NEUROTRANSMITTER RELEASE FROM VIABLE PURELY CHOLINERGIC TORPEDO SYNAPTOSOMES

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Received September 7,1976

Viable synaptosomes from the electric organ of Torpedo have been prepared and partially purified. The synaptosomes contain about 100 fold more acetylcholine (Ach) than do mammalian synaptosomes, synaptic vesicles and mitochondria. The Torpedo synaptosomes release Ach by K depolarization in the presence of Ca ions, and manifest an ionophore-mediated Ca-dependent Ach release. These results demonstrate that the synaptosomes contain the neurosecretion apparatus in a functional viable state. Since this preparation uniquely contains only one neurotransmission system (cholinergic), it is most suitable for structural and functional investigations of neuro transmission.

The isolation of synaptosomes from CNS tissue has provided an excellent in vitro preparation for study of the presynaptic processes at the molecular level (1). Due to the variety of neurotransmitters contained in the synaptosomes, the experimental results obtained with these preparations represent an average of a population of different types of nerve endings. The electric organ of the elasmobranch Torpedo, which is richly innervated, contains only cholinergic synapses and is thus most suitable for biochemical investigations of synaptic transmission. While it was not possible in the past to obtain sufficient yields of synaptosomes from this tissue (2), Dowdall and Zimmerman (3) recently reported success in preparing nerve endings from the electric organ of Torpedo Marmorata.

For such a preparation to be useful in an investigation of the structural and functional aspects of the Ach release mechanism, it must be viable; that is, like in intact tissue, it must release Ach upon the introduction of Ca into the nerve endings (4,5). The present work describes the preparation and fractionation of viable synaptosomes from the electric organ

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

of Torpedo ocellata. These syanptosomes release Ach by K depolarization in the presence of Ca ions, and also manifest an ionophore-mediated Ca-dependent Ach release.

EXPERIMENTAL

Preparation and fractionation of synaptosomes. Torpedo ocellata were caught live off the coast of Tel Aviv and maintained in sea water aquaria up to four months prior to use. Synaptosomes from the electric organ of the elasmobranch Torpedo were prepared by a procedure similar to that described by Whittaker (1) for mammalian tissue, using 0.8M glycine in the homogenization buffer as suggested by Dowdall and Zimmerman (2).

The electric organ (30-60 gr) was excised from fish which had been prechilled at 4°C for 30 min. The excised tissue, 10% w/v, in 0.8M glycine 1mM EGTA pH 6.6 was blended in a omni-mixer (Sorvall) for half a minute, and then homogenized in a lucite glass homogenizer (6 passages at 880 rpm). The homogenized material (H) was centrifuged for 10 min at 1000g. The resultant pellet (P_1) was examined and discarded, and the supernatant (S_1) was further centrifuged at 17,5000g for 1 hr. The pellet resulting from this fast centrifugation (P_2) was resuspended by one passage at 150 rpm with the lucite glass homogenizer, and loaded on the density gradient. Density gradient centrifugation was performed with a discontinuous gradient in a SW40 rotor spun at 25K rpm for 1 hr. There were six layers in the gradient tubes (a_1 to a_6) each containing 1mM EGTA and one of the following concentrations of sucrose: 0, 0.15, 0.3, 0.55, 0.8, 1.6M. Fractions a_1 to a_5 also contained glycine so that their osmolarity was equal to that of the homogenization buffer.

Assays. Whole tissue Ach was TCA extracted (6) whereas the fractionated organ was extracted by boiling at pH 4.0 (1). Ach was assayed with the guinea pig ileum bioassay (7). Protein was determined according to Lowry (8) and Acetylcholinesterase (AchE) was assayed according to Ellman (9).

