Biochemical evidence that acetylcholine release from cholinergic nerve terminals is mostly vesicular

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Received 17 May 1985

The nature of the intraterminal compartments from which acetylcholine (ACh) is released following presynaptic stimulation was investigated. This was pursued by examining the effects of the anticholinergic drug 2-(4-phenylpiperidino)cyclohexanol (AH5183) on the release of newly synthesized [^{3}H]ACh and of endogenous ACh from purified cholinergic nerve terminals (synaptosomes) which were isolated from the electric organs of *Torpedo*. Preincubation of the synaptosomes, with AH5183 (1-10 μM), does not affect either the intraterminal synthesis of [^{3}H]ACh or the uptake of its precursors, but results in a marked inhibition (85%) of the release of the newly synthesized [^{3}H]ACh. However, when AH5183 is added following the accumulation of [^{3}H]ACh in the nerve terminals, it does not affect [^{3}H]ACh release. AH5183 also has no effect on the release of preformed endogenous ACh. These findings, together with the previous in vitro demonstrations that AH5183 is a potent inhibitor of ACh uptake into isolated cholinergic vesicles, suggest that most of the synaptosomal ACh is secreted by a vesicular mechanism.

Acetylcholine *Cholinergic synaptic vesicle* *Synaptosome* Torpedo *AH5183*

1. INTRODUCTION

The vesicular hypothesis, which asserts that quantal release of ACh from cholinergic nerve terminals arises from the fusion of synaptic vesicles with the presynaptic membrane (review [1,2]), has been challenged by the cytoplasmic theory according to which the presynaptic cytosol is the immediate source of the released ACh [3–5]. To resolve this controversy, it is pertinent that the ratio of vesicular to non-vesicular release is determined directly. This can be achieved by examining the effects on presynaptic release of compounds which specifically block ACh uptake into cholinergic synaptic vesicles.

Previous studies suggest that the drug AH5183, which causes neuromuscular blockade [6], inhibits ACh release by interfering with the storage of newly synthesized ACh in cholinergic synaptic vesicles [7,8]. This assertion has recently been confirmed by Parsons and co-workers [9], who demonstrated that AH5183 is a potent inhibitor (IC_{50} = 40 nM) of ACh uptake into isolated *Torpedo* synaptic vesicles and by Howard and collaborators who demonstrated that AH5183 inhibits the loading of ACh into storage vesicles in PC12 cells [10] and blocks the evoked release of newly synthesized ACh from these cells [11].

Here, we investigated the subcellular source of the ACh which is released from isolated *Torpedo* cholinergic nerve terminals (synaptosomes) by examining the effects of AH5183 on the release of preformed and newly synthesized ACh. Our findings indicate that AH5183 blocks the secretion of most of the releasable ACh (85%) which accumulated in the nerve terminals following the addition of the drug, but does not inhibit the release of the ACh which was formed prior to its addition.

Abbreviations: ACh, acetylcholine; AH5183, 2-(4-phenylpiperidino)cyclohexanol

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2. EXPERIMENTAL

2.1. *Preparation and radiolabeling of* Torpedo *synaptosomes*

Torpedo ocellata were caught live off the coast of Tel Aviv during the autumn and winter and were maintained in seawater aquaria up to 2 months prior to use. Cholinergic synaptosomes (fraction a_2) were purified from the homogenates of fresh *Torpedo* electric organs as described [12]. The synaptosomes (1.5–2.0 mg protein/ml) were then diluted 2-fold with modified *Torpedo* buffer (modified TB: 250 mM NaCl, 4.8 mM KCl, 2.4 mM $MgCl_2$, 0.1 mM EGTA, 10 mM glucose, 260 mM sucrose, 1.2 mM Na phosphate buffer; pH 7.2) and incubated at 25°C for 1 h. Radiolabeling was initiated by the addition of choline (10 μM), acetate (50 μM) and [^{3}H]acetate (2 μCi/ml), followed by a 75 min incubation at 25°C. The amount of [^{3}H]acetate taken up by the nerve terminals was determined by filtration through GF/C filters as in [13]. The extent of intraterminal conversion of [^{3}H]acetate to [^{3}H]ACh was determined as follows: the radioactivity taken up by the synaptosomes was extracted from the GF/C filters with 3 ml of 0.1 M HCl (10 min at 90°C), after which it was lyophilized and resuspended in 0.5 ml of 0.2 M Na phosphate, pH 7.5. The [^{3}H]ACh content of the lyophilizate was determined by the assay of Johnson and Russell [14] which is based on determination of the partitioning of the radioactivity between an acidic buffer and a toluene-based scintillator prior to and following the addition of exogenous acetylcholinesterase. The yield of this procedure (>90%) was determined utilizing an [^{3}H]acetate-labeled [^{3}H]ACh standard. When the effect of AH5183 on [^{3}H]acetate uptake and [^{3}H]ACh synthesis was examined it was added 15 min before the radiolabeled precursor. Results presented are means ± SD of the indicated number of experiments.

