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Influence of Monovalent Cation Transport on Anabolism of Glycosphingolipids in Cultured Human Fibroblasts†

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ABSTRACT: We have reported [Saito, M., Saito, M., & Rosenberg, A. (1984) *Biochemistry 23*, 1043–1046] that the monovalent cationic ionophore monensin reduced the incorporation of labeled galactose into oligosaccharidyl glycosphingolipids (globotriaosylceramide, globotetraosylceramide, and gangliosides) and induced a cellular accumulation of glucosyl- and lactosylceramide in cultured diploid human fibroblasts. We have undertaken further studies on the effects of monensin and made comparison with the effects of related monovalent cation transporters on plasma membrane glycosphingolipid anabolism in human fibroblasts. Our results demonstrate that ionic flux can markedly influence glycosphingolipid synthesis, and they indicate that, like glycoprotein, the sites of glycosylation of the initial, precursor glycosphingolipids are different from the sites of higher glycosylation. At a concentration of 10^{-7} M, monensin induced the maximum inhibition of incorporation of labeled galactose into polyglycosyl sphingolipids: globotriaosylceramide, globotetraosylceramide, and gangliosides; increased incorporation of labeled galactose into glucosyl- and lactosylceramide was clearly evident, and their content rose measurably in the cell at concentrations of monensin as low as 10^{-8} M. These effects of monensin were reversible. Incorporation of labeled galactose into higher glycosylated neutral glycosphingolipids and gangliosides slowly resumed, and the accumulated glycosylceramide diminished after removal of monensin from the culture medium. Ouabain (plasma membrane Na^+,K^+-ATPase inhibitor) and A23187 (Ca^{2+} ionophore) also caused a rapid increase in incorporation of labeled hexose into glucosylceramide and decreased its incorporation into higher neutral glycosphingolipids and into gangliosides. The effects of the K^+ ionophore valinomycin and the lysosome-disrupting cation NH_4^+ were different from those of ouabain and monensin and showed a general enhancement of incorporation of labeled galactose into all glycosphingolipids and no accumulation. These findings suggest that manipulation of ionic flux may variably regulate anabolism and, consequently, the composition of plasma membrane glycosphingolipids.

Monensin, a monovalent cationic ionophore, has been shown to arrest intracellular transport of newly synthesized proteoglycans, secretory proteins, and plasma membrane glycoproteins within the Golgi complex [for a review, see Tartakoff (1983)]. Therefore, monensin inhibits some of the posttranslational modifications of proteins (Tartakoff, 1979; Tartakoff et al., 1981; Tajiri et al., 1980; Nishimoto et al., 1982; Townsend & Benjamins, 1983; Crine & Defour, 1982) and the synthesis of mucopolysaccharides (Goldberg & Toole, 1983), which are believed to occur in the Golgi apparatus.

Elongation of sugar chains in glycosphingolipids through stepwise addition of monosaccharide units to the nonreducing end of the lengthening oligosaccharide chain by a "multiglycosyltransferase complex" (Roseman, 1970; Li & Li, 1982; Basu & Basu, 1982; Kishimoto, 1982; Ledeen, 1983) has been shown to occur mainly in the Golgi apparatus (Keenan et al., 1974; Wilkinson et al., 1976; Richardson et al., 1977; Pacuszka et al., 1978). We have demonstrated that manipulation of cationic flux by monensin, the monovalent cationophore, influences glycosphingolipid synthesis in human fibroblasts, and we have obtained suggestive evidence that the sites of glycosylation of glucosyl- and lactosylceramide are different from those of higher glycosylated glycosphingolipids (Saito et al., 1984), which is not consistent with the multiglycosyltransferase concept. In this study, we have further analyzed the monensin

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effects on glycosphingolipid synthesis in cultured human diploid fibroblasts and examined the effects caused by other, related cationophoric compounds or by inhibition of the physiological K^+-transporting system, plasma membrane sodium and potassium ion adenosinetriphosphatase (Na^+,K^+-ATPase). The results suggest that manipulation of monovalent cation transport very effectively influences specific glycosphingolipid synthesis in cultured diploid human fibroblasts.

