The ratio of [^{3}H]-ACh released to [^{14}C]-ACh accumulated was calculated to be 33. The calculation of this ratio of ACh released to ACh accumulated assumed that exogenous ACh releases surplus ACh and not transmitter that is available for release by nerve impulses; this assumption is probably correct (Brown *et al.*, 1970; Collier & Katz, 1970). If the calculation of the amount of [^{3}H]-ACh released by ACh had been made from the specific radioactivity of total tissue ACh, or from the specific activity of releasable ACh, the ratio of ACh released to ACh accumulated would have been greater than the ratio calculated, because the conditions of the experiments were such that surplus ACh was labelled to higher specific radioactivity than was releasable ACh.

The ratio of the amount of surplus [^{3}H]-ACh released from nerve endings to the amount of exogenous [^{14}C]-ACh accumulated by nerve endings would also be greater than the ratio that was calculated if part of the [^{14}C]-ACh accumulation measured was the result of

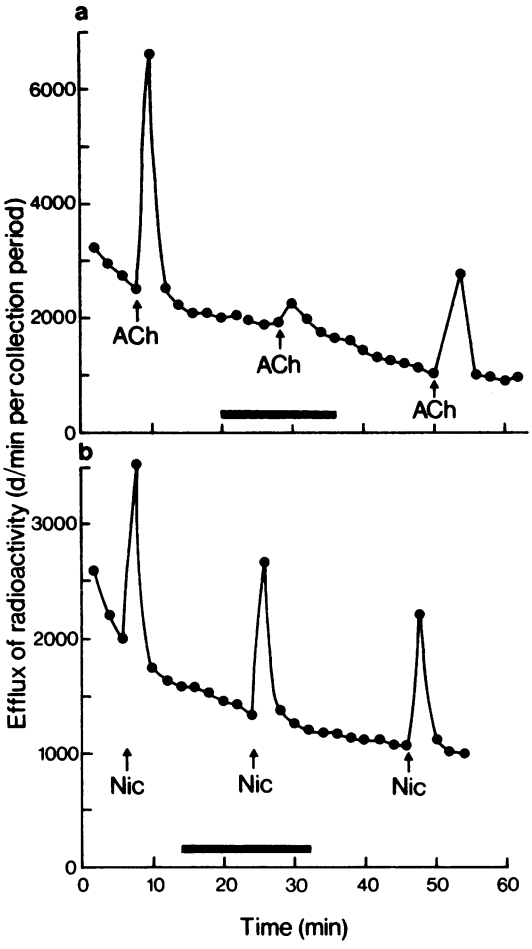

Figure 4 Effects of atropine (a) 50 μM and (b) 200 μM (present during the time indicated by the horizontal bars) on the release of surplus [^{3}H]-acetylcholine induced by 50 nmol of (a) acetylcholine (ACh) and (b) nicotine (Nic). Experimental conditions were the same as for Figure 2; (a) and (b) are separate experiments.

