CHOLINERGIC NERVE TERMINALS CONTAIN ASCORBIC ACID WHICH INDUCES Ca^{2+}-DEPENDENT RELEASE OF ACETYLCHOLINE AND ATP FROM ISOLATED *TORPEDO* SYNAPTIC VESICLES

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1. Introduction

We have shown that a soluble, low-molecular weight, alkali-labile and oxidation-sensitive factor, prepared from the electric organ of *Torpedo ocellata*, induces Ca^{2+}-dependent acetylcholine (ACh) release from isolated *Torpedo* synaptic vesicles [1,2]. Diverse activities of ascorbic acid in the brain have been reported [3–8]. These include effects on metabolism of biogenic amines [9] and on release of neurotransmitters from isolated mammalian vesicles [10].

We therefore tested the possibility that ascorbic acid may be the soluble factor present in *Torpedo* and thus a naturally-occurring mediator of ACh release in vivo.

2. Experimental

2.1. *Preparation of synaptic vesicles and the soluble factor*

Crude (P_3) and purified (SV) synaptic vesicles were prepared as in [11].

When the soluble factor was prepared, the electric organ was homogenized (15%, w/v) in 1 mM EGTA (pH 6.6) and centrifuged as in [1]. The S_3 fraction was passed through an Amicon PM-10 filter and the filtrate lyophilized and kept at −20°C up to several weeks.

2.2. *Preparation of synaptosomal cytoplasm*

The synaptosomal fraction $a_2 + a_3$ [12,13] (~20 mg protein) was homogenized vigorously in water and proteins were precipitated by trichloroacetic acid (5%, v/v). The clear extract served for both ascorbic acid determination and activity measurements (vide infra). In the latter case, extract obtained from 5 mg protein was used per ml reaction mixture.

2.3. *Measurement of ACh and ATP release*

This was carried out as in [11], except that the reaction was conducted in 30 mM Tris–HCl (pH 7.2) which contained KCl (100 mM), NaCl (20 mM), EGTA (1 mM), dithiothreitol (DTT) (20 μM), glycine (480 mM), apyrase (0.25 mg/ml), and the designated concentrations of ascorbic acid or the *Torpedo* soluble fractions. The vesicles (2–4 nmol ACh/ml; 0.6–1 nmol ATP/ml) were preincubated with the buffer for 2 min, after which release was induced by addition of $CaCl_2$. The concentration of free Ca^{2+} was calculated according to [14].

2.4. *Assays*

The vesicular ACh and ATP were assayed as in [15,16]. Ascorbic acid was determined by the micromethod of [17] on trichloroacetic acid-extracted fractions. Protein was determined according to [18].

3. Results

The electric organ of *Torpedo ocellata* was found to contain ascorbic acid (200 ± 70 nmol/g tissue in the homogenate), 85–95% of which was recovered in the S_3 fraction. The amount of ascorbic acid in S_3 was 20 ± 6 nmol/mg protein.