Materials and Methods

Fibroblast Culture. Human diploid skin fibroblasts (GM3440), obtained from the Institute for Medical Research (Camden, NJ), were cultured in 10 mL of growth medium consisting of 83% Dulbecco's modified Eagle's medium (Gibco), 15% fetal calf serum (Gibco), and 2% penicillin–streptomycin (5000 IU and 5 mg/mL, respectively, Flow Laboratories) in 75-cm^2 plastic tissue culture bottles (Falcon) at 37 °C in a 5% CO_2 atmosphere in air. To observe the effects of monensin (Calbiochem), confluent fibroblasts (10–13 days after seeding) were incubated wih 10^{-9}–10^{-6} M monensin in the culture medium for 1–43 h. The effects of other compounds were studied by incubating confluent fibroblasts with 10^{-6} M ouabain (Sigma), 10^{-6} M A23187 (Sigma), 10^{-6} M valinomycin (Sigma), or 10^{-3} M NH_4Cl (Sigma) in the culture medium for 18 h. For labeling the cells with [^{3}H]Gal,[1] confluent fibroblasts were incubated with [^{3}H]Gal (11.5 Ci/mmol, New England Nuclear) in the culture medium at a concentration of 1.5×10^{-6} Ci/mL.

Isolation and Identification of Glycosphingolipids. Fibroblasts in 75-cm^2 plastic tissue culture bottles were washed 3 times with 2 mL of 0.15 M NaCl–0.01 M sodium phosphate buffer, pH 7.4 (PBS), scraped with a rubber policeman, and centrifuged at 600*g* for 5 min. Total lipids were extracted from the pellets 3 times with 1 mL of chloroform–methanol (2:1 v/v). The isolation of glycosphingolipids was done according to a method (Irwin & Irwin, 1979) modified as described previously (Saito & Rosenberg, 1982). The gangliosides were separated by silica gel G high-performance thin-layer chromatography (HPTLC) plates (E. Merck) with chloroform–methanol–0.25% $CaCl_2$ in water (65:35:8 v/v/v) and visualized by resorcinol reagent. Neutral glycosphingolipids were developed in chloroform–methanol–water (65:25:4 v/v/v) and visualized by anthrone reagent. Glucosylceramide and galactosylceramide were separated on HPTLC plates presprayed with 1.5% sodium borate and developed in chloroform–methanol–water–15 M NH_4OH (280:70:6:1 v/v/v/v). For the determination of each ganglioside and neutral glycosphingolipid, direct densitometric measurement was performed in a Kratos (Schoeffel) densitometer after TLC development. The isolation of glycosphingolipids from cells labeled by [^{3}H]Gal was done as described above. The patterns of radioactivity were obtained by scraping each 2 mm wide strip from TLC plates, transferring the samples into scintillation vials, adding 5 mL of Aquasol (New England Nuclear), sonicating, and counting in a Beckman scintillation spectrometer. Glucosylceramide was purified from a total lipid extract of splenic tissue from a subject with Gaucher's disease (Kuske & Rosenberg, 1972). Galactosylceramide, lactosylceramide, trihexosylceramide, and globoside were purchased from Supelco, Inc., Bellefonte, PA. G_{M1} and G_{D1a} were prepared from bovine brain, and G_{M3}, G_{M2}, and G_{D3} were prepared from chick liver, Tay-Sachs diseased brain, and a methanol extract of buttermilk, respectively, by the method of Momoi et al. (1976).

Results

Effects of Different Concentrations of Monensin on Glycosphingolipids of Human Fibroblasts. Confluent human fibroblasts were incubated with [^{3}H]Gal for 18 h in the presence of different concentrations of monensin in the culture medium, and each glycosphingolipid from these cells was analyzed as described under Materials and Methods. Figure 1a,b shows the effect of monensin on the amount of the glycosphingolipids, and Figure 1c,d shows the radioactivity incorporated into each glycosphingolipid fraction. Cellular content of GlcCer and LacCer was increased at 10^{-8} M or higher concentrations of monensin. The amounts of other neutral glycosphingolipids and gangliosides were essentially unchanged. Increased incorporation of [^{3}H]Gal into GlcCer and LacCer was observed while inhibition of incorporation was observed in other, more highly glycosylated glycosphingolipid fractions. At a concentration of 10^{-7} M, monensin induced the maximum inhibition of incorporation of [^{3}H]Gal into $GbOse_3Cer$, $GbOse_4Cer$, and gangliosides. Increased incorporation of [^{3}H]Gal into GlcCer and LacCer was clearly evident at 10^{-7} M monensin and was greater above this concentration.

