Glycolipids and Transmembrane Signaling: Antibodies to Galactocerebroside Cause an Influx of Calcium in Oligodendrocytes

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Abstract. This is the first study to provide evidence that one function for the surface glycolipid galactocerebroside (GalC) is participation in the opening of Ca^{2+} channels in oligodendroglia in culture. This glycolipid is a unique differentiation marker for myelin-producing cells; antibodies to GalC have been shown to markedly alter oligodendroglial morphology via disruption of microtubules (Dyer, C. A., and J. A. Benjamins. 1988. *J. Neurosci.* 8:4307–4318). This study demonstrates that extracellular EGTA blocks anti-GalC-induced disassembly of microtubules in oligodendroglial membrane sheets, demonstrating that an influx of extracellular Ca^{2+} mediates the cytoskeletal changes. The Ca^{2+} influx was examined directly by loading oligodendroglia with the fluorescent dye Indo-1 in defined medium, and measuring changes in Ca^{2+} in individual cells with a laser cytometer. Upon addition of anti-GalC IgG, a marked sustained increase in intracellular Ca^{2+} occurred in 80% of the oligodendroglia observed. EGTA blocked the increase, indicating the increase is due to an influx of extracellular Ca^{2+}, and not due to release from intracellular stores. The effect is specific, since Ca^{2+} levels remain normal in oligodendroglia treated with nonimmune IgG; astrocytes do not respond to the anti-GalC. The Ca^{2+} response in oligodendrocytes is dependent on concentration of antibody and GalC on the oligodendroglial membrane surface. The Ca^{2+} influx is not mediated by voltage-sensitive Ca^{2+} channels: it is not blocked by cadmium, and depolarization with K^+ does not mimic the response. The kinetics of the response suggest that second messenger–mediated opening of Ca^{2+} channels is involved.

Lipids have received increasing attention for their role in receptor-mediated signaling across cell membranes. In particular, glycolipids presumably associated with transmembrane proteins have been reported to participate in ligand binding and signaling; for example, cholera toxin binds to GM_1 ganglioside leading to activation of adenylate cyclase (see Fishman, 1982, for review). Glycolipids are involved in initiating such events as mitogenesis, morphogenesis, and cell recognition (Sharom and Grant, 1978; Grant and Peters, 1979; Hakomori, 1981; Spiegel and Wilchek, 1981; Thompson and Tillack, 1985; Facci et al., 1988; Curatolo, 1987; Bansal and Pfeiffer, 1989). However, little is known about the mechanisms underlying these effects.

We have focused on the function of galactocerebroside (GalC), a glycolipid highly enriched on the surface of myelin-producing cells. Oligodendrocytes are the only cells in the central nervous system that express this lipid; although it is used widely as a unique marker for differentiation of oligodendrocytes, its role in the specialized membrane produced by these cells is not known. Several studies over the last decade indicate that antibodies to GalC can alter oligodendroglial morphology and myelination, providing indirect evidence that this glycolipid is involved in signal transduction (Diaz et al., 1978; Dorfman et al., 1979; Dyer and Benjamins, 1988; 1989). This study provides direct evidence for participation of GalC in the opening of calcium (Ca^{2+}) channels in oligodendroglia, indicating that this glycolipid has an essential function in signal transduction and in regulation of intracellular events via modulation of Ca^{2+} levels. Oligodendroglia have been reported to contain voltage-operated channels that conduct either Cl^- or K^+ ions, but not Ca^{2+} ions (Barres et al., 1988; Soliven et al., 1988; Sontheimer et al., 1989; Barres et al., 1989). Recently, Castros et al. (1990) demonstrated that several neurotransmitters induce receptor-mediated mobilization of Ca^{2+} in oligodendroglia; however, it is not yet known whether these increases are due to influx or release from intracellular stores. To our knowledge, ours is the first report demonstrating Ca^{2+} influxes in oligodendroglia via plasma membrane channels.

Oligodendrocytes grown in culture in the absence of neurons elaborate large membrane sheets, providing an ideal system in which to study the organization of the specialized membrane produced by oligodendrocytes. These membrane sheets contain an extensive cytoskeleton as well as the major myelin components myelin basic protein (MBP),[1] proteolipid protein, 2',3'-cyclic nucleotide 3'-phosphohydrolase,

1. *Abbreviations used in this paper*: MBP, myelin basic protein; VOC, voltage-operated channel.

myelin associated glycoprotein, sulfatide, and GalC. We have shown previously that binding of antibodies specific for GalC to oligodendroglial membranes causes dramatic changes in the organization of these cells (Dyer and Benjamins, 1988; 1989). Briefly, surface anti-GalC:GalC complexes redistribute into areas directly overlying cytoplasmic MBP domains in the sheets. Subsequently, microtubular networks depolymerize, MBP domains fuse, and actin filament reorganization occurs. With continued exposure to the antibody over a period of days, the membrane sheets contract. This effect is reversible; membrane sheets reextend after removal of the antibody. Microtubule depolymerization is a key early event in these changes. Because increases in intracellular Ca^{2+} are known to cause microtubule depolymerization as well as alterations in cytoskeletal:plasma membrane interactions in other cell types, we examined whether anti-GalC caused changes in Ca^{2+} levels leading to membrane reorganization.

Materials and Methods

Oligodendrocyte Cultures

Cultures were prepared from cerebra of 2-d-old mice as previously described (Dyer and Benjamins, 1988). Briefly, primary cultures were plated in 75-cm^2 flasks and grown for 7–10 d in DME (Gibco Laboratories, Grand Island, NY) containing 10% calf serum (Hyclone Laboratories, Logan, UT). Small, dark process-bearing cells were knocked free of the bed layer and plated on 25-mm-diam coverslips. These cells were grown in chemically defined medium (CDM) (Bottenstein, 1986) overnight, in nine-tenths CDM and one-tenth 10% calf serum DME for the next day, and then four-fifths CDM and one-fifth 10% calf serum DME for the remaining days in culture. Cultures were used at ~26 d after birth. Under these conditions, our cultures contain between 30 and 50% oligodendrocytes based on morphology and immunocytochemical staining with anti-GalC. The remaining cells are predominantly astrocytes, with some microglia and fibroblasts also present.

