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Glycosphingolipids of skeletal muscle: II. Modulation of Ca^{2+}-flux in triad membranes by gangliosides

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Abstract

Membrane vesicles of rabbit skeletal muscle were prepared and separated by sucrose density gradient centrifugation. The fractions obtained (in the order of increasing density) were sarcolemma (SL), T-tubules (TT), sarcoplasmic reticulum (SR1 and SR2) and triads/mitochondria (Tr/M) as characterized by their specific marker enzymes, ligand binding, and ion flux activities. The distribution of neutral glycosphingolipids and gangliosides in these membrane preparations has been documented in the preceding paper (J. Müthing, U. Maurer, U. Neumann, B. Kniep, and S. Weber-Schürholz, *Carbohydr. Res.*, (1988) 135–145). G_{M3}(Neu5Ac) is the dominant ganglioside, neolacto-series gangliosides are moderately expressed and ganglio-series gangliosides were found in minor quantities, however, all showing different qualitative and quantitative membrane-type specific patterns. The voltage dependent Ca^{2+}-channels of skeletal muscle reside prevalently in the triad enriched membrane fractions deduced from highest binding capacity of 1,4-dihydropyridines. Calcium channel complexes of triads were reconstituted into unilamellar phospholipid vesicles of 400 nm defined size and the active $^{45}Ca^{2+}$-uptake into intravesicular space was measured after incorporation of muscle specific gangliosides into the outer vesicle lipid bilayer in parallel to control liposomes without gangliosides. G_{M3}(Neu5Ac) strongly increased the uptake of $^{45}Ca^{2+}$ (+285%) whereas G_{M3}(Neu5Gc) severely inhibited the ion flux (−61%). Neolacto-series gangliosides evoked miscellaneous effects upon $^{45}Ca^{2+}$-flux depending on isomeric sialic acid configuration, oligosaccharide size and fatty acid chain length of the ceramide portion. VI^{3}Neu5Ac–*n*LcOse$_6$Cer (C_{24}-fatty acid), IV^{3}Neu5Ac–*n*LcOse$_4$Cer (C_{16}-fatty acid) and IV^{6}Neu5Ac–*n*LcOse$_4$-

Abbreviations: DHP, dihydropyridines; GSL(s), glycosphingolipid(s); HPTLC, high performance thin-layer chromatography; SL, sarcolemma; SR, sarcoplasmic reticulum; Tr/M, triads/mitochondria; TT, T-tubules. The nomenclature of sialic acids follows the suggestions of Reuter and Schauer [1], Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid. The designation of glycosphingolipids was performed according to the IUPAC–IUB recommendations [2] and the nomenclature of Svennerholm [3]. Gangliotetraosylceramide or GgOse$_4$Cer, Galβ1-3GalNAcβ1–4Galβ1–4Glcβ1–lCer; lacto-*N*-*neo*tetraosylceramide or *n*LcOse$_4$Cer, Galβ1–4GlcNAcβ1-3Galβ1–4Glcβ1-lCer; lacto-*N*-*nor*hexaosylceramide or *n*LcOse$_6$Cer, Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–lCer; G_{M3}, II^{3}Neu5Ac–LacCer; G_{M1}, II^{3}Neu5Ac–GgOse$_4$Cer; G_{D1a}, IV^{3}Neu5Ac, II^{3}Neu5Ac–GgOse$_4$Cer; G_{D1b}, II^3(Neu5Ac)$_2$–GgOse$_4$Cer; G_{T1b}, IV^{3}Neu5Ac, II^3(Neu5Ac)$_2$–GgOse$_4$Cer.

