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Research Article

A New Hypothesis on the Participation of Neurolectins of Synaptic Vesicles in the Secretion of Neurotransmitters

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Abstract.

Specific neurolectins, binding inositol (VL-I) and N-acetyl-D-glucosamine (VL- NAGA) are isolated from synaptic vesicles of chicken brain. It has been shown that VL-I and VL-NAGA appear to be glycoproteins, consisting of 6 and 13% of carbohydrates, relatively. For the determination of their neurolectin activity, the presence of Ca2+ ions is necessary, pH optimum is 7.0-7.4. It has been shown that inositol and inositol phospholipids inhibit hemagglutinating activity of VL-I. Based on the biochemical indices of vesicular neurolectins and the role of inositol phospholipids in the organization of synaptic membranes, we offered the model of VL-I and VL-NAGA neurolectins participation in the mechanisms of neuro- transmitters secretion.

Key words: the brain; synaptic vesicles; neuorolectins

Introduction

In recent years the neurolectins of animal brain have been widely studied. A number of neurolectins differing by carbohydrate specificity were isolated [1-4]. The physical-chemical properties of these neurolectins were studied and the attempts of identifying their possible functional role have been made [5-7].

Based on the available literature data, neurolectins of the brain can participate in the processes of synaptogenesis, of metabolic signals transmission [8], cellular adhesion [9], modulation of the activity of a number of enzymes [10], formation of vesicles on plasmatic membranes [11], etc.

Unfortunately, the data on neurolectins of the brain are limited, the detection of new neurolectins and establishment of their possible role in the functioning of neural cells appear to be one of the urgent tasks of modern neurochemistry.

Earlier the presence of neurolectins in subcellular fractions of chicken brain has been shown [12]. A special attention was attracted by neurolectins of synaptic vesicles, specifically binding inositol (VL-I) and N-acetyl-D-glucosamine (VL-NAGA). Taking into account the role of synaptic vesicles in the storage and secretion of neurotransmitters, we suggest the participation of revealed neurolectins in the processes of fusion of the vesicles with presynaptic membrane and secretion of their content. The aim of the given research is the isolation and purification of the neurolectins of synaptic vesicles of chicken brain and the establishment of their possible functional role in the processes of synaptic transmission.

The Methods of the Research

The brain of Leghorn hens were used in the experiment. Fraction of synaptic vesicles was obtained from brain homogenate using differential centrifugation according to De Robertis method [13].

Proteins with neurolectin activity have been extracted by means of 0.5% solution of triton X-100, prepared on 40 mM phosphate buffer (pH 7.4), containing 0.9% solution of NaCl and an inhibitor of proteinase phenylmethylsulfonyl fluoride (PMSF) in the final concentration 0.01mM.

Lectin activity was determined in 2% suspension of trypsinized rabbit erythrocytes on U-like titration plate [14]. Lectin specific activity (SA) was calculated using the formula:

SA=T-1/C, where T-1 – protein titer, C – protein concentration (mg/ml) [15].

Carbohydrate-binding lectin ability was defined using hapten inhibitory method [16].

The amount of carbohydrate in glycoproteins was determined by Colb and Kamishnikov [17]. The amount of proteins was determined by Lowry et al. method [18]. The data obtained were statistically processed by Student method [19].

The Results

In the first series of the experiment the solutions with different contents have been tested for the extraction of proteins with maximum lectin activity. It has been established that protein fraction of synaptic vesicles of chicken brain is characterized by the highest lectin activity extracted by means of phosphate buffer, containing 0.5% of non-ionic detergent triton X-100. Specific neurolectin activity of extracted protein fraction makes up 254, which is on average 45 times higher than the second and third versions according to their activity.

In further experiments a phosphate buffer was used, containing non-ionic detergent triton X-100 in final concentration 0.5%. For the revealing of different neurolectin types in extracted solution, we undertook a study of their carbohydrate specificity (Table 1).

