Inhibition by Botulinum Toxin of Depolarization-Evoked Release of [^{14}C]Acetylcholine from Synaptosomes *in vitro*

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1. Cerebral-cortex synaptosomes were shown to synthesize [^{14}C]acetylcholine after incubation with [^{14}C]choline, and 25 mM-KCl released [^{14}C]acetylcholine (but not [^{14}C]choline) into the medium by a Ca^{2+} -dependent and Mg^{2+} -sensitive process. 2. The K^{+} -stimulated release of [^{14}C]acetylcholine was inhibited by more than 80% after preincubation of the synaptosomes with 10^5 mouse lethal doses of botulinum toxin/ml. [^{14}C]Choline uptake, [^{14}C]acetylcholine synthesis, intrasynaptosomal K^{+} and occluded lactate dehydrogenase were unaffected by the toxin. It also failed to prevent the K^{+} -stimulated release of [^3H]noradrenaline and [^{14}C]glycine from synaptosomes. 3. Fractionation of hypo-osmotically shocked synaptosomes revealed that more than 75% of the radioactive acetylcholine was in the cytoplasmic compartment, although the vesicle pellet contained more total acetylcholine than the cytoplasmic pool. Consequently the specific radioactivity of acetylcholine in the cytoplasmic pool was almost 5 times that of the vesicles. This distribution was unaffected by preincubation with botulinum toxin. It is concluded that the toxin acts directly on the release of acetylcholine, rather than influencing its storage. 4. After K^{+} -stimulation, toxin-inhibited synaptosomes contained increased amounts of total acetylcholine, which suggests that its rate of synthesis is controlled by depolarization rather than release.

Synaptosomes were discovered in an attempt to isolate the particle that stored acetylcholine in the synaptic region. This endeavour had particular urgency at the time, because of the proposal that the small vesicles seen aggregated in the pre-terminal region contained a fixed amount (the quantum) of acetylcholine. The stability of the pre-terminal region of cerebral-cortex synapses, such that they survived homogenization, was an unexpected impediment to the separation and isolation of synaptic vesicles. However, this obstacle to the study of the storage forms of neurotransmitters turned out to have useful consequences. It has become apparent that the isolated nerve terminals, or synaptosomes, as they are now called, retain a surprising degree of metabolic and functional integrity. This offered the possibility that neurotransmitter release mechanisms could be studied with the preparation *in vitro*. The advantages of using synaptosomes compared with other, perhaps more physiologically appealing, preparations is that only the pre-terminal region retains an organized structure, so that uptake and other metabolic activities may be investigated in conjunction with release, with some assurance that only events in the relevant regions are being measured.

The use of synaptosomes for studies on neurotransmitter release depends on the previous demonstration of a number of functional characteristics. These in-

clude showing that the terminal limiting membrane is substantially intact (Marchbanks, 1967), that transmitter precursors such as choline can be taken up (Marchbanks, 1968) and that the transmitter (in this case acetylcholine) can be synthesized and stored within the terminal (Marchbanks, 1969*b*). In order to demonstrate transmitter release under physiological conditions it is necessary to show that the synaptosome membrane can be depolarized (Blaustein, 1975), though earlier experiments (Marchbanks, 1969*a*; Blaustein *et al.*, 1972; De Bellerocche & Bradford, 1972), in which transmitter release was stimulated by depolarizing agents, were suggestive in this respect. Finally, the whole exercise is more persuasive if a specific inhibitor of transmitter release can be shown to act in synaptosomes.

In this paper we describe an experimental system in which the release on depolarization of [^{14}C]acetylcholine from synaptosomes can be conveniently studied by using acetylcholine stores prelabelled with [^{14}C]choline. Botulinum toxin was found to inhibit this release specifically, and, since its physiological action is to prevent acetylcholine release (Burgin *et al.*, 1949; Brooks, 1956; Harris & Miledi, 1971), this demonstration serves to support the credibility of the system for studying physiological release processes, as well as providing some information about the mechanism of action of the toxin. A preliminary

report has been given (Wonnacott & Marchbanks, 1975b).

Methods

Preparation and preincubation of synaptosomes

Synaptosomes were prepared from scraped guinea-pig cerebral cortices by the method of Gray & Whittaker (1962), except that the centrifugal field used to sediment the mitochondrial pellet was decreased to 10000g and the pellet was washed once (Whittaker *et al.*, 1964). The synaptosome (P₂B) fraction was removed from the gradient with a Pasteur pipette and diluted slowly with an equal volume of incubation medium (see below). The synaptosomes were sedimented by centrifuging at 30000g for 20 min. The pellet was resuspended in incubation medium to give a final concentration of 1–2 mg of protein/ml, and allowed to equilibrate with the medium by standing on ice for 10 min before incubation. The incubation medium contained 10 mM-sodium phosphate buffer, pH 7.2, 3 mM-KCl, 2 mM-MgSO₄, 2 mM-CaCl₂, 180 mM-NaCl and 10 mM-glucose.

After equilibration the suspension was divided into two or more fractions, and to one sample botulinum toxin type A was added to give a final concentration of 10⁵ mouse LD₅₀/ml (1 mouse LD₅₀ kills 50% of mice; 0.01 µg of crude toxin equalled 1 mouse LD₅₀). The other synaptosome samples served as controls and received an equivalent volume of the solvent only, or of boiled toxin (inactivated by heating for 15 min in a boiling-water bath; Burgen *et al.*, 1949) or no addition. The suspensions (contained in stoppered tubes) were incubated for a total of 60 min at 37°C. After 30 min, 2 µl of [¹⁴C]choline (50 µCi/ml)/ml was added to the tubes so that during the last 30 min of the preincubation period botulinum toxin and [¹⁴C]choline were present simultaneously. At the end of the preincubation, the synaptosomes were precipitated by centrifugation at 4000 rev./min for 5 min in an MSE bench centrifuge, and resuspended in standard medium. Each suspension was subdivided into two or more equal volumes and the synaptosomes were sedimented again. This wash eliminated more than 99% of the radioactivity in the medium (see the Results section), which would otherwise have interfered with the determination of radioactivity released from the synaptosomes.

