Vol. 80, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 14, 1978 Pages 547-552

FACTORS REQUIRED FOR CALCIUM DEPENDENT ACETYLCHOLINE RELEASE FROM ISOLATED TORPEDO SYNAPTIC VESICLES

Daniel M. Michaelson, Irit Pinchasi and Mordechai Sokolovsky

The George S. Wise Center for Life Sciences

Department of Biochemistry, Tel-Aviv University

Tel-Aviv, Israel

Received November 7,1977

The release of acetylcholine (Ach) from Torpedo synaptic vesicles has been investigated. Factors have been found which induce Ca^{+2} dependent Ach release from the synaptic vesicles. In the absence of these factors, the vesicles are not affected by Ca^{+2}. Addition of a soluble factor to the vesicles induces a Ca^{+2}-dependent release of their Ach. This secretion is enhanced by a non-vesicular membranous component which, by itself, does not induce the Ca^{+2}-dependent release. These results demonstrate that vesicular Ach release may be studied in vitro and thus will enable the study, at the molecular level, of the biochemical events underlying neurotransmission.

Cholinergic neurotransmission has been studied very extensively. However, little is known about the biochemical mechanisms underlying acetylcholine (Ach) release. The quantal nature of Ach release shown by electrophysiological studies (1,2) strongly suggests that the synaptic vesicles, which reside in the presynaptic cytoplasm and contain the neurotransmitter, play a physiological role in this process.

It has been shown that Ca^{+2} is essential for Ach release (3). Furthermore, Miledi (4) has shown that Ach release may be induced by direct intracellular injection of Ca^{+2}. The mechanism by which elevated cytoplasmic levels of Ca^{+2} trigger this process is not yet known. Since synaptic vesicles seem to be involved in Ach release, it is of great interest to investigate whether in vitro, Ca^{+2} may induce Ach release from synaptic vesicles and whether other subcellular components may affect this process.

The electric organ of the elasmobranch Torpedo is extensively

---

Abbreviations: Ach, Acetylcholine; AchE, Acetylcholinesterase; EGTA, Ethyleneglycol-bis-(β-Aminoethyl ether)N,N'-Tetraacetic acid.

0006-291X/78/0803-0547$01.00/0

innervated with purely cholinergic synapses, and is thus very suitable for molecular investigations of synaptic transmission. Highly purified synaptic vesicles can be prepared from the electric organ (5,6,7). This is the only vesicle preparation available, which contains only one type of neutrotransmitter, and is thus most fitting for the investigation of the biochemistry of presynaptic release.

In the present study we investigated the Ca^{+2}-dependent release of Ach from Torpedo synaptic vesicles. It is shown that Ca^{+2}-dependent Ach release may be induced by the addition of non-vesicular factors to the synaptic vesicles.

EXPERIMENTAL

Preparation of synaptic vesicles and subcellular fractions. Torpedo ocellata were caught alive off the coast of Tel-Aviv and maintained in sea water aquaria up to six months prior to use. While in captivity the Torpedos were fed with live Blenny which they readily devoured.

Subcellular fractions of the Torpedo electric organ were prepared as previously described (8,9). Crude synaptic vesicles were obtained by centrifugation of S_2 at 240,000g for 1 hr. The supernatant (S_3) was kept and used later in the Ach release experiments. The pellet (P_3), which constituted the crude synaptic vesicles, was resuspended in 0.8M glycine. Further purification of the P_3 synaptic vesicles was carried out by discontinuous sucrose density gradient centrifugation (to be published). The purified synaptic vesicles (I_2) band between the 0.2 and 0.4M sucrose layers. This fraction (I_2) contains virtually all the P_3-derived Ach and ATP (800 nmoles Ach/mg protein, the ratio of Ach to ATP was 2±1), and was shown by electron microscopy to be highly enriched in synaptic vesicles.

Assays. Ach was extracted by standard procedures (10,11) and assayed with the guinea pig ileum bioassay (12). ATP was determined by the luciferin luciferaze method employing a procedure similar to that of Holmsen et al (13). The photomultiplier case and sample holder were built according to Hastings and Weber (14). Protein was determined according to Lowry et al (15) and Acetylcholinesterase (AchE) was assayed according to Ellman et al (16).