Antibodies

Polyclonal rabbit anti-GalC was produced and characterized according to Benjamins et al. (1987). IgG antibodies were purified from heat-inactivated sera (56°C for 30 min) on a protein A–Sepharose column as described in Dyer and Benjamins (1988). Nonimmune rabbit serum was obtained from bleeds taken before immunization with GalC and the IgG purified as described for anti-GalC IgG. Monoclonal IgGs against α- and β-tubulins were obtained from East Acres Biologicals (Southbridge, MA). Second antibodies, goat anti-rabbit IgG conjugated to fluorescein (GAR-FITC) and goat anti-mouse IgG conjugated to rhodamine (GAM-TRITC) were purchased from Organon Technika (Malvern, PA).

Immunofluorescent Staining

Shakeoff cultures grown on coverslips were stained for GalC by exposure to anti-GalC for 15 min at 37°C followed by exposure to GAR-FITC (1:40) for 15 min at 37°C. Cultures were fixed with 4% paraformaldehyde for 5 min. Cells double-labeled for tubulin after GalC staining were permeabilized with 0.05% saponin in PBS for 10 min and stained for tubulin using mouse mAbs to α- and β-tubulins (1:10) for 15 min followed by GAM-TRITC (1:40) for 15 min. Cells were viewed with a Leitz Orthoplan 2 fluorescent microscope and were photographed with 400 ASA film.

Calcium Studies

Changes in intracellular free Ca^{2+} were monitored using the Ca^{2+}-sensitive dye, Indo-1 (Bijsterbosch et al., 1986; Gray et al., 1987). Ca^{2+} fluxes in individual oligodendrocytes were examined using an ACAS laser cytometer (Meridian, East Lansing, MI). Cultures were loaded with 1 μm Indo-1AM (Molecular Probes, Inc., Eugene, OR) in serum-free DME for 45–60 min at 37°C. They were washed free of excess Indo-1AM and placed in a coverslip holder designed to fit into the microscope stage of the laser cytometer. Experiments were performed in serum-free DME. Individual oligodendrocytes were identified by their characteristic morphology with phase optics. The cells chosen for analysis were not in contact with surrounding cells. The selected cell was then image-scanned with fluorescence optics to determine the distribution of Indo-1 within the cell. The computer converts fluorescence intensity into color, which is the output image. Ca^{2+} changes were analyzed by a line-scanning technique; two points were identified on either side of the cell that span the area of greatest fluorescence intensity at 485 nm. These points define the line through the cell that the laser scanned repeatedly during the analysis. The analysis consists of 15–20 initial scans to establish a base line; after addition of antibody (or other agent) 200 to 300 scans are performed to follow any changes in fluorescence. Indo-1 was excited at 388 nm and Ca^{2+}-bound Indo-1 was monitored at 405 and unbound Indo-1 was monitored at 485 nm. Fluorescence values taken at 405 and 485 during each scan are reported as a ratio of 405/485. Measurement of absolute Ca^{2+} concentrations is problematic in this system; calculated resting Ca^{2+} concentrations vary depending on the individual cell examined and the amount of ethanol used to construct calibration curves. We estimate resting intracellular free Ca^{2+} concentrations to be between 5 and 120 nM, with increases from resting values ranging between 300 and 500 nM in the presence of anti-GalC. For example, in one oligodendrocyte, using a calibration curve constructed with 20% ethanol, we obtained a resting value of 10–15 nM Ca^{2+}, which rose to ~500 nM after addition of anti-GalC.

Results

Extracellular Ca^{2+} Is Required for Microtubular Depolymerization by Anti-GalC

We examined whether extracellular Ca^{2+} plays a role in the microtubule depolymerization in anti-GalC-treated oligodendrocytes by exposing cultures to anti-GalC alone or to anti-GalC in the presence of 2 mM EGTA for 15 min at 37°C. Fig. 1 *a* demonstrates normal microtubular structures within oligodendroglial membrane sheets. In the presence of EGTA, the majority of oligodendrocytes treated with anti-GalC and second antibody had patched surface GalC (Fig. 1 *b*), but depolymerization of microtubules did not occur as evidenced by networks positively stained for tubulin (Fig. 1 *c*). After GalC surface patching in the presence of 1.8 mM extracellular Ca^{2+} (Fig. 1 *d*), the majority of oligodendrocytes show extensive microtubule depolymerization (Fig. 1 *e*), as previously described (Dyer and Benjamins, 1989). Thus, these results demonstrate that extracellular Ca^{2+} is required for microtubule depolymerization induced by anti-GalC.

Anti-GalC Causes a Dramatic Rise in Intracellular Ca^{2+} in Oligodendroglia

Ca^{2+} fluxes in Indo-1-loaded oligodendroglia were examined fluorometrically using a Meridian ACAS laser cytometer (see Materials and Methods). Pseudocolor image scans of a representative oligodendrocyte before and after addition of anti-GalC are shown in Fig. 2. The increase in intracellular free Ca^{2+} is noted by the changes in color at 485 nm (unbound Indo-1) and at 405 nm (Ca^{2+}-bound Indo-1). The increase in intracellular Ca^{2+} appears to be highest in one area of the cell body, possibly over the nucleus. It is difficult to assess relative changes in various regions of the cell body and membrane sheets, because the fluorescent intensity at a given point is a summation through the thickness of the cell. While the surface of the entire cell, including membrane sheets, is stained by anti-GalC (Fig. 1), it is possible that Ca^{2+} entry is localized in distinct regions. Indeed, changes in Ca^{2+} are

