J. Physiol. (1974), **240**, *pp.* 125–134
With 1 *text-figure*
Printed in Great Britain

STORAGE AND RELEASE OF ACETYLCHOLINE IN A SYMPATHETIC GANGLION

BY R. I. BIRKS* AND S. J. G. FITCH
From the Physiology Department, McGill University, Montreal, Canada

(*Received 14 December 1973*)

SUMMARY

1. The hypotheses of preferential release of newly synthesized acetylcholine (ACh) and two compartment storage of transmitter in the cat superior cervical ganglion have been re-examined by testing, first, the assumption that ganglionic ACh stores do not alter during a 20 min rest following 60 min preganglionic nerve stimulation at 20/s, and secondly, the implication that the rate of ACh release should be high near the onset of activity and decline to a lower rate with time irrespective of the frequency of stimulation.

2. The ganglionic ACh stores were found to increase by 38 ± 8 % within 20 min following 60 min preganglionic nerve stimulation at 20/s, and this extra ACh was releasable.

3. The rate of ACh release from ganglia perfused with cat plasma and stimulated at 4/s increased over the first 5 min of stimulation to reach a 27 % higher rate that was maintained.

4. Correction of the original data to allow for the post-activation increase in ACh stores suggests that newly synthesized ACh equilibrates with most of the preformed stores. The time course of ACh release at 4/s does not support the two compartment model as currently formulated.

5. These findings resolve in part a conflict between the physiological data and a recent hypothesis for ACh storage based on ganglion morphology.

INTRODUCTION

A recent electron microscopic study of synaptic structure in the cat superior cervical ganglion (Birks, 1974) has demonstrated that the populations of synaptic vesicles at resting boutons de passage can be markedly reduced by patterns of preganglionic nerve stimulation that do not deplete bouton stores of ACh. Stimulation for 20 min at frequencies of 1, 4 and 20/s, reduced the numbers of vesicles by 56, 54 and 75 % respectively. If

* Medical Research Associate of the Medical Research Council of Canada.

ACh is stored in vesicles in the resting ganglion it may be concluded that as much as 75% of the ACh stores of an active ganglion may be extravesicular and presumably cytoplasmic. In addition it was found that with mild or brief synaptic activation the fractional depletion of vesicles was considerably greater than the fraction of ganglionic ACh stores that could have been released. Taken together these and other findings suggested that ACh might be released from a cytoplasmic pool rather than directly from vesicles.

One consequence of this hypothesis is that, since choline acetyltransferase is located in the cytoplasm (Fonnum, 1967, 1968), newly synthesized ACh in active ganglia would be expected to equilibrate rapidly with the cytoplasmic pool (i.e. with about 75% of the bouton stores at 20/s stimulation), and probably less rapidly with the vesicular pool. However, the data of Collier & MacIntosh (1969) and Collier (1969) on ganglia loaded with radioactive choline suggest that newly synthesized ACh equilibrates with a much smaller fraction of transmitter stores, at most 30% of the total.

The aim of the present experiments has been to examine this question in further detail. The results indicate that previous estimates need to be corrected and that newly synthesized ACh may not be released in preference to preformed stocks.

METHODS

The methods of perfusion, preganglionic nerve stimulation, recording of nictitating membrane contractions and bio-assay of ACh were similar to those described by Birks & MacIntosh (1961).

Cats weighing 2·5–3·5 kg were used. Anaesthesia was induced with ethyl chloride or halothane and maintained with chloralose (100 mg/kg).

Two perfusion fluids were used. One had the same composition as that used by Collier (1969) and it contained in g/l. NaCl 8·2, KCl 0·42, $CaCl_2$ 0·24, $NaHCO_3$ 1·35, glucose 1·0 and choline chloride $1{\cdot}5 \times 10^{-3}$. No anticholinesterase agent was added to this fluid. The second fluid was heparimized cat plasma. It was prepared by arterial bleeding of lightly etherized cats into bottles containing heparin sodium to give a final concentration of 75 i.u./ml. Following separation of the plasma, eserine sulphate was added to a final concentration of 2×10^{-5} g/ml. Both fluids were equilibrated at room temperature with 3·5% CO_2 in O_2 before use. Perfusion of the ganglion was always started 15 min before the start of a test, and the flow rate was maintained between 0·2 and 0·4 ml./min.

