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Glutamate Transport and Not Glutamate Receptor Binding Is Stimulated by Gangliosides in a Ca^{2+}-Dependent Manner in Rat Brain Synaptic Plasma Membranes

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Abstract: Crude as well as purified synaptic plasma membrane (SPM) preparations were analyzed for the influence of the ganglioside galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl ceramide (GM1) on high-affinity binding of L-[^{3}H]glutamate. Assayed in two different buffer systems, SPM consistently exhibited increased (40–50%) binding upon incubation with GM1 plus Ca^{2+}, as compared to controls without GM1. Incorporation experiments with ^{3}H-labeled GM1 proved trypsin-stable insertion of GM1 into SPM, with a maximum incorporation of four times the endogenous amount (35 nmol/mg of protein). The observed increase in glutamate binding was not due to a change in the affinity of the binding sites, but to a change in the number of binding sites, and it was absolutely dependent on the presence of Ca^{2+}. A pharmacological profile of the GM1/Ca^{2+}-stimulated glutamate binding is presented. The original classification of the stimulatory effect as an effect on glutamate receptor binding had to be revised to take into account the observed temperature sensitivity of the ganglioside effect, its sensitivity to high osmolarity and to ultrasonication, and the lack of binding stimulation after detergent treatment of membranes or after receptor solubilization. Vesicular space measured in both SPM preparations was found to be around 7 μl/mg of protein, in ganglioside-treated as well as in control membranes. From the data, it is concluded that a special, Na^+- and Cl^--independent form of glutamate transport into resealed membrane vesicles is stimulated by gangliosides in the presence of Ca^{2+}. **Key Words:** Glutamate transport—Ganglioside—GM1—Synaptic plasma membrane—Calcium—Na^+/Cl^--independent transport. **Hollmann M. and Seifert W.** Glutamate transport and not glutamate receptor binding is stimulated by gangliosides in a Ca^{2+}-dependent manner in rat brain synaptic plasma membranes. *J. Neurochem.* **53,** 716–723 (1989).

Triggered by several speculations in the literature (Rahmann et al., 1975, 1976; Svennerholm, 1980) and by experiments with hippocampal slices in our laboratory (Wieraszko and Seifert, 1985; Seifert et al., 1986) which suggested that gangliosides might be involved somehow in the modulation of synaptic transmission, we set out to investigate the influence of gangliosides on parameters of glutamatergic neurotransmission. In a previous report (Hollmann and Seifert, 1986), we demonstrated that exogenously applied galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl ceramide (GM1), as well as other gangliosides, in the presence of Ca^{2+} is able to stimulate glutamate binding to rat brain synaptic plasma membranes (SPM). In consideration of the observed pharmacological profile, this stimulation originally was thought to be due to an increase in receptor binding sites on the SPM.

However, recent reports by several investigators suggested the possibility that glutamate binding to SPM measured in Cl^--containing buffers to some extent might represent transport into resealed membrane vesicles rather than receptor binding (Fagg and Lanthorn, 1985; Bridges et al., 1986; Kessler et al., 1987; Zaczek et al., 1987). Additionally, experiments in our group revealed the existence in SPM of a novel glutamate transport mechanism, which is active even in Cl^--free buffers (Hollmann et al., 1988). Thus, the question arose whether Ca^{2+}/ganglioside-stimulated glutamate binding represented receptor binding or transport of

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Abbreviations used: AP4, 2-amino-4-phosphonobutyrate; cSPM, crude synaptic plasma membranes; GM1, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminyl)-galactosylglucosyl ceramide; PSD, postsynaptic density; pSPM, purified synaptic plasma membranes; SPM, synaptic plasma membranes; Tris-Ac, Tris-acetate buffer; Tris-Cl, Tris-chloride buffer.

glutamate, or both. In this article, we provide evidence that the stimulation by gangliosides of glutamate binding to SPM is due solely to an increase in transport of glutamate into resealed membrane vesicles.