In one experiment, the [¹⁴C]choline was replaced by a mixture of [¹⁴C]glycine and [³H]noradrenaline [at final concentrations of 14 µM (0.8 µCi/ml) and 0.2 µM (1.0 µCi/ml) respectively] during the preincubation. In this case, 1 µM-ascorbic acid and 1 µM-EDTA were included in the incubation medium to protect the amine. Other constituents were unchanged.

Measurement of release

The washed synaptosomes were resuspended in medium containing 5 µM-eserine (to protect released acetylcholine from enzymic hydrolysis) and transferred to 25 ml beakers, which were incubated in a shaking water bath. The media at this stage were adjusted by additions or omissions as indicated in the Results section. When large increments in the KCl concentration were used, osmolarity was maintained constant by a corresponding decrease in NaCl concentration. Samples (1 ml) were withdrawn to Eppendorf micro reaction tubes at the required intervals and were rapidly centrifuged (10000g for 30 s in a Janetzi TH11 micro-centrifuge). The supernatant fluid was transferred to tubes kept on ice containing 5 µl of [³H]acetylcholine internal standard (approx. 5 pmol; 30000 d.p.m./pmol) and 0.1 ml of 50% (w/v) trichloroacetic acid, to give a final concentration of 4.5%. The pellets were washed by resuspension in 1 ml of cold incubation medium containing 5 µM-eserine and recentrifuging. The supernatant phase was discarded and the pellet resuspended in 1 ml of 5% (w/v) trichloroacetic acid, and 5 µl of internal standard was added to each sample.

The experiments to investigate the release of noradrenaline and glycine were similar except that eserine was omitted from the medium, but ascorbic acid (1 µM) and EDTA (1 µM) were added. Smaller samples (0.2 ml) were removed and, after precipitation of the synaptosomes as described for the acetylcholine experiments, 0.1 ml of the supernatant fraction was transferred to a scintillation vial. The synaptosome pellet was washed in cold medium and treated with 0.15 ml of 5% trichloroacetic acid. A proportion of the acid extract (0.1 ml) was taken for determination of radioactivity; and protein was measured in the precipitate. The ³H and ¹⁴C present in the medium and pellet were taken as a measure of the released or accumulated noradrenaline and glycine respectively, although obviously the values may have also included breakdown products.

Fractionation of synaptosomes

The compartmentation of acetylcholine within the synaptosome was examined after preincubation with and without botulinum toxin. At the end of the preincubation period, 0.5 ml samples of the synaptosome suspensions were withdrawn, rapidly centrifuged as above, and the synaptosome pellets were washed with 1.0 ml of incubation medium. The washed pellets were subjected to hypo-osmotic shock by resuspension in 2.5 ml of ice-cold water containing 5 µM-eserine. After 5 min on ice, the samples were centrifuged at 130000g for 60 min to sediment the ruptured synaptosomes and vesicles, leaving the soluble cytoplasmic constituents in the supernatant frac-

tion, which was decanted, and 50% trichloroacetic acid was added to give a final concentration of 5%. The pellet was resuspended in 1.0ml of 5% trichloroacetic acid. Internal standard was added in both cases and the samples were subsequently extracted and assayed in the same way as others.

Extraction and determination of acetylcholine and choline

After at least 30min on ice, the trichloroacetic acid-treated samples were centrifuged at 10000g for 30s in a Janetzi TH11 micro-centrifuge and the supernatant fraction was decanted into clean tubes. The trichloroacetic acid precipitates of the synaptosome pellets were retained at 4°C for protein determination. Acetylcholine and choline were first extracted from the supernatants by the method of Fonnum (1969) as modified by Marchbanks & Israël (1971). The freeze-dried extracts were then re-extracted by the method of Goldberg & McCaman (1973) in order to remove any residual trichloroacetic acid, and other contaminants, which interfere with the subsequent assay of acetylcholine, by redissolving the residue in 20μl of 100mM-propanediol/HCl buffer, pH 8.0, and shaking with 50μl of heptan-3-one, containing 20mg of tetraphenylboron/ml. After a brief centrifugation (10000g for 30s), 45μl of the organic layer was removed to clean tubes and shaken with 50μl of 0.4M-HCl. The organic phase was removed with a syringe and discarded, and 45μl of the remaining aqueous solution removed and divided into two equal portions before vacuum evaporation. One sample was then subjected to t.l.c. (Marchbanks & Israël, 1971) to measure [¹⁴C]acetylcholine and [¹⁴C]choline, and the total acetylcholine in the duplicate sample was measured by radio-enzymic assay (Wonnacott & Marchbanks, 1975a). Blanks were derived from 1.0ml of incubation medium containing eserine, and the assay method was modified to compensate for the presence of eserine by increasing the pH and acetylcholinesterase concentration of the second phosphorylation step.