Time Course of Effects of Monensin. Figure 2 shows the time course of the effects of monensin on glycosphingolipid anabolism. Fibroblasts were incubated with [^{3}H]Gal in the presence or absence of 10^{-6} M monensin in the culture medium for the time periods shown. One hour after adding monensin, the inhibition of the incorporation of [^{3}H]Gal into $GbOse_3Cer$ (32% of control), $GbOse_4Cer$ (39% of control), and gangliosides (30–50% of control) was already evident. Although inhibition of incorporation into G_{M3} was not observed after 18 h (Saito et al., 1984), inhibition was detected after treatment with 10^{-6} M monensin for 1–7 h. The cellular content of GlcCer and LacCer increased gradually under incubation with 10^{-6} M monensin. About a 1.5-fold increase of GlcCer and LacCer was observed after 5- and 7-h incubation, respectively. The content of other glycosphingolipids was essentially unchanged.

Fate of Accumulated GlcCer and LacCer after Removal of Monensin. Table I shows radioactivity incorporated into glycosphingolipids and glycosphingolipid content before and after removal of monensin. When the 10^{-7} M monensin-treated cells were, after 19 h, further incubated in medium without monensin for 24 h, the accumulated GlcCer decreased and LacCer increased (Table I, row 3). LacCer decreased after another 72-h incubation in the medium without monensin (Table I, row 4). Decrease of GlcCer was not evident when the cells were incubated for as long as 43 h in the presence of monensin (Table I, row 2). The accumulated radioactivity in GlcCer after 19-h monensin treatment decreased, and the radioactivity in other glycosphingolipids increased slowly after monensin was removed from the medium (Table I, rows 3 and 4). After pretreatment with 10^{-7} M monensin for 19 h, the cells were incubated in medium containing [^{3}H]Gal for 24 h in the presence or absence of 10^{-7} M monensin (Table I, rows 5 and 6). The radioactivities incorporated into $GbOse_3Cer$ and some gangliosides were higher in the absence of monensin.

Effects of Other Cation-Transport Systems on Glycosphingolipids in Human Fibroblasts. Human diploid fibroblasts were incubated for 18 h in medium containing [^{3}H]Gal plus the ionophores or the energy-dependent cation-transport inhibitor shown in Table II, and the glycosphingo-

[1] Abbreviations: Gal, galactose; GlcCer, glucosylceramide; LacCer, lactosylceramide; $GbOse_3Cer$, globotriaosylceramide; $GbOse_4Cer$, globotetraosylceramide; G_{M3}, sialosyllactosylceramide; G_{M2}, sialosylgangliotriaosylceramide; G_{M1}, sialosylgangliotetraosylceramide; G_{D3}, disialosyllactosylceramide; G_{D1a}, disialosylgangliotetraosylceramide.

Table I: Reversible Effects of Monensin on Glycosphingolipids in Cultured Human Skin Fibroblasts