Measurement of Ach release from synaptosomes. Since the Torpedo synaptosomes contain a small fraction of the total AchE activity (see Results section), Ach is rapidly hydrolysed upon release to the external medium. Thus, the kinetics of Ach release from Torpedo synaptosomes were monitored by withdrawing samples at different times, and measuring their Ach content which, as described above, is equal to that retained within the synaptosomes. The experiments were performed in the following manner: 0.5 ml of synaptosomes, which contained approximately 0.6 mg protein, were diluted tenfold with 1.2 mM phosphate buffer, pH 6.9, containing 250 mM NaCl, 5.3 mM KCl, 10 mM sucrose, 2.8 mM Mg, 266 mM glycine and 10 μM EGTA. After dilution the synaptosomes were incubated at 25°C, and 0.5 ml samples were periodically collected and their synaptosomal Ach extracted and assayed. In experiments employing an ionophore, a small aliquot of a stock solution (1 mg/ml in ethanol) was added to the buffer prior to dilution. When the effect of Ca ions were sought, 5λ of 1M $CaCl_2$ was added per one ml of diluted synaptosomes. The effect of K depolarization was examined by a slightly different procedure: the synaptosomes were diluted fivefold with the above buffer, and later diluted twofold with a high K buffer with or without Ca ions. The high K buffer contained 390 mM KCl, 10 mM glucose, 2.8 mM $MgCl_2$, 10 μM EGTA and 1.2 mM phosphate buffer at pH 6.9.

RESULTS

The intact electric organ contained about 700 nmole Ach/gr organ. After homogenization in 0.8 M glycine 1 mM EGTA pH 6.6 the homogenate con-

TABLE 1

Distribution of acetylcholine, protein and acetylcholinesterase among subcellular fractions of the electric organ of Torpedo

Fraction*	Acetylcholine	Protein	Acetylcholinesterase
H	52	100	100
S_1	41	73	60
P_1	12	21	36
S_2	10	42	10
P_2	25	24	42
a_2	8	2	4

*See text for explanation of the various fractions

Legend to Table 1 Ach, protein and AchE distribution in the fractionated electric organ of Torpedo ocellata. Ach values are percentage of total, 100% being 590 nmole/gr organ. The protein and AchE are expressed as percentage of the homogenate (H) which contained 19.5 mg protein/gr tissue and 0.75 AchE units/gr tissue.

tained, on the average 55% of the total Ach. The decrease was caused by the breaking of some of the nerve endings and the consequent exposure of Ach to AchE. It was found that EGTA reduced this decrease in Ach content. Thus, homogenization in the absence of EGTA results in an homogenate which

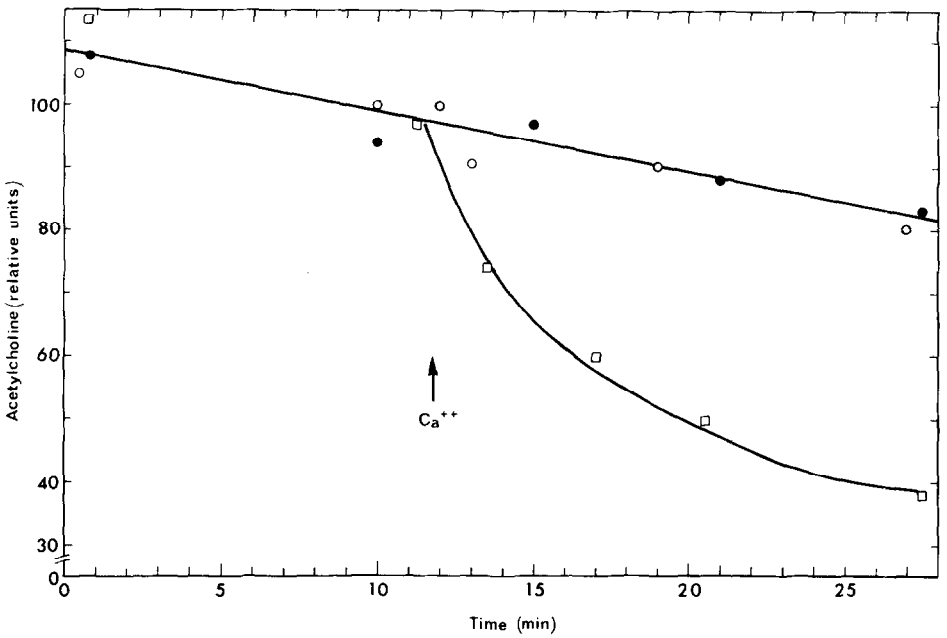

Fig. 1. Ca-dependent A21387-mediated Ach release from Torpedo synaptosomes; • represents untreated synaptosomes, □ synaptosomes with A21387 (10 μg/ml) to which 5 mM Ca was added as indicated, o synaptosomes without A21387 to which Ca was similarly added.

contains only about 11% of the total electric organ Ach. Sheridan et al. (3) obtained similar results in the absence of EGTA.