After the radioenzymic assay of acetylcholine, the samples, which contained ³H, ¹⁴C and ³²P (which were determined simultaneously), were counted for radioactivity in a Nuclear Enterprises 8312 scintillation spectrometer with toluene (containing 5g of PPO (2,5-diphenyloxazole) and 0.2g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]/litre)/Triton X-100/2M-NH₃ (100:50:1, by vol.) as scintillant. The counting efficiencies for these radioisotopes were determined in three windows selected to offer the best compromise between maximum efficiency and minimum overlap. From the counting efficiencies, the radioactivity of each radioisotope could be derived from the composite c.p.m. of the three radioisotopes measured together by solving for D_a, D_b and D_c in

the following system of equations, by using an Olivetti P101 Calculator:

$$\begin{aligned}C_1 &= E_{1a}D_a + E_{1b}D_b + E_{1c}D_c \\C_2 &= E_{2a}D_a + E_{2b}D_b + E_{2c}D_c \\C_3 &= E_{3a}D_a + E_{3b}D_b + E_{3c}D_c\end{aligned}$$

where C is the total c.p.m. in the window denoted by the subscript 1, 2 or 3, D is the d.p.m. of the radioisotope denoted by the subscript a, b or c, and E is the counting efficiency of a given radioisotope (a, b, c) in a given window (1, 2, 3). The average efficiencies of ³H, ¹⁴C and ³²P in their main windows were 13%, 66% and 85% respectively; the ³H cross-over into the ¹⁴C and ³²P windows was 9% and 0.6%, the ¹⁴C cross-over into the ³H and ³²P windows was 0.5% and 11%, and the ³²P cross-over into the ³H and ¹⁴C windows was 0% and 4% respectively.

The recovery of [³H]acetylcholine in samples subjected to t.l.c. was between 40 and 60% and constant (within 5%) for any experiment. The [¹⁴C]-acetylcholine and [¹⁴C]choline found in each sample was corrected for any incomplete recovery and also for losses or over-estimations due to the hydrolysis of acetylcholine as shown by its associated internal standard. Results for both synaptosome pellets and media were expressed as d.p.m./mg of protein; for media, this calculation was based on the mean protein concentration of the individual synaptosome suspension. This manipulation makes the results for pellet and medium directly comparable, and compensates for differences in synaptosome concentration in the separate flasks. The radio-enzymic assay yielded values of d.p.m. of [³H]phosphorylcholine, derived from the [³H]acetylcholine internal standard, and d.p.m. of [³²P]phosphorylcholine from the acetylcholine in the sample. After subtraction of the ³²P-labelled blank, the [³²P]phosphorylcholine was corrected for recovery by reference to the internal standard and converted into pmol of acetylcholine by division by the specific radioactivity of the [³²P]ATP used. Results were expressed as pmol/mg of protein.

K⁺ determination

The K⁺ content of synaptosomes before and after incubation was measured on acid extracts of samples of synaptosome suspension after gel filtration (Marchbanks, 1967), by using an EEL flame photometer.

Measurement of lactate dehydrogenase

Lactate dehydrogenase was used as an estimate of the amount of entrapped cytoplasm in sealed synaptosomes (Johnson & Whittaker, 1963), and was measured in synaptosome suspensions before and after incubation by the method of Johnson (1960), modified for the determination of the occluded enzyme by Marchbanks (1967).

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine plasma albumin as standard. The procedure was adapted to measure protein in trichloroacetic acid precipitates by dissolving the pellets in 3.0M-NaOH and lowering the NaOH concentration of the alkaline Na₂CO₃ solution so that the same final concentration was obtained.

Materials

All radiolabelled compounds were from The Radiochemical Centre, Amersham, Bucks., U.K. Acetyl[methyl-³H]choline was prepared from [³H]-choline by incubation with acetic anhydride (Schuberth & Sundwall, 1967). Crude botulinum toxin (type A) was provided by Dr. J. Keppie, Microbiological Research Establishment, Porton Down, Wilts., U.K. It was dissolved in 2% (w/v) sodium phosphate, pH 6.2, containing 0.2% gelatin, at a concentration of approx. 10⁷ mouse LD₅₀/ml.

Safety

Experiments with botulinum toxin were undertaken with extreme caution. During experiments with the toxin, access to the laboratory was restricted. The active toxin was handled within a fume cupboard and protective clothing was worn at all times. All apparatus and materials that had come into contact with the toxin were immersed in boiling dilute HCl for at least 30 min to inactivate it (Burgen *et al.*, 1949).

Results

Synthesis of [¹⁴C]acetylcholine and release of [¹⁴C]-acetylcholine and [¹⁴C]choline from synaptosomes in low-K⁺ medium

Synaptosomes were preincubated at 37°C for 30 min with [¹⁴C]choline (2.5 μM; 0.1 μCi/ml) in order to label the intrasynaptosomal stores of choline and acetylcholine. At the end of this period, the synaptosomes were sedimented, washed and resuspended in medium lacking choline, but containing eserine. Analysis of the synaptosomes at this time for labelled acetylcholine and choline revealed some variability in the amount of [¹⁴C]choline accumulated by different synaptosome preparations [8920 ± 2080 (S.E.M., *n* = 8) d.p.m./mg of protein], but the proportion of the total label present in acetylcholine was more consistent [68.7 ± 1.9% (S.E.M., *n* = 8)]. The external concentration of [¹⁴C]choline during the preincubation was 2.5 μM; therefore these results are comparable with the findings of Haga (1971) and Yamamura & Snyder (1972), who used 1.5 μM- and 0.5 μM-choline respectively. Both groups reported that the proportion of label in acetylcholine decreases as the choline concentration is raised, so it was advantageous that the added choline concentration should be kept as low as possible in these experiments.

