Differential inhibition of the release of endogenous and newly synthesized acetylcholine from *Torpedo* synaptosomes by presynaptic muscarinic receptors

S. Luz, I. Pinchasi and D.M. Michaelson*

Department of Biochemistry, George S. Wise Faculty of Lifes Sciences, Tel-Aviv University, Ramat Aviv 69978, Israel

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Activation of *Torpedo* presynaptic muscarinic acetylcholine (ACh) receptors with the agonist oxotremorine (20 μM) results in the inhibition of Ca^{2+}-dependent release of endogenous ACh from *Torpedo* synaptosomes. This effect is reversed by the muscarinic antagonist atropine (1 μM) which, by itself, has no effect. In contrast, under the same conditions the amount of newly synthesized radiolabeled [^{3}H]ACh released is not affected by muscarinic ligands. These findings suggest that presynaptic muscarinic inhibition in the *Torpedo* is due to interference with the mobilization of ACh from a storage pool.

Torpedo *Acetylcholine* *Synaptosome* *Muscarinic acetylcholine receptor* *Presynaptic inhibition*

1. INTRODUCTION

Acetylcholine (ACh) release from *Torpedo* cholinergic nerve terminals is regulated by presynaptic muscarinic ACh receptors (AChR) [1–3], which inhibit release by interfering with the coupling, within the nerve terminal, between the increased cytoplasmic Ca^{2+} and ACh secretion [2,4]. The nature of the intraterminal event that is blocked by muscarinic agonists is not known.

In the nerve terminal ACh is present in several compartments which are defined according to their subcellular localization (e.g., vesicular and cytoplasmic ACh) and metabolic state. In the *Torpedo* [3] and numerous other cholinergic preparations [5–8] newly formed ACh is released preferentially following stimulation of the nerve terminal, implying that the newly synthesized and endogenous ACh are compartmentalized within the nerve terminal.

We here examined the possibility that muscarinic activation has a differential effect on the synaptosomal release of newly synthesized and of endogenous ACh. The experimental approach was to radiolabel the newly formed ACh and then measure the effect of muscarinic ligands on the release of the endogenous ACh, which was assayed chemically, and of the radiolabeled newly synthesized ACh. The results obtained indicate that under the conditions employed, muscarinic agonists inhibit the release of endogenous ACh but have no detectable effect on the amount of newly synthesized ACh released.

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 sea water aquaria up to 3 months prior to use. Cholinergic synaptosomes (fraction a_2) were purified from the homogenate of fresh *Torpedo* electric organs by differential and density gradient centrifugation as in [9]. The synaptosomes (1.2–2.4 mg protein/ml) were

* To whom correspondence should be addressed

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diluted 2-fold with modified *Torpedo* Krebs Ringer (TKR) (250 mM NaCl; 4.8 mM KCl; 2.4 mM $MgCl_2$; 0.1 mM EGTA; 10 mM glucose; 260 mM sucrose; 20 mM HEPES, pH 7.2) which contained 10 μM choline, 10 μM acetate and either [^{3}H]choline (1 μCi/ml) or [^{3}H]acetate (1 μCi/ml). The synaptosomes were then incubated at 25°C for 2 h and subsequently transferred to 4°C for up to 3 h prior to use. Control experiments revealed that the profiles of ACh and [^{3}H]ACh release were not affected by storage of the synaptosomes at 4°C for up to 3 h. Protein was determined as in [10].

2.2. *Measurement of synaptosomal release and of newly synthesized radiolabeled acetylcholine*

Release experiments were performed in a continuous flow apparatus [11] in which the synaptosomes (0.3–0.7 mg protein) were loaded onto a GF/C filter (Whatman) mounted on a holder (25 mm diameter; 0.5 ml volume) and washed with 20 ml of modified TKR. A baseline was then established by flowing 3 ml of modified TKR (at a flow rate of 1 ml/min), which were collected as 0.5 ml fractions. Release was induced by flowing 3 ml of a K^+ buffer which contained 2 mM Ca^{2+} followed by 3 ml of modified TKR. The K^+ buffer was similar to the modified TKR, except that it contained 125 mM KCl and 130 mM NaCl. When the effect of muscarinic ligands was examined, they were added 2 min (atropine) and 1 min (oxotremorine) prior to K^+ depolarization.

The levels of endogenous ACh release were measured by the chemiluminescence method in [12], 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. Release of newly synthesized radiolabeled ACh was monitored by liquid scintillation spectrometry. In several experiments, the synaptosomal acetylcholinesterase (AChE) was inhibited with phospholine iodide (100 μM for 30 min). The amount of endogenous ACh liberated under these conditions was analyzed by monitoring the increase in the choline content of the eluted fractions following the addition of exogenous active AChE [12]. The amount of [^{3}H]acetate-ACh released was determined by examining the effect of the added AChE on the extraction of the eluted radioactivity from an acidic buffer to an organic phase [13].