Table 1 presents the distribution of Ach in the fractions obtained by differential centrifugation. Most of the homogenate Ach remains in the supernatant of the slow centrifugation and is pelleted by the fast centrifugation. In the sucrose gradient centrifugation, the fraction a_2 is the most enriched in Ach, containing about 8% of the total electric organ Ach or about 30% of the Ach loaded onto the gradient. About 4% of the total AchE is pelleted by the fast centrifugation (P_2). The sucrose gradient fraction a_2 contains a small fraction of the total AchE with specific activity similar to that of P_2 (Table 1). Fraction a_2 was examined by electron microscopy and found to be composed of sac-like structures containing synaptic vesicles and occasional mitochondria.

The viability of the preparation was examined by introducing Ca ions into the synaptosomes by means of a specific ionophore (10,11) and K depolarization, and measuring the induced Ach release. The synaptosome bound Ach is not released by the addition of Ca ions in mM concentrations.

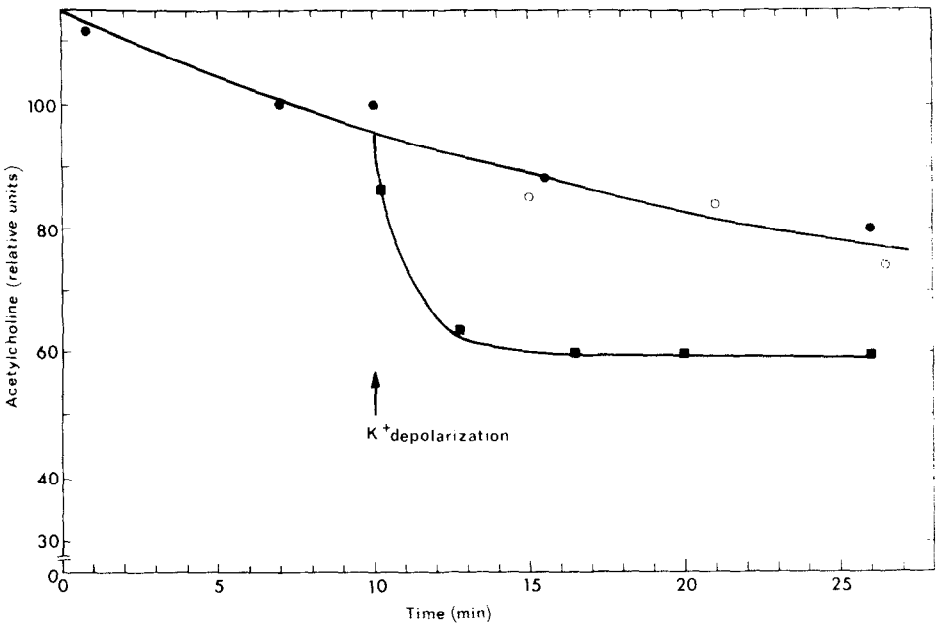

Fig. 2. K-stimulated Ach release from Torpedo synaptosomes. At the designated time the external K was raised from 4.2 mM to 197 mM while maintaining the buffer osmolarity. ■ represents stimulation in the presence of 5 mM Ca, o K-stimulation in the absence of Ca and • represents nonstimulated material.

However, when Ca is added to synaptosomes preincubated with the ionophore A21387, a fast Ach release is observed in which about 30% of the total Ach is released (Figure 1). The release seems to be biphasic; the first phase has a half life of the order of 1 min, while the second is much slower and only slightly faster than the leak rate from the untreated material. The Ca effect is observed at 0.4 mM Ca and is maximal at levels higher than 1 mM. In control experiments it was shown that the ionophore itself has no releasing effect. Figure 2 illustrates the effect of K depolarization on the Torpedo synaptosomes. When the buffer ionic composition is isoosmotically changed from 4.2 mM KCl to 197 mM KCl, in the presence of Ca ions, a rapid Ach release of about 30% of the total Ach is observed. The effect is slightly slower than that induced by A21387 and is monophasic. K depolarization in the absence of Ca ions yields no detectable Ach release (Fig. 2). The effect is observed at 0.4 mM Ca and is maximal at approximately 1 mM Ca.