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Cer (C_{16}-fatty acid) strongly enhanced the $^{45}Ca^{2+}$-flux (+208, +162, and +120%, respectively), whereas IV^{3}Neu5Ac–*n*LcOse$_4$Cer (C_{24}-fatty acid), VI^{3}Neu5Ac–*n*LcOse$_6$Cer (C_{16}-fatty acid) and IV^{6}Neu5Ac–*n*LcOse$_4$Cer (C_{24}-fatty acid) slightly reduced $^{45}Ca^{2+}$-flux (−3, −6, and −17%, respectively). Out of all gangliosides tested in this study, G_{M1} showed the strongest stimulatory effect (+327%). G_{D1a} and G_{T1b} gave rise to remarkable flux-stimulation of +283 and +255%, respectively, whereas G_{D1b} exhibited only a slightly positive effect (+38%). This data suggest a functional role of gangliosides in subcellular muscle membranes giving strong evidence that gangliosides are capable of modulating the cytosolic calcium level of muscle, which regulates muscle contraction. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: Membrane fractionation; Ca^{2+}-channel; Ion flux; DHP-receptor; Ganglioside subcellular distribution

1. Introduction

Gangliosides, sialic acid-containing glycosphingolipids (GSLs), constitute a family of complex membrane components and are found in virtually all cells of vertebrate organisms. GSL biosynthesis starts in the endoplasmic reticulum with the formation of ceramide, which then is glycosylated stepwise in the Golgi complex [4]. G_{M3} is the basic ganglioside of the ganglio-series and predominantly expressed in non-neuronal tissue. It represents the branching point at which ganglioside biosynthesis continues via sequential addition of monosaccharide and sialic acid residues to the growing oligosaccharide chain yielding a-, b- and c-series gangliosides. Gangliosides are highly enriched in nervous tissue with their more complex derivatives G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} being particularly prevalent [5]. The diverse structures of gangliosides provide biological specificity for numerous putative cellular functions such as cell-cell recognition, cell growth regulation, development, and differentiation [6–8]. Gangliosides suppress various immune activities [9–11] and are suggested to modulate cell growth through influencing the function of receptors, transducers, or transporters which are essential for cell growth regulation [12,13].

Gangliosides added exogenously to culture media are taken up by a wide range of cells in vitro [14]. Delivered to cells this way, gangliosides show a variety of biological effects depending on the cell type. Such effects are, e.g. reduction of growth rate of fibroblasts [15–17], inhibition of lymphoproliferation [18–22] and modulation of CD4 from helper T lymphocytes [23,24], suppression of cytotoxic activity of natural killer cells [25,26], inhibition of monocyte accessory function [27], and modulation of receptor phosphorylation and associated kinases [28–32]. On the other hand, exogenous gangliosides stimulate the proliferation of various neuronal cells [33–36] and suggest a perspective for use as therapeutic agents in the treatment of neural problems [37–39]. Thus, gangliosides cause opposite effects on cell growth and are considered as bimodal regulators of positive and negative signals for cell growth [40,41].

Subcellular membrane preparations of a crude mitochondrial fraction of rat brain have been used to investigate effects of various ganglio-series gangliosides (namely G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}) on the activity of (Na^+,K^+)-ATPase [43]. All tested gangliosides activated (Na^+,K^+)-ATPase obviously due to modifications of the membrane lipid environment surrounding the enzyme. The facilitating effect on the (Na^+, K^+)-pump of muscle fibres by using a brain derived ganglioside mixture has been reported [44] and the positive effect of ganglioside G_{M3} on the activity of reconstituted Ca^{2+}-ATPase from rabbit skeletal muscle sarcoplasmic reticulum has been recently described [45]. The nervous system communicates with muscles by way of motor neurons. Their signals trigger an action potential in the sarcolemma (SL), and this electrical excitation spreads rapidly into the T-tubules (TT), which connect with the sarcoplasmic reticulum (SR) via TT/SR junctional complexes called triads (Tr) (Fig. 1). The intracellular triad junction is purported to be the link between the signal process of the SL and the release of Ca^{2+} from the terminal cisternae of the SR resulting in muscle contraction. All the mentioned membrane fractions carry specific enzyme marker activities and channel proteins, which are highly sensitive to their specific ligands and which can be further detected by their ion flux activities. The 1,4-dihydropyridines (DHP) are potent blockers of voltage dependent Ca^{2+}-channels (= DHP receptors),