The ACh content of ganglia was determined by bio-assay on the cat blood pressure preparation of the supernatant from minced ganglia following 90 min extraction in 10% aqueous tricholoroacetic acid (TCA) and subsequent removal of TCA with ether. In experiments in which comparison of ACh content of the test ganglion with the untreated contralateral ganglion was required the control ganglion was removed 30 min before the start of the test on the other ganglion. The test ganglion unless otherwise stated was always excised starting at the post-ganglionic pole to permit stimulation to continue to the last possible moment before extraction. ACh in

samples of effluent, eserinized plasma in the present experiments, was assayed against appropriate dilution of ACh standards made up in the plasma perfusion fluid.

In some experiments in which it was required to block ACh synthesis, hemicholinium (HC-3) 5 mg/kg was given by vein 5 min before the test.

Stimulation of the preganglionic nerve trunk was either via platinum wire electrodes or silver, silver-chloride electrodes (Birks & MacIntosh, 1961). Current pulses of 1 msec duration were used. Contractions of the nictitating membrane were recorded as a monitor of the effectiveness of the stimulation.

Values given in the text and legends indicate the mean ± S.D.

RESULTS

A. *ACh accumulation in ganglia following activity*

In their analyses of the relative contributions of preformed and newly synthesized ACh to the release of transmitter in active ganglia, the protocol adopted by Collier & MacIntosh (1969) and by Collier (1969) was as follows. The preganglionic nerve trunk was stimulated at 20/s for 60 min during perfusion with Locke solution or plasma containing radioactive choline. The procedure labelled 80–90 % of the neuronal ACh stores. Labelled choline, but not labelled ACh, was then washed out of the ganglion during a 20 min perfusion at rest with unlabelled choline. The release of labelled and unlabelled ACh was determined next in a further period of 20/s stimulation during perfusion in the presence of an anticholinesterase drug. Labelled ACh stores at the conclusion of the test were determined following extraction of the test ganglion. Labelled ganglion ACh stores at the onset of the test were estimated by adding the total labelled ACh released in the test to the amount in the residual labelled store. The unlabelled ACh stores at the onset and conclusion of the test were calculated from the total ACh in the control contralateral ganglion less the respective labelled amounts, determined above. In other words it was assumed that the ACh stores of the test ganglia, which are known to be unaltered immediately after 60 min stimulation at 20/s (Birks & MacIntosh, 1961), remained unaltered during the ensuing 20 min rest period prior to the test stimulation.

Later work has indicated in fact that under certain circumstances the ACh stores of a ganglion may alter following synaptic activity. Thus Rangachari, Khatter & Friesen (1969) found that following 4 min stimulation at 60/s the ganglionic ACh stores increased by 32 % 4 min following the end of stimulation; whereas 4 min following the termination of 30 min stimulation at 20/s the ACh stores of the active ganglion had increased by 14 %. Using a more complex procedure of alternate stimulation and rest periods Bourdois, McCandless & MacIntosh (1970) found an even greater increase in ganglionic ACh stores following the activity. The purpose of the present experiments was primarily to test the above assumption of

Collier & MacIntosh (1969) and Collier (1969) under the specific conditions of their experiments.

(1) *ACh accumulation following varying periods of 20/s stimulation and 20 min rest*

For these experiments the test ganglia were perfused with Locke without an anticholinesterase agent, and all were rested for 20 min post-stimulation before removal of the test ganglion and extraction of its ACh. The duration of 20/s stimulation was either 10, 30, or 60 min. The latter period, followed by the 20 min rest, corresponds to the procedure of Collier & MacIntosh (1969) and Collier (1969). As shown in Table 1, the ACh stores of the stimulated ganglia increased following the activity and rest as compared with the control unstimulated, unperfused ganglia. The extent of the increase tended to become greater as the duration of the stimulation was increased, reaching a peak of $138 \pm 9\%$ following 60 min stimulation.