DISCUSSION

In this communication we report the preparation and partial purifica-

tion of viable synaptosomes from the electric organ of Torpedo ocellata. The synaptosomes contain synaptic vesicles, and Ach at a concentration about 100 fold more than that of a mixed population of synaptosomes. They also contain lactic dehydrogenase which is a cytoplasmic marker, and succinic dehydrogenase which is a mitochondrial marker (Michaelson and Sokolovsky, in preparation). The yields of the preparation, in terms of synaptosomal Ach per mg protein, was greatly increased by adding EGTA to the homogenization buffer. The reason for the EGTA stabilization is still uncertain, although a likely explanation is that by sequestering Ca ions it prevents Ach secretion which may be induced during tissue homogenization.

The Ca-dependence of Ach release from the synaptosomes, demonstrated by means of K depolarization and the ionophore A21387, is of great importance. It shows that the synaptosomes contain the components required for neurosecretion in a functional form and that they probably maintain a resting potential. In both the K-depolarization and the ionophore-mediated experiments, the secretion is maximal at mM levels of Ca which are similar to those required for neurosecretion in vivo. In addition, both methods of inducing neurosecretion effect the release of the same fraction, about 30%, of the total synaptosomal Ach. It is of interest that isolated synaptic vesicles from the Torpedo electric organ contain 46% of the total Ach (12). Assuming that the distribution of Ach in the synaptosomes between the cytoplasm and the synaptic vesicles is similar to that of the unfractionated organ, it follows that the amount of Ach released in our experiments corresponds to that contained within more than half the synaptic vesicles. In accordance with this, electron microscopy revealed a substantial reduction in the number of synaptic vesicles in synaptosomes induced to release Ach as compared to synaptosomes not so induced (unpublished observations).

The experiments described here demonstrate that viable synaptosomes may be prepared from the electric organ of Torpedo. The fact that this tissue uniquely contains only one neurotransmission system makes the preparation most suitable for biochemical studies of presynaptic aspects of neurotransmission. This fact enables the expansion of the study of the molecular mechanisms underlying cholinergic transmission. The Torpedo synaptosomes may serve as a good starting point for experiments in which subsynaptosomal components will be fractionated and recombined to reconstitute the release process.

ACKNOWLEDGEMENT

We thank Moshe Moldevan for excellent technical assistance and Eli Lilly Co. for donating the A21387.

REFERENCES

1. Hebb, C.O. and Whittaker, V.P. (1958) J. Physiology 142, 187-196.
2. Sheridan, M.N., Whittaker, V.P. and Israel, M. (1966) Zeitschrift fur Zellforschung 74, 291-307.
3. Dowdall, M.J. and Zimmerman, H. (1976) Exp. Brain Res. 24, 8-9.
4. Rubin, R.P. (1970) Pharmacological Rev. 22, 389-428.
5. Miledi, R. (1973) Proc. R. Soc. Lond. B. 183, 421-425.
6. MacIntosh, F.C. and Perry, W.L.M. (1961) Methods in Medical Research R.W. Gerrard (Ed) 3, 78-96.
7. The Edinburgh Staff, (1970) Pharmacological experiments on isolated preparations (Eds. E. and S. Livingstone) London.
8. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
9. Ellman, G.L., Courtney, K.D., Andres, V. and Featherstone, K. (1961) Biochemical Pharmac. 7, 88-95.
10. Reed, P.W. and Lardy, H.A. (1972) J. Biol. Chem. 247, 6970-6977.
11. Pressman, B.C. (1973) Federation Proceedings 32, 1698-1703.
12. Whittaker, V.P., Essman, W.B. and Dowe, G.H.C. (1972) Biochem. J. 128, 833-846.