IS PRESYNAPTIC ACETYLCHOLINE RELEASE ACCOMPANIED BY THE SECRETION OF THE SYNAPTIC VESICLES CONTENTS?

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Received 25 January 1978

1. Introduction

The subcellular origin of the acetylcholine (Ach) released upon presynaptic stimulation is not yet known. This question is being investigated by an examination of the synaptosomal constituents which are released following stimulation. Purely cholinergic synaptic vesicles [1–3] and synaptosomes [4–7] may be prepared from the electric organ of *Torpedo*. The *Torpedo* synaptosomes release Ach by K^+ depolarization in the presence of Ca^{2+} and manifest an ionophore-mediated Ca^{2+}-dependent Ach release [6,7]. *Torpedo* synaptic vesicles contain Ach as well as ATP, the latter is present at a concentration of about 1 molecule/5 Ach molecules [8].

The possibility that the synaptic vesicle contents are being secreted during presynaptic release was examined by measuring the extrasynaptosomal levels of adenosine nucleotides following Ca^{2+}-dependent Ach secretion from the *Torpedo* synaptosomes. It was found that Ach release is not accompanied by the release of adenosine nucleotides, thus implying that the synaptic vesicles contents are not fully secreted during presynaptic release.

2. Experimental

Torpedo synaptosomes were prepared as in [6,7]. Prior to the measurement of Ach release, the synaptosomes were pelleted by centrifugation at 35 000 × *g* for 30 min, then resuspended in 1.2 mM phosphate buffer, pH 6.9, which contained 250 mM NaCl, 5.3 mM KCl, 10 mM glucose, 2.8 mM $MgCl_2$, 266 mM glycine, 10 μM EGTA and 100 μM phospholine iodide, an acetylcholinesterase inhibitor. The synaptosomes were incubated at 4°C for 20 min followed by 10 min at 25°C. When the effect of Ca^{2+} was being investigated, 5 μl 0.4 M $CaCl_2$ were added per ml synaptosomes and the sample was incubated for an additional 5 min at 25°C. Control samples were similarly incubated except that no Ca^{2+} was added. The synaptosomes were then cooled to 4°C and pelleted by a 10 min centrifugation at 35 000 × *g*. The supernatants were collected and assayed for Ach [6] and for ATP, ADP and AMP [9]. In experiments employing the ionophore A23187, 10 μl stock solution (1 mg/ml in ethanol) were added per ml synaptosomes 10 min prior to the addition of Ca^{2+}. The effect of K^+ was examined by a 2-fold dilution of the synaptosomes with a high K^+ buffer, with or without Ca^{2+}. The high K^+ buffer contained 330 ml KCl, 10 mM glucose, 2.8 mM $MgCl_2$, 10 μM EGTA, 100 μM phospholine iodide and 1.2 mM phosphate buffer, at pH 6.9.

3. Results and discussion

We examine here the levels of external adenosine nucleotides following Ca^{2+}-dependent Ach release from *Torpedo* synaptosomes. To minimize nucleotide hydrolysis, the synaptosomes were rapidly centrifuged following stimulation; this pelleted about 90% of the synaptosomes. The extent of nucleotide hydrolysis was estimated by adding adenosine nucleotides to the synaptosomes during the release experiments, at a concentration similar to that of the released Ach. It

was found that about 85% added ATP, 70% added ADP and 50% added AMP were recovered in the supernatant as adenosine nucleotides. The remainders were recovered as adenosine and other catabolites. Thus, had the vesicular ATP been released from the synaptosomes, it would have been detected. The addition of ATP, tritium labeled on the adenosine moiety, revealed that following incubation with synaptosomes, all the breakdown products were recovered in the supernatant.

As shown in fig.1, the addition of Ca^{2+} by itself to the synaptosomes induces some Ach release which was further enhanced either by preincubation with the ionophore A23187 or by K^+ depolarization in the presence of Ca^{2+}. It should be noted that at shorter incubation times, Ca^{2+} does not induce Ach release [7]. Interestingly, Ca^{2+} did not induce the release of either ATP, ADP or AMP (fig.1). Taking into consideration the limits of detection, we may infer that if there were any ATP or ADP released, their ratios to that of Ach would have been less than 1–10. Similarly, AMP could not have been released at levels higher than 1–7 Ach molecules.

The possibility that lower nucleotide levels were released, which were not detectable by the above technique, was also examined. The synaptosomes were loaded onto a glass-bead column packed on top

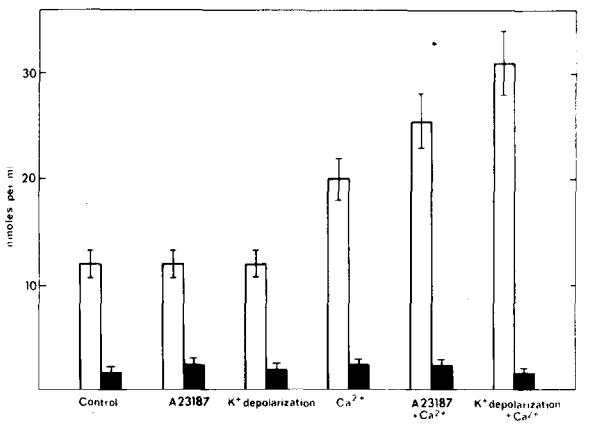

Fig.1. Ca^{2+}-dependent Ach and adenosine nucleotides release from *Torpedo* synaptosomes. The white areas represent the concentration of Ach recovered in the supernatant following centrifugation of the stimulated synaptosomes. The black areas similarly represent the concentration of adenosine nucleotides.