MATERIALS AND METHODS

Chemicals

L-[^{3}H]Glutamate (53 Ci/mmol) was obtained from Amersham. [^{3}H]H_2O (1 mCi/g) and [^{14}C]inulin (2.4 mCi/g) were purchased from New England Nuclear, as was [*galactose*-6-^{3}H(N)]GM1 (7 Ci/mmol). The latter was supplied in methylene chloride/ethanol/water (20:10:1), sealed under argon in silanized ampoules, and shipped on dry ice. It was stored at −20°C until use. When aliquots for experiments were removed from the stock solution, the stock was regassed with nitrogen before further storage. Aliquots to be used in experiments were dried under a gentle stream of nitrogen without heating and the residue taken up in assay buffer. Purified unlabeled GM1 was a generous gift from FIDIA (Abano Terme, Italy); before use, it was dialyzed extensively first against 2 m*M* EGTA-KOH, pH 7.0, and then three times against water, followed by lyophilization. All inhibitors used were from Sigma (St. Louis, MO, U.S.A.), except DL-threo-β-hydroxyaspartic acid and 2-amino-4-phosphonobutyric acid, which were from Calbiochem (San Diego, CA, U.S.A.). All other chemicals were of analytical grade or of the highest purity commercially available, and were purchased from either Merck (Darmstadt, F.R.G.) or Sigma.

Preparation of SPM and postsynaptic densities (PSDs)

Crude cortex SPM (cSPM) from 3-month-old male Wistar rats were prepared according to the differential centrifugation method of Enna and Snyder (1975), whereas purified SPM (pSPM) were prepared by the sucrose density gradient procedure of Jones and Matus (1974). All final membranes were washed three times with assay buffer [either 50 m*M* Tris-acetate (Tris-Ac), pH 7.1, or 50 m*M* Tris-chloride (Tris-Cl), pH 7.1] and stored frozen in assay buffer at −80°C at a protein concentration of 2–4 mg/ml for up to 2 months without significant loss of any of the glutamate binding properties.

PSDs were prepared from cSPM according to Fagg and Matus (1984). Briefly, 4 mg of pSPM were pelleted and resuspended in 6 ml of 1% Triton X-100. The suspension was stirred for 30 min at 0°C and then layered on top of a 30-ml cushion of 1 *M* sucrose. Centrifugation for 60 min at 105,000 *g* resulted in the pelleting of PSDs, which were washed three times with 35 ml of Tris-Ac before use in the glutamate binding assay.

Receptor solubilization

Glutamate receptors were solubilized as described by Koshija (1985). Briefly, cSPM were pelleted and resuspended in 10 m*M* 3-[(3-cholamidopropyl)dimethylamino]-1-propanesulfonate (CHAPS)/200 m*M* NH_4SCN/1 m*M* $MgSO_4$/50 m*M* Tris-Ac, pH 7.1, at a protein concentration of 1.5 mg/ml. The membranes were stirred at 37°C for 30 min, followed by ultracentrifugation for 60 min at 150,000 *g*. The supernatant was adjusted to 0.5% Triton X-100 and dialyzed against 0.5% Triton X-100/1 m*M* EDTA/50 m*M* Tris-Ac, pH 7.1, for 24 h with two buffer changes.

Analysis of ganglioside incorporation

cSPM (120 μg) were incubated with 10 n*M* [^{3}H]GM1 plus 50 μ*M* unlabeled GM1 in a total volume of 300 μl of Tris-Ac/5 m*M* calcium acetate at 37°C for time periods ranging from 1 to 120 min. Incubation was stopped by centrifugation for 10 min at 12,800 *g*. The pellet was resuspended in 1 ml of 0.25% trypsin/0.02% EDTA/phosphate-buffered saline, pH 7.1, and incubated for 20 min at 37°C. Samples were centrifuged as above and the pellets solubilized in 1% sodium dodecyl sulfate/20 m*M* EDTA-NaOH, pH 8.0, for 10 min at 95°C. Radioactivity was counted in aliquots of both the solubilized pellets and the trypsinization supernatant, as described above. TLC analysis of the labeled [^{3}H]GM1 was performed on a sample of the [^{3}H]GM1 before incubation with SPM, and on a sample of total gangliosides isolated by the method of Svennerholm and Fredman (1980) from SPM incubated with [^{3}H]GM1. No labeled bands other than GM1 could be detected, and no breakdown products were seen; also, no labeled material remained at the origin of sample application or migrated with the solvent front.