The release of the labelled compounds into medium containing 3 mM-KCl was reasonably consistent: approx. 1600 d.p.m. of acetylcholine (Table 1) and 1300 d.p.m. of choline/mg of protein were present in the eserine-containing medium after 20 min. However, extrapolation of the release curves to zero time indicates the presence of approx. 500 d.p.m. of

Table 1. Summary of the inhibitory action of botulinum toxin on [¹⁴C]acetylcholine release

Synaptosomes were preincubated with active or boiled botulinum toxin and [¹⁴C]choline. After being washed, they were incubated for 20 min in medium containing the K⁺ concentration indicated. The [¹⁴C]acetylcholine released during this period was determined. Each row gives the results of a separate synaptosome preparation.

[¹⁴ C]Acetylcholine released (d.p.m./mg of protein)				Inhibition (%) $\frac{(b-d)}{(b-c)}$	Fractional change with 25mm-K ⁺		Inhibition (%) after correction for different store size
Control		+Botulinum			Control (b/a)	+Botulinum (d/c)	
3mm-K ⁺ (a)	25mm-K ⁺ (b)	3mm-K ⁺ (c)	25mm-K ⁺ (d)				
1150	2480	1100	1280	86.7	2.15	1.16	80.0
1310	2900	1300	909	124.5	2.21	0.70	108.0
1480	5530	1600	2690	72.3	3.74	1.67	63.5
1210	2500	1540	1880	64.6	2.06	1.22	62.4
2040	4400	1650	1860	93.4	2.16	1.13	84.9
			Mean	88.3	2.46*	1.17*	80.1
			S.E.M.	10.4	0.32	0.15	7.7

* Significantly different (*P* < 0.01, Student's *t* test, two-tailed).

Fig. 1. Effect of time and of increasing concentrations of KCl on the release of $[^{14}\text{C}]\text{acetylcholine}$ (a) and $[^{14}\text{C}]\text{choline}$ (b).

Synaptosomes were preincubated with $[^{14}\text{C}]\text{choline}$ in medium containing 3 mM-KCl, washed and then incubated in medium containing eserine and the following KCl concentrations: $\circ$, 3.0 mM; Δ , 13.0 mM; $\bullet$, 20.0 mM; $\blacktriangle$, 28.5 mM. Samples were removed, and the medium was isolated by centrifugation and analysed for $[^{14}\text{C}]\text{acetylcholine}$ (a) and $[^{14}\text{C}]\text{choline}$ (b). (c) shows the radioactivity released in 20 min plotted against KCl concentration: $[^{14}\text{C}]\text{acetylcholine}$ ($\bullet$), $[^{14}\text{C}]\text{choline}$ ($\circ$). Each point represents a single determination.

$[^{14}\text{C}]\text{choline}$, but no $[^{14}\text{C}]\text{acetylcholine}$, initially (see Figs. 1 and 3). This choline presumably is derived from preincubation medium that was not completely

Fig. 2. Effect of increasing KCl concentrations on the synaptosome stores of $[^{14}\text{C}]\text{acetylcholine}$ (a) and $[^{14}\text{C}]\text{choline}$ (b).

Experimental details and symbols are the same as for Fig. 1 except that the isolated synaptosomes were analysed for $[^{14}\text{C}]\text{acetylcholine}$ (a) and $[^{14}\text{C}]\text{choline}$ (b) after incubation with the specified concentration of KCl.

eliminated when the synaptosomes were washed, and the unstimulated output with time is probably leakage from poorly sealed or slowly deteriorating synaptosomes.

Effects of high K^+ concentration

The effect of increased concentrations of K^+ after preincubation is shown in Fig. 1. Increasing the K^+ concentration produced successive increases in the $[^{14}\text{C}]\text{acetylcholine}$ released; plotting the d.p.m. of $[^{14}\text{C}]\text{acetylcholine}$ released/mg of protein in 20 min against the K^+ concentration gave an almost linear relationship up to 30 mM-KCl. This suggested that KCl is a reliable and reproducible method of releasing acetylcholine. The release of $[^{14}\text{C}]\text{choline}$ from the same synaptosomes was also examined. Changes in the KCl concentration failed to alter the concentration of $[^{14}\text{C}]\text{choline}$ in the medium (Fig. 1); this indicated that K^+ was acting in a specific manner and not producing a general disruptive effect on the synaptosome membrane.

Both $[^{14}\text{C}]\text{acetylcholine}$ and $[^{14}\text{C}]\text{choline}$ concentrations tended to decrease in the synaptosome pellet during the release period (Fig. 2). The rate of decline was similar at all KCl concentrations, but the tissue concentrations of $[^{14}\text{C}]\text{acetylcholine}$ were lower at the higher concentrations. This observation is

Fig. 3. Ca^{2+} -dependence of the release of $[^{14}\text{C}]$ acetylcholine (a) and $[^{14}\text{C}]$ choline (b)

Experimental details are the same as for Fig. 1 except that Ca^{2+} was in some cases omitted from the final incubation medium. $\circ$, 3 mM-KCl, 2 mM- Ca^{2+} ; Δ , 25 mM-KCl, 2 mM- Ca^{2+} ; $\bullet$, 3 mM-KCl, no Ca^{2+} ; $\blacktriangle$, 25 mM-KCl, no Ca^{2+} ; $\square$, 3 mM-KCl, no Ca^{2+} , 5 mM-EGTA; $\blacksquare$, 25 mM-KCl, no Ca^{2+} , 5 mM-EGTA.