row	treatments	glycosphingolipid radioact. (cpm/mg) [glycosphingolipid content (nmol/mg)][a] GlcCer	LacCer	$GbOse_3Cer$	$GbOse_4Cer$	G_{M3}	G_{M2}	G_{M1}	G_{D3}	G_{D1a}
1	[^{3}H]Gal, 10^{-7} M monensin, 19 h	3728 [2.40]	1799 [0.42]	436 [1.67]	150 [0.40]	3320 [4.66]	70 [0.47]	18 [0.09]	54 [0.25]	36 [0.33]
2	same as row 1, plus Gal, 10^{-7} M monensin, 24 h	994 [2.90]	3513 [2.28]	642 [1.30]	121 [0.31]	4183 [5.52]	100 [0.34]	17 [0.08]	32 [0.20]	29 [0.27]
3	same as row 1, plus Gal, no monensin, 24 h	761 [0.97]	2794 [1.48]	1475 [1.93]	184 [0.35]	4818 [5.73]	129 [0.54]	71 [0.12]	31 [0.20]	20 [0.28]
4	same as row 1, plus Gal, no monensin, 96 h	489 [1.14]	843 [0.37]	1972 [1.92]	483 [0.42]	4859 [4.76]	531 [0.64]	57 [0.12]	116 [0.32]	236 [0.38]
5	10^{-7} M monensin, 19 h, plus [^{3}H]Gal, 10^{-7} M monensin, 24 h	7064 [3.13]	12459 [1.70]	3328 [1.21]	426 [0.29]	10515 [5.24]	277 [0.64]	86 [0.10]	151 [0.17]	134 [0.25]
6	10^{-7} M monensin, 19 h, plus [^{3}H]Gal, no monensin, 24 h	1831 [1.15]	7473 [1.13]	10531 [1.93]	1180 [0.32]	11783 [5.16]	578 [0.66]	128 [0.09]	159 [0.28]	114 [0.33]

[a] The radioactivity (cpm/mg of protein) and content (nmol/mg of protein) (in brackets) of each glycosphingolipid were analyzed as described in the text after the following treatments: (1) fibroblasts were labeled with [^{3}H]Gal (1.5×10^{-6} Ci/mL) for 19 h in medium containing 10^{-7} M monensin; (2) fibroblasts were labeled with [^{3}H]Gal for 19 h in medium containing 10^{-7} M monensin, washed, and incubated for 24 h in medium containing 5×10^{-6} M Gal and 10^{-7} M monensin; (3) fibroblasts were labeled with [^{3}H]Gal for 19 h in medium containing 10^{-7} M monensin, washed, and incubated for 24 h in medium containing 5×10^{-6} M Gal without monensin; (4) fibroblasts were labeled with [^{3}H]Gal for 19 h in medium containing 10^{-7} M monensin, washed, and incubated for 96 h in medium containing 5×10^{-6} M Gal without monensin; (5) fibroblasts were incubated in medium containing 10^{-7} M monensin without [^{3}H]Gal for 19 h and then labeled with [^{3}H]Gal for 24 h in the preesence of 10^{-7} M monensin; (6) fibroblasts were incubated in medium containing 10^{-7} M monensin without [^{3}H]Gal for 19 h and then labeled with [^{3}H]Gal for 24 h in the absence of monensin. The values are the average of two cultures. A maximum difference of ±5% was observed.

Table II: Effects of Cationophores, Na^+,K^+-ATPase Inhibitor, and NH_4^+ on Anabolism of Glycosphingolipids of Cultured Human Fibroblasts[a]

glycosphingolipids	radioactivity ([^{3}H]Gal) incorporated into each glycosphingolipid (% of control) monensin	valinomycin	ouabain	A23187	NH_4Cl
GlcCer	1169 ± 339	248 ± 19	227 ± 67	180 ± 15	169 ± 52
LacCer	885 ± 85	126 ± 17	55 ± 14	130 ± 11	191 ± 53
$GbOse_3$-Cer	27 ± 6	179 ± 12	79 ± 6	50 ± 10	211 ± 63
$GbOse_4$-Cer	16 ± 4	133 ± 28	55 ± 14	28 ± 11	180 ± 53
G_{M3}	87 ± 12	156 ± 20	73 ± 11	81 ± 24	199 ± 45
G_{M2}	18 ± 1	131 ± 15	66 ± 18	47 ± 18	257 ± 59
G_{M1}	36 ± 3	259 ± 82	62 ± 16	22 ± 9	206 ± 20
G_{D3}	24 ± 9	401 ± 44	25 ± 15	69 ± 19	297 ± 62
G_{D1a}	16 ± 11	276 ± 49	62 ± 8	44 ± 11	233 ± 38

[a] Fibroblasts were labeled with [^{3}H]Gal for 18 h in medium containing 10^{-6} M monensin, 10^{-6} M ouabain, 10^{-6} M A23187, 10^{-6} M valinomycin, and 10^{-3} M NH_4Cl, respectively, and the glycosphingolipids were analyzed. The values are means ± SD for triplicate cultures.