Glutamate binding assay

L-[^{3}H]Glutamate binding to SPM was measured by a filtration assay. All buffers were sterile-filtered before use in order to avoid artifacts by bacterial glutamate transport. SPM (100 μl; ~60–100 μg of protein) were incubated in assay buffer (see above) at 37°C (in some experiments at 0°C) for 60 min in a total volume of 310 μl, with L-[^{3}H]glutamate added to give a final concentration of 50 n*M*. Glutamate concentrations ranging from 5 μ*M* to 6,600 μ*M* were employed in those experiments where binding constants were determined; in these cases, concentrations above 200 μ*M* L-[^{3}H]glutamate were achieved by addition of unlabeled L-glutamate. Gangliosides, salts, and/or glutamate binding inhibitors to be tested were added to the incubation mix from 10-fold concentrated stock solutions prepared in assay buffer. Membrane addition started the incubation period, which was stopped by the addition of 5 ml of ice-cold assay buffer, followed by rapid filtration (<10 s) onto nitrocellulose filters (25 mm diameter, Schleicher and Schuell no. BA85). Filters were washed once with ice-cold assay buffer, solubilized in scintillator (ACS, Amersham), and counted in a Packard CA 2000 liquid scintillation counter. Specific binding was calculated as the difference between total binding and unspecific binding which was measured in the presence of a 2,000-fold excess (100 μ*M*) of unlabeled L-glutamate. Unspecific binding typically amounted to 20–30% (Tris-Cl) or 60–70% (Tris-Ac) of the total binding. All determinations were done in triplicate, with SEM values being less than 10%.

Solubilized receptors were measured by the method of Koshija (1985). Briefly, 100 μl of solubilization supernatant (~20–50 μg of protein) were incubated as described above for SPM. Receptor-bound and free L-[^{3}H]glutamate were separated by rapid filtration through GF/B-type glass fiber filters (Whatman) which had been precoated by immersion into 0.3% polyethyleneimine, pH 10, for 16 h. Filters were washed three times with ice-cold assay buffer, rocked in scintillator for 8 h, and counted as described above.

Estimation of intravesicular space

Intravesicular volumes of resealed vesicles in 1-ml aliquots (~0.6–1 mg of protein) of SPM preparations were measured utilizing the differential distribution of membrane-permeable [^{3}H]H_2O and membrane-impermeable [^{14}C]inulin tracer in membrane pellet versus supernatant, according to the method

described by Rudnick (1977). Protein content of the samples was estimated according to the method of Peterson (1977).

RESULTS

Incorporation of gangliosides into SPM

In order to check whether gangliosides under our incubation conditions would become stably incorporated into SPM, we employed the trypsinization criterion of Radsak et al. (1982): only that portion (around 55%) of the total membrane-associated ganglioside that resisted trypsin treatment (see Materials and Methods) was considered to be integrally incorporated into the lipid bilayer. Trypsin-stable incorporation of GM1 was very rapid (see Fig. 1). After 1 min, it had already reached about 65% of the saturation value found after 90 min. Trypsin-labile association of GM1 showed a different time course. It reached its maximum within a few seconds, too quick to be resolved by the method employed, and then declined gradually, probably reflecting the increasing trypsin-stable incorporation of GM1 into the membranes (data not shown). The maximum value of incorporation (35 nmol of GM1/mg of protein) was reached after 90 min. Assuming the endogenous GM1 content of SPM to be around 9 nmol/mg of protein (Skrivanek et al., 1982), this incorporation represents a considerable increase in GM1, to nearly 400% of the endogenous level. Furthermore, GM1 incorporation into membranes was found not to be dependent on the temperature of incubation.

Stimulation of glutamate binding by gangliosides plus Ca^{2+}

As has been shown in a previous paper (Hollmann and Seifert, 1986), glutamate binding to cSPM was saturated within 30 min in both Tris-Ac and Tris-Cl buffers. Binding values measured in Tris-Cl were consistently about five times higher than those measured in Tris-Ac. Supplementation of the assay buffer with millimolar concentrations of Ca^{2+} resulted in an increase in binding (Hollmann and Seifert, 1986; Hollmann et al., 1988).