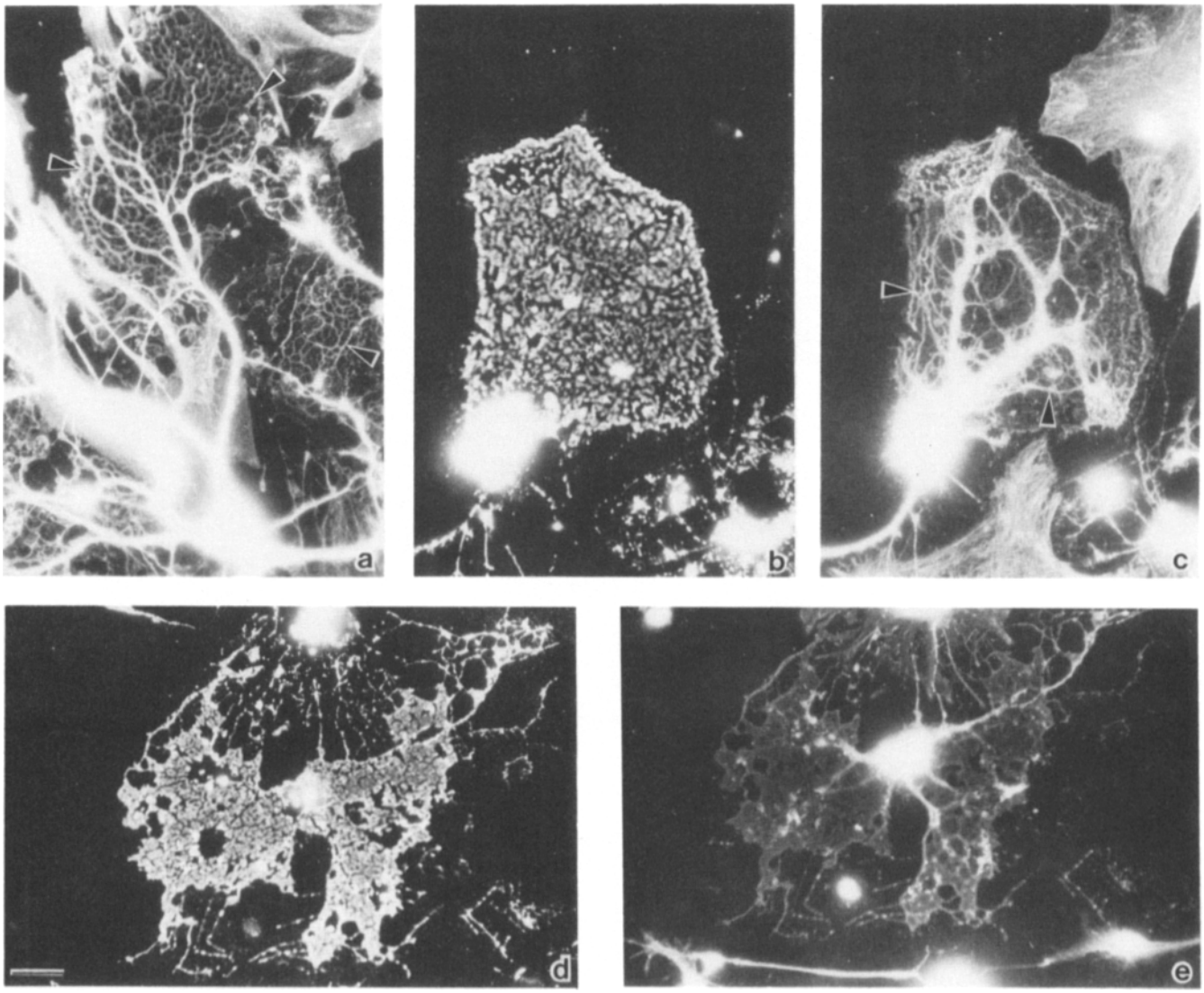

Figure 1. Extracellular Ca^{2+} is required for microtubule depolymerization by anti-GalC. Cultures were incubated with anti-GalC IgG alone (470 μg/ml), or anti-GalC plus 2 mM EGTA for 15 min at 37°C. Cultures then were washed and GAR-FITC was added for 15 min at 37°C; the EGTA-treated cultures remained in solutions containing EGTA until fixation. Cultures were fixed for 5 min with 4% paraformaldehyde, permeabilized with 0.05% saponin, and double-stained for tubulin with mouse monoclonal IgG against tubulin and GAM-TRITC. (*a*) Normal tubulin staining in oligodendrocyte membrane sheets (*arrowheads*). (*b*) Oligodendrocyte treated with anti-GalC and second antibody in medium treated with EGTA (2 mM) shows patched surface GalC. (*c*) Many internal microtubule structures (*arrowheads*) present in membrane sheet of cell shown in *b*. (*d*) Oligodendrocyte treated with anti-GalC and second antibody in medium containing 1.8 mM extracellular Ca^{2+} shows surface GalC patches. (*e*) Loss of majority of microtubule structures in membrane sheet of cell shown in *d*. Bar, 20 μm.

seen in areas of the membrane sheets in many cells with Indo-1 as well as Fluo-3, another Ca^{2+}-binding fluorescent dye (not shown). Further analysis with nuclear staining and confocal optics will be needed to address this issue.

Anti-GalC induced a marked rise in intracellular Ca^{2+} in ~80% of the oligodendroglia examined (Table I). A typical curve is demonstrated in Fig. 3. The time between addition of anti-GalC and the Ca^{2+} response varied markedly from cell to cell (Table I), with the shortest response time ~20 s and the longest time 360 s. Once the intracellular Ca^{2+} started to rise, the time taken for the response to reach a maximum was more constant, averaging 95 s (Table I). Changes in intracellular Ca^{2+} due to anti-GalC appear to be sustained increases; we observed one oligodendrocyte for as long as 30 min, at which time Ca^{2+} levels were still increased (not shown). Preliminary data show that Fab fragments elicit responses similar to intact IgG, indicating that cross-linking of the GalC molecules is not required to trigger the Ca^{2+} response.