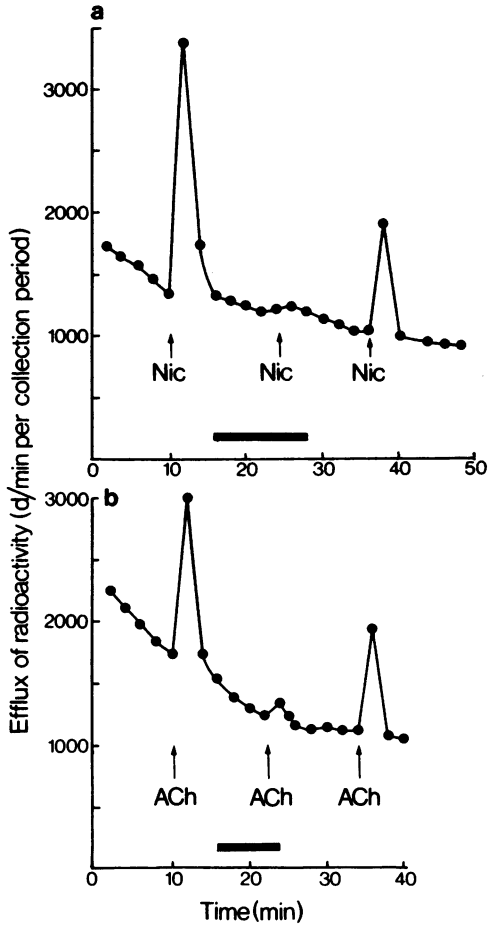

Figure 5 Effects of hexamethonium 200 μM (present during the time indicated by the horizontal bars) on the release of surplus [^{3}H]-acetylcholine induced by 50 nmol of (a) nicotine (Nic) and (b) acetylcholine (ACh). Experimental conditions were the same as for Figure 2; (a) and (b) are separate experiments.

non-specific binding of ACh or represented [^{14}C]-ACh uptake into structures other than preganglionic nerve endings. Other experiments (Katz, Salehmoghaddam & Collier, 1973) have shown that chronic decentralization does not reduce the amount of exogenous ACh accumulated by ganglia exposed to ACh for 90 min, which indicates that under those conditions ACh accumulation was primarily by structures other than cholinergic nerve terminals (see also Kuhar & Simon, 1974). In the present experiments, the exogenous ACh was injected close to the ganglion so that the time of exposure of the tissue to [^{14}C]-ACh was as short as possible; under these conditions, the site at which exogenous ACh accumulates is not known.

There is now evidence suggesting that mammalian brain and the electric organ of *Torpedo* can synthesize from radioactive choline a small pool of ACh that is of much greater specific radioactivity than is the rest of the tissue's ACh (Barker, Dowdall, Essman & Whittaker, 1970; Richter & Marchbanks, 1971; Marchbanks & Israël, 1971, 1972; Barker, Dowdall & Whittaker,

1972). It could be argued that such a pool of endogenous ACh might exchange with exogenous ACh, but if this were so in the present experiments, the [^{3}H]-ACh released (4844 d/min) in exchange for the 8.6 pmol of [^{14}C]-ACh accumulated (see Table 2) would have to have been from a pool of ACh with a specific radioactivity of 563 d min^{-1} pmol^{-1}. It is not possible that ACh of this specific radioactivity could have been synthesized in these experiments because the specific radioactivity of choline used was 222 d min^{-1} pmol^{-1}.

The results of experiments which measured the uptake of exogenous ACh by ganglia that had, or had not, first accumulated surplus ACh were consistent with the conclusion that surplus ACh release by ACh does not result from ACh exchange. If the exchange occurred, it might be expected that accumulation of exogenous ACh would have been greater in ganglia with surplus ACh. This was not so.

The previous suggestion (Collier & Katz, 1970) that the mechanism by which ACh releases ACh from ganglia might be similar to that by which certain amines release noradrenaline from sympathetic nerves appears to be wrong; for example, under appropriate conditions, a molecule for molecule exchange of metaraminol for noradrenaline can be demonstrated (Porter, Torchiana, Totaro & Stone, 1967).

The results of McKinstry & Koelle's (1967b) experiments suggest that ACh-blocking agents block the release of surplus ACh release evoked by carbachol, as if release might involve presynaptic cholinoceptors. The present experiments are consistent with this, but the mechanism involved could not be clearly defined as nicotinic or muscarinic. Both pilocarpine, which exerts primarily muscarinic effects, and nicotine, which exerts nicotinic effects, released surplus ACh; thus activation of either type of receptor appears to evoke ACh release. The effect of ACh in releasing surplus ACh might, therefore, be expected to be reduced, but not completely blocked by either atropine, which is anti-muscarinic, or by hexamethonium, which is anti-nicotinic; however, either antagonist alone effectively blocked the effect of ACh. If the presynaptic cholinoceptor involved in the phenomenon of surplus ACh release by ACh were sensitive to blockade by either atropine or hexamethonium, and if nicotine mimics the action of ACh, either antagonist might be expected to block this action of nicotine; however, only hexamethonium prevented the effect of nicotine. Further speculation about the mechanism by which ACh releases surplus ACh is unwarranted without more experimental evidence.

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