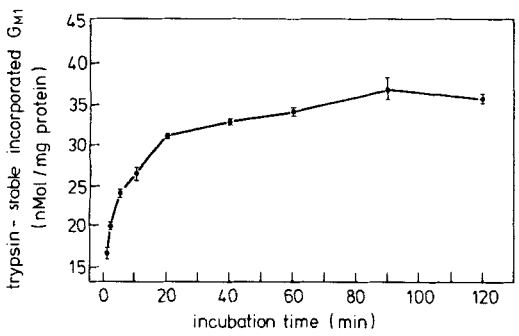

FIG. 1. Time course of trypsin-stable GM1 incorporation into rat brain cSPM. cSPM (120 μg of protein) were incubated in Tris-Ac at 37°C with 10 n*M* [^{3}H]GM1 plus 50 μ*M* unlabeled GM1 in the presence of 5 m*M* Ca^{2+} for the times indicated. Data represent means ± SEM (n = 3).

Addition of gangliosides produced a further, concentration-dependent (Hollmann and Seifert, 1986) increase in glutamate binding in both buffer systems, but did so only in the presence of Ca^{2+}; gangliosides alone had no significant modulatory influence on glutamate binding (Fig. 2). Ca^{2+} was effective in permitting binding stimulation by gangliosides in concentrations ranging from about 100 μ*M* to 10 m*M*; below 100 μ*M*, there was no effect on glutamate binding, and above 10 m*M*, Ca^{2+} inhibited binding. When SPM were preincubated with gangliosides, followed by a second incubation with glutamate plus Ca^{2+}, the same binding stimulation was observed as compared to those experiments where SPM were simultaneously incubated with GM1/Ca^{2+} plus glutamate. The latter procedure then was chosen as the standard procedure for most of the further experiments because of its simplicity. As has been shown earlier (Hollmann and Seifert, 1986), all of the gangliosides tested produced binding stimulation in the presence of Ca^{2+}, monosialogangliosides as well as polysialogangliosides. However, we now found that asialo-GM1, a derivative of GM1 lacking the sialic acid moiety, did not exhibit any stimulatory effect on glutamate binding. Similarly, none of several molecules structurally related to gangliosides (glucocerebroside, galactocerebroside, ceramide, sialyllactose, or free sialic acid) was effective in the stimulation of binding. For all further experiments, GM1 was chosen, because the insertion of GM1 into plasma membranes had already been proven to result in biologically active GM1 (Moss et al., 1976), and because GM1 could be obtained in highly purified form.

Kinetics and pharmacology of GM1/Ca^{2+}-dependent glutamate binding

The increase in binding to SPM produced by gangliosides plus Ca^{2+} was due to an increase in β_{max}, the maximum number of binding sites. If, however, the binding site measured represents a carrier protein (see Discussion), the observed increase would correspond to an increase in V_{max}, the maximum transport velocity. In contrast, the affinity of the binding sites was unchanged, as shown in Table 1 for both buffer systems.

Table 2 outlines the pharmacological specificity of the glutamate binding sites measured in the presence of GM1/Ca^{2+}. Inhibition values of a series of glutamate receptor agonists, antagonists, and some transport inhibitors appear to be very similar to those found for basal binding (Hollmann et al., 1988). The only compounds exhibiting significantly different inhibition values of GM1/Ca^{2+}-stimulated binding as compared to basal binding were 2-amino-4-phosphonobutyrate (AP4) and L-glutamic acid-γ-hydroxamate, which both were clearly more potent inhibitors for the GM1/Ca^{2+}-stimulated binding.

Comparison of different membrane preparations

GM1/Ca^{2+}-dependent binding stimulation could be found in both cSPM and pSPM (Fig. 2A and B). GM1 alone in all preparations tested produced either none

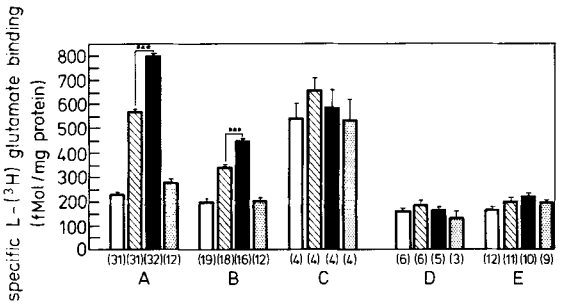

FIG. 2. Comparison of the influence of 5 m*M* Ca^{2+} (hatched bars), 5 m*M* Ca^{2+} plus 100 μM GM1 (filled bars), or 100 μM GM1 (stippled bars) on basal L-[^{3}H]glutamate binding (open bars) to SPM. **A:** cSPM at 37°C. **B:** pSPM at 37°C. **C:** PSDs at 37°C. **D:** cSPM at 0°C. **E:** pSPM at 0°C. Incubation was done as described in Materials and Methods. The numbers of experiments are in parentheses, and all experiments were done in triplicate. Statistical significance of differences was calculated using the Wilcoxon test: ***$p < 0.001$.