consistent with the rapidity of K^+ depolarization in releasing acetylcholine, a finding also reported by Haga (1971). It was concluded from the radioactivity data that the stores of acetylcholine were not exhausted during the period of incubation with high concentrations of K^+ . This was an important consideration in the selection of an appropriate KCl concentration for examining evoked acetylcholine release as a routine. As a compromise between maximal release of $[^{14}\text{C}]$ acetylcholine with minimal perturbation of the control system, 25 mM-KCl was selected for subsequent experiments. A summary of the release data (Table 1, columns a and b) illustrates the consistency of K^+ depolarization; despite variations in the absolute amount of $[^{14}\text{C}]$ acetylcholine released, 25 mM-KCl more than doubled the output in every instance. For this reason and because of the simple manipulations involved in raising the KCl concentration, this method of evoking acetylcholine

Fig. 4. Mg^{2+} -sensitivity of the release of $[^{14}\text{C}]$ acetylcholine (a) and $[^{14}\text{C}]$ choline (b)

Experimental details are the same as for Fig. 1 except that Mg^{2+} was at a higher concentration in the final incubation medium in some cases (2 mM- Ca^{2+} was present throughout). $\circ$, 3 mM-KCl, 2 mM- Mg^{2+} ; Δ , 25 mM-KCl, 2 mM- Mg^{2+} ; $\bullet$, 3 mM-KCl, 10 mM- Mg^{2+} ; $\blacktriangle$, 25 mM-KCl, 10 mM- Mg^{2+} ; $\square$, 3 mM-KCl, 20 mM- Mg^{2+} ; $\blacksquare$, 25 mM-KCl, 20 mM- Mg^{2+} .

release was adopted. Attempts to release $[^{14}\text{C}]$ acetylcholine from synaptosome suspensions and beds by electrical stimulation, by using concentric gold-ring electrodes and grid electrodes respectively, were unsuccessful.

Ca^{2+} -dependence and Mg^{2+} -sensitivity of K^+ -stimulated $[^{14}\text{C}]$ acetylcholine release

To establish that the release process in synaptosomes conforms to the physiological requirement for Ca^{2+} it was investigated in the absence of Ca^{2+} and in the presence of increased Mg^{2+} concentrations, since in both these conditions the physiological release of

acetylcholine is severely curtailed (Del Castillo & Engbaek, 1954; Katz & Miledi, 1965).

The presence of 5mM-EGTA [ethanedioxybis-(ethylamine)tetra-acetate], added to chelate any Ca^{2+} arising from the synaptosomes, severely disturbed release under control conditions of both [^{14}C]acetylcholine and [^{14}C]choline (Fig. 3), so the influence of Ca^{2+} was examined by incubating the synaptosomes in medium lacking Ca^{2+} , but with no chelating agent added. The presence of endogenous Ca^{2+} in the synaptosome preparation might permit some release, but this was expected to be considerably less than observed at the optimal Ca^{2+} concentration. The results (Fig. 3) showed that the 25mM- K^{+} -stimulated release of [^{14}C]acetylcholine was abolished; this confirms the Ca^{2+} -dependence of [^{14}C]acetylcholine release from synaptosomes in this system.

The effect of high Mg^{2+} concentrations on the evoked release of [^{14}C]acetylcholine was less convincing (Fig. 4); at 10mM- MgSO_4 the K^{+} -stimulated release of [^{14}C]acetylcholine was almost as high as the

normal control (2mM- Mg^{2+}), but at 20mM- MgSO_4 there was a 40% decrease.

Influence of botulinum toxin on the synthesis and release of acetylcholine

Initially the toxin was added immediately after resuspension of the washed synaptosomes in high- or low- K^{+} medium, but it did not affect [^{14}C]acetylcholine release under these conditions. It has been demonstrated both *in vivo* and *in vitro* that there is a latent period after addition of the toxin before any effects are observed (Burgin *et al.*, 1949; Boroff *et al.*, 1974). From the results of experiments *in vitro* with

Fig. 5. Effect of botulinum toxin on the synaptosome stores of [^{14}C]acetylcholine (a) and [^{14}C]choline (b)

Synaptosomes were preincubated with boiled or active botulinum toxin and [^{14}C]choline. After washing, they were incubated in medium containing the KCl concentration indicated. Samples were withdrawn at the stated time-intervals and the synaptosomes isolated by centrifugation for analysis. $\circ$, 3mM-KCl; $\bullet$, 25mM-KCl; $\triangle$, 3mM-KCl+botulinum; $\blacktriangle$, 25mM-KCl+botulinum.

Fig. 6. Effects of botulinum toxin on the release of [^{14}C]acetylcholine (a) and [^{14}C]choline (b)

Experimental details are the same as for Fig. 5 except that [^{14}C]acetylcholine (a) and [^{14}C]choline (b) in the incubation medium were determined. $\circ$, 3mM-KCl; $\bullet$, 25mM-KCl; $\triangle$, 3mM-KCl+botulinum; $\blacktriangle$, 25mM-KCl+botulinum.

similar concentrations of toxin, 1 h was selected as a suitable time-period for preincubation of the synaptosomes with the botulinum toxin. However, in view of the deteriorating viability of the preparation during a prolonged incubation it was decided to perform the [14 C]choline loading during the latter half of this preincubation; the results indicate that there was no significant interference with [14 C]choline uptake or [14 C]acetylcholine synthesis (see Fig. 5). Because botulinum toxin binds very firmly to tissue during the first 5–20 min of the latent period (Burgen *et al.*, 1949; Simpson, 1971) the synaptosomes could be sedimented and washed as normal after the preincubation, without loss of inhibition by toxin of release.

