Influence of endogenous GM1 ganglioside on TrkB activity, in cultured neurons

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Abstract We verified the hypothesis that changes in the endogenous GM1 ganglioside density in the environment of TrkB, receptor of brain-derived neurotrophic factor, can affect receptor activity, and focused on rat cerebellar granule cells expressing both GM1 and TrkB. Changes of the amount of GM1 associated to immunoprecipitated TrkB and of receptor tyrosine phosphorylation were evaluated after treatment with phorbol-12-myristate-13-acetate (1 μM, 7 min), reported to affect the plasma membrane distribution of endogenous gangliosides in the same cells. After treatment, the amount of GM1 associated to receptor and TrkB phosphorylation decreased by about 40%. The amount of associated GM1 decreased by about 33% also after concomitant treatment with phorbol ester and brain-derived neurotrophic factor, but in this case the neurotrophin was unable to enhance receptor tyrosine phosphorylation. These results for the first time suggest that changes in the amount of endogenous GM1 in the environment of TrkB can modulate receptor activity, and offer new clues for a better understanding of physiological and pathological events of the nervous system.

Key words: Brain-derived neurotrophic factor tyrosine kinase receptor; GM1 ganglioside; Brain-derived neurotrophic factor; Cerebellar granule cell; Detergent-resistant membrane domain

1. Introduction

Neurotrophins (NT), a family of polypeptides promoting the differentiation and survival of both central and peripheral neurons, include molecules such as the brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), neurotrophin-3, and neurotrophin-4/5. Neurotrophins initiate signal transduction in responsive cells by ligand-induced dimerization and activation of a receptor tyrosine kinase of the tyrosine kinase (Trk) family [1]. The existence of a relationship between gangliosides and NT receptors has been documented (for a review on the argument see [2]). The knowledge that gangliosides enhance NT action [2], that GM1 ganglioside interacts with the NGF receptor [3,4], and that endogenous GM1 co-localizes with the BDNF receptor in neuronal membrane domains [5], suggests the importance of these interactions in physiological functions and pathological events of the nervous system, even though the underlying mechanisms have not been completely clarified. Although it is known that exogenously administered gangliosides can affect Trk activity [3], the influence of endogenous gangliosides, and in particular of changes in their local concentration in the receptor environment, is unknown. We decided to investigate this issue and, starting from the hypothesis of a possible correlation between GM1 ganglioside and TrkB, the BDNF receptor, we carried out the investigation using rat cerebellar granule cells, known to express both the glycolipid and the protein [6,7]. Our approach consisted of monitoring changes of the amount of endogenous GM1 associated with TrkB and, on the other side, of the receptor activity, consequent to a stimulus able to modify the distribution of endogenous gangliosides at the level of the plasma membrane. This modification was brought about by the addition of a phorbol ester, a procedure that we recently set up using the same cellular system [7].

2. Materials and methods

2.1. Materials

TLC plates and common chemicals were from Merck (Darmstadt, Germany). Basal modified Eagle's medium, fetal calf serum (FCS; heat-inactivated before use), poly-L-lysine, 1-α-D-arabinofuranosylcytosine, gentamycin, Triton X-100, phorbol-12-myristate-13 acetate (PMA) and crystalline bovine serum albumin were obtained from Sigma (St. Louis, MO, USA). BDNF was from Alexis (Laufelfingen, Switzerland). Standard GM1 ganglioside was extracted and purified from calf brain [8]. Secondary antibodies and enhanced chemiluminescence (ECL) detection kit were from Amersham International (Amersham, UK). Horseradish peroxidase (HRP)-conjugated cholera toxin B subunit (CTB) was from List Biological (Vandell Way, CA, USA). The anti-TrkB antibody used in this study for immunoprecipitation experiments was obtained from albino rabbits immunized with an HPLC-purified synthetic peptide (TrkB-peptide) representing a 14-amino acid residue derived from the C-terminus of TrkB protein [3]. This antibody can cross-react weakly with TrkA and TrkC protein. For this reason, for immunoblotting experiments we used an anti-TrkB antibody (794) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), which is more specific than ours towards TrkB, even if its titer is weaker. Anti-phosphotyrosine (TyrP) antibodies (4G10) were from Upstate Biotechnology (Lake Placid, NY, USA).