2.2. *Measurements of synaptosomal release of acetylcholine and [^{3}H]acetylcholine*

Release experiments were performed at 25°C in a continuous flow apparatus [15] in which synaptosomes (0.5–0.7 mg protein) prelabeled with [^{3}H]acetate were loaded onto a GF/C filter (Whatman) mounted on a holder (25 mm diameter, 0.5 ml volume) and washed with 20 ml modified TB (flow rate 2 ml/min). A baseline was then established by flowing 5 ml modified TB (flow rate 1 ml/min), which were collected in 0.5 ml fractions. Release was induced by flowing 8 ml K^+-modified TB which contained 5 mM Ca^{2+}, after which the synaptosomes were lysed by flowing 3 ml water. The K^+ buffer was similar to modified TB, except that it contained 125 mM KCl and 125 mM NaCl. In all the experiments the synaptosomes were loaded onto the continuous flow apparatus 75 min after the initiation of radiolabeling and stimulated 15 min later.

The amounts of preformed endogenous ACh were measured by the chemiluminescence method developed by Israel and Lesbats [16], which is based on conversion of the choline, produced by hydrolysis of the released ACh, to betaine and H_2O_2 and detection of the latter by the luminol-peroxidase system. [^{3}]ACh release was monitored by liquid scintillation spectrometry. Control experiments in which an [^{3}H]acetate-labeled preparation was treated with the acetylcholinesterase inhibitor phospholine iodide (100 μM for 30 min) revealed that most of the released choline (>75%) and [^{3}H]acetate (>90%) moieties are secreted as ACh and [^{3}H]acetate-ACh, respectively. Since the contribution of newly formed [^{3}]ACh to the total ACh released is less than 1% [15], these measurements enable separate and parallel investigation of the effects of modulators on the release of endogenous ACh and radiolabeled [^{3}]ACh.

When the effect of AH5183 on release was examined it was added to the preparation either 105 min before stimulation (e.g. 15 min before the addition of [^{3}H]acetate) or 15 min prior to stimulation (e.g. 75 min after the initiation of radiolabeling) and was present throughout the release experiment.

The ACh and [^{3}H]ACh release data are presented as percent of total. The total was obtained by adding the amounts of synaptosomal neurotransmitter which were released by stimulation to those liberated subsequently by hypoosmotic lysis. Results are the means ± SD of the indicated number of experiments. Protein was determined according to Bradford [17].

3. RESULTS

Incubation of *Torpedo* synaptosomes with

[^{3}H]acetate (50 μM) and choline (10 μM) resulted in the accumulation of radioactivity within the nerve terminals (716 ± 162 pmol [^{3}H]acetate/mg protein following a 75 min incubation, $n = 5$) and in the intrasynaptosomal synthesis of [^{3}H]ACh (510 ± 102 pmol/mg protein, $n = 3$). K^+-depolarization of the labeled synaptosomes in the presence of Ca^{2+} (5 mM) resulted in a rapid ($t_{1/2} \simeq 1$ min) Ca^{2+}-dependent release of [^{3}H]ACh (107 ± 37 pmol/mg protein, $n = 5$) and ACh (13.6 ± 3 nmol/mg protein, $n = 5$) which corresponded respectively to 14.9 ± 5% of the accumulated radioactivity and 13.8 ± 3% of the endogenous synaptosomal ACh (fig.1).

Incubation of the synaptosomes with AH5183 (5 μM) for 15 min prior to the addition of [^{3}H]acetate and choline resulted in a marked reduction of [^{3}H]ACh release to 15 ± 4% of control ($n = 5$) but had no effect on the release of preformed endogenous ACh (98 ± 19% of control, $n = 4$) (fig.1). Similar inhibitions were observed when the synaptosomes were incubated with AH5183 (5 μM) for a longer duration (30 min) prior to the addition of [^{3}H]acetate, and when either 10 μM or 1 μM AH5183 was utilized.