Table III: Effects of Cationophores, Na^+,K^+-ATPase Inhibitor, and NH_4^+ on the Glycosphingolipid Content of Cultured Human Fibroblasts[a]

glycosphingolipids	amounts of each glycosphingolipid (% of control) monensin	valinomycin	ouabain	A23187	NH_4Cl
GlcCer	315 ± 15	107 ± 25	112 ± 13	111 ● 14	111 ± 22
LacCer	211 ± 27	98 ± 19	104 ± 14	120 ± 20	94 ± 12
$GbOse_3$-Cer	101 ± 17	102 ± 9	101 ± 12	115 ± 9	95 ± 9
$GbOse_4$-Cer	84 ± 2	86 ± 4	95 ± 13	105 ± 16	103 ± 18
G_{M3}	95 ± 6	97 ± 15	109 ± 1	97 ± 10	110 ± 17
G_{M2}	97 ± 9	85 ± 5	99 ± 2	121 ± 13	98 ± 4
G_{M1}	88 ± 5	98 ± 37	92 ± 6	97 ± 15	102 ± 6
G_{D3}	97 ± 120	120 ± 12	97 ± 14	84 ± 15	112 ± 13
G_{D1a}	97 ± 15	91 ± 7	86 ± 16	93 ± 8	101 ± 10

[a] Conditions are the same as for Table II.

lipids were analyzed as described under Materials and Methods. Ouabain enhanced the incorporation of [^{3}H]Gal after epimerization into GlcCer but decreased the incorporation of [^{3}H]Gal into other glycosphingolipids. A23187 showed similar effects to ouabain except that the incorporation of [^{3}H]Gal into LacCer was not inhibited. Valinomycin and NH_4Cl showed overall enhancement of the incorporation of [^{3}H]Gal into glycosphingolipids. The quantity of glycosphingolipids was essentially unchanged after incubation with ouabain, A23187, valinomycin, or NH_4Cl (Table III).

Discussion

We have shown previously that 10^{-5} M monensin inhibited the incorporation of [^{3}H]Gal into $GbOse_3Cer$, $GbOse_4Cer$, and gangliosides (except G_{M3}) and increased both radioactivity incorporated and the cellular content of GlcCer and LacCer (Saito et al., 1984). Since the primary sites of action of monensin appear to be within the Golgi complex, and monensin slows down intracellular transport of newly synthesized proteoglycans, secretory proteins, and membrane glycoproteins (Tartakoff, 1983), we predicted a separation of the intracellular locus of the initial and subsequent glycosylations of glycosphingolipids and the inhibition of translocation of glycosphingolipid from an initial glycosylation site [endoplasmic reticulum (ER) or the proximal face of Golgi] to a subsequent glycosylation site (the distal face of Golgi) by monensin (Saito et al., 1984). The results of detailed study of the effects of monensin on glycosphingolipids appear to support this hypothesis. At 10^{-7} M, monensin showed maximum inhibitory activity toward the incorporation of [^{3}H]Gal into $GbOse_3Cer$, $GbOse_4Cer$, and gangliosides (Figure 1c,d). This concentration is close to that required for inhibition of sulfation of glycos-