Measurement of [14 C]acetylcholine release from synaptosomes preincubated with and without botulinum toxin (at a final concentration of 10^5 mouse LD $_{50}$ /ml) showed that the toxin prevented the increase in release resulting from 25 mM-KCl in the control (Fig. 6). At low K $^{+}$ concentrations there was no difference in release between botulinum-preincubated and control suspensions. Further, the toxin exerted no influence on the release of [14 C]choline (Fig. 6) and, as noted above, the tissue contents of [14 C]choline and [14 C]acetylcholine were unaffected (Fig. 5).

The inhibition of [14 C]acetylcholine release by botulinum toxin was very reproducible and statistically significant; the results of a series of experiments are summarized in Table 1. On average, the toxin was effective in preventing almost 90% of the K $^{+}$ -evoked release. In some instances the phosphate buffer containing gelatin, in which the toxin was suspended, or heat-inactivated toxin was added to the control flasks; in these cases the normal uninhibited response to high K $^{+}$ was observed, indicating that the inhibition is a property of the un-denatured toxin.

Effects of botulinum toxin on synaptosomal metabolism and integrity

Intrasyntosomal K $^{+}$ and lactate dehydrogenase activity were measured during the course of the experiments (Table 2) in order to assess the structural and functional state of the synaptosomes. The mean synaptosomal K $^{+}$ content was approx. $0.2 \mu\text{mol/g}$ of protein. This is within the reported range (Haga, 1971; Richter & Marchbanks, 1971*b*). The final values appear somewhat higher than those of the P $_2$ B fraction; this probably reflects the recovery of the synaptosomes and consequent uptake of K $^{+}$ after the prolonged preparative procedures when leakage was likely to have occurred. Intracellular concentrations were unaffected by incubation in 25 mM-KCl, and preincubation with botulinum toxin did not seem to have any influence on the K $^{+}$ content. The occluded lactate dehydrogenase (as a percentage of the total enzyme activity) decreased by less than 10% during

Table 2. K $^{+}$ content and lactate dehydrogenase activity of synaptosomes

Synaptosome fraction (P $_2$ B)	After release period					
	After preincubation		Control		+Botulinum	
	Control	+Botulinum	3 mM-K $^{+}$	25 mM-K $^{+}$	3 mM-K $^{+}$	25 mM-K $^{+}$
K $^{+}$ ($\mu\text{mol/g}$ of protein)	0.154 ± 0.036	0.192 ± 0.027	0.235 ± 0.015	0.240 ± 0.054	0.187 ± 0.040	0.219 ± 0.036
Occluded lactate dehydrogenase ($\mu\text{mol/min per mg}$ of protein)	0.187 ± 0.031	0.180 ± 0.025	0.173 ± 0.039	0.116 ± 0.017	0.253 ± 0.048	0.200 ± 0.037
Occluded lactate dehydrogenase (%)	81.8 ± 2.5	79.6 ± 7.6	87.2 ± 1.8	71.1 ± 10.2	88.2 ± 4.1	86.4 ± 3.3

Synaptosomes were incubated as described in the legend to Table 1 and samples were removed at different stages for determination of intrasyntosomal K $^{+}$ and lactate dehydrogenase activity as described in the Methods section. Results are the means $\pm$ S.E.M. of the values from three separate synaptosome preparations.

Fig. 7. Effects of botulinum toxin on the release of $[^3\text{H}]$ noradrenaline (a) and $[^{14}\text{C}]$ glycine (b)

Synaptosomes were preincubated with boiled or active botulinum toxin, $[^3\text{H}]$ noradrenaline and $[^{14}\text{C}]$ glycine. After washing, they were incubated in medium containing the KCl concentration specified. Samples were removed at the stated times and the radioactivity in the medium was determined. The radioactivity released is expressed as a percentage of the mean tissue store. For $[^3\text{H}]$ noradrenaline, the mean tissue store of radioactivity was 9000 d.p.m./mg of protein for the 3 mM-KCl and 25 mM-KCl controls and 7000 d.p.m./mg for the botulinum-treated conditions. The mean tissue store of $[^{14}\text{C}]$ glycine was 5000 d.p.m./mg for the controls and 2500 d.p.m./mg for the botulinum-treated conditions. ○, 3 mM-KCl; ●, 25 mM-KCl; △, 3 mM-KCl + botulinum; ▲, 25 mM-KCl + botulinum. Each point is the mean of duplicate determinations, which differed by $3.15 \pm 0.92\%$ (S.E.M., $n = 16$) in the case of $[^3\text{H}]$ noradrenaline and $2.26 \pm 0.46\%$ (S.E.M., $n = 16$) in the case of $[^{14}\text{C}]$ glycine. Release when considered as a percentage of the control release at each time-point was significantly greater in the presence of 25 mM-KCl than in the respective 3 mM-KCl control ($P < 0.05$ for $[^3\text{H}]$ noradrenaline in the presence of both boiled and active toxin; $P < 0.001$ and $P < 0.02$ for $[^{14}\text{C}]$ glycine in the presence of boiled and active toxin respectively; Student's t test, two-tailed).

the incubation and was unaffected by botulinum toxin. The values derived from high-KCl control suspensions were rather erratic and often lower than those obtained under other conditions. However, lower values were not consistently observed, and in the presence of bound botulinum toxin high KCl did not influence the values for occluded lactate dehydrogenase.

Effects of botulinum toxin on other putative transmitters

In order to determine the specificity of botulinum toxin with regard to the inhibition of the release of acetylcholine, its effect on the release of $[^3\text{H}]$ noradrenaline and $[^{14}\text{C}]$ glycine from synaptosomes was examined. Synaptosomes were loaded with the labelled compounds in the presence of active or boiled toxin, and their release in response to 3 mM- and 25 mM-KCl was measured. Raising the KCl concentration was effective in releasing both compounds

in the absence of botulinum toxin (although to a smaller extent, relative to the control output, than observed with acetylcholine).