2.2. Cell cultures and cell treatment

Cerebellar granule cells, obtained from 8-day-old Sprague-Dawley rats (Charles River), were prepared and cultured according to [9]. Glial proliferation was prevented by adding cytosine arabinofuranoside (final concentration, 10 μM) 18–20 h after plating. Cells were grown on 100-mm plastic dishes coated with poly-L-lysine and used for the experiments after 6 days in vitro. Cells were treated with PMA according to a procedure able to exert a change of ganglioside distri-

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Abbreviations: BDNF, brain-derived neurotrophic factor; CTB, cholera toxin B subunit; ECL, enhanced chemiluminescence; HRP, horseradish peroxidase; NGF, nerve growth factor; NT, neurotrophins; PKC, protein kinase C; PMA, phorbol-12-myristate-13 acetate; Trk, tyrosine kinase; TrkB, brain-derived neurotrophic factor tyrosine kinase receptor; TyrP, phosphotyrosine

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bution in the plasma membrane of cerebellar granule cells [7], with minor modifications. Briefly, the cells were preincubated in serum-free medium for 1 h at 37°C and then treated for 7 min with PMA (1 μM), or with BDNF (100 ng/ml), or both. After treatment, the cells were harvested and submitted to immunoprecipitation, as described below.

2.3. Assessment of the total content of GM1 in cultured cells

The total GM1 ganglioside content was assessed in control and in PMA-treated cells as described [7]. Briefly, gangliosides were extracted and purified from a pool of cells prepared from five 60-mm diameter dishes. The fractionation and quantification of GM1 was accomplished by analytical HPLC using a photodiode-array detector and peak integration, as described [7,10].

2.4. Immunoprecipitation

Immunoprecipitation was performed according to [3], the only difference being that 1% Triton X-100, instead of Nonidet P-40, was used in the lysis buffer. Cell lysates were normalized for the protein content and immunoprecipitated with an anti-TrkB antibody, as described [3].

2.5. Assessment of the amount of GM1 ganglioside co-precipitated with TrkB

After immunoprecipitation with anti-TrkB antibodies, an aliquot of the immunoprecipitate was submitted to lipid extraction [11]. The extract was submitted to TLC (solvent system chloroform/methanol/$CaCl_2$, 0.2%, 50:42:11 by vol.). The identification of GM1 was accomplished using the immunostaining with HRP-conjugated CTB, already described [3], followed by ECL detection. Quantification of GM1 was accomplished by comparison with known amounts of standard ganglioside.

2.6. Assessment of tyrosine phosphorylation of TrkB

An aliquot of the immunoprecipitate obtained with anti-TrkB antibodies from cell lysates, was submitted to electrophoresis on sodium dodecylsulfate (SDS)/8% polyacrylamide gels, transferred to polyvinylidene difluoride membranes and probed with anti-TyrP antibody. Detection was performed according to the manufacturers' directions (ECL, Amersham). Manufacturers' specified protocols were also used to strip anti-TyrP antibody from the blots and to reprobe the membranes with antibodies against TrkB. In order to quantitatively evaluate the effects exerted by cell treatments, the tyrosine phosphorylation of each sample was normalized for the TrkB content of the same sample. For this purpose, films of the ECL-visualized immunoblots were quantified by densitometry. Then, the digitized band in the blot obtained with anti-TyrP was divided by the digitized band of the same sample in the blot obtained with anti-TrkB.

3. Results

3.1. Amount of GM1 ganglioside co-precipitated with TrkB receptor

The amount of GM1 co-precipitated with TrkB, from lysates of control cells, was 12 ± 1 pmol/mg cell proteins (Fig. 1). We could detect neither TrkB protein nor GM1 in the immunoprecipitate when the immunoprecipitation was carried out in the presence of TrkB-immunogen, added at the final concentration of 1 mg/ml. Using pre-immune serum instead of anti-TrkB antibody and submitting the cells to the immunoprecipitation procedure, the amounts of GM1 and TrkB recovered in the immunoprecipitate were below the detection limit of the technique used.

The assessment of GM1 in the immunoprecipitate was performed after cell treatment with PMA. In this case the amount of GM1 co-precipitated with the receptor was lower (7.2 ± 0.9 pmol/mg cell proteins) than in control cells. A lower amount of GM1 associated with the receptor (8 ± 1 pmol/mg cell proteins) was also detected in cells after treatment with PMA and BDNF.

As a control, the possible effect of the PMA treatment on the total GM1 content of cells was also evaluated. The results showed that, under the experimental conditions adopted, the content of GM1 (data not shown) was not affected by the treatment with the phorbol ester, confirming results already reported [7].