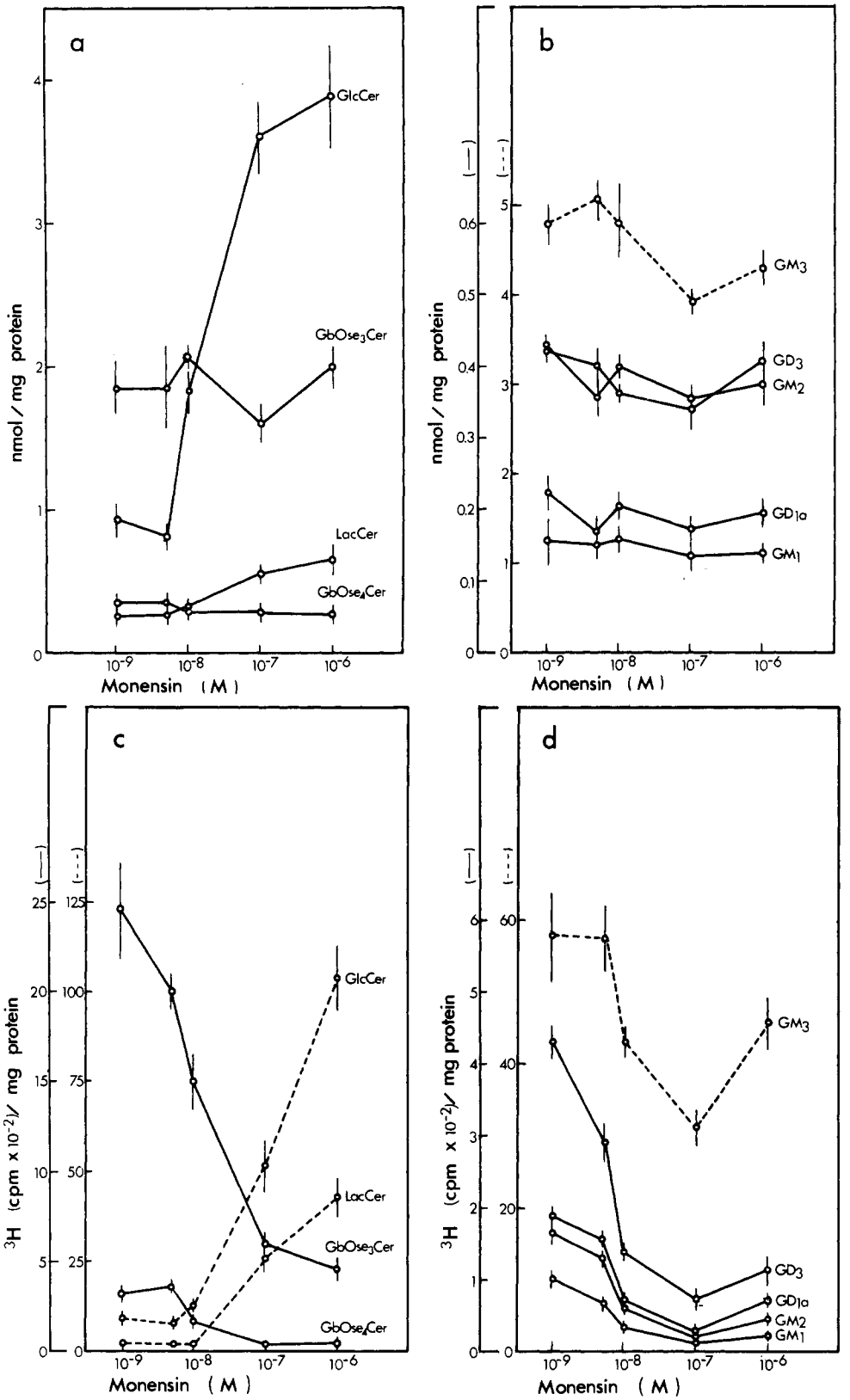

FIGURE 1: Dose-dependent effect of monensin on glycosphingolipids of human skin fibroblasts. Human fibroblasts were incubated for 18 h in culture medium containing various concentrations of monensin and [^{3}H]Gal (1.5×10^{-6} Ci/mL), and their glycosphingolipids were analyzed as described under Materials and Methods. (a) Cellular content (nmol/mg of cell protein) of each neutral glycosphingolipid. (b) Cellular content (nmol/mg of cell protein) of each ganglioside. (c) Radioactivity (cpm/mg of cell protein) incorporated into each neutral glycosphingolipid. (d) Radioactivity (cpm/mg of cell protein) incorporated into each ganglioside. Values are mean ± SD for triplicate cultures. Values of control (without monensin) were essentially the same as those of 10^{-9} M monensin treated samples.

