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INTERACTION OF ISOLATED SYNAPTIC VESICLES WITH SYNAPTIC JUNCTIONAL COMPLEXES ISOLATED FROM THE RAT BRAIN

G. N. Kryzhanovskii, I. M. Antonikov, and R. N. Glebov

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The mechanisms (and methods of regulation under normal and pathological conditions) of secretion of mediators and hormones are under intensive study at the present time. Attempts to develop a model of exocytosis of mediators or hormones *in vitro*, essentially to study interaction of isolated secretory granules with the corresponding cell membranes, or more precisely, with the inner surface of the cell membranes of gland cells or with the presynaptic membrane (pre-SM), are consequently interesting [3]. During interaction between chromaffin granules (CG) and the fraction of adrenal cell membranes in the presence of Mg-ATP (phosphorylation substrate) and Ca^{++} (5 μM) liberation of labeled exogenous catecholamines and secretion satellites, namely ATP, dopamine-β-hydroxylase, and chromogranin [8], into the incubation medium, and also Ca-dependent phosphorylation of certain blocks of granules, and to a lesser degree, of plasmalemma proteins [9] are observed. Junction formation was recorded in these experiments by the sedimentation method: by the appearance of a new peak in the sucrose density gradient [10].

A similar approach has been used in relation to isolated brain synaptic vesicles (SV). It has been found [11], for instance, that interaction between SV and the synaptic membranes fraction of rat brain leads to acetylcholine release into the incubation medium. This effect can probably be explained by the formation of a junction between SV and the "everted" synaptosomal plasmatic vesicles present in the fraction used. Such interaction, induced by Mg-ATP, calmodulin (CaM) and Ca^{++} (1-5 μM) may lead to fusion of SV with the inner surface of the synaptolemma (with the pre-SM), as has been shown electron-microscopically [5].

The aim of this investigation was to study interaction of isolated SV with synaptic junctional complexes (the fraction consisting of the active zone of synapses) from rat brain by turbidimetry, a method not previously used. This method is basically suitable for recording the fusion (aggregation) of isolated SV with the pre-SM, a component of synaptic junctional complexes, quickly and over a period of time.

EXPERIMENTAL METHOD

The fraction of isolated SV was isolated from whole brain (without the cerebellum) of rats weighing 150-200 g bythe method described previously [2], suspended in 0.25 M sucrose, 20 mM Tris-HCl, pH 7.4, and used after a single freezing (−20°C) and thawing. The fraction of synaptic junctions (SJ) was obtained from whole rat brain (without the cerebellum) by the

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method in [12]. All solutions were adjusted to pH 7.1 (at 20°C) with the aid of NaOH. Brain tissue from 30 rats was homogenized in 9 volumes of 0.32 M sucrose, 1 mM $MgCl_2$, 50 μM $CaCl_2$ in a homogenizer with Teflon pestle. The homogenate was centrifuged (10 min, 900 g) and the residue washed twice with 5-7 volumes of 0.32 M sucrose; the supernatants were pooled and centrifuged for 15 min at 12,000g. The residue of unpurified synaptosomes was resuspended in 5 volumes of 0.32 M sucrose and centrifuged for 10 min at 900g, and the supernatant was centrifuged for 20 min at 14,000g. The washed unpurified synaptosomes were subjected to osmotic shock, by resuspending the residue in 9 volumes of 10 μM $CaCl_2$ solution (three fractions). The suspension was kept for 30 min at 0-2°C, then rehomogenized and centrifuged for 25 min at 35,000g. The residue thus obtained was resuspended in 54 ml of 0.32 M sucrose, 50 μM $CaCl_2$ and layered above a stepwise sucrose gradient, consisting of equal volumes of 0.85, 1.0, and 1.2 M sucrose, containing 50 μM $CaCl_2$, after which the gradient was centrifuged for 80 min at 23,000 rpm in a CW-27 rotor (Beckman L-5-65, Austria). Material on the boundaries between the layers of 0.85-1.0 and 1.0-1.2 M sucrose, and also the whole of the 1 M sucrose layer were removed with the aid of a Pasteur pipet, and the suspension was diluted with 2 volumes of 50 μM $CaCl_2$ and then centrifuged for 25 min at 35,000g. The resulting residue was the fraction of unpurified synaptosomal membranes.