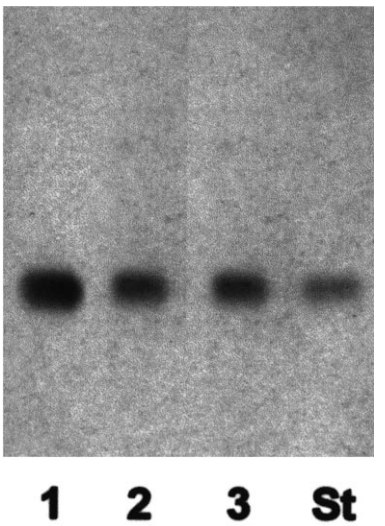

Fig. 1. Assessment of the amount of GM1 ganglioside in immunoprecipitates obtained with anti-TrkB antibody from lysates of cerebellar granule cells. Total lipids were extracted from an aliquot of the immunoprecipitates as described in the text. The extracts were submitted to TLC, followed by immunostaining with HRP-conjugated cholera toxin B subunit and ECL detection. Lane 1: GM1 co-precipitated with TrkB from control cells; lane 2: GM1 co-precipitated with TrkB from cells treated with 1 μM PMA for 7 min; lane 3: GM1 co-precipitated with TrkB from cells treated with PMA and 100 ng/ml BDNF for 7 min; St: 1 pmol standard GM1 ganglioside.

3.2. Tyrosine phosphorylation of TrkB

After electrophoresis of the immunoprecipitate obtained from control cells, followed by Western blotting, the probing with anti-TyrP antibodies showed the presence of a positive band of about 145 kDa, corresponding to the mass of TrkB (Fig. 2) [6]. The same analyses were performed on cells treated with PMA and with PMA+BDNF. As a control also the effect of BDNF was checked. The membranes used for probing with anti-TyrP antibodies were submitted to stripping and reprobed with anti-TrkB antibodies. The results showed that the amount of TrkB in the immunoprecipitates was similar in all the samples (Fig. 2). Tyrosine phosphorylation of each sample was normalized for the TrkB content of the same sample by calculating the ratio: intensity units of immunoreactivity to anti-TyrP/intensity units of immunoreactivity to anti-TrkB, measured from the scanned images of the ECL films, as above described. The value of the normalized phosphorylation in control cells was 0.45 (arbitrary units). In the following this value will be referred to as the basal phosphorylation level. Upon treatment with PMA, normalized tyrosine phosphorylation was strongly reduced to 0.32. When cells were treated with PMA and BDNF, the value (0.29) was comparable with the figure obtained after PMA treatment alone. On the contrary, and as expected, the phosphorylation was strongly enhanced (2.3-fold with respect to the basal phosphorylation level) upon cell treatment with BDNF.

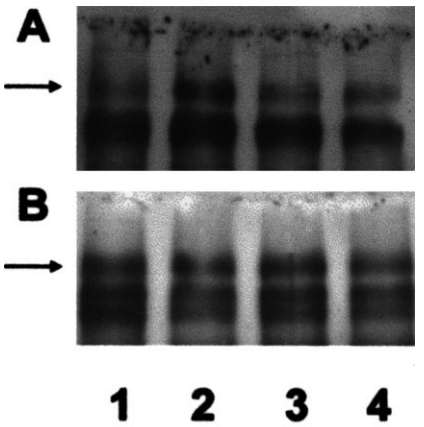

Fig. 2. Immunoblot analysis of TrkB with anti-phosphotyrosine and with anti-TrkB antibodies. The immunoprecipitates obtained with anti-TrkB from lysates of cerebellar granule cells were submitted to SDS/8% polyacrylamide gel electrophoresis. Panel A: Western blot analysis was performed with anti-phosphotyrosine antibody (4G10 from Upstate Biotechnology) and an ECL detection system. Panel B: The same membranes were stripped and immunoblotted with anti-TrkB (794, Santa Cruz) antibody. Lane 1: Control cells; lane 2: cells treated with 100 ng/ml BDNF for 7 min; lane 3: cells treated with 1 μM PMA for 7 min; lane 4: cells treated with PMA and 100 ng/ml BDNF for 7 min. The arrow indicates the molecular weight (145 kDa) of TrkB.

4. Discussion

This investigation shows that the treatment of cerebellar granule cells with PMA is followed by a remarkable change both in the GM1 ganglioside amount associated with TrkB and in the receptor activity, either basal or stimulated by BDNF. To reach this conclusion, tyrosine phosphorylation state of the receptor, and the content of GM1 ganglioside, were assessed in immunoprecipitates obtained with anti-TrkB antibodies from cells after different treatments. The co-precipitation of GM1 ganglioside with TrkB was expected. In fact, there have been reports of the occurrence of a tight association between GM1 and Trk receptors [3]. Moreover, recent data [5] show that TrkB is localized, along with other Trk receptors, in those GM1-enriched domains of neuronal plasma membranes which are involved in pivotal events such as signal transduction. It is likely that the cell lysis carried out by cold Triton X-100, a detergent widely used to isolate membrane domains [12,13], does not completely disrupt the bilayer assembly of granule cell plasma membrane, leaving detergent-resistant TrkB-containing membrane rafts. Therefore, antibodies directed against TrkB cause the receptor to precipitate together with its glycolipid environment. The evaluation of this possibility and that TrkB-GM1 interaction occurs within specialized membrane domains is not the aim of the present study, and deserves further investigation.