NN	Carbohydrates	Minimal concentration of carbohydrate,inhibitinglectinactivity, mM
1	D-galactose	100
2	D-glucose	75
3	N-acetyl- glucosamine	36
4	D-xylose	75
5	D-lactose	100
6	Inositol	18

Table 1. *Carbohydrate specificity of neurolectins of synaptic vesicles in chicken brain*

It has been established that in protein fraction, extracted from synaptic vesicles of chicken brain several neurolectins different by their carbohydrate specificity were found. The highest affinity neurolectins show to inositol and N-acetyl-D-glucosamine, making it possible to isolate individual protein fractions with lectin activity from the total extract of chicken brain vesicles by means of affinity chromatography on a column with N-acetyl-glucosamine-sepharose 4B (Sigma, USA) and on column, containing trypsinized erythrocytes, fixed by means of glutaraldehyde [20]. The neurolectins were eluted from the column with erythrocytes 50 mM with inositol solution, while from the column filled with N-acetyl-glucosamine-sepharose 4B – with N-acetyl-glucosamine. After affinity chromatography the eluate was tested on carbo- hydrate specificity (Table 2).

NN	Carbohydrates	Minimalconcentrationof carbohydrate, inhibiting neurolectinactivity, mM
1	D-galactose	100
2	D-glucose	100
3	N-acetyl- glucosamine	100
4	D-xylose	100
5	D-lactose	100
6	Inositol	18

Table 2. *Carbohydrate specificity of eluate of protein fractions of synaptic vesicles of chicken brain after the immobilization on the column with N-acetyl-glucosamine-sepharose 4B*

In the eluate with N-acetyl-glucosamine-sepharose 4B, only VL-I was detected.

The both neurolectins appear to be glycoproteins: the content of total carbohydrates in them made up 6 and 13%, respectively. Maximum neurolectin activity was revealed at pH 7.0- 7.4.

The role of Ca^{2+} ions in the manifestation of hemagglutination of neurolectins VL-I and VL-NAGA was studied in special experiments. According to the literature data, membrane neurolectins are especially sensitive to Ca^{2+} ions [21]. This suggestion was not confirmed in our experiments on EDTA and EGTA (Table 3).

NN	Reagents	Minimal concentration of reagent, completely inhibiting neurolectin activity, mM	
		VL-1	VL-NAGA
1	EDTA	0.70	1.1
2	EGTA	0.35	0.55

Table 3.*The impact of EDTA and EGTA on specific activity of neurolectins of synaptic vesicles of chicken brain*

Taking into account a leading role of Ca^{2+} ions in the release of neurotransmitters from synapticvesicles[22, 23] andapredominant localization of inositolphospholipids in the synaptic membranes [24], it is possible to hypothesize the participation of inositol binding lectin VL-I in the processes of the secretion of neurotransmitters from the synaptic vesicles. Regarding this, special experiments were carried out using hapten-inhibitory approach. The different fractions of phospholipids were used as haptens. The neurolectins were predominantly incubated on titration plates with individual representatives of phospholipids (phosphatidylcholine, phosphatidylinositol, phosphatidylserine, sphingomyelin, phosphatidylethanolamine, phosphatidic acid). After this 2% suspension of rabbit trypsinized erythrocytes was added and the development of hemagglutination was observed. The data are given in Table 4.

NN	Phospholipids	Minimal amount of phospholipids, completely inhibiting lectin activity (mg/150 mkl)	
		VL-1	VL-NAGA
1	Phosphatidylinositol	0.032	+
2	Phosphatidylcholine	0.016	0.016
3	Phosphatidylethanolamine	+	+
4	Phosphatidylserine	+	+
5	Sphingomyelin	0.020	+
6	Phosphatidic acid	0.026	+

Note: "+" – agglutination

Table 4. *The impact of phospholipids on specific activity of neurolectins VL-I and VL-NAGA*

As it has been found, neurolectins show higher sensitivity to EGTA as compared to EDTA. The concentration of EGTA completely inhibiting hemagglutinating activity of VL-I and VL-NAGA was 2 times less, than EDTA concentration. As known, EDTA predominantly binds magnesium ions, while EGTA shows a high affinity to Ca^{2+} ions. Hence follows that neurolectins VL-I and VL-NAGA of synaptic vesicles of chicken brain need the presence of Ca^{2+} ions for the manifestation of their biological activity. However, the both neurolectins behave differently depending on the presence of Ca^{2+} ions in theincubation medium. So, in thepresence of Ca^{2+} ionstheneurolectin activity of VL-Idoes not change, while neurolectin VL-NAGA increases.