3. RESULTS

Incubation of *Torpedo* synaptosomes with [^{3}H]choline or [^{3}H]acetate resulted in their accumulation within the nerve terminal (0.7 ± 0.08 nmol [^{3}H]choline and 1.2 ± 0.14 nmol [^{3}H]acetate/mg protein following a 2-h incubation; N = 6) and, as was previously shown, in their conversion to [^{3}H]ACh [11,14]. K^+ depolarization in the presence of Ca^{2+} of [^{3}H]choline-prelabeled synaptosomes resulted in liberation of choline (13 ± 7 nmol/mg protein; N = 18) and [^{3}H]choline (45 ± 15 pmol/mg protein, N = 18) (fig. 1). K^+ depolarization in the absence of Ca^{2+} and with Ca^{2+} by itself had no effect. Similar results were obtained with [^{3}H]acetate-labeled synaptosomes (22 ± 11 pmol [^{3}H]acetate released/mg protein; N = 7). Pretreatment of [^{3}H]acetate-labeled preparation with phospholine iodide (an inhibitor of AChE) resulted in the recovery of most of the released choline (> 75%) and [^{3}H]acetate (> 90%) moeities as ACh and [^{3}H]acetate-ACh, respectively. The amount of radioactive material released was calculated based on the specific activity of the

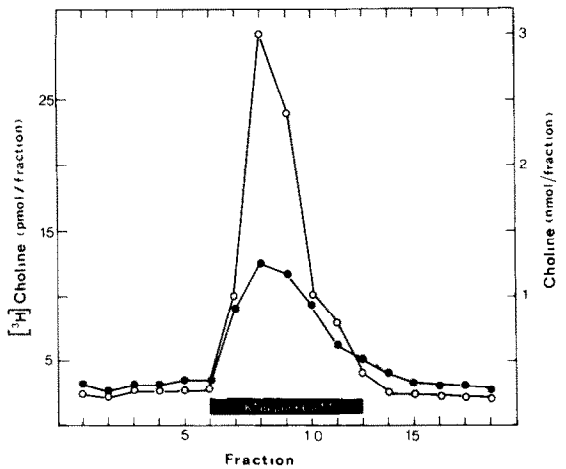

Fig. 1. Ca^{2+}-dependent K^+-depolarization-mediated release of endogenous synaptosomal choline (○) and [^{3}H]choline (●). Prelabeled *Torpedo* synaptosomes (0.7 mg protein) were loaded onto a continuous flow apparatus, stimulated by KCl (125 mM) in the presence of Ca^{2+} (2 mM), and the choline and [^{3}H]choline released were collected and assayed as described in section 2.

externally added precursors and therefore corresponds only to the newly formed [^{3}H]ACh synthesized from these precursors during the preincubation, and not to the newly synthesized ACh which was formed from endogenous precursors. The contribution of the newly formed [^{3}H]ACh to that of the total ACh release is 0.35 ± 0.2% (N = 18) (note different scales in fig. 1), thus enabling a separate and parallel investigation of the effects of modulators of release on these ACh compartments.

Activation of the presynaptic muscarinic AChR with the agonist oxotremorine (20 μM) caused a marked diminution in the amount of endogenous ACh released (54 ± 13% of control; N = 5). This effect was completely reversed by the muscarinic antagonist atropine (1 μM) which, by itself, had no effect (fig. 2A). It should be noted that the potency of oxotremorine varies throughout the year so that during the winter 10 μM caused maximal inhibition (>90%) of release whereas during the spring 50 μM of oxotremorine had only a partial effect. The reason for this seasonal variation is not yet known.

In contrast to the above findings, oxotremorine (up to 100 μM) and atropine (up to 10 μM), under the same conditions, had no detectable effect on the amount of newly synthesized [^{3}H]ACh released (fig. 2B). The amount of [^{3}H]ACh released in the presence of oxotremorine (20 μM) and atropine (1 μM) was, respectively 90 ± 7% (N = 5) and 100 ± 10% (N = 3) of control.