Measurement of Ach release from synaptic vesicles. The crude (P_3) and purified (I_2) synaptic vesicles contain a small fraction of the total AchE activity. Thus, upon release of the vesicular Ach to the external media, it is rapidly hydrolysed. Furthermore, since under the conditions of our experiments there was no detectable synthesis of Ach (results not shown), the Ach retained in a synaptic vesicles suspension is only that which has not been released. The kinetics of vesicular Ach release were monitored by withdrawing samples at different time intervals after stimulation and measuring their Ach content. The experiments were performed in the following manner: synaptic vesicles (1.5-3 nmoles Ach/ml) were preincubated with the appropriate subcellular fractions for 5 min at 25°C. The incubation buffer was 5 mM Tris pH 7.4 which contained 30 mM NaCl, 30 mM KCl, 0.65 mM $MgCl_2$, 0.2 mM EGTA and 730 mM glycine. Ach release was induced by the addition of $CaCl_2$ and/or ATP. The concentration of free Ca^{+2} in the reaction mixture which contained 0.2 mM EGTA and 0.65 mM $MgCl_2$, as well as endogenous Ca^{+2} and Mg^{+2} present in the subcellular fractions, was calculated by the method of Portzehl et al (17).

RESULTS

Ca^{+2}, at concentrations up to 1 mM, did not induce Ach release either from the purified (I_2) or from the crude (P_3) synaptic vesicles. However, ATP did cause some Ach release, the extent of which varied in different preparations. On the average, ATP induced the release of 10% of the Ach contained in the purified synaptic vesicles and 25% of that contained in the crude vesicles. This effect was concentration dependent: it was half maximal at about 0.5 mM ATP, and reached a plateau at 1 mM ATP. At all ATP concentrations the release was immediate and was completed within less than 30 sec. A similar pattern was observed in the presence of both ATP and Ca^{+2}.

Since Ca^{+2} had no effect on the synaptic vesicles, we examined the possibility that non-vesicular factors are required for the Ca^{+2}-dependent release. Figure 1 illustrates the effect of Ca^{+2} on the release of Ach from crude synaptic vesicles preincubated with the soluble fraction S_3. In the presence of S_3, Ca^{+2} induced a gradual release of virtually all the vesicular Ach ($t_{\frac{1}{2}} \sim 4$ min.). This release was half maximal at about 50 μM Ca^{+2}, and its extent was a function of the concentration of S_3 (Fig. 2); Maximal effect was obtained at about 35 μg S_3 protein per ml. S_3 by itself did not induce any Ach release. The effect of ATP on synaptic vesicles preincubated with S_3, was similar to that observed with the vesicles only. Similarly to what was shown for Ach release from Torpedo synaptosomes (9), Sr^{+2} (0.2 mM) and Ba^{+2} (0.4 mM) can replace Ca^{+2} in evoking vesicular Ach release.

The releasing effect of Ca^{+2} did not depend on the addition of external ATP (up to 0.3 mM). On the contrary, when Ca^{+2} (150 μM) was added together with nmolar levels of ATP, its releasing effect was greatly diminished. However since S_3 contained intrinsic ATP (about 3×10^{-5}M in the reaction mixture) the Ach release induced by Ca^{+2} in the absence of added ATP could still be dependent on the ATP present in S_3. To examine this possibility the concentration of ATP in S_3 was reduced by prior treatment with apyrase. The releasing effect of Ca^{+2} was unaltered after this treatment, even though the concentration of free ATP in the reaction mixture was less than 5×10^{-8}M.

S_3 was inactivated by either boiling for 20 min. or by dialysis. The dialysed S_3 could not be reactivated by the addition of either ATP (0.4 mM), or GTP (0.1 mM) or cAMP (0.1 mM) or cGMP (0.033 mM).