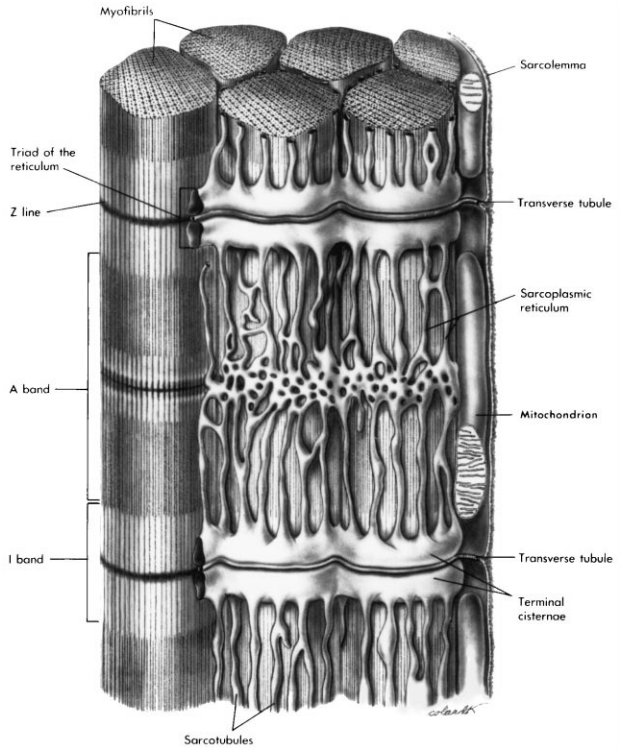

Fig. 1. Architecture of a part of a muscle fibre according to electron microscopy data. The assembly of the sarcolemma, sarcoplasmic reticulum, T-tubules, triads, and mitochondria is shown. The longitudinal sarcotubules are confluent with transverse elements called the terminal cisternae. A slender T-tubule extending inward from the sarcolemma is flanked by two terminal cisternae to form the so-called triads of the reticulum. The location of these with respect to cross banded pattern of the myofibrils varies from species to species. In frog muscle, depicted here, the triads are at the Z-line. In mammalian muscle there are two to each sarcomere, located at the A–I junctions (drawn from ref. [42], reproduced by kind permission of Chapman and Hall).

which are localized predominantly in the triad fraction [46] and to lesser extent in the T-tubules [47]. The modulation of the DHP-sensitive Ca^{2+}-channels from rat gastric mucosa by ganglioside G_{M1} has been described by Slomiany et al. [48]. The aim of our study was to elucidate the influence of muscle relevant gangliosides on voltage dependent Ca^{2+}-channels, which are enriched in the triad membrane fraction of skeletal muscle. Profound qualitative as well as quantitative differences in GSL expression were demonstrated for the lipid environment of ion channel proteins in SL, TT, SR and Tr enriched membrane preparations as shown in the preceding paper [49]. Specific stimulatory effects of gangliosides towards triad Ca^{2+}-channels reconstituted into phospholipid vesicles are shown in this study.

2. Results

Distributions of gangliosides in muscle membrane fractions.—As demonstrated in the preceding paper [49] quantitative and qualitative different expression of G_{M3}, neolacto- and ganglio-series gangliosides (all substituted with Neu5Ac) was detected in SL, TT, SR and Tr/M membrane preparations. The dominant ganglioside G_{M3} of rabbit muscle distributed to about 64% in the SL and ca. 10% in the TT, SR and Tr/M fractions, respectively. IV^3Neu5Ac–*n*LcOse$_4$Cer decreased in the order SL > TT, Tr/M > SR, whereas IV^6Neu5Ac–*n*LcOse$_4$Cer was found to be restricted to the SL and Tr/M vesicles. Low quantities of ganglio-series gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}, showing an identical relative distribution pattern within respective fractions, were elicited in SL and TT vesicles, and considerable less amounts were found in the Tr/M and SR preparations in this order.