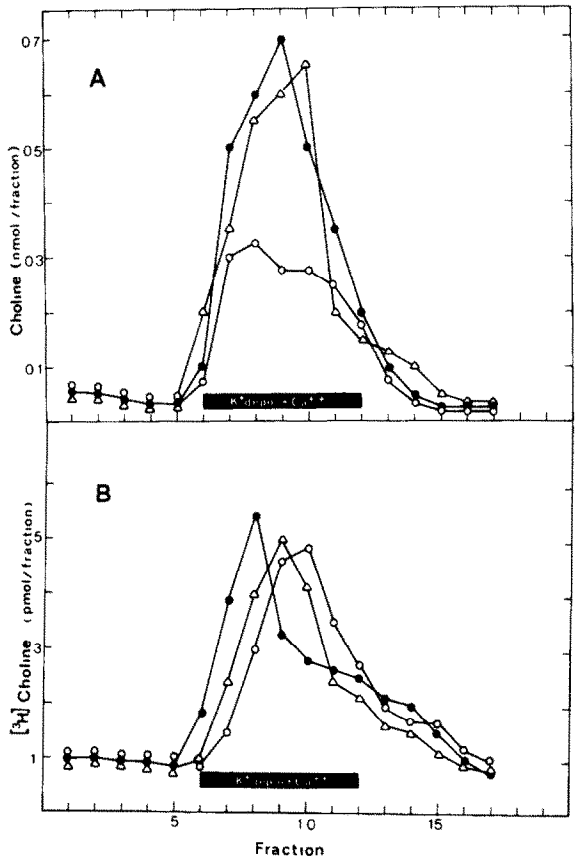

Fig. 2. The effect of oxotremorine and of exotremorine plus atropine on Ca^{2+}-dependent K^+-depolarization-mediated release of endogenous (A), and radiolabelled (B) synaptosomal choline. The experiment was performed as in fig. 1, except that the amount of synaptosomes used was 0.3 mg protein. (●,○,△) K^+-depolarization in the presence of Ca^{2+}, Ca^{2+} and oxotremorine (20 μM), and Ca^{2+} with oxotremorine (20 μM) and atropine (1 μM), respectively.

4. DISCUSSION

We here report that activation of presynaptic muscarinic AChR inhibits the release, from *Torpedo* synaptosomes, of endogenous ACh but not of newly synthesized [^{3}H]ACh. There seems to be a small delay in the release of [^{3}H]ACh, which is caused by oxotremorine and reversed by atropine (fig. 2B). Further studies with finer kinetic resolution are required in order to delineate this possibility.

Here, the newly formed [^{3}H]ACh was synthesized within 2 h after the introduction of the labeled precursors. In [3] it was shown that incubation of electric organ tissue blocks with [^{3}H]precursor for 6 h at 25°C, followed by an overnight incubation at 7°C without precursors results in [^{3}H]ACh release which is inhibited by oxotremorine. This difference may be due to the synaptosomal preparation and assaying conditions. Alternatively, it may suggest that, in *Torpedo*, the time of transfer of the newly synthesized [^{3}H]ACh to the muscarinic-sensitive pool is greater than 2 h and less than 18 h.

It is not yet known how the varied response of the endogenous and newly formed ACh pools to muscarinic ligands relates to the well-known ACh compartments, namely, the synaptic vesicles and

presynaptic cytoplasm. Most of the non-labeled endogenous ACh is in the bound pool [15,16] and is presumably vesicular. It is therefore tempting to suggest that the synaptic vesicles, in particular fraction VP_1, which is not readily labeled with newly synthesized [^{3}H]ACh [17], are the target of the muscarinic agonists, which presumably interfere with their mobilization from a storage pool to a readily available state. Since muscarinic inhibition in the *Torpedo* seems to be mediated by prostaglandins [18], it will be of interest to study the possible involvement of prostaglandins in synaptic vesicle mobilization.

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REFERENCES

[1] Kloog, Y., Michaelson, D.M. and Sokolovsky, M. (1978) FEBS Lett. 95, 331-334.
[2] Michaelson, D.M., Avissar, S., Kloog, Y. and Sokolovsky, M. (1979) Proc. Natl. Acad. Sci. USA 76, 6336-6340.
[3] Dunant, Y. and Walker, A.I. (1982) Eur. J. Pharmacol. 78, 201-212.
[4] Michaelson, D.M., Avissar, S., Ophir, I., Pinchasi, I., Angel, I., Kloog, Y. and Sokolovsky, M. (1980) J. Physiol. 76, 505-514.
[5] Collier, B. (1969) J. Physiol. 205, 341-352.
[6] Potter, L.T. (1970) J. Physiol. 206, 145-166.
[7] Molenaar, P.C. and Polak, R.L. (1975) in: Metabolic Compartmentation and Neurotransmission (Berl, S. et al., eds) pp. 641-649.
[8] Charkin, L.W., Marchbanks, R.M., Mitchell, J.F. and Whittaker, V.P. (1972) J. Neurochem. 19, 2727-2736.
[9] Michaelson, D.M. and Sokolovsky, M. (1978) J. Neurochem. 30, 217-230.
[10] Bradford, M. (1976) Anal. Biochem. 72, 248-254.
[11] Morel, N., Israel, M., Manaranche, R. and Lesbats, B. (1979) in: Progress in Brain Research (Tucek, S. ed) vol. 49, pp. 191-202.
[12] Israel, M. and Lesbats, B. (1981) Neurochem. International 3, 81-90.
[13] Johnson, C.D. and Russell, R.L. (1978) Anal. Biochem. 621, 229-238.
[14] Morel, N., Israel, M., Manaranche, R. and Mastour-Frachon, P. (1977) J. Cell Biol. 75, 43-55.
[15] Marchbanks, R.M. and Israel, M. (1971) J. Neurochem. 18, 439-448.
[16] Weiler, M., Roed, I.S. and Whittaker, V.P. (1982) J. Neurochem. 38, 1187-1191.
[17] Zimmerman, H. (1979) in: Progress in Brain Research (Tucek, S. ed) vol. 49, pp. 141-151.
[18] Pinchasi, I., Shanietzki, B., Schwartzmann, M., Raz, A. and Michaelson, D.M. (1982) in: Presynaptic Receptors Mechanism and Function (deBelleroche, J. ed) pp. 114-129. Ellis Horwood, Chichester.