The fraction of synaptosomal membranes was suspended for 30 sec in 10 ml of 0.2% Triton X-100, containing 0.5% dextran T-500 and 1 mM EDTA, pH 7.5 (20°C), after which 10 ml of cold Freon-113 was quickly added, and the mixture homogenized for 2 min at 0-4°C. The resulting suspension was centrifuged for 10 min at 3000g, the top layer was drawn off with a Pasteur pipet, without disturbing the intermediate suspension on the boundary between the two layers, after which it was diluted with 3 or 4 volumes of 50 μM $CaCl_2$ and then centrifuged (25 min, 35,000g). The residue was suspended in 2 ml of 0.32 M sucrose, and allowed to stand for 15 min at 0-4°C. Solutions of the substances were then added to the medium up to a final concentration of 1.2 M sucrose, 0.05% Triton X-100, and 0.02% dextran sulfate (final volume of suspension 8 ml). The suspension was then layered above a gradient consisting of equal volumes of 1.3, 1.4, 1.5, and 2.0 M sucrose, containing 0.05% Triton X-100 and 0.02% of dextran sulfate in each layer, after which an equal volume of 0.32 M sucrose was layered above the uppermost phase. This complex gradient was centrifuged for 3 h at 23,000 rpm in the same rotor. The maximum of the material was distributed at the boundary between the 0.32-1.2 and 1.5-2 M sucrose layers and in the residue. Material was sampled at the boundary between layers 1.5-2 M sucrose, diluted with 4 volumes of cold distilled water, and centrifuged for 20 min at 27,000 rpm in a type 30 rotor. The resulting residue was a purified SJ fraction. The SJ fraction was suspended in 0.25 M sucrose, 20 mM Tris-HCl, pH7.4, and kept at −20°C. The once frozen and thawed preparation was used for investigation.

Protein was determined by Lowry's method. The bovine brain CaM was generously provided by V. A. Tkachuk (All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow). The ATP-Na_2 used in the work was obtained from Reanal (Hungary) and neutralized to pH 7.4.

The intensity of scattering of light (I_{sc}) of the suspension used was measured at an angle of 90°, at a wavelength of 650 nm, and at 37°C (with constant mixing) on an MPF-4 spectrofluorometer (Hitachi, Japan) [1, 2].

EXPERIMENTAL RESULTS

Analysis of data in the literature shows that Ca^{++} ions (in micromolar concentrations) and Mg-ATP (in millimolar concentrations) are possible effectors of exocytosis; the latter, moreover, is evidently essential as the substrate of Ca-CaM-dependent phosphorylation of the membranes of SV and pre-SM [3, 5]. Ca^{++} ions also facilitate adhesion and fusion of SV with pre-SM [3]. However, it has been shown [5] that in the presence of SV and Mg-ATP only the CaM–Ca^{++} complex induces Ca-dependent phosphorylation of membrane proteins and release of labeled exogenous mediators into the incubation medium, as well as aggregation of vesicles, accompanied also by a decrease in size of the SV; under these circumstances the CaM blocker trifluoperazine, and the Ca-dependent protein kinase blockers diazepam and diphenylhydantoin inhibited the first two processes mentioned above. Hence it follows that homologous membrane-membrane interaction may also lead to release of the contents of vesicles.

The experiments showed that addition of 1 mM ATP and 1.0-o.5 mM $MgCl_2$ to the SV suspension (20 μg protein/ml) led to an increase in I_{sc}. This fact can be explained by Mg-induced aggregation of SV due to the presence of free Mg^{++} ions in the solution of Mg-ATP. In addi-

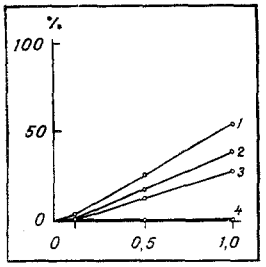

Fig. 1

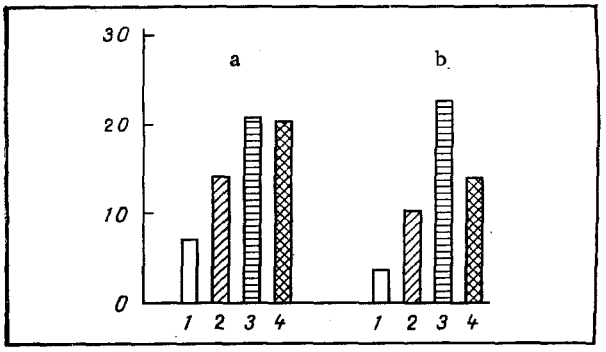

Fig. 2

Fig. 1. Action of effectors on I_{sc} of SV suspension. Abscissa, concentration of chelating agents (in mM); ordinate, inhibition of effect of change in I_{sc} of SV suspension (60 μg protein/ml) induced by 1 mM Mg-ATP (1, 3) or 1 mM $MgCl_2$ (2, 4). 1, 2) Inhibition by EDTA, 3, 4) inhibition by EGTA. Results of five or six experiments shown.