Examination of the tissue stores revealed that lower concentrations of $[^3\text{H}]$ noradrenaline and $[^{14}\text{C}]$ glycine were present in the samples that had been preincubated with the active toxin. This was especially apparent for glycine; evidently the presence of the toxin interfered with the accumulation of the compounds by synaptosomes. In order to compensate for these discrepancies in the store size, the release data was recalculated as a percentage of the mean store size (which was the average of the tissue measurements made throughout the release period). Fig. 7 demonstrates that botulinum toxin did not inhibit the K^+ -evoked release of noradrenaline or glycine when this was considered as a percentage of the store size. Calculation of the $[^{14}\text{C}]$ acetylcholine release data in the same way (Table 1, column 8) confirmed that botulinum toxin inhibited acetylcholine release.

Total synaptosomal acetylcholine

Total acetylcholine was measured by a radio-enzymic procedure. The dilution factors involved in the extraction of the choline bases resulted in the assay of samples at the limit of sensitivity of the assay (50 pmol). Consequently, the determinations from a number of synaptosome preparations have been pooled (Table 3). There is no significant difference between the contents of acetylcholine in the low-K⁺ samples after incubation with and without botulinum toxin, and the high-K⁺ control samples exhibit no significant decrease in the total store size. However, when release was inhibited by preincubation with botulinum toxin, there was an increment in the tissue acetylcholine in the presence of 25 mM-KCl. These results show that botulinum toxin has no direct effect

Table 3. Total acetylcholine in synaptosomes

Synaptosomes were preincubated with boiled or active botulinum toxin, washed and incubated in medium containing the KCl concentration indicated for between 5 and 30 min. They were then isolated and assayed for acetylcholine by the radio-enzymic procedure. Values are the means $\pm$ s.e.m. of the numbers of determinations given in parentheses.

K ⁺ concn. (mM)	Acetylcholine (pmol/mg of protein)	
	Control	+Botulinum
3	316 $\pm$ 33 (16)	395 $\pm$ 66 (15)
25	284 $\pm$ 69 (10)	537 $\pm$ 77 (10)*

* Significantly different from the 3 mM-K⁺ control ($P < 0.01$, Student's *t* test, two-tailed).

on acetylcholine synthesis or its total store size in the isolated nerve terminal. However, it is plausible that the toxin influences the compartmentation of acetylcholine within the synaptosome, and such an effect might not be apparent when measuring total values. A preliminary attempt was made to investigate this possibility; after preincubation with [¹⁴C]choline and active or boiled botulinum toxin, a portion of the synaptosome suspension was removed, washed and resuspended in cold water containing eserine. This procedure was designed to rupture the synaptosomes, releasing the cytoplasmic pool of acetylcholine. The aqueous suspension was then centrifuged at a speed sufficient to sediment the ruptured membranes and synaptic vesicles, leaving the cytoplasmic acetylcholine (protected from acetylcholinesterase by the added eserine) in the supernatant fraction. The two pools (pellet and supernatant) were subsequently analysed for [¹⁴C]acetylcholine, [¹⁴C]choline and total acetylcholine (Table 4).

In the control condition, less than 25% of the [¹⁴C]acetylcholine and 10% of the [¹⁴C]choline was found in the pellet. Preincubation with botulinum toxin did not alter the distribution of either labelled or total acetylcholine, which confirms the inference from measurements of total synaptosomal acetylcholine that the toxin has no effect on the synthesis or storage of acetylcholine. The specific radioactivities of the different pools of acetylcholine (Table 4) were calculated from the measurements of [¹⁴C]acetylcholine and total acetylcholine made on duplicate extracts of a single sample, so that they could be directly related. The specific radioactivity of the cytoplasmic acetylcholine was almost 5 times that of the vesicular pool.

Table 4. Compartmentation of acetylcholine and [¹⁴C]choline

Synaptosomes were preincubated with boiled or active botulinum toxin and [¹⁴C]choline. They were then washed, hypo-osmotically shocked and centrifuged at 130000g for 60 min. The fractions were analysed for [¹⁴C]acetylcholine, [¹⁴C]choline and total acetylcholine. Values are the means $\pm$ s.e.m. (or range) of the numbers of determinations given in parentheses.

	Control			+Botulinum		
	(% of total)			(% of total)		
	Total	Pellet	Supernatant	Total	Pellet	Supernatant
[¹⁴ C]Choline (d.p.m./mg of protein)	5620 $\pm$ 1050	9.71 $\pm$ 1.93 (8)	90.3 $\pm$ 1.9 (8)	5840 $\pm$ 892	8.20 $\pm$ 0.60 (6)	91.8 $\pm$ 0. (6)
[¹⁴ C]Acetylcholine (d.p.m./mg of protein)	3870 $\pm$ 314	16.1 $\pm$ 3.3 (8)	83.9 $\pm$ 3.3 (8)	3340 $\pm$ 434	14.7 $\pm$ 2.3 (6)	85.3 $\pm$ 2.3 (6)
Total acetylcholine (pmol/mg of protein)	674 $\pm$ 178	57.6 $\pm$ 16.2 (2)	42.4 $\pm$ 10.2 (3)	599 $\pm$ 99	62.9 $\pm$ 11.5 (2)	36.7 (1)
Specific radioactivity of acetylcholine (d.p.m./pmol)	5.74 $\pm$ 1.76	1.08 $\pm$ 0.85	8.34 $\pm$ 1.21*	5.56 $\pm$ 1.38	2.22 $\pm$ 0.35	7.72*

* Significantly higher ($P < 0.001$, Student's *t* test, two-tailed) than values for the pellets.