Controls were performed to demonstrate the specificity of the anti-GalC effect. No increases in intracellular Ca^{2+} were observed in astrocytes as a result of anti-GalC treatment; the cells were exposed to 940 μg/ml of IgG purified from anti-GalC serum (Table I, Fig. 3). Ionomycin (0.1 mM) was added near the end of the analysis to demonstrate an influx of Ca^{2+} was possible (Fig. 3). Nonimmune rabbit IgG (940 μg/ml)

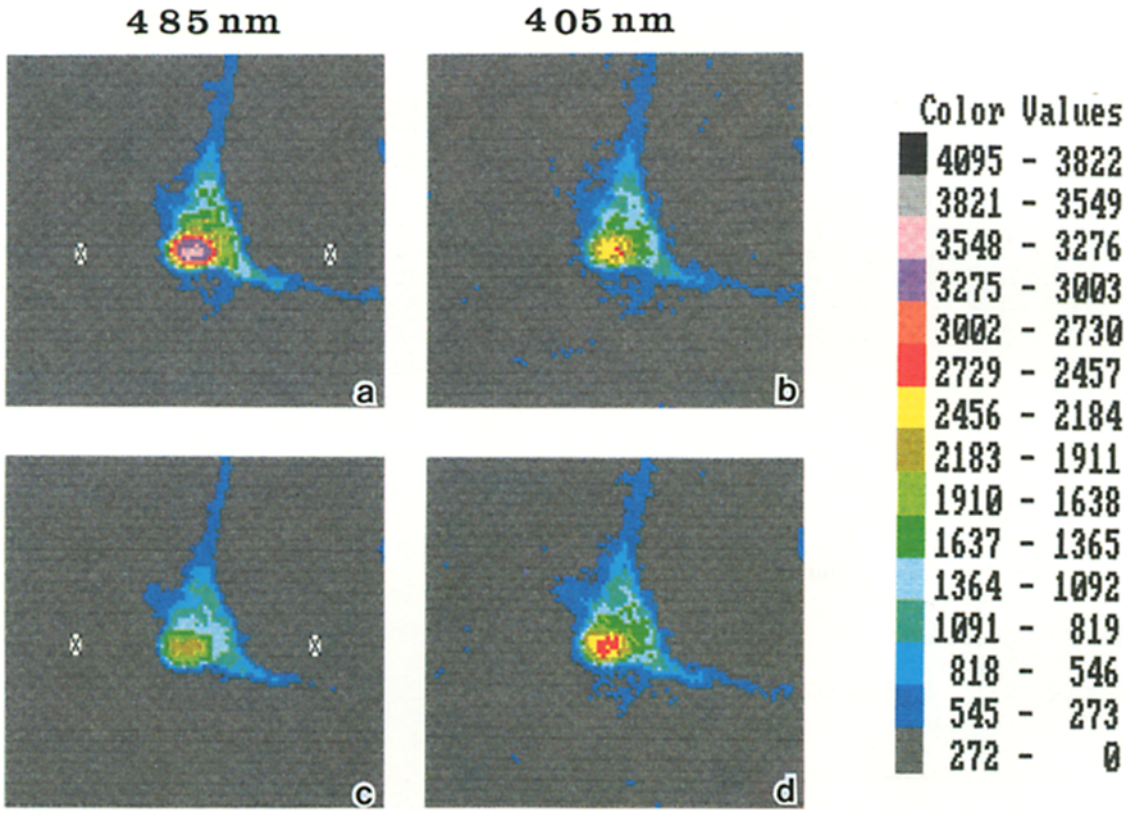

Figure 2. Anti-GalC increases levels of intracellular free Ca^{2+}. Cultures were loaded with 1 μM Indo-1 in serum free medium for ~45 min. An oligodendrocyte was located using phase optics and then image-scanned using fluorescence optics. The computer converts fluorescence intensities into colors; the scale is shown on the right side of the figure. The Xs define the line through the cell that is repeatedly scanned by the laser during the analysis. Anti-GalC (940 μg/ml) increases levels of intracellular Ca^{2+}, as shown by decreased fluorescence at 485 nm (unbound Indo-1) and increased fluorescence at 405 nm (Ca^{2+}-bound Indo-1). (*a* and *b*) Resting cell scanned at 485 and 405 nm, respectively. (*c* and *d*) Same cell 7 min after addition of IgG fraction (940 μg/ml) containing anti-GalC antibodies.

did not induce any alteration in Ca^{2+} concentration within oligodendroglia (Table I).

Ca^{2+} Response Depends on Anti-GalC and GalC Concentrations

We have examined the effect of various dilutions of anti-GalC on the Ca^{2+} response (Table I). A 1:10 or 1:20 dilution of anti-GalC IgG (approximately equivalent to 940 and 470 μg/ml, respectively, of IgG purified from anti-GalC serum) resulted in increases in intracellular Ca^{2+} in about four out of five oligodendrocytes. The times taken for the response to occur and to reach a maximum, and the magnitude of the responses were not significantly different for these two dilutions. However, a 1:40 dilution (approximately equivalent to 240 μg/ml of purified IgG from anti-GalC serum) did not

Table I. Response Times and Magnitude of Intracellular Ca^{2+} Fluxes

Cell type	*n*	Agent	Time to response	Time for response to reach maximum	Ratio change
			s		
Oligodendrocyte	14*	Anti-GalC (1:10, 1:20)	93 ± 103 (20–360)‡	95 ± 33	1.02 + 0.36
Oligodendrocyte	3	Anti-GalC (1:40)	No response		
Oligodendrocyte	5	EGTA, anti-GalC (1:10), Ca^{2+}-free ionomycin	6 ± 5§	70 ± 20	0.43 ± 0.03
Oligodendrocyte	3	Ionomycin	5 ± 3	32 ± 21	1.33 ± 0.25
Oligodendrocyte	3	RIgG	No response		
Astrocyte	3	Anti-GalC	No response		

Glia were exposed to the above agents and the fluxes in intracellular free Ca^{2+} were recorded. Values reported are the means ± SDs.
* A total of 24 oligodendrocytes exposed to anti-GalC were analyzed; 19 showed increases in intracellular Ca^{2+}, and 5 no response. Of the 19 positive responses, 5 were not included in this analysis due to problems in data collection.
‡ Range of times to response observed.
§ These intracellular free Ca^{2+} fluxes were observed after addition of 0.1 μM Ca^{2+}-free ionomycin.