Fig. 2. Interaction of SV with brain synaptic junctional complexes. Vertical axis — change in I_{sc} of suspension of a fraction induced by 1.5 mM Mg-ATP + 1.0 mM ATP (in % of initial level of I_{sc}). SV) 130 μg protein/ml, SJ) 100 μg protein/ml, CaM) 140 μg protein/ml (CaM 8.5 μM). Action of Mg-ATP on SJ (1), SV (2), SJ + SV (3) and expected additive sum of changes for SJ and SV (4). Results of three or four experiments shown. a) Without CaM, b) in the presence of 8.5 μM CaM.

tion, considering the data cited above [5], it can be postulated that the true Mg-ATP complex, evidently in the presence of contaminating Ca^{++} ions and membrane-bound CaM, can also behave as an additional aggregation factor. The action of Mg-ATP on swelling of SV [1], which may also make its contribution to the observed effect, must also be taken into account. In fact, addition of 80 μM dicyclohexylcarbodiimide, which abolishes Mg-ATP-induced swelling of SV [1], in the present experiments led to an increase in amplitude of the change in I_{sc} (by 20%), evidence of a contribution (negative) of swelling to the recorded changes.

Introduction of stages of their additional purification into the existing method of isolation of SV, and removal of contaminating bivalent cations with EDTA helped to mask the effects of swelling against the background of a stronger process of aggregation.

It will be clear from Fig. 1 that Mg-induced aggregation of a suspension of brain SV was abolished by the chelating agent EDTA, but not by EGTA, which specifically binds Ca^{++} ions. However, Mg-ATP-induced aggregation of the SV suspension was abolished by both EDTA and EGTA, although to a lesser degree. These facts suggest that low Ca^{++} ion concentrations are essential for aggregation of vesicles to proceed in the presence of Mg-ATP as the possible substrate for Ca-dependent phosphorylation.

The results of experiments to record interaction between isolated SV and SJ from the brain in the presence of Mg-ATP and 8.5 μM brain CaM (Ca^{++} ions in trace quantities are present in the SV preparation, as is clear from Fig. 1). It was found necessary to use quite high concentrations of SV and SJ, for in this particular case, besides heterologous membrane-membrane interaction, homologous interaction also takes place. The process was started by addition of Mg-ATP. In the presence of the above-mentioned effectors, the observed change in I_{sc} of a mixture of SV and SJ (Fig. 2b, 3) exceeded the expected value obtained as a result of additive summation of changes in SV and SJ separately (Fig. 2b, 4). This indicated that heterologous membrane-membrane interaction of SV and SJ did in fact take place. Incidentally, it was not observed in the absence of CaM (Fig. 2a, 3, 4).

It was thus possible to record Mg-ATP-induced, and also, evidently, Ca-CaM-induced specific interaction between brain SV and SJ by the method of turbidimetry. However, despite the outward simplicity of the method, the study of the effect of pharmacologic agents on this process may prove difficult because of the difficulty of interpretation of the data [2]. It follows from these and other investigations [5] that Ca^{++} ions and Mg-ATP are cofactors for the initiation of mediator exocytosis by nerve endings. We also know that exocytosis of catecholamines (and their satellites in CG) by chromaffin cells of the adrenals requires Mg-ATP as well as Ca^{++}, as has been proved by the method of "electrical" [7] or "chemical" (action of detergents) [4, 6] breakdown of the cell membranes, so that the contents of the cyto-

sol could be changed without disturbing the secretion apparatus. Mg-ATP is essential as the substrate either for contractile ATPases, to enable the secretory granules to move toward the site of exocytosis, or for Ca (CaM)-dependent phosphorylation of the granules on their making contact with the plasmalemma, and to facilitate adhesion and release of the contents of the granules into the extracellular medium.

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STABILIZING EFFECT OF α-TOCOPHEROL ON SYNAPTOSOMES EXPOSED TO PHOSPHOLIPASE A_2

E. N. Erin, V. I. Skrypin, L. L. Prilipko, and V. E. Kagan

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The stabilizing action of the tocopherols in biological membranes is linked with their ability to inactivate the lipid radicals of lipids [13], to quench singlet molecular oxygen [11], and to organize the lipid bilayer of biomembranes by Van der Waals' interaction with unsaturated acyl groups of phospholipids [8]. It has recently been demonstrated that α-tocopherol (TP) can protect synaptosomal membranes against the harmful action of phospholipase A_2 (PLA_2), as is shown, for example, by restoration of the transmembrane potential (TMP) and the microviscosity of synaptosomal membranes, modified by the action of PLA_2 [2, 5]. However, the concrete molecular mechanisms of the stabilizing effect of TP on synaptosomal membranes have not yet been explained.

It has been shown that the harmful action of PLA_2 on synaptosomal membranes, assessed as the change in TMP, can be quantitatively modeled by the action of free fatty acids (FFA) and is independent of equimolar additions of lysophospholipids [2, 5]. It has also been shown that complexes of TP with FFA are formed in homogeneous systems, and also in lipid bilayers [10]. Investigation of the nature of these complexes in solutions has shown that they own their formation to interaction of a fatty acid with the chromane ring of TP [9]. 4-Methyl-2,6-di-tert-butylphenol (ionol) and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC), aromatic

All-Union Mental Health Research Center, Academy of Medical Sciences of the USSR, Moscow. Institute of Physiology, Bulgarian Academy of Sciences, Sofia.

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