or only a marginal stimulation which was statistically not significant ($p > 0.1$). Interestingly, stimulation by GM1/Ca^{2+} was absent in PSDs (Fig. 2C), which also lacked any significant stimulation by Ca^{2+}. Similarly, solubilized glutamate receptor preparations, which exhibited high specific binding (392 ± 83, 847 ± 153, and 549 ± 70 fmol/mg of protein in Tris-Ac for solubilized receptors from rat cortex, hippocampus, and cerebellum, respectively) also lacked binding stimulation both by Ca^{2+} and by GM1/Ca^{2+}.

The GM1/Ca^{2+}-dependent increase in glutamate binding showed some species specificity, because cSPM prepared from cow or guinea pig brain did not exhibit binding stimulation, although Ca^{2+} alone increased binding comparable to its action on rat cSPM preparations (data not shown).

Influence of temperature and osmolarity on the GM1 effect

Lowering the assay temperature to 0°C resulted in a complete abolition of Ca^{2+}- as well as GM1/Ca^{2+}-induced stimulation of glutamate binding in Tris-Ac buffer (Fig. 2D and E). Identical results were obtained when SPM were preincubated with GM1/Ca^{2+} at 37°C, followed by the glutamate binding assay performed at 0°C. The same was true for the Tris-Cl buffer system, where at 0°C, in addition to the above effects, even basal binding was drastically reduced to about 5–10% of its value at 37°C (data not shown). These experiments indicated that part of the observed binding might represent a highly temperature-sensitive transport process, instead of receptor binding which generally has only a low temperature coefficient (Kanner, 1978; Pin et al., 1984).

Raising the osmolarity of the incubation mix by the introduction of increasing concentrations of sucrose resulted in a gradual decline and finally complete abolition of the GM1/Ca^{2+}-dependent binding stimulation (Fig. 3). Basal binding was not affected by sucrose. The same influence of sucrose had been observed before on the Ca^{2+}-induced binding stimulation (Hollmann et al., 1988). The capacity of SPM to exhibit GM1/Ca^{2+}-stimulated binding could not be regained when sucrose-treated SPM were washed free of sucrose, emphasizing that the action of sucrose was not reversible.

Vesicle size in SPM preparations and the influence of ultrasonication on vesicles, glutamate binding, and the GM1/Ca^{2+} effect

Because the influence of osmolarity on glutamate binding described above suggested the existence of

TABLE 1. *Effect of ganglioside GM1 in the presence of Ca^{2+} on the kinetic constants of glutamate binding to rat brain SPM, measured in Tris-Cl and Tris-Ac buffer*

	K_D (n*M*)	B_{max} (pmol/mg of protein)
Tris-Cl buffer		
Ca^{2+} (control)	1,429 ± 233	57 ± 10
GM1/Ca^{2+}	1,488 ± 241	98 ± 16
Tris-Ac buffer		
Ca^{2+} (control)	1,646 ± 99	19.7 ± 1.3
GM1/Ca^{2+}	1,565 ± 73	25.2 ± 1.3

cSPM were incubated with 50 n*M* L-[^{3}H]glutamate for 60 min at 37°C. The concentration of Ca^{2+} was 5 m*M*, and that of GM1 50 μM and 100 μM in Tris-Cl and Tris-Ac, respectively. Data are means ± SEM of three experiments done in triplicate.