aminoglycans (3×10^{-8} M) (Tajiri et al., 1980), secretion of fibronectin or procollagen (10^{-7} M) (Uchida et al., 1979), and hyaluronate synthesis (10^{-7} M) (Goldberg & Toole, 1983), which are supposed to be caused by Golgi disruption. Maximum increase in the incorporation of [^{3}H]Gal into GlcCer and LacCer was observed at a concentration as high as 10^{-6} M monensin. As the absolute amounts of GlcCer and LacCer reached almost the maximum at a concentration of 10^{-7} M

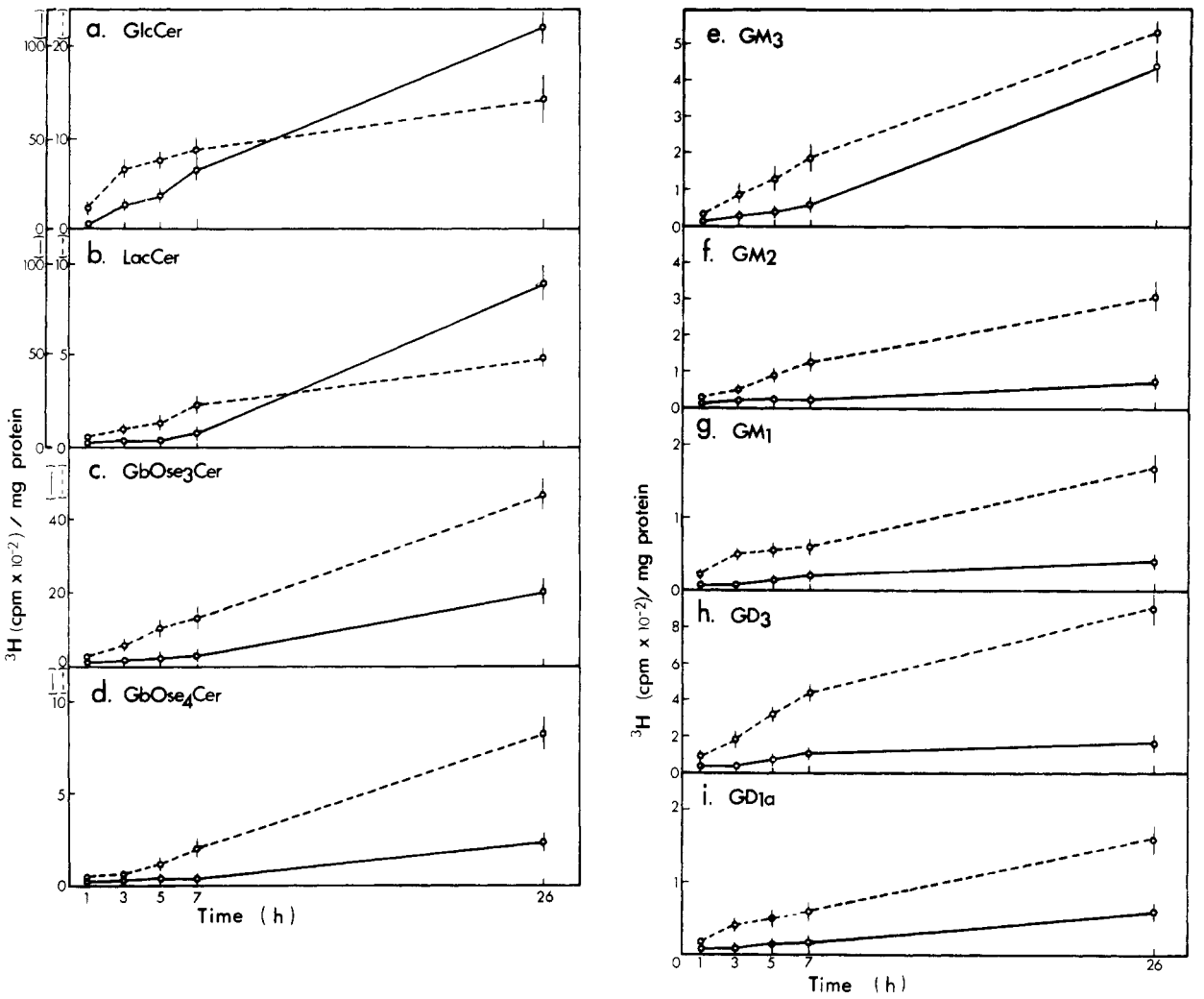

FIGURE 2: Time course of the effect of monensin on [^{3}H]Gal incorporation into glycosphingolipids. Human fibroblasts were incubated in culture medium containing [^{3}H]Gal (1.5×10^{-6} Ci/mL) with 10^{-6} M monensin (—) or without monensin (---), and their glycosphingolipids were analyzed as described under Materials and Methods. Values are the radioactivity (cpm/mg of protein) incorporated into (a) GlcCer, (b) LacCer, (c) $GbOse_3Cer$, (d) $GbOse_4Cer$, (e) G_{M3}, (f) G_{M2}, (g) G_{M1}, (h) G_{D3}, and (i) G_{D1a} and are expressed as mean ± SD for triplicate cultures.

monensin, it might be possible that the specific radioactivity of UDP-Glc also is augmented at still higher concentrations of monensin. A time-course study showed that the effects of monensin on some glycosphingolipids were already in evidence within 1 h (Figure 2). These results agree with the postulate that the effects of monensin on glycosphingolipids are caused by Golgi disruption that is visibly evident within seconds (Tartakoff, 1983). These effects of monensin on glycosphingolipids were at least partially reversible (Table I). The GlcCer accumulated by monensin treatment appeared to be reutilized as the substrate for further glycosylation after removing monensin from the medium; accumulated GlcCer decreased and higher glycosylated glycosphingolipids increased. This result indicates the possibility that GlcCer accumulates in the site of synthesis (ER or Golgi apparatus). It is possible that the site of synthesis of GlcCer is different not only from those of higher glycosylated glycosphingolipids but also from that of LacCer. The subcellular localization of the accumulated GlcCer by monensin and that of glucosyltransferase activity are now under