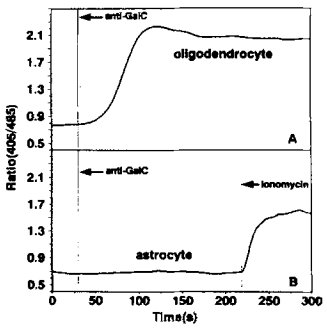

Figure 3. Anti-GalC triggers marked rise in intracellular free Ca^{2+} in oligodendrocytes but not in astrocytes. (*a*) Anti-GalC (940 μg/ml) induces a threefold increase in the 405/485 ratio in an oligodendrocyte. The increase reaches a maximum ~90 s after addition of anti-GalC and remains close to that level for the remainder of the analysis. (*b*) No increase in intracellular free Ca^{2+} results from treating an astrocyte with anti-GalC (940 μg/ml). After ~4 min, 0.1 μM ionomycin was added, triggering a rapid increase in intracellular Ca^{2+}.

cause any increase in intracellular Ca^{2+} in oligodendroglia. Thus, the amount of antibody added to the cells determines whether or not a response is observed.

Live cultures were stained with a series of dilutions of anti-GalC to determine what staining patterns were observed at antibody concentrations which induce a Ca^{2+} response (Fig. 4). Solid staining of membrane sheets was observed in the majority of oligodendrocytes stained with a 1:10 dilution of anti-GalC (Fig. 4 *a*). A 1:20 dilution resulted in patched GalC surface staining in the majority of oligodendrocytes (Fig. 4 *b*). Punctate staining was seen on oligodendroglia at dilutions of 1:30 and greater; this staining became fainter and fainter as the antibody was further diluted (Fig. 4, *c* and *d*). Thus, at the antibody concentrations that elicit a Ca^{2+} response, most of the oligodendrocytes show solid or patched distribution of anti-GalC:GalC complexes. With lower levels of antibody and resultant punctate staining, the Ca^{2+} response does not occur.

In addition, on a given coverslip stained with one dilution of anti-GalC, a few oligodendrocytes stain either more or less intensely than the majority of oligodendrocytes. For example, the majority of oligodendroglia stained with a 1:20 dilution of anti-GalC IgG show patched GalC (similar to Fig. 4 *b*), while the remaining are stained solidly (similar to Fig. 4 *a*) or with a punctate pattern (similar to Fig. 4 *d*). These results indicate that the concentration of GalC varies in cultured oligodendrocytes. Thus, if an oligodendrocyte has a relatively small amount of GalC expressed on its surface, the amount of anti-GalC binding to its surface may not be enough to trigger a rise in intracellular Ca^{2+}. This may be why we observe an 80% response rate at a 1:10 or 1:20 dilution of anti-GalC.