(2) *ACh accumulation following 20/s stimulation for 60 min and varying rest periods*

For these experiments all the ganglia retained their blood supply intact. As expected from earlier work (Birks & MacIntosh, 1961) there was no increase in ACh stores in the ganglia stimulated for 60 min and removed without delay (Table 1). The increase in ACh stores following a 20 min rest was $138 \pm 8\%$, the same as with the perfused ganglia. When the rest period was reduced to 5 min the increase was less, but not significantly so ($P > 0{\cdot}5$). Also there was no further increase when the rest period was extended beyond 20 min; but the extra ACh was maintained in the ganglion, and in one test it was found to be still present 180 min after the activity. In another experiment following a 20/s 60 min stimulation and 20 min rest, the test ganglion was stimulated for a second time at 20/s, this time for 20 min. The test ganglion was removed at the end of this second stimulation and found to contain 127% of the ACh of the unstimulated control, well within the range of values for the ganglia not exposed to the second test. These effects on neuronal transmitter stores do not appear to occur at lower rates of activation; for in one experiment the ganglion ACh stores were unaltered following 60 min stimulation at 4/s followed by a 20 min rest.

(3) *Release of accumulated ACh*

For these experiments the ganglia retained their blood supply intact. The experiments were divided into two groups. In one group the ganglia were stimulated for 20 min at 20/s following blockade of ACh synthesis by HC-3 and their ACh stores were then determined. In the other group the

TABLE 1. Effects of activity and post-activation rest on ACh stores in ganglia

Treatment	ACh as % ACh in control ganglion, min stimulated or rested						
	0	5	10	20	30	60	180
Stimulated 20/s for period indicated with 20 min rest*	—	—	119 ± 24 (3)	—	126 ± 22 (3)	138 ± 9 (6)	—
Stimulated 20/s, 60 min with rest indicated†	104 ± 4 (3)	127 ± 26 (3)	—	138 ± 8 (4)	—	144 ± 16 (3)	140 (1)

* Perfused choline-Locke.
† Unperfused.
Values are mean ± S.D. (no. of experiments).

ACh stores of the ganglia were first increased by stimulation at 20/s for 60 min followed by a 20 min rest. HC-3 was injected 5 min before the end of the rest period. The ganglia were then stimulated for 20 min at 20/s and their ACh stores determined. The ACh stores in five ganglia of the first group were depleted to 34 ± 8 % of the control untreated, unstimulated contralateral ganglia, a value which compares well with the 29 ± 9 % found by Birks & MacIntosh (1961) in earlier similar experiments. The depletion in the five other ganglia that had been subjected to prior stimulation and rest was significantly less ($P < 0{\cdot}05$) following 20 min stimulation at 20/s, the value being 48 ± 6 % of the control. When allowance was made for the 38 % increase in ganglionic ACh stores prior to the test stimulation with HC-3 the actual depletion of ACh stores resulting from the test in these last experiments was to 35 % of this increased value. Thus, the extra ACh that accumulates following activity and rest appears to form part of the releasable ACh stores of the ganglia.

B. *The time course of ACh release from active ganglia*

In previous studies the time course of ACh release from the ganglion appears only to have been followed at 20/s stimulation. Because different effects of stimulation on synaptic morphology occur following stimulation at 4/s as compared with 20/s it appeared worth while to examine the time course of ACh release at the lower frequency. Furthermore the characteristics of the ACh output curve with respect to time at 20/s have been interpreted to suggest that the ACh stores exist in two fractions, one representing about 15 % of the total and considered to be more 'readily releasable' than the remainder or 'depot' ACh which comprises the majority of the stocks (Birks & MacIntosh, 1961). It would be expected on this hypothesis that the shape of the output curve with time should be qualitatively similar at lower frequencies of activation.

For these experiments the ganglia were perfused with eserinized plasma; four were stimulated at 20/s and five at 4/s for 30 min. The venous effluent in each group of experiments was collected successively into seven 1 min, two 1·5 min, two 5 min and one 10 min samples. The results of the two groups of experiments are shown in Fig. 1. The 0–1 min sample at 20/s and at 4/s contained only a small amount of ACh relative to the later samples. This is because of the delay between the release of transmitter at the boutons and its washout in the effluent (cf. Birks & MacIntosh, 1961). No attempt to allow for this lag was made. At 20/s the output rate was highest in the 1–2 min sample and thereafter declined to a steady level within 5 min. However, a second small peak of output occurred between 5 and 7 min. These results are similar to those of Birks & MacIntosh, except that the second peak is more prominent here because of

the more detailed fractionation of effluent at these times. The value of the initial peak rate was $30 \pm 9{\cdot}2$ as compared with $31 \pm 6{\cdot}6$ ng/min in the Birks & MacIntosh study; but the steady maintained level was lower, $16{\cdot}6 \pm 3{\cdot}4$ as compared with $27{\cdot}8 \pm 7{\cdot}7$ ng/min. On the other hand the steady rate compares with that found by Matthews (1966) who expressed his values as percentage control ganglion ACh content, the figure being 6·8 %/min. The mean value for ACh content in the present experiments was 290 ng giving a figure of 6·0 %/min.

