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QUARTERLY

Glycolipid–protein interaction in the mechanism of signal transduction: Studies with a photoactivable ganglioside analogue*✪

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An increasing body of evidence suggests that glycolipid domains are present on the plasma membrane surface of mammalian cells and play a key role in signal transduction. We have investigated the modulation of glycolipid–protein interaction consequent to a specific event occurring at the plasma membrane. For this purpose, a new photoactivable, radioactive derivative of G_{M1} ganglioside, carrying a phenyldiazirine fatty acid labelled with ^{125}I, has been used with rat cerebellar granule cells in culture. Upon incubation of photoactivable G_{M1} with the cells followed by illumination, several proteins become radioactive and were detectable on the two dimensional-electrophoresis, which points to their interaction with the ganglioside. Upon addition of cytotoxic doses of glutamate, known to induce indirectly the activation of protein kinase C (PKC), one of the proteins crosslinked by photoactivable G_{M1} in control cells of molecular mass about 92 kDa and pI about 4, was not anymore detectable; this suggests its exclusion from the glycolipid domains. On the contrary, another protein, of about 15 kDa and pI 6.5, previously not crosslinked, was interacting with the ganglioside derivative after glutamate treatment. Comparable effects were exerted by phorbol-2-myristate-3-acetate, which directly induces the activation of PKC. These results show that PKC activation, a key step of inbound trans-membrane signalling, affects the interaction between glycolipids and proteins at the plasma membrane surface,

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Abbreviations: PMA, phorbol-2-myristate-3-acetate; BME, modified Eagle's basal medium; FCS, fetal calf serum; PKC, protein kinase C; [^{125}I]TID-G_{M1}, 3-(trifluoromethyl)-3-(*m*-[^{125}I]iodophenyl)-diazirine G_{M1} ganglioside.

possibly within a mixed domain. The dynamic modulation of ganglioside–protein interaction may affect the involvement of glycolipid domains in membrane-located events such as signal transmission and lipid/protein sorting.

Gangliosides, sialic acid-containing glycosphingolipids, are typical components of the plasma membrane where they are asymmetrically located in the outer leaflet [1]. Through their lipid portions, they share the dynamic flow of the lipid components of the membrane. A peculiar feature of glycosphingolipids is that they undergo lateral phase separation with formation of glycolipid-enriched domains [2]. An increasing body of evidence indicates that glycolipid domains are supramolecular structures accomplishing the correlation among membrane architecture, dynamics and function [3–5]. The interest for this subject increased after the glycolipid-enriched domains isolated from cell membranes were found to show a concomitant, peculiar enrichment in proteins [5–8], suggesting their involvement in the mechanism of signal transmission and lipid/protein sorting. In particular, G_{M1} ganglioside has been suggested to be a glycolipid marker of these structures [8–11]. To investigate this issue, in the past years photoactivable and fluorescent derivatives of gangliosides have been synthesized and used for photolabeling of membrane proteins [11–13]. In this paper we describe the use of a ganglioside derivative carrying the diazirine photoactivable probe in the ceramide portion of the molecule. We utilized this product to study the ganglioside–protein interactions in rat cerebellar granule cells, and the changes consequent to a specific membrane event, i.e. activation of the protein kinase C exerted by addition of phorbol-2-myristate-3-acetate.

MATERIALS AND METHODS

Chemicals. Solvents and silica gel HPTLC plates were from Merck (Darmstadt, F.R.G.); Modified Eagle's basal medium (BME) and foetal calf serum (heat-inactivated before use) were from Irvine (Santa Ana, CA, U.S.A.). Poly-L-lysine, 1-α-D-arabinofuranosylcytosine, NeuAc, and crystalline bovine serum albumin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

^{125}Iodine (5 mCi, 350–600 Ci/ml), [^{14}C]-methylated protein standard for electrophoresis and autoradiographic films were from Amersham. All chemicals for electrophoresis were from Bio-Rad.

G_{M1} ganglioside was extracted and purified as earlier described [14]. G_{M1}, tritium labelled at the 3-position of the long chain base, was prepared as described by Sonnino *et al.* [15]. Photoactivable ^{125}I-labelled G_{M1} ganglioside, [^{125}I]TID-G_{M1}, 3-(trifluoromethyl)-3-(*m*-[^{125}I]-iodophenyl)diazirine G_{M1} ganglioside (200 Ci/mmol) was synthetized according to Brunner [16], with minor modifications.