The possibility that factors other than S_3 are required for Ach release, was examined by comparing the extent of the S_3-metiated Ca^{+2}-

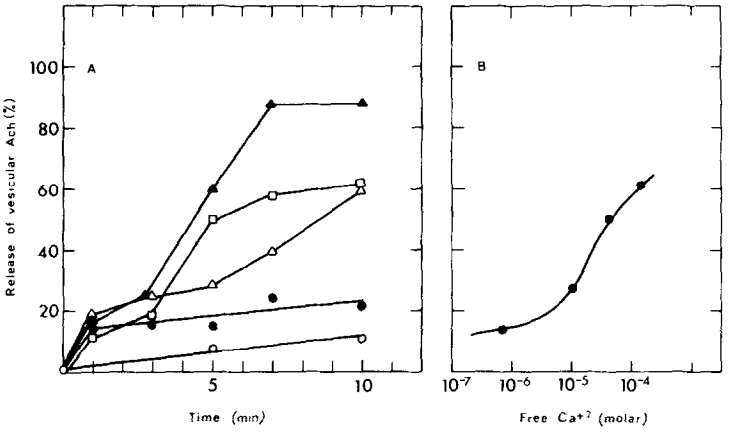

Fig. 1. A. Ca^{+2}-dependent Ach release from crude synaptic vesicles (P_3) in the presence of the soluble component S_3. The reaction mixture contained 0.1 mg protein/ml of P_3, 35 µg protein/ml of S_3, and the following free Ca^{+} concentrations: no Ca^{+} (o-o); 0.6 µM (●-●); 10 µM (Δ-Δ), 50 µM (□-□) and 150 µM (▲-▲). B. Dose-response curve of the Ca^{+2}-induced Ach release. The points shows are taken from Fig. 1A, 5 min. after the addition of Ca^{+2}.

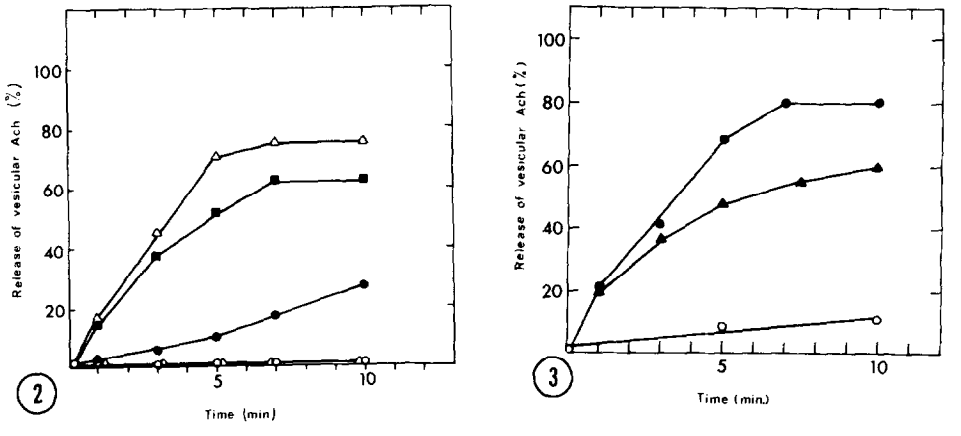

Fig. 2. The effect of various concentrations of S_3 on the Ca^{+2}-stimulated Ach release from crude synaptic vesicles (P_3). 0.1 mg protein/ml of P_3 were preincubated for 5 min. with the following concentrations of S_3: no S_3 (o-o); 0.2 (Δ-Δ); 4 (●-●); 12 (■-■) and 36 (▲-▲) µg protein/ml. Ach-release was then induced by the addition of 150 µM free Ca^{+2} ions at t=0.

Fig. 3. A comparison of the Ca^{+2}-stimulated Ach release from crude (P_3) and purified (I_2) synaptic vesicles. P_3 (●-●) and I_2 (▲-▲) at a concentration corresponding to 2 nmole Ach/ml were incubated with 35 µg protein/ml of S_3. Ach-release was induced by the addition of 150 µM free Ca^{+2} ions at t=0. (o-o) corresponds to the above experiment (average of P_3 and I_2) in the absence of Ca^{+2}.

dependent Ach release from both the purified and the crude synaptic vesicles. Figure 3 illustrates the results obtained from such an experiment. A smaller fraction of the Ach is released from the purified vesicles as compared to the crude ones. The extent of the difference varied in different preparations. The S_3- dependent Ca^{+2}-stimulated Ach release from the purified vesicles was enhanced by the addition of the membranes, contained in the crude vesicle fraction and not in the pure one.