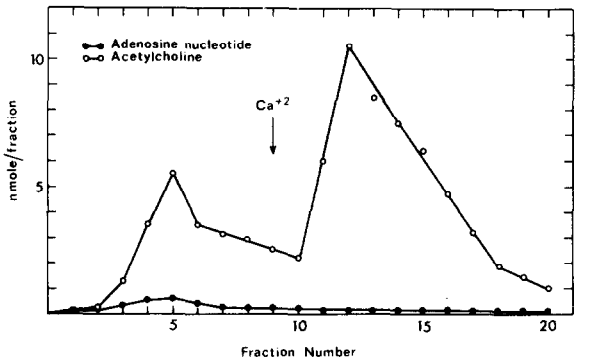

Fig.2. Ca^{2+}-dependent A23187-mediated release of Ach and adenosine nucleotides from *Torpedo* synaptosomes adsorbed to glass beads. One ml of synaptosomes (containing 100 nmol Ach) were loaded onto a 2 ml column which contained controlled pore glass beads (Sigma, CPGB2500 -200) packed on top of a 0.45 μm millipore filter. The column was then washed with 10 ml 1.2 mM phosphate buffer, pH 6.9, which contained 250 mM NaCl, 5.3 mM KCl, 10 mM glucose, 2.8 mM $MgCl_2$, 266 mM glycine, 10 μM EGTA, 100 μM phospholine iodide and 10 μg/ml A23187. Followed by a similar buffer which contained in addition 2 mM $CaCl_2$. One ml fractions were collected at a flow rate of 1 ml/min. The fractions were then assayed for Ach, and adenosine nucleotides.

of a 0.45 μm millipore filter. The column was then washed with buffer which contained the ionophore A23187, followed by a similar buffer which contained in addition 2 mM Ca^{2+}. Most of the synaptosomes adhered to the column. A small fraction, probably the smaller synaptosomes, did not adsorb onto the column and was thus collected in the first fractions (fig.2). The addition of Ca^{2+} to the ionophore-containing elution buffer induced the release of Ach in an amount similar to that released from free synaptosomes in solution. As shown in fig.2, Ca^{2+} did not induce the release of any adenosine nucleotides. Similar results were obtained when the synaptosomes were stimulated by K^+ depolarization in the presence of Ca^{2+} (data not shown). The brief exposure of the synaptosomes to the elution buffer resulted in a high recovery of added ATP, ADP and AMP as adenosine nucleotides, following their passage through the column. Consequently, we may infer that if there were any nucleotides released with Ach, their amount would have been less than 1 molecule/20 Ach molecules. Direct examination of the extrasynaptosomal ATP following stimulation, by the luciferin-luciferase method [10], confirmed the above conclusion.

The results reported here agree with [10,11] where following presynaptic stimulation of innervated fragments of the electric organ, ATP was released post-synaptically. However, with another preparation, the neuromuscular junction, an association has been observed between Ach secretion and the release of adenosine nucleotides [12]. A depolarization-induced release of ATP from the chemically heterogenous rat brain synaptosomes has been demonstrated [13]. The difference between *Torpedo* and rat synaptosomes may suggest that in the brain ATP is released as a neurotransmitter in its own right.

Our observation is that although the synaptic vesicles contain ATP, none is released from the synaptosomes following stimulation. This suggests that the vesicle contents are not fully secreted during presynaptic release. Thus, this process differs from hormone secretion, where it has been shown that ATP and other vesicular contents are co-released with the hormone and in a stochiometry similar to that present in the secretory vesicles [14,15].

It is not yet clear whether Ach is released from the presynaptic cytoplasm by a gating mechanism, or whether it is released directly from the synaptic vesicles by a selective process which does not permit the secretion of the vesicular ATP. Preliminary electron microscopic observations (to be published) indicate that parallel to the synaptosomal Ach release, fusion occurs between the synaptic vesicles and the presynaptic membrane. Thus, it it likely that the synaptic vesicles release their ATP and/or its breakdown products into the nerve terminal cytoplasm. The vesicular Ach is either released into the presynaptic cytoplasm or secreted directly into the external medium.

Acknowledgements

I thank Professor M. Sokolovsky for his support and valuable discussion and M. Moldovan for excellent technical assistance. This work was supported in part by the US-Israel Binational Science Foundation (BSF grant number 1035).

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