TABLE 2. *Pharmacological profile of GM1/Ca^{2+}-stimulated L-[^{3}H]glutamate binding*

	Inhibition of GM1/Ca^{2+}-stimulated binding (%)
Glutamate receptor agonists	
L-Glutamate	100
Quisqualate	95 ± 5
Ibotenate	95 ± 6
L-Aspartate	82 ± 6
L-Homocysteinate	99 ± 8
D-Glutamate	55 ± 6
L-Cysteine sulfinate	81 ± 9
AP4	81 ± 8
N-Methyl-D-aspartate	0 ± 7
Kainate	−3 ± 5
Glutamate receptor antagonists	
D-α-Amino adipinate	90 ± 9
L-α-Amino adipinate	96 ± 6
2-Amino-5-phosphonovalerate	81 ± 6
Glutamic acid diethylester	19 ± 6
Glutamate transport inhibitors	
L-Glutamic acid-γ-hydroxamate	71 ± 6
D-Aspartate	41 ± 7
DL-*threo*-β-Hydroxyaspartate	7 ± 6
DL-Aspartic acid-β-hydroxamate	11 ± 10

cSPM were incubated for 60 min at 37°C. All inhibitors were tested at 100 μM, [Ca^{2+}] was 5 m*M*, and [GM1] was 100 μM. All data are given in percent; inhibition by L-glutamate arbitrarily was set at 100%. Data are means ± SEM of two determinations done in triplicate.

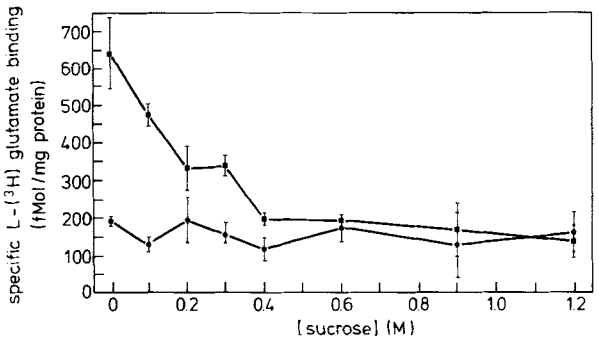

FIG. 3. Influence of increasing amounts of sucrose on basal (●) and GM1/Ca^{2+}-stimulated (■) L-[^{3}H]glutamate binding. cSPM were incubated for 60 min at 37°C in Tris-Ac with sucrose added (0–1.2 *M*). [Ca^{2+}] = 5 m*M*, [GM1] = 100 μM. Data represent means ± SEM of three determinations done in triplicate.

transport into membrane vesicles, a phenomenon which can be abolished by osmotically compressing vesicles, we set out to measure vesicular volumes under different conditions in our SPM preparations. We found that cSPM, as well as pSPM, preparations contain considerable amounts of vesicles. The intravesicular space is not changed significantly by incubation with either Ca^{2+}, GM1/Ca^{2+}, or GM1. However, intravesicular volumes were reduced by approximately 50% after 5 s of ultrasonication with a Branson microtip sonicator or after incubation with 0.3 *M* sucrose (Table 3). Freezing and rethawing of SPM preparations had only a minor influence on intravesicular volumes, reducing them by about 15% (data not shown).

After 5 s of ultrasonication of cSPM, the GM1/Ca^{2+}-dependent stimulation of glutamate binding was greatly reduced; it dropped from 892 ± 31 to 433 ± 46 fmol/mg of protein. A similar reduction had been found for Ca^{2+}-stimulated binding (Hollmann et al., 1988), whereas basal binding was not significantly affected. Washing of sonicated SPM preparations did not return the level of GM1/Ca^{2+}-dependent binding to control values, and, at the same time, it did not return the intravesicular space in those membrane preparations to the control value.

DISCUSSION

As has been reported by several groups, gangliosides become stably incorporated into plasma membranes when added exogenously to membranes or intact cells (Morgan and Seifert, 1979; Toffano et al., 1980; Schwarzmann et al., 1983). Furthermore, this incorporation leads to functionally correct insertion of gangliosides as judged by the ability of incorporated ganglioside GM1 to function as receptor for cholera toxin, resulting in increased cyclic AMP levels in cell cultures (Moss et al., 1976). The big increase in GM1 to 400% of the endogenous GM1 level, which we found in our incorporation experiments, represents an increase of 80% in total membrane ganglioside content, as calculated on the assumption that GM1 constitutes about 20% of the endogenous total gangliosides of rat cortex (Skrivanek et al., 1982). It seems feasible that this big change might influence the properties of the membrane itself or of intramembrane proteins, such as the glutamate receptor or the glutamate carrier.

