EFFECTS OF SYNAPTIC PLASMA MEMBRANES ON RELEASE OF ACETYLCHOLINE FROM SYNAPTIC VESICLES

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Abstract—The influences of synaptic plasma membranes on release of acetylcholine (ACh) from synaptic vesicles isolated from rat brain were examined. In the presence of ATP, Mg++ and Ca++ but absence of cytoplasm from the nerve endings, the synaptic plasma membranes did not increase ACh release indicating absence of a stimulating factor which is known to be present in the cytoplasm. In presence of ATP, Mg++, Ca++ and the cytoplasm, the synaptic plasma membranes inhibited ACh release from the synaptic vesicles in high K+ medium, though not in high Na+ medium. Binding of Ca++ by the synaptic plasma membranes was dependent on ATP, inhibited by Na++ and stimulated by K+. Thus, the synaptic plasma membranes may inhibit ACh release in high K+ medium due to reduction in the concentration of free Ca++. Acetylcholine (ACh) is mainly present in nerve terminals, and is stored in synaptic vesicles (1–3). A miniature end plate potential (m.e.p.p.) has been detected in the neuromuscular junction by an electrophysiological technique, and such led to the idea of quantal release of ACh (4, 5). Release of ACh has been suggested to occur by exocytosis; that is, the membranes of the synaptic vesicles may fuse with synaptic plasma membranes and the contents of the synaptic vesicles may be released into the extracellular space (6, 7).

Previously we studied release of ACh from synaptic vesicles from rat brain; the release of ACh from the vesicles in the presence of ATP, Mg++, and Ca++ (10⁻⁵ M) was stimulated by addition of cytoplasm from the nerve endings and this stimulation was completely blocked by EGTA (8). The release of ACh in presence of the cytoplasm was regulated by a low concentration of Ca++ and this may be a partial reaction of the transmitter release under physiological conditions. In this work we examined the effects of synaptic plasma membranes on the release of ACh from the vesicles as one step of reconstitution of a system for ACh release in intact cells.

MATERIALS AND METHODS

Synaptic vesicles and plasma membranes were prepared by a slight modification of the method of Gray and Whittaker (1) as follows: the crude mitochondrial fraction (P₂) obtained from rat brain was suspended in distilled water (4 ml/g original tissue) and centrifuged at 12,000 × g for 40 min. Then, the supernatant (W₂) was recentrifuged at 100,000 × g for 40 min and the resulting supernatant was used as cytoplasm from the nerve endings. The precipitate (crude synaptic vesicles) was suspended in water, and layered onto 0.1 M sucrose instead of 0.4 M sucrose (9) and centrifuged at 58,000 × g for 90 min.
The white material on 0.1 M sucrose was collected and centrifuged at 100,000×g for 40 min. The resulting pellet was suspended in basic medium (10 mM tris-HCl buffer pH 7.4, 120 mM KCl, 20 mM NaCl, 10⁻⁴ M eserine) and recentrifuged at 100,000×g for 30 min. The pellet was used as the vesicle fraction. Synaptic plasma membranes were obtained by discontinuous sucrose gradient centrifugation of the synaptosome fraction (P₂ B) pretreated with hypotonic medium (10-12). Sonicated synaptic plasma membranes were prepared as follows: a suspension of the synaptic plasma membranes in water at a concentration of 5 mg protein/ml was sonicated in a Kontes K-88140 sonicator at 23,500 cycles/sec in an ice water bath 6 times for 30 sec periods at 30 sec intervals.

Ca-binding was examined by a method described previously with ⁴⁵Ca and a Millipore Filter (HA 0.45 m/μ pore size) (13-14). Release of ACh was assayed with frog abdominal rectus muscle (15) as described in our previous paper (8) and protein was determined by the method of Lowry et al. (16).

**RESULTS**

Release of ACh from the vesicles is slow in the presence of ATP, Mg⁺⁺ and Ca⁺⁺, but as already reported, the release is stimulated by further addition of cytoplasm from the nerve endings and 60-70% of the ACh in the vesicles is released during incubation for 5 min at 20°C (8).

We found that the synaptic plasma membranes had no effect on the slow release which was observed in the presence of ATP, Mg⁺⁺ and Ca⁺⁺ but in the absence of the cytoplasm.