It has been established that among the studied phospholipids only phosphatidylcholine (0.016 mg/150 mkl) blocked VL-NAGA lectin activity, while VL-I was characterized by a broad spectrum of sensitivity to phospholipids. The complete inhibition of the activity was shown after a preliminary incubation with phosphatidylinositol (0.032 mg/mkl), phosphatidylcholine (0.016 mg/150 mkl), sphingomyelin (0.02 mg/150 mkl) and phosphatidic acid (0.026 mg/150 mkl). It shouldbenotedthatthe synaptic vesicles themselves also show a hemagglutinating activity. The incubation of synaptic vesicles with 2% suspension oferythrocytesinducedtheir hemagglutination. Along with phospholipids, the effect was blocked by inositol. It follows from this that inositol-sensitive centers of VL-I neurolectin are located on the surface of the vesicles and can interact with inositol of inositolphospholipid of presynaptic membrane.

Discussion of the Results

Until recently the problem of neurotransmitters secretion from synaptic vesicles remains open. The available hypotheses have no direct confirmations. The theory of actomyosin complex participation in the release of neurotransmitters did not find support [23]. Taking into account the above-said, as well as our experimental data on the presence of inositol- and N-acetyl-D- glucosamine binding lectins, we offer the hypothesis of the participation of VL-I and VL-NAGA lectins in the release of neurotransmitters from the synaptic vesicles.

As it has been shown above, VL-I and VL-NAGA neurolectins appear to be glyco- proteins, needing Ca ions for the manifestation of activity. The both lectins are membrane proteins. Unlike VL-NAGA, VL-I is embedded in vesicle membrane so that its active center faces the cytoplasm – to the inner side of presynaptic membrane. It became possible to prove experimentally. Without homogenization the native vesicles agglutinate trypsinized erythr- ocytes of rabbit. The agglutination was blocked by means of specific haptens – inositol and phosphatidylinositol. Based on our hypothesis, by means of VL-I neurolectins, the vesicles anchor on the presynaptic membrane as carbohydrate residue of inositolphospholipid inosit, which based on the literature data is oriented towards the cytoplasm [24] (Fig. 1).

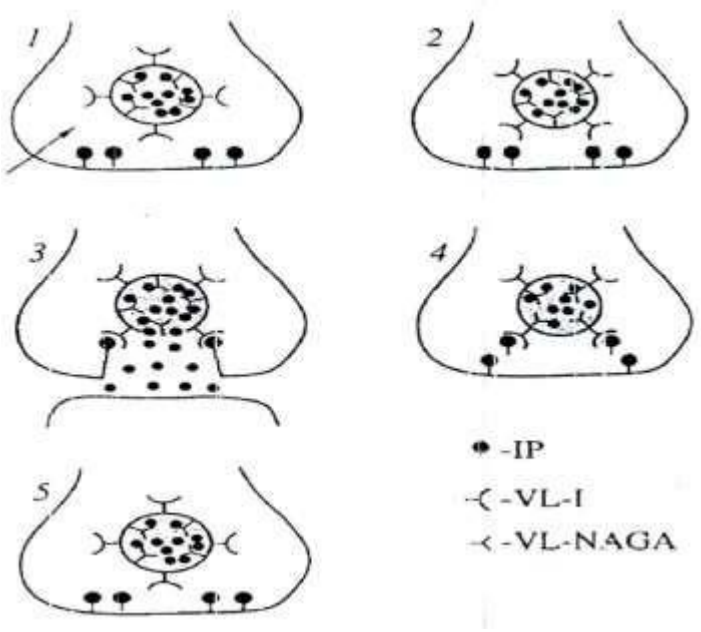

Figure 1. *Hypothetical scheme of lectin VL-I and VL-NAGA participation in the process of Neurotransmitters secretion*

At the same time the fusion of presynaptic membrane and vesicle takes place with the vesicular lumen formation. Through this lumen Ca^{2+} are actively moving from the synaptic gap. The formation of analogous structure has been shown in electron-microscopic experiments [23].

1 – synapse at rest; 2 – anchoring of vesicular neurolectin VL-I by inositolphospholipid of presynaptic membrane; 3 – The formation of presynaptic gap and the secretion of neuro- transmitters; 4 – Splitting of vesicles bound with inositol triphosphate (IP) and the recovery of presynaptic membrane; 5 – Splitting of inositol triphosphate from synaptic vesicles and the transmission of synapse at rest. Reference designations: IP - inositol triphosphate; VL-I – vesicular inositolspecific neurolectin; VL-NAGA – vesicular N-acetyl-D- glucosamine-specific neurolectin.