Modulation by gangliosides of the activity of membrane proteins has been reported in several cases. Bremer et al. (1984, 1986) found inhibition of the tyrosine kinase activity associated with the receptors for epidermal growth factor and platelet derived growth factor, and Tsuji et al. (1985) described a new protein kinase which is stimulated by ganglioside/Ca^{2+}. Inhibition of protein kinase C, accompanied by stimulation of the calmodulin-dependent protein kinase, was reported by Goldenring et al. (1985), Kim et al. (1986), and Yu et al. (1986), and activation of Mg^{2+}-ATPase as well as Ca^{2+}-ATPase (Caputto et al., 1977) and stimulation of Na^+,K^+-ATPase (Leon et al., 1981) have been demonstrated. Recently, Nagata et al. (1987) showed that the uptake into isolated rat ganglia of α-aminoisobutyric acid, a nonmetabolizable amino acid, is enhanced by exogenously added gangliosides.

Taking into consideration all these well-documented effects of gangliosides on plasma membrane enzymes, receptors, and transport proteins, we were not surprised to find a stimulation of glutamate binding to SPM by gangliosides/Ca^{2+}. Because we used Na^+-free buffers, we assumed this binding to be a receptor binding phenomenon and not a transport phenomenon, because high-affinity transport of glutamate generally was believed to be strictly Na^+-dependent (Balcar and Johnston, 1972; Vincent and McGeer, 1980; Baudry and Lynch, 1981; Cross et al., 1986). Accumulating evidence for the existence of a Cl^--dependent glutamate transport system (Fagg and Lanthorn, 1985; Bridges et al., 1986; Kessler et al., 1987; Zaczek et al., 1987)

TABLE 3. *Intravesicular volumes of SPM preparations and influence of different treatments*

Type of membrane	Pretreatment	Intravesicular volume (μl/mg of protein)
cSPM	—	7.9 ± 0.3[a] (12)
pSPM	—	6.1 ± 0.2 (9)
cSPM	Ca^{2+}	6.5 ± 0.2[a] (3)
cSPM	GM1/Ca^{2+}	6.1 ± 0.2 (3)
cSPM	GM1	8.1 ± 0.1 (3)
cSPM	5 s US	3.7 ± 0.2 (6)
cSPM	0.3 *M* sucrose	3.2 ± 0.3 (3)

SPM were incubated for 60 min at 37°C with 1.6 × 10^6 dpm [^{3}H]H_2O, in the presence or absence of different additives, as specified in the table ([Ca^{2+}] = 5 m*M*, [GM1] = 100 μM, US = ultrasonication before incubation). After the incubation, 0.7 × 10^6 dpm [^{14}C]inulin was added to each sample, which was vigorously mixed and centrifuged for 10 min at 12,800 *g*. Aliquots of pellets and supernatants were counted for radioactivity using a double label program. Data are means ± SEM, with the number of separate experiments given in parentheses.

[a] Data from Hollmann et al. (1988).

led us to repeat our binding studies in a Cl^--free buffer system. However, by using Tris-Ac instead of Tris-Cl, we measured the same GM1/Ca^{2+}-dependent binding stimulation, a finding which seemed to support our view that we were dealing with receptor binding.

In addition, the pharmacological profile of the GM1/Ca^{2+}-stimulated binding (Table 2) suggested that the observed binding was to either the quisqualate or the AP4 subtype of the glutamate receptor family, because these two compounds produced almost complete inhibition of binding. *N*-Methyl-D-aspartate and kainate, the agonists at the other two known glutamate receptor subtypes (Foster and Fagg, 1984), were totally inactive. We furthermore found that of four known uptake inhibitors, only L-glutamic acid-γ-hydroxamate showed appreciable inhibition.

However, some observations, such as the pronounced temperature dependence of the ganglioside effect (Fig. 2), raised doubts whether the "receptor modulation" hypothesis was correct. Therefore, we increased the osmolarity in our incubation mix in order to reduce osmotically any possible compartment into which glutamate transport could occur. This experimental criterion for the existence of transport previously had been applied successfully by several workers (Rudnick, 1977; Kanner, 1978; Marvizon et al., 1981; Pin et al., 1984). The lack of any GM1/Ca^{2+}-dependent binding stimulation after hyperosmotic treatment of SPM (Fig. 3) provided an important indication of the involvement of a transport process.