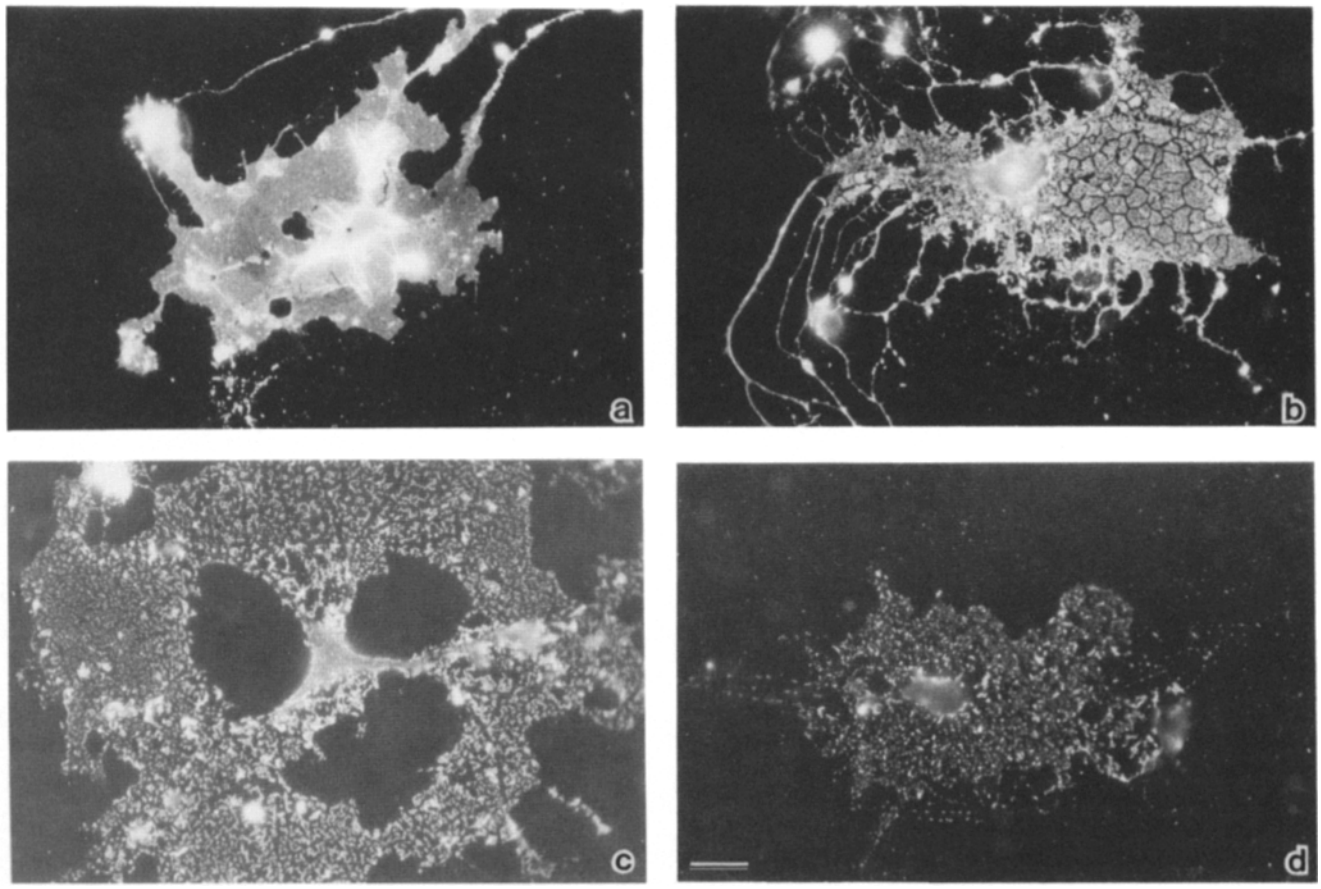

Figure 4. Differential staining patterns of GalC on oligodendroglial membrane sheets is dependent on concentration of anti-GalC. Glial cultures were exposed to various dilutions of anti-GalC for 15 min at 37°C followed by GAR-FITC (1:40) for 15 min at 37°C. The cells then were fixed and mounted for viewing. (*a*) A 1:10 dilution of anti-GalC IgG (940 μg/ml) solidly stains the majority of oligodendroglial membrane sheets. (*b*) GalC is patched on the majority of membrane sheets exposed to a 1:20 dilution of anti-GalC (470 μg/ml). (*c*) A 1:30 dilution of anti-GalC IgG (310 μg/ml) results in small GalC patches and punctate staining on the majority of oligodendroglial membrane sheets. (*d*) A 1:40 dilution of anti-GalC IgG (240 μg/ml) stains the majority of membrane sheets in a punctate pattern. Bar, 20 μm.

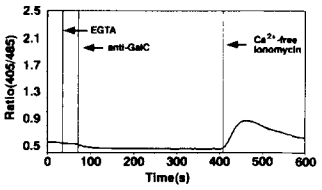

Figure 5. Increase in intracellular free Ca^{2+} by anti-GalC is result of influx and not release from intracellular stores. The source(s) of increased intracellular Ca^{2+} in oligodendroglia was investigated by adding EGTA (2 mM) to the medium before addition of anti-GalC (940 μg/ml). After addition of EGTA, no rise in intracellular Ca^{2+} was observed as a result of addition of anti-GalC. About 6 min after addition of anti-GalC, the culture was treated with Ca^{2+} free ionomycin (0.10 μM); this induced an immediate small increase in intracellular Ca^{2+}.

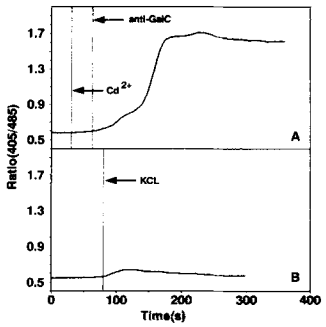

Figure 6. VOCs are not involved in the anti-GalC Ca^{2+} response in oligodendroglia. The possible role of VOCs was investigated by adding cadmium, which blocks VOCs. (*a*) Representative Ca^{2+} response in oligodendrocyte treated with 500 μM Cd^{2+} and then anti-GalC (n = 2). (*b*) The possible role of VOCs was investigated by depolarizing membranes with high K^+. Representative Ca^{2+} response observed when KCl was increased to 60 mM; a small transient rise in intracellular Ca^{2+} was triggered which does not mimic the anti-GalC Ca^{2+} response (n = 3).

Increase in Intracellular Ca^{2+} in Anti-GalC-treated Oligodendroglia Is Due to Influx and Not Release from Intracellular Stores

From our immunocytochemical studies, we showed that extracellular Ca^{2+} is required for microtubule depolymerization induced by anti-GalC; this indicated that an influx of Ca^{2+} is occurring as a result of anti-GalC binding. However, release from intracellular stores may be occurring as well. Thus, 2 mM EGTA was added 30 s before addition of anti-GalC (940 μg/ml IgG) to reduce extracellular Ca^{2+} to a low level. Addition of EGTA blocked the effect; none of the five oligodendrocytes examined under these conditions showed an increase in intracellular Ca^{2+} following addition of anti-GalC (Table I, Fig. 5). Therefore, the increase observed in the presence of extracellular Ca^{2+} must be due to an influx of Ca^{2+} into the cell and not to release from intracellular stores. This was confirmed by treating oligodendrocytes with 0.1 μM Ca^{2+}-free ionomycin after exposure to EGTA and anti-GalC; a transient increase in intracellular Ca^{2+} was observed which decreases to an elevated resting level (Fig. 5). The magnitude of the increase was about half of the anti-GalC increase and about one-third of that when ionomycin is added in the presence of extracellular Ca^{2+} (Table I). This increase was probably from internal stores since the extracellular Ca^{2+} was chelated with EGTA, as indicated by the lack of influx after addition of anti-GalC. These results demonstrate that release from intracellular stores is possible and that anti-GalC is not triggering such a release.