Cell cultures. Cerebellar granule cells, obtained from 8-day-old Sprague-Dawley rats (Charles River), were prepared and cultured according to Gallo *et al.* [17]. Glial proliferation was prevented by adding cytosine arabinofuranoside (final concentration, 10 μM) 18–20 h after plating. Primary cultures were grown for 8 days in 60-mm culture plastic dishes coated with poly-L-lysine. At that time the cerebellar cultures contained more than 95% neurons and less than 5% glial cells.

Treatment of cells with [^{125}I]TID-G_{M1}. [^{125}I]TID-G_{M1} was dried from metanol by flushing with N_2 and dissolved in 2 ml BME. After removal of the medium followed by rapid washing, the cells were incubated at 8°C for 2 h in the presence of 10^{-6} M [^{125} I]TID-G_{M1}. At the end of incubation cells were washed three times with 3 ml (each time) of BME and then incubated (4 times, 5 min each time) with BME containing 0.2% bovine se-

rum albumin, at 37°C. This treatment removes the ganglioside derivative loosely adhering to the cells, and leaves photoactivable ganglioside in the so called "serum stable" form of association [12].

Binding and metabolism of [^{125}I]TID-G_{M1}. [^{125}I]TID-G_{M1} was dissolved in the medium for cell culture (10^{-6} M final concentration). After 2 h incubation at 8°C and successive treatment with fetal calf serum and/or with trypsin (trypsin stable form of associated ganglioside), different amounts of the G_{M1} derivative were found associated to the cells. A parallel experiment was performed using [^{3}H]G_{M1} ganglioside.

The metabolic processing of [^{125}I]TID-G_{M1} was assessed as described by Sonnino *et al.* [12] and all the experiments were carried out without exposing the cells to UV light (see below). The cells were harvested by scraping, centrifuged and subjected to lipid extraction [14]. The extracted lipids were separated by HPTLC (solvent system: chloroform/methanol/water, 60:35:8, by vol.) and TLC plates subjected to autoradiography. In parallel experiments, cells in culture were incubated in a medium containing tritium-labelled G_{M1} (10^{-6} M) instead of [^{125}I]TID-G_{M1}.

Identification of granule cells proteins interacting with [^{125}I]TID-G_{M1} after photoactivation. Cells subjected to the above treatment were irradiated for 5 min at 4°C with a UV lamp (HG 100, 125 W, Jelosil, Milan, Italy), harvested by scraping with a rubber scraper in 320 mM sucrose, EDTA 0.1 mM in phosphate buffer, 1 mM, pH 7.2, and centrifuged (8000 × *g*, 10 min). The pellet was homogenized in the same solution, centrifuged at 1000 × *g* for 15 min (three times) and the pooled supernatants were centrifuged at 100000 × *g* for 1 h. The pellet obtained, from now on called the "enriched membrane fraction", was submitted to two-dimensional electrophoresis. For this purpose a buffer containing 9.5 M urea, 2% NP-40, 2% ampholytes and 100 mM dithiothreitol was used for isoelectric focusing, performed according to Dessi *et al.* [18], using the BioRad Mini-protean II; 12% gels (SDS/PAGE) were used for the second dimension, and the gel slabs submitted to autoradiography. The total protein pattern of granule cells was assessed by silver staining.

Effect of glutamate or phorbol-2-myristate-3-acetate. Cells were incubated with [^{125}I]TID-G_{M1}, as above described. The medium in the dish was replaced by Locke's solution containing glutamate (100 μM, without Mg^{2+}) or, in separate experiments, containing PMA (1 μM). Cells were then irradiated with UV light and processed for identification of radiolabeled proteins after 15 min incubation with glutamate or after 5 min incubation with PMA.

Other assays. Protein content was determined by the methods of Lowry *et al.* [19].

RESULTS

The viability of cells, after exposure to 10^{-6} M [^{125}I]TID-G_{M1} at 8°C for up to 5 h, was good and identical to that of cells incubated with native G_{M1} ganglioside.

