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Endoglycoceramidase treatment inhibits synchronous oscillations of intracellular Ca^{2+} in cultured cortical neurons

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Summary: Gangliosides are major components of nerve cell membranes and are especially rich in synaptic areas. In order to evaluate the role of endogenous gangliosides in synapse formation, endoglycoceramidase (EGCase) was used to remove oligosaccharides of gangliosides from the cell surface. We have reported previously that synapse formation between cultured rat cerebral cortical neurons can be estimated by the synchronous oscillation of synaptic activity monitored by fura-2 calcium imaging. Continuous application of endoglycoceramidase (EGCase) together with its activator protein dose-dependently decreased the frequency of synchronous oscillations without any morphological changes in neurons and their neurites. The result suggests that oligosaccharides liberated from glycosphingolipids on cultured cortical cell surface with EGCase are important for synapse formation between cortical neurons.

Gangliosides, a type of glycosphingolipids, are abundant in the nervous system (1) and project their oligosaccharide moieties in extracellular space. Since gangliosides are highly localized in the synaptic region (2,3), they have been suggested to play important roles in synaptic transmission and synapse formation (4-7). However, the precise functions of gangliosides have not yet been defined.

We have found that continuous application of K-252b (8), an ecto-protein kinase inhibitor, to cultured cells blocks their synchronous firing (9,10) and significantly decreases the number of synapses identified morphologically using electron microscopy (10). Since it has been reported that ecto-protein kinase activity is regulated by a specific type of ganglioside, GQ1b (11), the

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correlation of ecto-protein kinase activity to the gangliosides, especially extracellular sugar moieties, is also of great interest.

Recently Ito and Yamagata found a novel enzyme, endoglycoceramidase (EGCase) (12,13), which specifically hydrolyzes the glycosidic linkage between the oligosaccharide and ceramide of glycosphingolipids but does not act on cerebrosides, phospholipids or glycoproteins (12,13). Using activator protein (14,15) for stimulating of EGCase, it is now possible to remove oligosaccharides of glycosphingolipids from the cell surface without any damage to living cells (16,17). This new method was applied to study the synaptic function of the sugar moieties of gangliosides.

As previously described (5,6,14), neurons in the culture spontaneously formed networks and showed synchronous oscillations of $[Ca^{2+}]_{in}$, which represented bursts of action potentials (18), after around 7 Days in vitro (DIV). From morphological observation of the same culture by electron microscopy (EM), it was demonstrated that, 1) the development and maturation of synapses in the culture closely resembled that in vivo both morphologically and quantitatively (19), 2) the frequency of synchronous oscillations of spontaneous $[Ca^{2+}]_{in}$ transients also increased during development and was strongly correlated to the number of synapses in the cultures (20). Therefore, by simply measuring the frequency of $[Ca^{2+}]_{in}$ oscillations, it is possible to estimate synapse formation semi-quantitatively. In this report we investigated the effects of treatment of cultured cortical cells with EGCase in order to elucidate the biological functions of endogenous gangliosides on synapse formation.

Materials and Methods

Primary culture of rat cerebral cortical cells and the application of EGCase

Primary cultures of dissociated cerebral cortical neurons from 18-day fetal rats (Wistar) were prepared using a method described previously (9), which is a modification of the established method (21). After digestion of brain tissue with papain, cells were mechanically dispersed and plated on poly-ethylenimine (Sigma) coated coverslips. The culture was maintained in a humidified atmosphere of 93% air and 7% CO_2. The culture medium was Dulbecco's modified Eagle's medium (DEME: Gibco) containing 5% new born calf serum (Nakashibetsu Serum Center), 5% heat-inactivated horse serum (Gibco) and 1mM sodium pyruvate (Sigma).

Stock solutions EGCase and activator protein were in phosphate buffered saline (PBS) at concentrations of 1.25 mU/μl and 625 μM, respectively. The stock solutions were diluted with the culture medium and applied at 2 days in vitro (DIV) at final concentrations of 2 to 64 mU/ml (EGCase) and 1 to 32 μM (activator protein).

Assay for synapse formation (Optical monitoring of neuronal activity)

Functional synapse formation was observed by means of optical monitoring of intracellular Ca^{2+} concentration ($[Ca^{2+}]_{in}$) of cultured neurons loaded with the fluorescent Ca^{2+} indicator, fura-2 (9,10). Optical monitoring of $[Ca^{2+}]_{in}$ was carried out following the method of Ogura *et al.* (22), using video-assisted multi-site fluorometry equipment (23). Spontaneous synchronous oscillations of $[Ca^{2+}]_{in}$ were observed and their frequency was measured after 7 to 11 DIV.