investigation using a special subcellular fractionation arrangement (Rome et al., 1979). Ouabain and A23187 showed similar effects to monensin although less extreme at this concentration (Table II). Ouabain was reported to show a swelling of Golgi complex in neurons (Whetsell & Bunge, 1969) and partially to inhibit immunoglobulin secretion in myeloma cells (Tartakoff & Vassalli, 1977). The increased level of Na^+ or subsequent mobilization of Ca^{2+} might be important in this phenomenon. Although monensin has been shown to inhibit lysosomal activity (Grinde, 1983), the effects we observed on glycosphingolipids appear primarily to be due to Golgi disruption by action of this ionophore because valinomycin and NH_4Cl (both inhibitors of lysosomal activity) showed an entirely different set of effects from those of monensin. However, as valinomycin and NH_4Cl did not cause a quantitative accumulation of glycosphingolipids, it is possible that these reagents also affect the synthetic pathway, by a different mechanism from that influenced by monensin.

Recently, a similar effect of monensin on glycosphingolipids in neural cells was reported by others (Miller-Prodraza & Fishman, 1984). In general, it seems likely that the effects of monensin on glycosphingolipids are caused by a disruption of Golgi function altering the synthesis of glycosphingolipids directly or indirectly through their anabolic transport. If the transport of glycosphingolipids is affected by monensin, it is not unlikely that glycosphingolipids may be transported from the ER to the plasma membrane via the Golgi apparatus, in the same way as glycoproteins, or that intracellular glycolipid carrier proteins for glycosphingolipids become unavailable because of monensin treatment. It is clear from these studies that monovalent cation flux or inhibition of plasma membrane Na^+,K^+-ATPase, the physiological transporter of K^+ ions, may

have a remarkable influence on plasma membrane glycosphingolipid anabolism.

Registry No. G_{M3}, 54827-14-4; G_{D3}, 62010-37-1; G_{D1a}, 12707-58-3; G_{M2}, 19600-01-2; G_{M1}, 37758-47-7; LacCer, 4682-48-8; $GbOse_3Cer$, 71965-57-6; $GbOse_4Cer$, 11034-93-8; Ca, 7440-70-2; K, 7440-09-7; Na, 7440-23-5.

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