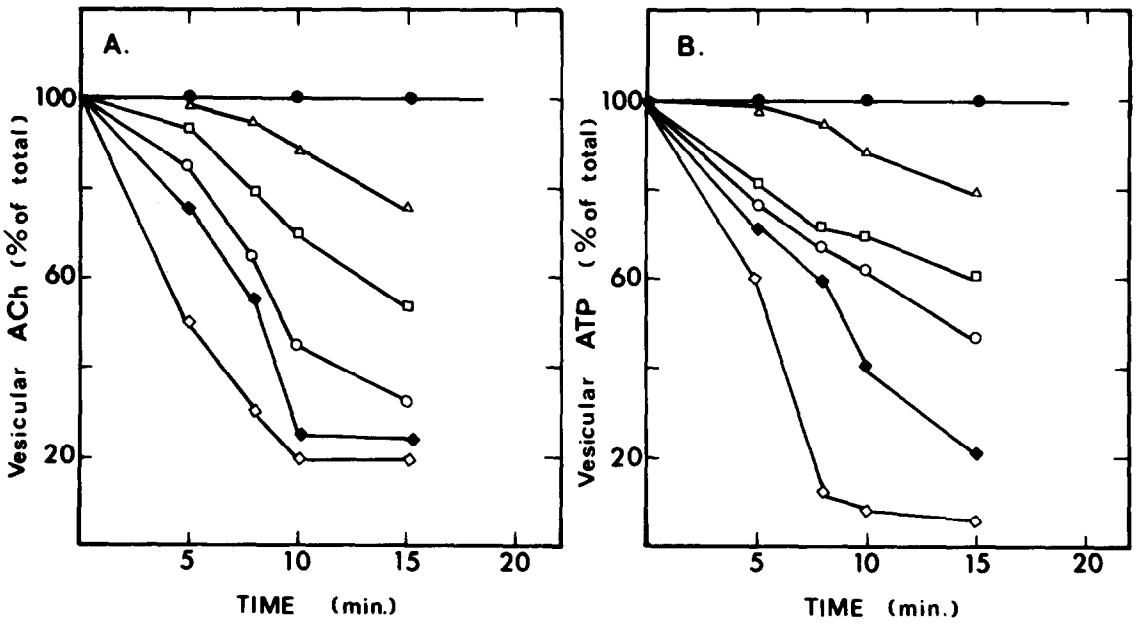

Fig.1. Kinetic profiles of the effects of various concentrations of L-ascorbic acid on vesicular ACh (A) and vesicular ATP (B). Release from P_3 was induced by 50 μM Ca^{2+} and the following L-ascorbic acid concentrations (μM): (● - ●) 0; (△ - △) 0.2; (□ - □) 0.5; (○ - ○) 1.0; (◇ - ◇) 5.0. (◆ - ◆) corresponds to 5.0 μM L-ascorbic acid and 50 μM Ca^{2+} added to purified (SV) synaptic vesicles.