By contrast, when AH5183 (10 μM) was added to prelabeled nerve terminals (75 min after the ad-

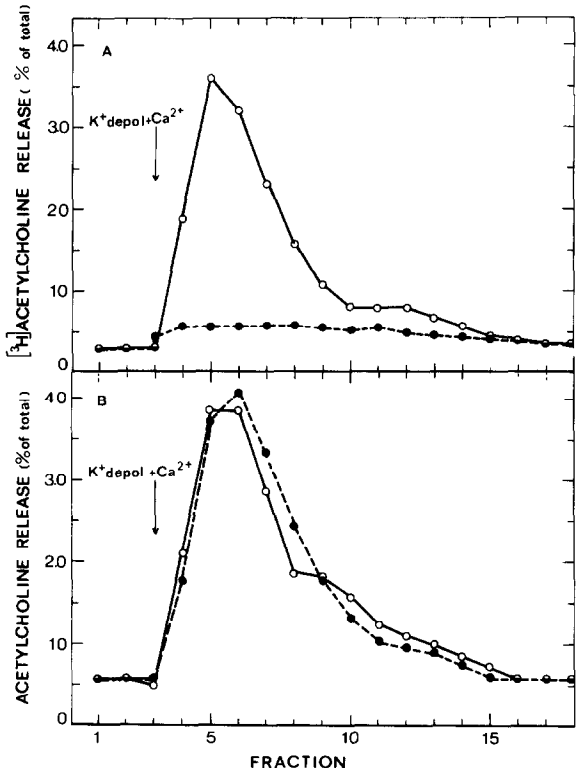

Fig.1. The effect of preincubation of purified *Torpedo* synaptosomes with AH5183 on the release of newly synthesized [^{3}H]ACh (A) and of preformed endogenous ACH (B). AH5183 (5 μM) was added to the synaptosomes 15 min prior to the addition [^{3}H]acetate (50 μM) and choline (10 μM). The synaptosomes were K^+ (125 mM) depolarized in the presence of Ca^{2+} (5 mM) 90 min after the addition of the radioactivity and the resulting [^{3}H]ACh and ACh release were measured as described in section 2. (○) Control; (●) synaptosomes preincubated with AH5183.

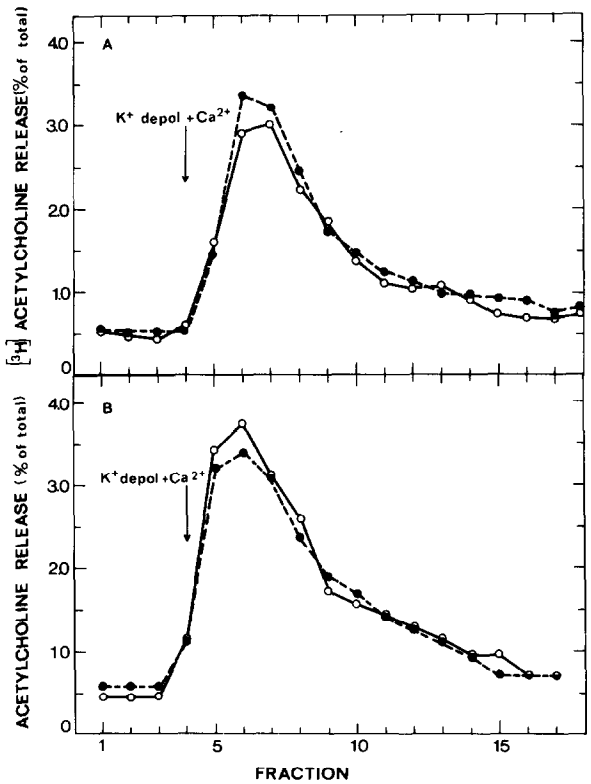

Fig.2. The effect of AH5183 on [^{3}H]ACh (A) and ACh (B) release from prelabeled *Torpedo* synaptosomes. The synaptosomes were labeled by incubation with [^{3}H]acetate (50 μM) and choline (10 μM) for 75 min after which they were loaded on to a continuous flow apparatus and stimulated at t = 90 min by K^+ (125 mM) depolarization in the presence of Ca^{2+} (5 mM) as described in section 2. AH5183 (10 μM) was added 75 min after the initiation of radiolabeling. (○) Control; (●) synaptosomes to which AH5183 was added.