Discussion

K⁺ concentrations of about 25 mM depolarize synaptosomes (Blaustein, 1975), and the release of acetylcholine would be expected as a result of this, and agrees with previous observations (Marchbanks, 1969a; De Belleruche & Bradford, 1972; Blaustein, 1975). The experimental system developed here uses radiolabelled acetylcholine to demonstrate the characteristics of the release process, because the ease of its detection enables more experimental flexibility. It is interesting that choline is not released by K⁺ depolarization, a result in agreement with the lack of choline release from electrically stimulated rat cortex reported by Richardson & Szerb (1974).

In agreement with the findings of Blaustein (1975) and De Belleruche & Bradford (1972) the release of [¹⁴C]acetylcholine was Ca²⁺-dependent. The dependency on Ca²⁺ is a point of similarity between transmitter release from synaptosomes and the classical physiological preparations. Along with other investigators (Blaustein, 1971) we have only been able to demonstrate a partial antagonism of release by high Mg²⁺ concentration.

Botulinum toxin at doses of 10⁵ mouse LD₅₀ caused 90% inhibition of the K⁺-stimulated release of [¹⁴C]acetylcholine. This large dose was used to minimize the latent period (Boroff *et al.*, 1974) and thus ensure the maximum inhibition with a minimum damage to the synaptosomes by an unduly prolonged preincubation. The effect of botulinum toxin on central-nervous-system synapses has not been unequivocally established. Although partial inhibition of acetylcholine release from cerebral-cortex slices has been reported (Molenaar & Polak, 1970), a pathological effect of botulinum toxin on the central nervous system has not been observed *in vivo*, presumably because the toxin does not cross the blood/brain barrier. Our results suggest that botulinum toxin in sufficient concentration at the terminal can completely inhibit the release of acetylcholine from cerebral-cortex synapses.

Synaptosomal concentration of K⁺ and occluded lactate dehydrogenase were unaffected by either K⁺ or botulinum toxin, so a general effect on membrane integrity can be excluded. The inhibition of release was not due to interference with the uptake of [¹⁴C]choline or the synthesis of [¹⁴C]acetylcholine (Fig. 5), so it must be concluded that the mechanism of release is directly affected. We take this as further evidence of the similarity of neurotransmitter release from synaptosomes to release from more intact systems, and therefore of the usefulness of the synaptosomal preparation as a model for transmitter-release mechanisms.

It is well established that release of acetylcholine increases acetylcholine synthesis (Richter & Marchbanks, 1971a) and it is noteworthy that the synthesis

was increased by K⁺ (Table 3), even though the toxin blocked the release. Since the release of [³H]noradrenaline and [¹⁴C]glycine was stimulated by K⁺ even in the presence of botulinum toxin, we can assume that the toxin does not affect the K⁺ depolarization of the terminals. It follows that the synthesis of acetylcholine must be stimulated by depolarization rather than by the consequent release of the transmitter. The mechanism of this coupling is clearly of interest from the point of view of the control of synthesis, and the action of botulinum toxin offers a useful way of distinguishing between the primary and secondary effects of depolarization.

Synaptosomes are heterogeneous with respect to the neurotransmitter. One of our hopes in investigating the effects of botulinum toxin on release was that it would block the release of transmitter from only the cholinergic fraction of the synaptosome population, thus imposing transmitter specificity on the experiments that we wish to perform. The toxin did not affect the K⁺-evoked release of [³H]noradrenaline or [¹⁴C]glycine and it therefore fulfils these, admittedly not very exhaustive, specificity tests. The consensus of results from studies *in vivo* is that botulinum toxin affects only cholinergic systems (Ambache, 1949, 1951; Vincenzi, 1967).

The compartmentation of both total and radioactive acetylcholine was examined before exposure to the depolarizing effects of K⁺. From these results (Table 4) some comment can be made about equating the pellet with the vesicle fraction. The [¹⁴C]choline content of the pellet might be cytoplasmic [¹⁴C]choline arising from supernatant trapped in the interstices of the pellet and from unruptured synaptosomes. The vesicular [¹⁴C]acetylcholine would thus be over estimated. Subtraction of the proportion of total [¹⁴C]choline in the pellet from the proportion of [¹⁴C]acetylcholine gives on average less than 10% as the proportion of total [¹⁴C]acetylcholine in the vesicles. This is the minimum limit that can be placed on the vesicular content of [¹⁴C]acetylcholine. Samples that were retained on ice for 40 min before centrifugation provided an estimate of the loss of vesicular acetylcholine due to leakage. [¹⁴C]Acetylcholine in the pellet was decreased by 10%, so a loss of 15% due to leakage is expected to have occurred during the 1 h centrifugation. This error approximately balances that attributable to cytoplasmic contamination, suggesting that the pellet and supernatant fractions provide reasonable estimates of the vesicular and cytoplasmic pools respectively. Therefore it is apparent that [¹⁴C]acetylcholine was predominantly located in the cytoplasm. This indicates that the recently synthesized, and therefore in this case highly labelled, acetylcholine is to be found principally in the cytoplasmic pool of the synaptosome, in agreement with other reports (Marchbanks, 1969b; Richter & Marchbanks, 1971b).

The disposition of the acetylcholine between vesicular or cytoplasmic stores was not affected by the toxin. The proposal that botulinum toxin exerts its effect by preventing vesicular storage of acetylcholine (see, e.g., Boroff *et al.*, 1974) therefore receives no support from our observations. We conclude that the toxin inhibits the process of release directly without affecting intermediate storage forms.

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