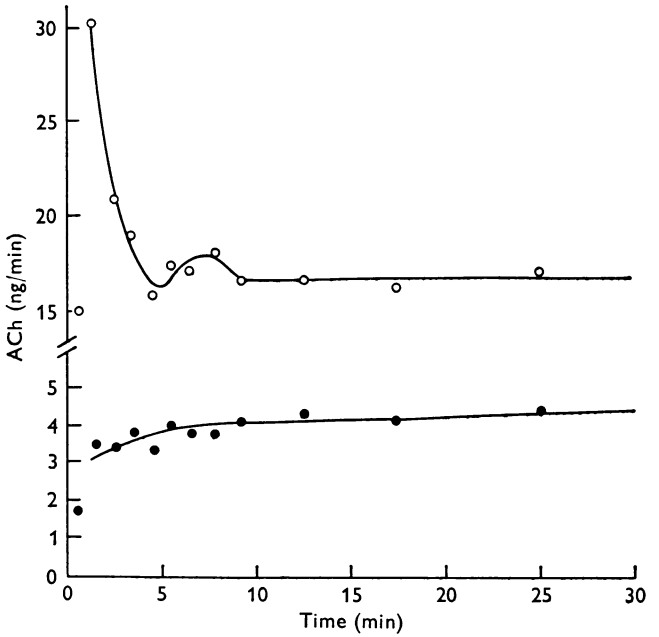

Fig. 1. Time course of ACh output from plasma-perfused ganglia. ○, mean values from four experiments at 20/s; ●, mean values for five experiments at 4/s. Note different ordinate scales for 4 and 20/s data.

Not only was the steady rate of output much lower in the 4/s experiments, as would be expected, but the shape of the curve was quite different. The initial output rate was the lowest and the rate increased with time to reach a steady level 5 min after the start of stimulation. The output rate in the 1–2, 2–3, 3–4, and 4–5 min samples was $3{\cdot}3 \pm 1{\cdot}3$ ng/min as compared with $4{\cdot}2 \pm 1{\cdot}0$ ng/min for the 8·5–10, 10–15, 15–20 and 20–30 min samples. The 1–5 min outputs were significantly lower than the later rates ($P < 0{\cdot}02$). The steady output rate was 25% of the rate at 20/s as found earlier by Birks & MacIntosh (1961).

DISCUSSION

Mixing of newly synthesized ACh with preformed stores

The post-activation increases in ganglion ACh stores that have been reported above confirm and extend earlier findings. The 14% increase following 30 min 20/s stimulation and a 4 min rest found by Rangachari *et al.* (1969) is consistent with the present data, and the long persistence of the increase following activity confirms the observations of Bourdois *et al.* (1970), although their test procedure was different from that used here.

It is of interest to recalculate the relative contributions of preformed and newly synthesized ACh to the release of ACh in the experiments of Collier (1969) on the basis of the present finding. Clearly in Collier's experiments the ACh content of the test ganglia following the loading procedure must have increeasd by about 38%. It appears safe to assume that most of this extra ACh would have been unlabelled, because if this were not the case then the sum of the total labelled ACh that was released in the test and the residual labelled ACh in the ganglion at the end of the test would have been anomalously high and it was not.

In Table 2 the data of Collier giving changes in the labelling of ACh stores and labelling of ACh outputs with time after the onset of stimulation are shown in columns 1–4. It was assumed that released labelled ACh would be rapidly replaced by new unlabelled ACh. Columns 5 and 6 show the corrected values for ganglionic unlabelled ACh after allowing for a 38% increase in ganglion contents. The corrected values for percentage labelled ACh in the ganglion with time after the onset of stimulation are compared in Table 3 with Collier's (1969) observed labelling of ACh outputs. Insofar as it may be legitimate to draw conclusions from data extracted and corrected in this way the calculations do not support the conclusion that newly synthesized ACh equilibrates with only a small fraction of the preformed stores. Indeed for the first 10 min of stimulation there may even have been a slight tendency for the newly synthesized transmitter not to contribute fully to release. With more prolonged stimulation there was a tendency in the opposite direction suggesting equilibration of new transmitter with about 60% of the old.