Binding of [^{125}I]TID-G_{M1} to cells and cross-linking were performed at low temperature in order to minimize endocytosis of the probe. In fact, under such conditions no internalization of G_{M1} has been detected [10]. After 2 h incubation at 8°C, 38 pmoles of [^{125}I]TID-G_{M1}/mg protein were bound to granule cells (serum stable form). A parallel experiment using [^{3}H]G_{M1} gave very similar results (30 pmoles/mg protein per 2 h at 8°C), indicating that association of the photoactivable ganglioside was comparable to that of isotopically labelled G_{M1} ganglioside.

After trypsin treatment, the amount of [^{125}I]TID-G_{M1} associated with the cells was 23 pmoles/mg protein. It is commonly accepted that the ganglioside molecules bound to the cells after this treatment are those correctly inserted into the lipid bilayer [20].

Metabolic experiments with [^{125}I]TID-G_{M1}

Figure 1 shows the autoradiography of TLC of the total ganglioside extract after treatment of granule cells with [^{125}I]TID-G_{M1}. Only one band, corresponding to the administered [^{125}I]TID-G_{M1}, was present after 2 h at 8°C, suggesting that under these conditions the ganglioside was still at the membrane and its metabolic processing had not yet started. On the contrary, after 2 h incubation at 37°C, other bands having a higher R_F, probably corresponding to [^{125}I]TID-derivatives of G_{M2} and G_{M3}, were found. A parallel experiment with [^{3}H]G_{M1} gave similar results (not shown), indicating that metabolic processing of [^{125}I]TID-G_{M1} and of [^{3}H]G_{M1} ganglioside were qualitatively similar.

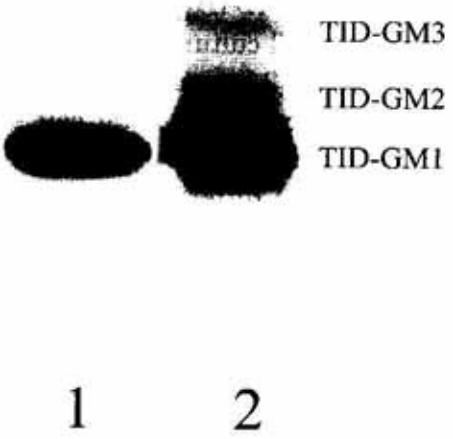

Figure 1. Thin-layer chromatography pattern of radioactive lipids extracted from granule cells: lane 1, incubation for 2 h with 10^{-6} M [^{125}I]TID-G_{M1} at 8°C; lane 2, as above followed by 2 h chase at 37°C.

Assessment of proteins interacting with [^{125}I]TID-G_{M1}

The protein pattern of the enriched membrane fraction prepared from granule cells, stained with silver, is shown in Fig. 2. The major proteins display a molecular mass of about 55 kDa and 45 kDa, with a pI of about 5. After incubation with [^{125}I]TID-G_{M1} and irradiation the photoactivable ganglioside covalently crosslinks neighbouring proteins, that thus become radiolabelled.

Experimental results, showing the proteins labelled after 2 h incubation of the cells at 8°C with [^{125}I]TID-G_{M1}, followed by photoactivation, are presented in Fig. 3, panel A. The Figure shows the autoradiography of the two-dimensional electrophoresis of the enriched