DISCUSSION

In this communication we report the presence of a soluble factor which induces Ca^{+2}-dependent Ach release from the purely cholinergic Torpedo synaptic vesicles. The stimulating activity of this soluble factor is enhanced by a non-vesicular membrane fraction, which by itself does not induce the Ca^{+2}-dependent release. The chemical nature and subcellular origin of these factors is not yet known. However, the observation that the activity of the soluble factor is lost by dialysis and destroyed by heat, tempts to suggest that it may be a polypeptide or a protein. The low levels of Ca^{+2} required for Ach release, and the finding that for optimal release both soluble and membranous factors are required, suggest that our findings may be an *in vitro* manifestation of the presynaptic release process.

It is of interest that the Ca^{+2}-induced Ach release does not require external ATP. Since the Torpedo synaptic vesicles contain high concentrations of ATP, this observation may suggest that the energy required for the release process is supplied from the vesicular ATP rather than from cytoplasmic pools. In the absence of Ca^{+2}, high levels of ATP cause the release of a small fraction of the vesicular Ach. The physiological significance of this effect is not yet clear.

Our findings are similar to those recently described by Hata et al (18) who demonstrated the presence of a soluble factor, possibly a low molecular weight peptide-like substance, which is required for Ach release from crude rat synaptic vesicles. However, Berl et al (19) suggested that Ca^{+2} may trigger neurosecretion via a high molecular weight actomyosin-like protein (neurostenin).

Recently, Davis and Lazarus (20) described an *in vitro* system for studying insulin secretion. The experiments described here demonstrate that the release of the neurotransmitter Ach from Torpedo synaptic vesicles may also be studied *in vitro*. We are presently attempting the purification of the Torpedo soluble and membranous factors which induce the Ach release,

so that their molecular properties and subcellular localization may be further investigated.

ACKNOWLEDGEMENT

We thank Professor Z. Selinger for his encouragement and Mrs. I. Ophir for excellent assistance. M. Sokolovsky is an established investigator of the Chief Scientist's Bureau, Ministry of Health, Israel. This work was supported in part by the U.S.-Israel Binational Science Foundation (BSF Grant number 1035).

REFERENCES

1. Del Castillo, J. and Katz, B. (1954) J. Physiol. London 124, 560-573.
2. Del Castillo, J. and Katz, B. (1956) Prog. Biophys. 6, 121-170.
3. Hall, Z.H. (1972) Ann. Rev. Biochem. 41, 925-954.
4. Miledi, R. (1973) Proc. R. Soc. B 183, 421-425.
5. Israel, M., Gautron, J. and Lesbats, B. (1968) C.R. Acas. Sc. Paris 266, 273-275.
6. Israel, M., Gautron, J. and Lesbats, B. (1970) J. Neurochem. 17, 1441-1450.
7. Whittaker, V.P., Essman, W.B. and Dowe, G.H.C. (1972) Biochem. J. 128, 833-846.
8. Michaelson, D.M. and Sokolovsky, M. (1976) Biochem. Biophys. Res. Comm. 73, 25-31.
9. Michaelson, D.M. and Sokolovsky, M. (1977) J. Neurochem. In press.
10. MacIntosh, F.C. and Perry, W.L.M. (1961) Methods in Medical Research (Ed. R.W. Gerrard) p. 78-96.
11. Hebb, C.O. and Whittaker, V.P. (1958) J. Physiology 142, 187-196.
12. The Edinburgh Staff (1970) Pharmacological experiments on isolated preparations (Eds. E. and S. Livingstone) London.
13. Holmsen, H., Holmsen, I. and Bernhardsen, A. (1966) Anal. Biochem. 17, 456-473.
14. Hastings, J.W. and Weber, G. (1963) J. Opt. Soc. Am. 53, 1410-1415.
15. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
16. Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, K. (1961) Biochemical Pharmac. 7, 88-95.
17. Portzehl, H., Caldwell, P.C. and Ruegg, J.C. (1964) Biochem. Biophys. Acta. 79. 581-591.
18. Hata, F., Kuo, C.H., Matsuda, T. and Yoshida, H. (1976) J. Neurochem. 27, 139-144.
19. Berl, S., Puszkin, S. and Nicklas, W.J. (1973) Science 179, 441-446.
20. Davis, B. and Lazarus, N.R. (1976) J. Physiology 256, 709-729.