Table 1

Effects of AH5183 on uptake of [^{3}H]acetate and synthesis of [^{3}H]acetylcholine by *Torpedo* synaptosomes

	Control (pmol/mg protein)	AH5183 (% of control)
[^{3}H]Acetate uptake	716 ± 162 (n = 5)	97 ± 8 (n = 3)
[^{3}H]Acetylcholine synthesis	510 ± 102 (n = 3)	106 ± 15 (n = 3)

[^{3}H]Acetate uptake and [^{3}H]acetylcholine synthesis following incubation (75 min at 25°C) of purified *Torpedo* synaptosomes with [^{3}H]acetate (50 μM) and choline (10 μM) were measured as described in section 2. AH5183 (5 μM) was added to the preparation 15 min prior to the initiation of uptake. Results presented are the means ± SD of the indicated number of experiments

dition of [^{3}H]acetate and 15 min prior to K^+ depolarization) it had no effect on the release of either [^{3}H]ACh (101 ± 14% of control, n = 4) or ACh (95 ± 5% of control, n = 3) (fig.2). Similar results were obtained when the effect of 20 μM AH5183 was examined and when the prelabeled preparation was incubated with AH5183 (1–10 μM) for a longer duration (30 min) prior to release (not shown).

The possibility that the selective inhibition of [^{3}H]ACh release induced by preincubation with AH5183 is due to a decrease in [^{3}H]acetate uptake and [^{3}H]ACh synthesis was examined by determining the effects of the drug on these processes. As shown in table 1, addition of AH5183 (5 μM) prior to the initiation of radiolabeling had no effect either on [^{3}H]acetate uptake or on its intrasynaptosomal conversion to [^{3}H]ACh (97 ± 8 and 106 ± 15% of the respective controls, n = 3).

4. DISCUSSION

Our findings show that preincubation of cholinergic nerve terminals with AH5183 inhibits the release of most of the [^{3}H]ACh which is synthesized subsequently (85%), but that release is unaffected when AH5183 is added following the accumulation of [^{3}H]ACh in the nerve terminal. The concentrations of AH5183 which were examined and found to have no effect on the release of preformed [^{3}H]ACh (1–20 μM) are up to 20-fold higher than those which inhibit release maximally when added prior to the accumulation of [^{3}H]ACh ($\geqslant$ 1 μM). This observation together with the finding that the release of preformed [^{3}H]ACh is unaffected even by prolonged incubations with AH5183 (30 min) imply that the observed time dependence of the effects of AH5183 on release is due to an intrinsic intraterminal process and not to the kinetics of the interaction of the drug with its target.

The known inhibitory effect of AH5183 on ACh uptake into isolated *Torpedo* synaptic vesicles [9] and the present finding that it does not affect either the intraterminal synthesis of ACh or the uptake of its precursors (table 1) suggest that AH5183 blocks synaptosomal release by inhibiting the translocation of ACh from the presynaptic cytosol into synaptic vesicles. Thus, most of the release (85%) is vesicular. The finding that AH5183 does not affect the release of preformed endogenous as well as radiolabeled ACh (figs.1B,2B), is consistent with this interpretation in that under these conditions the neurotransmitter has presumably been taken up by the vesicles prior to the addition of the drug.

The mechanisms underlying the release of the [^{3}H]ACh pool which is not inhibited by preincubation with AH5183 (15%) are not known. However, since it is not affected even by prolonged preincubation (up to 30 min) with high concentration (1–10 μM) of AH5183, it seems that this release is indeed mediated by ae different, presumably non-vesicular, mechanism. It is of interest to note that saturating levels of anticytoskeletal drugs also inhibit the release of about 80% of the releasable [^{3}H]ACh [18]. It is therefore tempting to suggest that cytoskeleton-disrupting drugs affect vesicular but not non-vesicular release.

The experimental approach which was undertaken here can be extended for the study of the rate of translocation of cytosolic ACh into synaptic vesicles. Indeed, preliminary findings (in preparation) which were obtained by examining the kinetics of the effects of AH5183 on *Torpedo* synaptosomes suggest that the transport of [^{3}H]ACh from the presynaptic cytosol into synaptic vesicles is relatively slow and lags behind its synthesis.

ACKNOWLEDGEMENTS

We thank Dr D.E. Bays of Glaxo Group Research Ltd for the gift of AH5183. This work was supported in part by the Familial Dysautonomia Foundation, The US-Israel Binational Science Foundation (BSF Grant no. 2410) and the Fund for Basic Research sponsored by the Israel Academy of Sciences and Humanities.

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