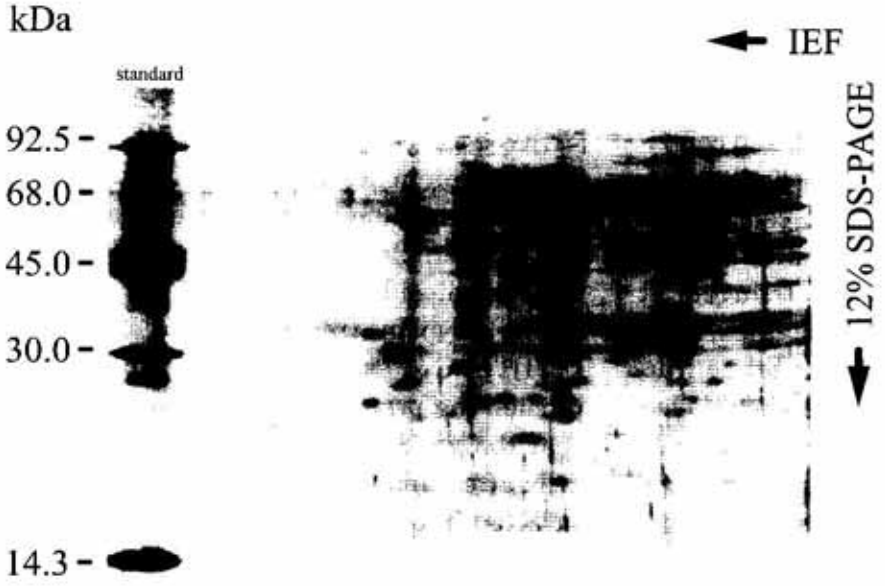

Figure 2. Protein pattern of an enriched membrane fraction prepared from granule cells, analysed by two-dimensional electrophoresis (pH range 4–9) and detected by silver staining.

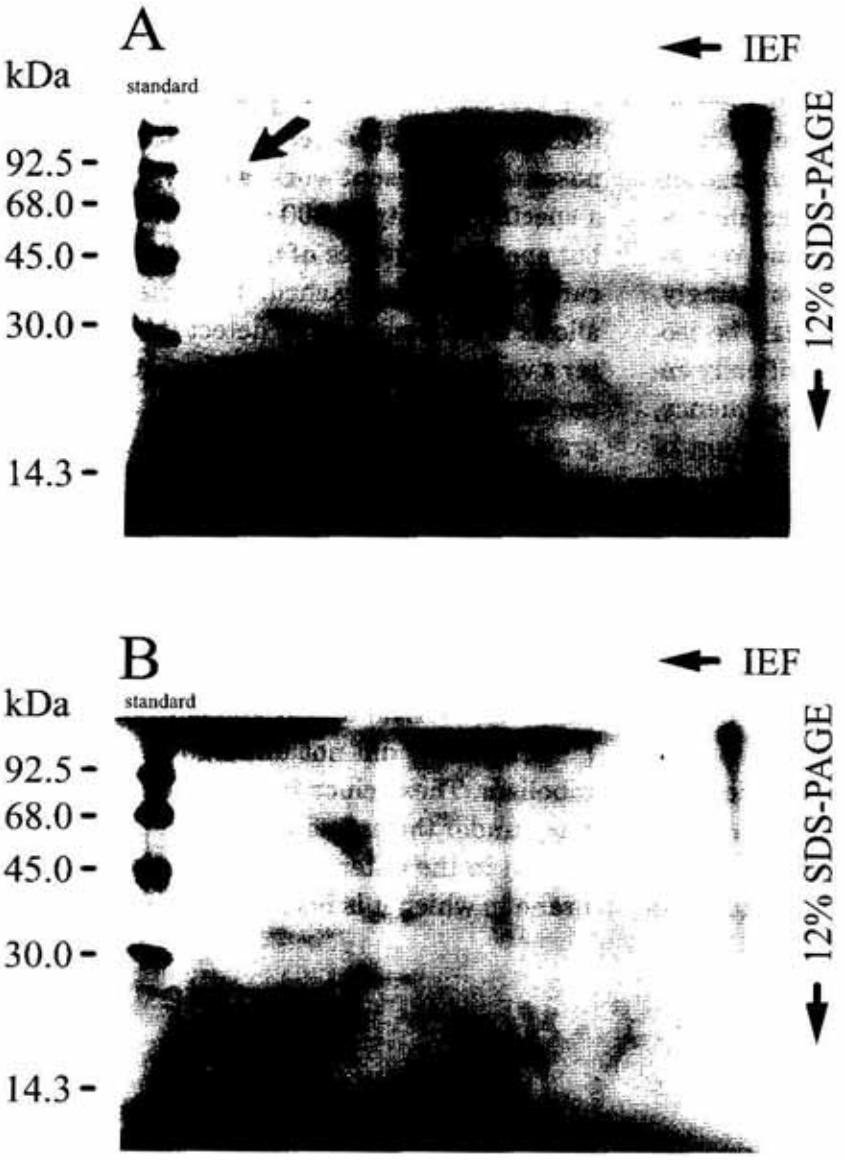

Figure 3. Pattern of radioactive proteins of an enriched membrane fraction prepared from granule cells, incubated for 2 h with 10^{-6} M [^{125}I]TID-G_{M1} at 8°C and analysed by two-dimensional electrophoresis (panel A). Panel B: after treatment with 100 μM glutamate.

membrane fraction carrying [^{125}I]TID-G_{M1} in the "serum stable" form of association to cells. Proteins of about 55, 39 and 30 kDa are the main radioactive spots present in the gel. In particular, as explained further on, a radioactive protein of 92 kDa and pI about 4, was also present. A large radioactive band, migrating at the gel front, was also evident. This band corresponds to a self-quenching ganglioside, not completely removed after lipid extraction, as the same behaviour is shown by the lipid extract (not shown).