Voltage-operated Ca^{2+} Channels Are Not Involved in the Anti-GalC Ca^{2+} Response

Since the increase in Ca^{2+} was shown to be due to an influx of extracellular Ca^{2+}, it was of interest to determine if voltage-operated Ca^{2+} channels (VOCs) were involved in the response. Cadmium, an inhibitor of VOCs (Barres et al., 1988), was added at a concentration sufficient to completely block VOCs (500 μM); this concentration failed to block the characteristic anti-GalC-induced rise in intracellular Ca^{2+} in two oligodendroglia (Fig. 6 *a*). Because VOCs are activated by depolarization of membranes with high K^+, the Ca^{2+} influxes elicited with high K^+ and anti-GalC were compared. Depolarization of oligodendroglial membranes with high K^+ resulted in an immediate small transient rise in intracellular Ca^{2+} (Fig. 6). Similar results were observed in all three oligodendrocytes analyzed. The magnitude of this increase is about one-tenth that of the anti-GalC Ca^{2+} increase. The Ca^{2+} channels opened under these conditions do not mimic the characteristic response of anti-GalC-activated Ca^{2+} channels. Our results indicate that it is unlikely that VOCs are primarily responsible for the Ca^{2+} influx induced by anti-GalC binding to GalC on oligodendroglia.

Discussion

We have demonstrated that GalC, a glycolipid present in the outer leaflet of oligodendroglial membranes, is a mediator in the signaling events initiated by the binding of anti-GalC; this signaling leads to an influx of Ca^{2+}. The Ca^{2+} influx, in turn, plays a role in the observed microtubule disassembly process, since extensive depolymerization is not observed when EGTA is present during the GalC patching process. Increases in intracellular free Ca^{2+} have been reported to cause microtubule disassembly in other cell types (Marcum et al., 1978; Yamamoto et al., 1985; Keith et al., 1986). The disassembly process is believed to occur via phosphorylation of tubulin and its associated proteins by Ca^{2+}, calmodulin-dependent kinases (Yamamoto et al., 1985). Thus, it is likely that similar mechanisms are occurring in the anti-GalC-treated membrane sheets.

We have noted that anti-GalC induces changes in oligodendroglial membranes similar to the patching and capping events that occur in lymphocytes (Dyer and Benjamins, 1988). The major difference between our study and the lymphocyte studies is that a glycolipid is the receptor in our system, whereas membrane proteins are involved in transducing signals in the majority of lymphocyte studies. Anti-GalC: GalC complexes redistribute into patches on the membrane surface that directly overlie cytoplasmic MBP domains within the membrane sheets (Dyer and Benjamins, 1988). These antibody:glycolipid complexes appear to be linked to MBP, probably via a transmembrane protein (Dyer and Benjamins, 1989). Similarly, transmembrane signals initiated by cross-linking a number of lymphocyte surface antigens cause cytoskeletal components to become associated with membrane at the sites of patches and caps (for review, see Braun and Unanue, 1983). Initial antigen cross-linking studies performed in the presence of $^{45}Ca^{2+}$ indicated that Ca^{2+} was the signal causing cytoskeletal changes in lymphocytes (for review, see Braun and Unanue, 1983). Indeed, investigators recently demonstrated using Indo-1 that intracellular free Ca^{2+} is increased after antibody binding to receptors on

lymphocytes (Finkel et al., 1987; Bijsterbosch et al., 1986; Ledbetter et al., 1987).

The increase in intracellular free Ca^{2+} in oligodendrocytes treated with anti-GalC is due to an influx and not to a release from intracellular stores. At least one other study had demonstrated that a ligand binding to a glycolipid in the outer leaflet of a membrane bilayer results in an influx of Ca^{2+}; the B subunit of cholera toxin upon binding to GM1 ganglioside on rat lymphocytes causes such a rise in intracellular Ca^{2+} (Dixon et al., 1987). In contrast, the same study showed that Con A, which binds to a number of glycolipids and glycoproteins, stimulated a release from intracellular stores as well as an influx of Ca^{2+}. These results suggest that the cholera toxin B subunit and concanavalin A produce increases in intracellular free Ca^{2+} via different mechanisms.

The mechanism of the Ca^{2+} response in oligodendrocytes treated with anti-GalC is currently unknown. It is unlikely that Ca^{2+}-permeable channels were formed by sublytic complement attack, since our cells were grown in heat-inactivated serum and were washed extensively before experiments were performed with purified IgG in serum-free medium. Interestingly, treatment of oligodendrocytes with anti-GalC and complement components C5b-9 elicited release of leukotrienes via activation of phospholipase A_2 (Shirazi et al., 1987). Although these investigators did not look directly at Ca^{2+} fluxes, they propose that entry of Ca^{2+} through C5b-9 channels caused activation of phospholipase A_2. In their study, anti-GalC alone did not cause leukotriene response (and presumably no Ca^{2+} influx). In contrast, we observe a marked Ca^{2+} influx in the presence of anti-GalC alone. This difference may be due to properties and concentration of the antibodies used, culture conditions, or other variables.

Sustained, rather than transient, increases in intracellular Ca^{2+} levels were observed in our anti-GalC-treated oligodendrocytes. The sustained increase, dependence on extracellular Ca^{2+}, and kinetics of the response are reminiscent of those observed after the addition of the B subunit of cholera toxin to lymphocytes containing GM_1 ganglioside (Dixon et al., 1987). In our case, the antibody would correspond to the B subunit, with binding to GalC triggering the influx of Ca^{2+}. Similar results in lymphocytes treated with antibodies without added complement have been reported; mechanisms other than nonspecific complement channel formation were concluded to have triggered the observed increases in intracellular free Ca^{2+} (Bijsterbosch et al., 1986). Instead, second messengers have been shown by electrophysiological and pharmacological methods to mediate this influx in cell types that do not use VOCs as their primary method of regulating Ca^{2+} entry (Meldolesi and Pozzan, 1987; Merritt and Rink, 1987; Penner et al., 1988). We have demonstrated that the Ca^{2+} influx in oligodendrocytes treated with anti-GalC does not appear to be regulated by VOCs. However, we cannot rule out the possibility that anti-GalC activates a small number of VOCs along with a large number of as yet unidentified Ca^{2+} channels.