The existence of resealed vesicles in SPM preparations has been described before by Jones and Matus (1974), Pin et al. (1984), and Bridges et al. (1986). In agreement with these reports, we could demonstrate the presence of vesicles (Table 3) in our membrane preparations by measuring intravesicular volumes. This confirmed an important prerequisite for any transport process, i.e., the existence of a so-called "trans" compartment, into which transport could occur. After hyperosmotic treatment, intravesicular space was reduced drastically, as was GM1/Ca^{2+}-dependent binding stimulation. This coincidence lent further support to the transport hypothesis. In a similar way, we were able to destroy intravesicular space in parallel with GM1/Ca^{2+}-dependent binding stimulation by employing ultrasonication. This result again indicated a glutamate transport process.

Finally, the lack of any GM1/Ca^{2+}-dependent binding stimulation in PSD preparations or solubilized receptor preparations (Fig. 2) can be interpreted in favor of the transport hypothesis, because both PSDs and solubilized glutamate receptors have been detergent-treated, a procedure which is likely to destroy all vesicles. In fact, no intravesicular space could be measured in SPM preparations after 0.5% Triton X-100 treatment. The impossibility of demonstrating GM1/Ca^{2+}-dependent binding stimulation in SPM from guinea pig or cow is difficult to understand, because even the well-known species differences in ganglioside composition of membranes are unlikely to explain this finding, as we found similar binding stimulation with all gangliosides tested. However, it has been noted in literature that membrane vesicles prepared from rat brain show "outside-out" orientation to an extent of almost 100% (Kanner and Schuldiner, 1987), whereas the majority of vesicles prepared from guinea pig brain by the same procedure exhibit "inside-out" orientation (Gill, 1982). Because gangliosides have a highly asymmetric distribution in plasma membranes, being confined to the outer leaflet of the lipid bilayer (Ledeen 1978), one might speculate that "inside-out" vesicles have different ganglioside incorporation kinetics as compared to "outside-out" vesicles. Alternatively, the difference might be due to the glutamate binding protein being asymmetrically situated in the membrane, thus being accessible for modulation by gangliosides only from one side of the bilayer.

With respect to the pharmacological profile of GM1/Ca^{2+}-dependent binding, serious doubts have been raised whether quisqualate and AP4 really are specific agonists of clearly defined glutamate receptor subtypes. In fact, AP4 is a powerful inhibitor of Cl^--dependent glutamate transport (Fagg and Lanthorn, 1985; Bridges et al., 1986; Zaczek et al., 1987), and quisqualate similarily was demonstrated to inhibit this process (Zaczek et al., 1987). Thus, our finding that inhibition by AP4 of GM1/Ca^{2+}-dependent binding is much higher than inhibition of basal binding can be interpreted in such a way that the GM1/Ca^{2+}-induced increment in glutamate binding represents a transport process. This interpretation is supported by the fact that the known glutamate transport inhibitor L-glutamic acid-γ-hydroxamate similarly shows higher inhibition of GM1/Ca^{2+}-stimulated binding than of basal binding.

Considering all the data discussed above, we conclude that the increase in glutamate binding produced by GM1/Ca^{2+} actually reflects glutamate transport into resealed membrane vesicles, and not the modulation of glutamate receptor binding. The observed increase thus is due to a change in V_{max}, the maximum transport velocity of the carrier. We do not know, however, whether this increase in V_{max} reflects a decrease in the cycle time of the carrier protein or an increase in the number of active transporters. Gangliosides alone do not influence transport, but Ca^{2+} alone does so, as described previously by us (Hollmann et al., 1988). In the presence of Ca^{2+}, a Na^+- and Cl^--independent glutamate transporter is activated which is different from all other glutamate transport systems described up to now (Hollmann et al., 1988). Thus, one may speculate that gangliosides selectively can stimulate only the active form of this carrier, and therefore are ineffective in the absence of Ca^{2+}, because no active carriers are present under these conditions.

It remains an open question whether the described stimulation by gangliosides of Na^+/Cl^--independent,

Ca^{2+}-dependent glutamate transport is of physiological relevance, because the results presented here have been obtained in an in vitro system. However, it may be of interest to note that we have preliminary data from experiments with primary cell cultures from rat cortex which indicate that GM1/Ca^{2+} stimulation of Na^+/Cl^--independent glutamate transport can be demonstrated in this system, too. Experiments along these lines are in progress.

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