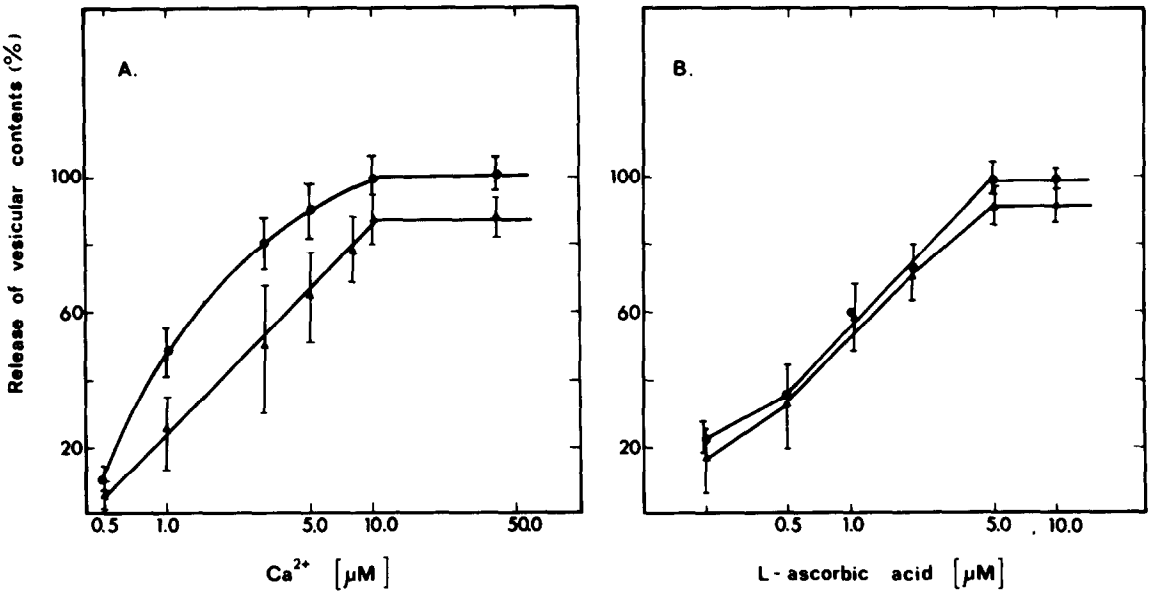

Fig.2. (A) Dose–response curves for the effect of Ca^{2+} on the vesicular ACh (▲) and ATP (●). The results shown were obtained 15 min after stimulation with Ca^{2+} in the presence of 5 μM L-ascorbic acid. (B) Dose–response curves for the effect of L-ascorbic acid on the vesicular ACh (▲) and ATP (●). The results plotted were observed 15 min after stimulation with 50 μM Ca^{2+} (fig.1). The points shown are the mean ± SD of ⩾ 4 expt for ACh and the mean ± the range of 2 expt for ATP.

Low concentrations of L-ascorbic acid induced a gradual, Ca^{2+}-dependent and dose-related increase in both the vesicular ACh and ATP (fig.1). Since the purified (SV) and the crude (P_3) synaptic vesicles were similarly affected by ascorbic acid (fig.1), the latter were routinely used in subsequent experiments. Half-maximal effect of L-ascorbic acid was found at ~1 μM (fig.2B). The effect of various Ca^{2+} concentrations, in the presence of saturating L-ascorbic acid levels (5 μM), is depicted in fig.2A. Half-maximal effects were observed at 3 μM Ca^{2+} (ACh release) and 1 μM Ca^{2+} (ATP release). High concentrations of Ca^{2+} alone ($\leqslant$ 1 mM) and of L-ascorbic acid alone ($\leqslant$ 0.50 mM) were ineffective. Neither Mg^{2+} nor external ATP were required. Furthermore, external ATP (150 μM) completely inhibited the releasing effect of 5 μM L-ascorbic acid and 50 μM Ca^{2+}. D-*iso* ascorbic acid also induced Ca^{2+}-dependent release, in concentrations comparable to those of L-ascorbic acid. NADH (0.5 mM) and glutathione (0.2 mM) were completely ineffective, suggesting that the effects of ascorbic acid are not due merely to its reducing capability.

The subcellular origin of the ascorbic acid present in S_3 is not known. We therefore examined the possibility that ascorbic acid may be present in the nerve terminal. The synaptosomal cytoplasm was indeed found to contain ascorbic acid (3 ± 1 nmol/mg protein), and to induce a marked decrease in vesicular ACh (75% after 15 min).

4. Discussion

The finding that ascorbic acid is present in the electric organ and that it can induce a Ca^{2+}-dependent release of the vesicular contents suggests that ascorbic acid is the active component of the *Torpedo* soluble factor [1,2]. This conclusion is supported by the following similarities between the soluble factor and ascorbic acid: low molecular weight; sensitivity to alkaline medium and oxidation; neither requires Mg^{2+} or ATP and both are inhibited by external ATP. In addition, preliminary findings show that they co-elute on an ion-exchange Dowex 1-X2 column.

Our results are similar to those in [10], where ACh and noradrenaline release from ascorbic acid-treated, Ca^{2+}-stimulated rat brain synaptic vesicles was shown. It should be noted, however, that Mg^{2+} and ATP are essential for ACh release from rat brain synaptic vesicles by both rat brain synaptosomal cytoplasm [19] and ascorbic acid [10]. This difference could mean that the molecular mechanisms involved in the release process from *Torpedo* and from mammalian synaptic vesicles are different. It is plausible that ATP present in *Torpedo* vesicles may serve as the energy source for the process.

The concentration of ascorbic acid in the nerve terminal is not known. The specific concentrations of ACh and ascorbic acid in the nerve terminal are 115 nmol/mg protein [13] and 3 nmol/mg protein, respectively. Assuming ACh at 20 mM in the nerve terminal [20], we obtain ~0.5 mM ascorbic acid, implying that the presently reported in vitro effects of ascorbic acid may also occur in vivo.

The physiological significance of the present finding is not yet known. It could be an in vitro manifestation of the ACh release process which, in vivo, gains its vectorial character by interactions with the presynaptic membrane. Alternatively, ascorbic acid may be involved in maintaining the balance between the vesicular and the cytoplasmic pools of ACh within the nerve terminal.

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