The formation of presynaptic lumen is followed by the secretion of neurotransmitters. It has been established that the stimulation of many receptors leads to the activation of enzyme phospholipase C [22, 25-27]. As a result of phospholipase C action, the splitting of inositol phospholipid into inositol-1, 4, 5-triphosphate (IP) and diacylglycerol takes place. Diacylglycerol remains in presynaptic membrane, while IP splits from the membrane as bound with vesicles. In a free form a part of IP contributes to the increase of Ca^{2+} ions concentration in the cytoplasm [28], while diacylglycerol activates proteinkinase C [29]. Consequently, along with the increase of cytoplasmic Ca^{2+} ions, the exit of neurotransmitters from the vesicles via presynaptic lumen accelerates (Fig. 1). Thus ends the first stage of neurosecretion and the phase of vesicles formation and their filling with neurotransmitters takes place. Unfortunately, it is yet difficult to judge the mechanism of fusion of presynaptic membrane ends and a possible role of neurolectins in this process until the establishment of neurolectins presence in presynaptic membrane. As to the formation of vesicles and the closure of their lumen, according to our scheme, VL-NAGA and Ca^{2+} ionsparticipatein thisprocess. Ithasbeenshown that at increasing Ca^{2+} ions concentration in the cytoplasm, the inhibition of binding IP with the receptor is observed [29, 30], while in our conditions – the disturbance of ligand-receptor binding (IP-VL- I). This process is also intensified at the expense of acidification of vesicular medium by the action of H^+-ATPase [31]. In acid medium ligand-receptor binding is disturbed [32]. In free form IP further enhances the release of Ca^{2+} ions and the increase of its concentration in the cytoplasm [24]. A high level of free Ca^{2+} ions in the cytoplasm dramatically stimulates the agglutination at the fusion of vesicles membrane ends with the participation of VL-NAGA. It should be noted that binding of inositolphospholipid IP by neurolectin VL-I vesicles, unlike the agglutinating activity of VL-NAGA, are not Ca^{2+}-dependent. The analogous results were also detected by other authors [30]. Only the addition of kalmedin to IP-receptor, secreted from the smooth muscles made the binding Ca^{2+}-dependent [32]. Naturally, the question arises about the identity of IP-binding receptor and neurolectin VL-I, also specifically binding IP. The evidence of their possible identity are the data of a number of authors that IP binding both with purified IP-receptorandwith VL-Iisinhibitedbyheparin[33]. Simultaneously, the bothreceptorsshow a high sensitivity to pH [34].

Vesicular lectin VL-NAGA unlike VL-I appears to be Ca^{2+}-sensitive. Ca^{2+}-ions promote the fusion of vesicular and presynaptic membrane ends. At the same time the transport of neurotransmitters enhances. An active transport is provided by vesicular H^+-ATPase. During action of H^+-ATPase an acidification of vesicular lumen took place, the metabolism of neurotransmitters with H^+ -ions enhanced and as it was noted above, IP release took place. Based on the data of some authors, exactly protein components (neurolectins ?) promote the fusion of vesicular membrane ends after neurotransmitters release [35].

It is clear that the proposed scheme needs the revision and biochemical justification of separate stages of the cycle of release and loading of vesicles by neurotransmitters, but the participation of neurolectins in the processes of neurotransmitters secretion and fusion of membranes should not cause adoubt.

Conclusion

Inositol (VL-I) and N-acetyl-D-glucosamine (VL-NAGA)-specific

neurolectins were isolated from chicken brain synaptic vesicles by affinity chromatography. The active center of VL-I is suggested to be oriented outside the vesicle cytoplasmic side, as the native vesicles agglutinated the trypsinized rabbit erythrocytes. This process was fully blocked bythe addition of phosphatidylinositol and inositol. Lectins VL-I and VL-NAGA are complex glycoproteins and high sensitive to pH, EDTA and EGTA. Taking into account the presence of phosphatidylinositol and phospholipase C in presynaptic membranes, we suggest the participation of synaptic lectins VL-I, VL-NAGA, H^+-ATPase and Ca^{2+} in neurotransmitter secretion and in subsequent reorganization of synapticstructures.

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