After treatment with 100 μM glutamate (Fig. 3, panel B), the membrane fractions were again submitted to electrophoresis and the gel slab to autoradiography. As the most remarkable difference after treatment with glutamate, the radiolabelled spot corresponding to a protein of about 92 kDa and pI of about 4, was not detectable anymore. In the meanwhile, another protein of about 15 kDa and pI 6.5, previously not detectable, was cross-linked by [^{125}I]TID-G_{M1}. Similar results were obtained after treatment with PMA (not shown).

DISCUSSION

Gangliosides are known to self associate, possibly through carbohydrate-carbohydrate interaction [3, 21] and this tendency is higher

in sphingomyelin environments [22]. In general, it has been reported that ganglioside segregation affects ganglioside–protein interactions, such as recognition by enzymes, interaction with toxins, as well as the conformation of integral membrane proteins, the mechanisms of lipid and protein sorting and regulation of receptors [4, 21–25]. Interestingly, glycolipid-enriched fractions that can be isolated from cell membranes are specifically enriched in proteins [26, 27]. As a consequence, it has been suggested that the formation of specialized glycolipid-enriched membrane domains can affect important membrane functions such as lipid/protein sorting and signal transmission [4]. A debated question is whether or not the lateral distribution in the membrane, the local enrichment in glycolipids and the interaction with proteins can be modulated [28, 29]. Starting from the hypothesis that the ganglioside–protein interaction is expected to change depending on specific events at the membrane level, we decided to investigate this subject using the photoactivable, radioactive derivative of G_{M1} ganglioside, [^{125}I]TID-G_{M1}. One of the main features of [^{125}I]TID-G_{M1} is its high specific radioactivity, due to the presence of ^{125}I. For the purposes of the present work, a derivative having a specific activity of 200 Ci/mmole was used, but specific activities of up to 2000 Ci/mmole can be currently reached. This characteristic allows in consequence to detect the results after a very short time of sample processing. We performed experiments using rat cerebellar granule cells in culture. The reason for this choice is based on the observation that neurons, and particular cerebellar granule cells, contain glycolipid-enriched domains where proteins involved in signal transmission are concentrated [6, 30]. Under the experimental conditions herein adopted for incubation of the photoactivable ganglioside with cells, [^{125}I]TID-G_{M1} did not undergo lysosomal catabolism. These clues indicate that [^{125}I]TID-G_{M1}, under the experimental conditions, is localised in the outer layer of the plasma membrane in which it is inserted with its lipid moi-

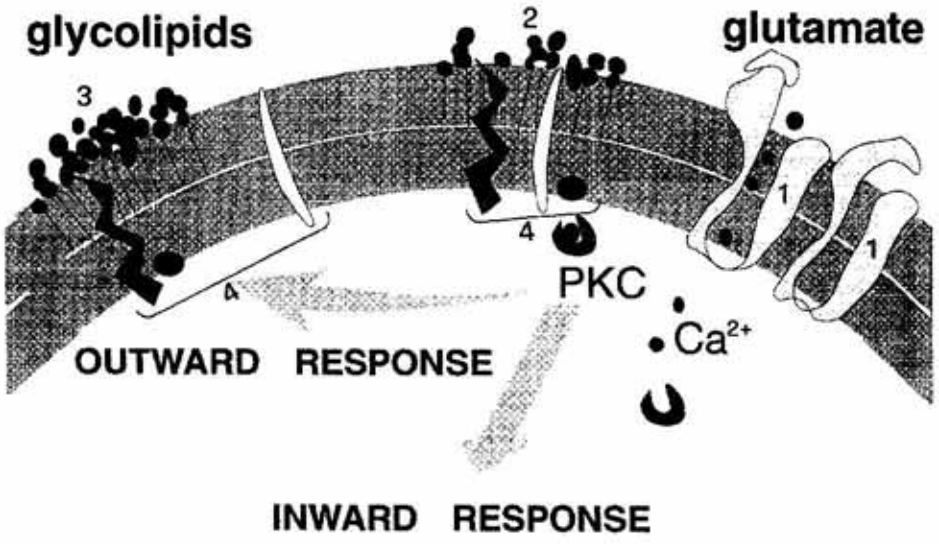

Figure 4. A possible mechanism for the modulation of glycolipid–protein interaction in signal transduction.