Since preliminary data show that Fab fragments trigger the influx with similar kinetics as intact IgG, this indicates that it is the specific binding of the antibody to the lipid that signals the Ca^{2+} influx and not cross-linking of the GalC molecules. If GalC is directly associated with a Ca^{2+} channel, then the binding of anti-GalC to GalC would immediately trigger the activation of the channel. This was not observed; instead the kinetics of the response indicate that a second messenger is involved. Several second messengers have been reported to regulate Ca^{2+} channels (for reviews, see Meldolesi and Pozzan, 1987; Rosenthal et al., 1988; Exton, 1988). In our system, the time between addition of anti-GalC and the initiation of the influx varied between 20 s and 6 min; however, the time taken to reach the maximum intracellular free Ca^{2+} concentration was more constant, ~100 s. One mechanism consistent with these data is that the amount of second messenger produced has to reach a critical concentration before triggering the opening of the Ca^{2+} channels. We have observed that high concentrations of antibody produce an influx in ~80% of the oligodendrocytes examined and that lower concentrations of antibody do not elicit any Ca^{2+} influx. Since oligodendroglia vary in the levels of GalC detected on the surface and since the Ca^{2+} response is lost as antibody is diluted, both the amount of antibody bound to the cell surface and the amount of surface GalC present appear to play a role in the Ca^{2+} response. It is likely that oligodendrocytes with low surface levels of GalC take longer to produce the amount of second messenger needed to activate the Ca^{2+} channels because the initial signal is small compared with cells with high surface levels of GalC.

Since the opening of the Ca^{2+} channels by anti-GalC took as long as 6 min in some cells, this suggests that in those cells few Ca^{2+} channels are normally present in the plasma membrane, with the delay in influx reflecting the time required to recruit additional Ca^{2+} channels from intracellular membrane compartments. Similar recruitment and activation from internal compartments have been reported for glucose transporters in adipocyte membranes (Kono et al., 1982; Simpson and Cushman, 1985), ion transporters in epithelia (Schwartz and Al-Awqati; 1986, Wade, 1986), and possibly Ca^{2+} channels in cortical astrocytes (Barres et al., 1989). This insertion and activation may depend upon a second messenger signaling system.

We cannot rule out the possibility that other mechanisms might account for the influx of Ca^{2+} after anti-GalC binding. It is possible that a minor population of antibodies directed against an oligodendrocyte-enriched antigen is present in our IgG fraction and is inducing the Ca^{2+} influx. However, this other reactivity would have to be against a minor component of the oligodendrocyte membrane since we do not observe any IgG reactivity on blots of rat brain homogenate or isolated myelin, and the IgG does not react with any other major glycolipid found in myelin (Benjamins et al., 1987). As discussed by Dixon et al. (1987) for interaction of the B subunit with GM_1 ganglioside, other possible mechanisms may be: (*a*) that Ca^{2+} transporters are induced to work in reverse as a result of anti-GalC binding, (*b*) that anti-GalC might in some way inhibit efflux of Ca^{2+} while allowing influx to continue, or (*c*) that anti-GalC itself induces the de novo formation of Ca^{2+} permeable pores. However, after anti-GalC binding, Ca^{2+} does not continue to rise unabated; instead, a new steady state is reached, and therefore mechanisms that have the capacity to check the rise in Ca^{2+} are favored. Since anti-GalC has been shown to be internalized rapidly in vesicles and then recycled, some extracellular Ca^{2+} may be entering oligodendrocytes via this mechanism (Dyer and Benjamins, 1988; Bradel et al., 1988).

This and our previous publications demonstrate that anti-GalC binding to GalC on oligodendrocytes results in a sequence of responses. Ca^{2+} entry and antibody internalization and recycling are initiated in the first few minutes after antibody addition. This is followed by patching of GalC and microtubule depolymerization, which take place after 2 h of continuous anti-GalC exposure (Dyer and Benjamins, 1987; 1989). Since Ca^{2+} is known to be important in exocytosis and possibly in endocytosis (Knight, 1987; Mohr and Fewtrell, 1987), it is likely that Ca^{2+} is also playing a role in the cycling of anti-GalC in oligodendrocytes. As shown in Fig. 1, Ca^{2+} influx is not required for GalC patching, but is required for microtubule depolymerization.

This study demonstrates that anti-GalC triggers the opening of Ca^{2+} channels in oligodendroglial membranes and that a rise in intracellular free Ca^{2+} is involved in the subsequent disassembly of microtubules. These microtubular structures may be expanded forms of the cytoplasmic channels found throughout myelin lamellae and at the internodes. After anti-GalC treatment, these membrane sheets contain only a few microtubular veins that are surrounded by solid staining of MBP; we propose that the areas of membrane sheet containing MBP are apposed membranes. Thus, exposure to anti-GalC results in membrane sheets that resemble compact myelin (Dyer and Benjamins, 1989). These data and the fact that continuous long-term exposure to anti-GalC results in reversible sheet contraction (Dyer and Benjamins, 1988) indicate that GalC is important in regulating membrane morphology. We do not know what physiologic conditions might cause Ca^{2+} entry and GalC patching. We propose there may be endogenous ligands mimicked by anti-GalC possibly on axonal membranes or extracellular matrix, which may have similar effects. We are examining antibodies and lectins to other surface components of oligodendroglia to characterize their effects on cell morphology and Ca^{2+} influxes. We are also in the process of identifying the second messenger that we propose mediates this propose as it is important for understanding how membrane assembly and maintenance is regulated by the oligodendrocyte.

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