**Fig. 1.** Effect of sonicated synaptic plasma membranes on release of ACh from the synaptic vesicles. The reaction mixture contained 2 mM ATP, 2 mM MgCl₂ and 10⁻² M CaCl₂ in the basic medium (See text). The total volume was 5 ml and the incubation temperature was 20°C. Release of ACh was expressed as a percentage of the total ACh in the vesicles before incubation. About 10 mg protein of synaptic vesicles containing about 1 m,umole ACh was used per each vessel. Each point represents mean value of at least three experiments. ○—○ control; ●—● with sonicated plasma membrane (1 mg protein/ml); ○—○ with the cytoplasm (1 mg protein/ml)
FIG. 2. Effects of synaptic plasma membranes on release of ACh in presence of the cytoplasm in media containing different salts. The reaction mixture and conditions were as described for Fig. 1. In exp. B, 20 mM KCl + 120 mM NaCl was used instead of 120 mM KCl + 20 mM NaCl in exp. A. ○ ○ control; ● ● with the cytoplasm (1 mg protein/ml); ○ ○ with the cytoplasm (1 mg protein/ml) and the plasma membranes (600 µg protein/ml).

FIG. 3. Ca-dependency of the release of ACh from the synaptic vesicles in presence of the cytoplasm. 1 mM EGTA and various concentrations of CaCl₂ were added to give Ca²⁺ concentrations of 10⁻⁸-10⁻⁶ M as indicated, and calculation was made by the method of Portzehl et al. (18). The incubation period was 20 min and 1 mg of cytoplasm protein was added per ml. Other conditions were the same as in Fig. 1.

FIG. 4. Ca-binding to the synaptic plasma membranes in media with different salt contents. Reaction mixtures were as for Fig. 1 except that 0.1 µCi ⁴⁰Ca per ml was added. The total volume was 2 ml. ○ ○ 120 mM KCl + 20 mM NaCl; ▲ ▲ 20 mM KCl + 120 mM NaCl; ○ ○ 120 mM KCl + 20 mM NaCl, without ATP.
Assuming that this could be due to too low a frequency of contact between plasma membranes or membrane components with the vesicles, we next tested synaptic plasma membranes that had been sonicated, but we found that the sonicated preparation was inhibitory rather than stimulatory as shown in Fig. 1. These results do not support the idea of the presence of a factor(s) in the synaptic plasma membranes, like that in the cytoplasm, that stimulates release of ACh.

Next we examined the effect of the synaptic plasma membranes on the fast release of ACh observed in presence of the cytoplasm with ATP, Mg++, and Ca++. As shown in Fig. 2, the plasma membranes did not stimulate ACh release, but were inhibitory in high K+ medium, though not in high Na+ medium.

The Ca-dependency of ACh release observed in the presence of the cytoplasm, ATP and Mg++ is shown in Fig. 3. It has been reported from our laboratory that ATP-dependent Ca-binding to the synaptic plasma membranes at a low concentration of Ca++ (3 × 10⁻⁷ M) was stimulated by K+ and blocked by Na+ (13, 14). Therefore, the inhibitory effect of the synaptic plasma membranes on the ACh release from the vesicles in high K+ medium may be due to reduction in the concentration of free Ca++. To examine this we measured the influences of K+ and Na+ on ATP-dependent Ca-binding to the synaptic plasma membranes with a higher concentration of Ca++ (10⁻⁵ M), which was the same as that used in the experiments on ACh release described above. As shown in Fig. 4, Na+ inhibited Ca-binding under the conditions tested.

DISCUSSION

The release of insulin, adrenaline and somatotropin from the respective storage granules was reported to be stimulated by the additions of the respective plasma membranes, and these reactions were postulated by Davis and Lazarus to be models of exocytosis (17). However, we did not detect any stimulatory effect of plasma membranes on ACh release from the synaptic vesicles. The result indicates that the nature of the ACh releasing system may be different from those of insulin and other systems. However, it is also possible that other factors or conditions may be required in order to observe the stimulatory effect of the plasma membrane on the ACh release. For example, some specific contact with a right orientation, but not random collision, between the vesicles and relevant area (active zone) of the plasma membrane may be necessary and it may be difficult to reproduce such specific contact in the in vitro cell free system. The possibility that the vesicle fraction is contaminated with plasma membranes cannot be excluded, although such would be small because we used 0.1 M sucrose to prepare the vesicles instead of 0.4 M sucrose to reduce contamination with plasma membranes (9).

Our findings suggest that the plasma membranes are important Ca++-storing sites. As already reported (12-14), the plasma membrane took up Ca++ ATP dependently in K+-medium and release of Ca++ from preloaded plasma membranes seemed to depend on Na+. Thus one role of the plasma membranes in the nerve endings may be to regulate Ca-dependent intracellular processes such as release of ACh from the vesicles and others, acting as a Ca-
acceptor or Ca-donor depending on changes in monovalent cations in their environment.

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