The arrival of a signal at the exoplasmic face of the plasma membrane (e.g. glutamate) and the binding to its receptor (1) induce Ca^{2+} influx. Ca^{2+}-induced translocation and activation of protein kinase C (PKC) classically exerts intracellular responses. As an alternative pathway, the cell may dispatch outbound signals by changing glycolipid segregation at the exoplasmic face of the plasma membrane e.g. by increasing locally the amount of glycolipids (cf. 2 and 3). Following this change, the pattern of proteins (4) associated to the glycolipid domains becomes altered, with possible repercussions on signal transduction processes.

ety, mimicking its endogenous counterpart. It is also worth noting that under different, appropriate conditions, [^{125}I]TID-G_{M1} is metabolized like isotopically tritium labelled-G_{M1}, which suggests that this derivative is normally recognized and metabolised by the metabolic machinery of the cells. After incubation with cells and crosslinking, of all the proteins present in the enriched membrane fraction prepared from the cells only a few were found to interact more closely with the ganglioside, becoming preferentially radiolabelled. In successive experiments we decided to study the effect of glutamate on the ganglioside-protein interaction. In fact, cytotoxic doses of glutamate added to cerebellar granule cells in culture trigger a series of events at the level of the plasma membrane. The opening of Ca^{2+} channels and the subsequent influx of Ca^{2+} induce PKC translocation and activation. On the other hand, the stimulation of metabotropic receptors starts the phosphoinositide cycle and may also contribute to PKC activation [31].

The data herein reported show that addition of glutamate brings about changes that affect the ganglioside-protein interaction. In fact, upon treatment with 100 μM glutamate, a protein of about 90 kDa normally interacting with the ganglioside, as shown by the experiments performed with control granule cells, was not anymore crosslinked by [^{125}I]TID-G_{M1}, while another protein, of about 15 kDa previously not interacting, was crosslinked. In order to verify whether or not this change of interaction was attributable to PKC activation, we carried out a similar experiment substituting glutamate with PMA, a drug inducing PKC activation in many cell types, granule cells included [32]. The results were comparable with those obtained with glutamate, thus confirming the involvement of PKC.

Taken together, these data show that PKC translocation from the cytosol to the endoplasmic side of the membrane affects the ganglioside-protein interaction at the plasma membrane surface. In particular, the interaction with a protein of 92 kDa, detected in control cells, is missing after PKC activation while another interaction, with a protein of 15 kDa, appears. It can be argued about the reasons leading to the change of the protein pattern in the G_{M1} environment, probably a lipid/protein domain, upon activation of the kinase. Since integral membrane proteins can interact with gangliosides [21, 28], a definite possibility is that protein phosphorylation, brought about by PKC, directly or indirectly triggers a change of their conformation or oligomerization state in the membrane, affecting their interaction with glycolipids. A relationship between protein conformation and interaction with gangliosides has been reported [21]. Moreover, it should be remembered that gangliosides, even if residing in the outer leaflet of the plasma membrane, can also directly interact with proteins facing the cytosolic side of the membrane [11], where the proteins, at this moment not yet identified, could be located.

In conclusion, PKC translocation from the cytosol to the endoplasmic side of the membrane, the central event of trans-membrane signalling, affects glycolipid domains and their interaction with proteins, possibly changing their functional organization. It could be argued that the transduction of the message triggered by glutamate (inbound trans-membrane signalling), is directly responsible for the changes in the organization of glycolipids at the outer leaflet of the plasma membrane. The results obtained with PMA, which is able to directly activate PKC bypassing the receptor system, comparable with those obtained with glutamate, rule out this possibility and at the same time suggest the existence of outbound trans-membrane signalling. Our hypothesis, illustrated in Fig. 4, is that the return of messages back to the exolayer may represent a general alternative to the well known inward-directed pathway, following the arrival of a signal at the cell surface.

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