It does not appear to be appropriate to discuss further the significance of the post-activation increase in ganglionic ACh until more is known of the state of bouton morphology under such conditions. The finding of a low initial output of ACh at 4/s stimulation as compared with the high initial output when stimulation was at 20/s is noteworthy, since the differences are not fitted easily to the simple idea of a small readily releasable pool of ACh in series or in parallel with a larger depot pool as

TABLE 2. Time distribution of labelled and unlabelled ACh in active ganglia

Time in min after start of test stimulation	1 Total ACh stores (estimated from control)*	2 Cumulative output of labelled ACh*	3 Labelled ACh stores*	4 Unlabelled ACh stores (column 1 − 3)†	5 Corrected total ACh stores‡	6 Corrected unlabelled ACh stores (column 5 − 3)
0	260	—	218 (84% control value)	42	359	141
5	260	36	182	78	359	177
10	260	53	165	95	359	194
20	260	73	145	115	359	214

* From Table 1, Fig. 1, and text of Collier (1969). All figures for ACh in ng.
† Assuming replacement of labelled ACh output (column 2) by unlabelled ACh.
‡ Correction based on data of Table 1, this paper.

TABLE 3. Time evolution of labelled ACh stores and ACh output in active ganglia

Time in min after start of test stimulation	Corrected labelled ACh stores in %*	% ACh output that was labelled†
0	61	70
5	51	60
10	46	33
20	40	28

* From Table 2, columns 3 and 5.
† From Collier (1969) Fig. 1 and text.

proposed by Birks & MacIntosh (1961). However, until more is known about the distribution between cytoplasmic and vesicular ACh at resting boutons, and the dynamic relationships between synthesis and release of transmitter in active ganglia the results are not possible to interpret with any degree of confidence. What seems clear, however, is that the relative contributions of preformed and newly synthesized ACh stores to the release of transmitter in the above circumstances are consistent with the hypothesis that ACh may be released from a cytoplasmic pool.

We are indebted to Dr M. W. Cohen and to Dr M. C. Mackey for their helpful comments on the manuscript, and to the Medical Research Council of Canada and the Muscular Dystrophy Association of Canada for financial support.

REFERENCES

BIRKS, R. I. (1974). The relationship of transmitter release and storage to fine structure in a sympathetic ganglion. *J. Neurocytol.* (in the Press).

BIRKS, R. I. & MACINTOSH, F. C. (1961). Acetylcholine metabolism in a sympathetic ganglion. *Can. J. Biochem. Physiol.* **39**, 787–827.

BOURDOIS, P. S., MCCANDLESS, D. L. & MACINTOSH, F. C. (1970). A prolonged after-effect of high-frequency stimulation in a cholinergic pathway. *Proc. Can. Fed. Biol. Sci.* **13**, 573.

COLLIER, B. (1969). The preferential release of newly synthesized transmitter by a sympathetic ganglion. *J. Physiol.* **205**, 341–352.

COLLIER, B. & MACINTOSH, F. C. (1969). The source of choline for acetylcholine synthesis in a sympathetic ganglion. *Can. J. Physiol. Pharmac.* **47**, 127–135.

FONNUM, F. (1967). The 'compartmentation' of choline acetyltransferase within the synaptosome. *Biochem. J.* **103**, 262–270.

FONNUM, F. (1968). Choline acetyltransferase binding to and release from membranes. *Biochem. J.* **103**, 389–398.

MATTHEWS, E. K. (1966). The presynaptic effects of quaternary ammonium compounds on the acetylcholine metabolism of a sympathetic ganglion. *Br. J. Pharmac. Chemother.* **26**, 552–566.

RANGACHARI, P. K., KHATTER, J. C. & FRIESEN, A. J. D. (1969). Effect of stimulation on acetylcholine content of a sympathetic ganglion. *Proc. Can. Fed. Biol. Sci.* **12**, 4.