Results and Discussion

The continuous presence of EGCase (20 mU/ml) together with its activator protein (10 μM) resulted in a decrease of the oscillation frequency, which is correlated with the number of synapses (20). In the case of shorter periods of EGCase application (during 2 to 3 DIV), the frequency was decreased to 64% of that of the control culture. Cultures treated for a longer period with EGCase

during 2 to 7 DIV showed a larger reduction in the frequency of synchronous $[Ca^{2+}]_{in}$ oscillations (14% of control frequency) (Fig.1). These effects on the frequency were dose-dependent (Fig.2). In observations by phase-contrast microscopy and immunostaining using anti-MAP2 or anti-neurofilament antibodies, we observed no significant morphological changes of neurons and their neurites. Therefore, in the presence of EGCase and its activator, synapse formation between cultured neurons was reduced but not neurite formation. No significant change of the frequency was observed with treatment by EGCase alone or activator protein alone. Therefore,it is unlikely that the effect originates from any contaminations in the preparation, since without activator EGCase was found to be inactive toward cell-surface glycosphingolipids (16,17). Moreover, when treatment by EGCase (and its activator protein) was stopped at 8 DIV, the synchronous oscillations of spontaneous $[Ca^{2+}]_{in}$ recovered and their frequency increased daily (Fig.3). This implied that the functional inhibition comes neither from suppression of neuronal viability nor from degeneration of neurons and their neurites. Thus, these data strongly suggest the possibility that the sugar moieties of gangliosides, which are hydrolyzed by EGCase activity, are necessary for synaptogenesis between cultured cortical neurons.

A conventional method to investigate the roles of gangliosides is observing morphological or functional changes in cells after the exogenous application of various kinds of gangliosides (24). A serious problem in this is the pre-existence of endogenous gangliosides on the cell surface. Moreover, it is not clear whether or not exogenous gangliosides are adsorbed to the cell surface from the culture medium. Thus, it is rather difficult to interpret such experimental results. The method used in the experiment to overcome this problem is to remove endogenous gangliosides from the cell surface. EGCase treatment has several advantages for elucidating the biological function of gangliosides using cultured cells. First of all, it remove sugar moieties of gangliosides but not those of glycoproteins. It is possible to inhibit specifically functions of the gangliosides and closely related components using this enzyme specific to glycosphingolipids. Secondly, since it does not damage cultured cells, we can test the long-term effects of gangliosides, such as effects on synapse formation. In other cell cultures, it has also been reported that EGCase treatments has

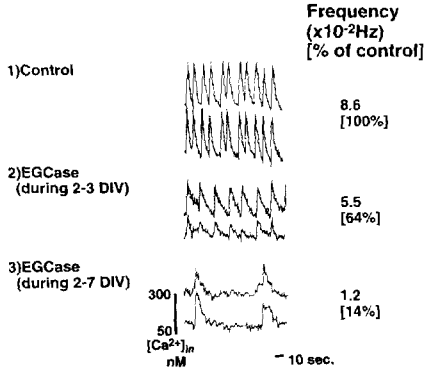

Fig.1.
The schedule of treatment with EGCase and its activator protein and their effects on synchronous oscillations of $[Ca^{2+}]_{in}$. The figure shows the periods of application of EGCase, the pattern and the frequency of synchronous oscillations of $[Ca^{2+}]_{in}$ transients at 7 DIV. The concentrations of EGCase and its activator were 20 mU/ml and 10 μM, respectively. The patterns of oscillation two neurons are shown for each experimental condition to demonstrate synchronization between neurons.

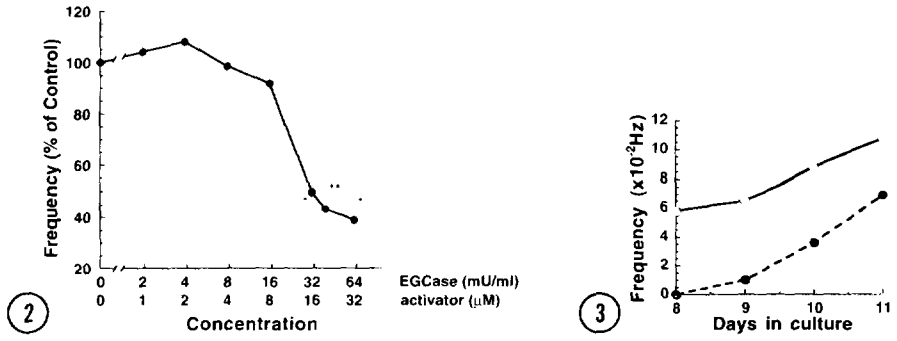

Fig.2.
Dose-dependent effects of EGCase and its activator protein on the frequency of synchronous oscillations of spontaneous $[Ca^{2+}]_{in}$. All frequencies were calculated from the patterns of synchronous oscillations observed at 8 DIV after treatment with EGCase during 2 to 8 DIV. Data represent the mean ± S.E.M.(error bars) from an experiment carried out in quintuplet except for the data points of 20 mU/ml (n=3) and 32 mU/ml (n=10) of EGCase concentration. *P<0.01, **P<0.001 compared to the untreated control (Student's t-test).

Fig.3.
Recovery of synchronous oscillations corresponding to spontaneous synaptic activity after treatment with EGCase. Cultured rat cortical cells were treated with 40 mU/ml EGCase and 20 μM activator proteins during 2 to 8 DIV. Cells were then incubated with EGCase free medium and spontaneous $[Ca^{2+}]_{in}$ oscillations were observed by video-assisted, multi-site Ca^{2+} fluorometry until 11 DIV (Closed circle). Open Circles indicate the changes in frequency of control cultures which are not treated with EGCase during culture periods.

no general toxic effects (14,16,17). In the present study (Fig.3), the neurons restarted formation of synaptic contacts after washing off EGCase. Therefore, a third important advantage is that it may now be possible to add defined molecular species of gangliosides to the culture to incorporate known populations of sugar moieties in the cell surface gangliosides. This possibility merits further investigation.

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