One of the difficulties to overcome, in investigating the role of endogenous GM1 on TrkB activity, was how to change the ganglioside density in the membrane region around the receptor, without making use of exogenous glycolipid. For this purpose, we employed short-term incubation with PMA, a treatment we recently showed to induce a modification of ganglioside exposure and segregation at the cell surface, as a consequence of protein kinase C (PKC) activation [7,14]. This approach gave the desired results (Fig. 1). In fact, upon PMA treatment, the amount of GM1 ganglioside co-precipitated with the receptor decreased with respect to control, untreated cells, indicating a decrease of its local density around TrkB. It can be argued that pretreatment with PMA could modify the amount of GM1 in the plasma membrane. However, PMA treatment did not affect the total ganglioside content of the cells, as shown by control experiments, and it is not reported to affect significantly the rate of fluid-phase endocytosis [7]. These data suggest that the treatment does not modify the content of GM1 at the exoplasmic surface of the cell, and strengthen the hypothesis that PMA elicits a redistribution of ganglioside within the plasma membrane, displacing it from the TrkB environment.

Most interestingly, the decrease was paralleled by a concomitant decrease of the tyrosine phosphorylation of TrkB. Furthermore, under these circumstances, i.e. when the amount of GM1 ganglioside associated with TrkB was lower, BDNF was no longer able to stimulate the strong phosphorylation of the receptor, normally exerted by this ligand (Fig. 2).

Is the effect of PMA on TrkB activity mediated by PKC or by the change in the ganglioside density near the receptor? The mediation by PKC would be conceivable, because it is known that its activation can affect NT receptors. In fact, it has been reported [15] that the proteolytic cleavage of the extracellular domain of the NGF receptor, TrkA, is regulated by PKC and by several receptor agonists (including NGF). As a consequence of the cleavage, the amounts of TrkA and of its phosphorylated form decrease. Although no similar effect of PKC on TrkB has been reported, a similar mechanism in our system could not be excluded a priori. However, the amount of immunoprecipitated TrkB protein in the different experiments was nearly identical. This observation rules out the occurrence of a PKC-stimulated cleavage of the receptor, at least under the experimental conditions of short-term treatment adopted here. Therefore, our data strongly suggest that the observed PMA-induced decrease of TrkB tyrosine phosphorylation is not due to a proteolytic degradation of the protein. Moreover, a kinase assay was carried out with radioactive ATP, as described [3], on immunocomplexes obtained from control and from PMA-treated cells. The analysis of the autoradiogram showed a decrease of TrkB phosphorylation in PMA-treated cells with respect to control, comparable to the decrease of tyrosine phosphorylation detected with anti-TyrP antibodies (data not shown). This result also rules out the possibility of a phosphorylation of TrkB serine or threonine residues exerted by PKC, and a possible consequent down-regulation of receptor phosphorylation.

Summing up, these observations open the alternative stimulating possibility that the decrease in the number of GM1 ganglioside molecules in the membrane area surrounding TrkB, induced by PMA treatment, is responsible for the decrease in receptor activity, basal or stimulated by BDNF. It is interesting to remember that an opposite change (increase) of GM1 concentration in the plasma membrane, obtained by addition of exogenous ganglioside, induces an increase in Trk activity, and rescues neuronal cells from apoptotic death, at least in part via Trk receptor dimerization and autophosphorylation [16]. Therefore, an appealing working hypothesis is that a decrease of endogenous GM1 in the membrane area surrounding TrkB prevents its dimerization, either basal or stimulated by BDNF. Needless to say, the evaluation of this hypothesis requires and deserves further experimental work.

Moreover, the observation that the change of ganglioside

association with the receptor is consequent to PKC activation, opens the possibility of a correlation between receptor modulation and trans-membrane signal transduction. It is also worth noting that BDNF is able to evoke PKC activation [17], thus also opening the possibility of a mechanism for receptor down-regulation.

Considered overall, the present investigation indicates for the first time that changes in the density of endogenous glycolipids in the environment of a receptor can modulate its activity, and thus offers intriguing new ideas towards an understanding of biochemical phenomena involved in physiological functions of the nervous system and in neurodegenerative disorders.

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