Modulation of $^{45}Ca^{2+}$-flux by gangliosides in triad membranes from skeletal muscle.—The Ca^{2+}-channels (= DHP receptors) prevalently reside in the Tr/M fraction. The triad junction is purported to be the link between the signal process of the SL and the release of Ca^{2+} from the terminal cisternae of the SR resulting in muscle contraction (Fig. 1). Thus, the Tr/M fraction, containing DHP-sensitive Ca^{2+}-channels, was chosen for $^{45}Ca^{2+}$-flux studies with regard to the modulatory potential of muscle relevant gangliosides. For this purpose, Ca^{2+}-channels were reconstituted into unilamellar phospholipid vesicles of 400 nm defined size. After ganglioside insertion into the outer lipid layer, $^{45}Ca^{2+}$-uptake into liposomes was quantified in parallel to control liposomes without gangliosides.

Modulation of $^{45}Ca^{2+}$-uptake by G_{M3}.—Two species of G_{M3}, i.e. G_{M3}(Neu5Ac), prepared from a G_{M3}(Neu5Ac) enriched ganglioside fraction of human granulocytes [50], and G_{M3}(Neu5Gc), isolated from a mouse–mouse hybridoma [51], both substituted with C_{24}- and C_{16}-fatty acids and C_{18}-sphingosine, were used for the first set of experiments. G_{M3}(Neu5Ac), the dominant ganglioside of skeletal muscle, showed a strong enhancing effect of $^{45}Ca^{2+}$-flux into vesicles (+285%) compared to controls without gangliosides (Fig. 2). Out of the tested gangliosides (see below), only G_{M3}(Neu5Gc) showed a severe and unequivocal inhibitory effect on the uptake of $^{45}Ca^{2+}$ into liposomes (−61%) compared to the BAY K 8644 derived background

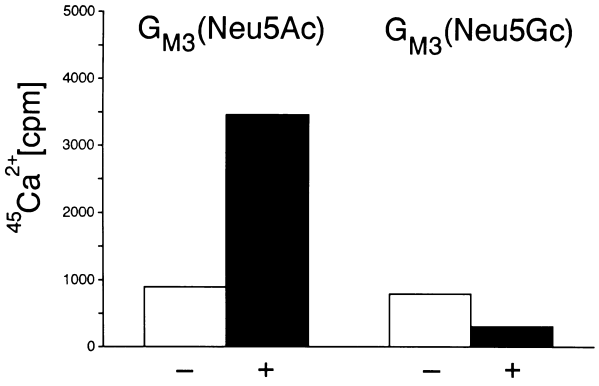

Fig. 2. Modulation of $^{45}Ca^{2+}$-flux by ganglioside GM3. Triad Ca^{2+}-channels were reconstituted into phospholipid vesicles and $^{45}Ca^{2+}$-uptake into liposomes was measured after ganglioside incorporation into the outer lipid layer (+) compared to controls without ganglioside (−). The standard deviation ranged within 10%.

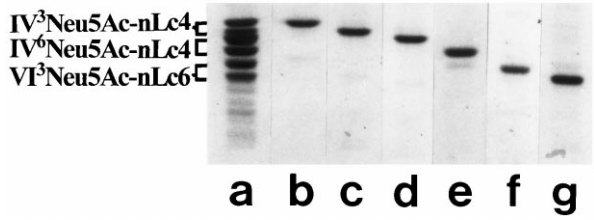

Fig. 3. Resorcinol stained thin-layer chromatogram of individual neolacto-series gangliosides used for $^{45}Ca^{2+}$-flux studies in liposomes. Lane a: 10 μg of a G_{M3}(Neu5Ac)-deprived ganglioside mixture from human granulocytes (starting material). Double bands of each ganglioside are due to C_{24}- and C_{16}-fatty acid substitution corresponding to upper and lower band, respectively; lane b: 1 μg IV^3Neu5Ac–*n*LcOse$_4$Cer (C_{24}-fatty acid); lane c: 1 μg IV^3Neu5Ac–*n*LcOse$_4$Cer (C_{16}-fatty acid); lane d: 1 μg IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{24}-fatty acid); lane e: 1 μg IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{16}-fatty acid); lane f: 1 μg VI^3Neu5Ac–*n*LcOse$_6$Cer (C_{24}-fatty acid); lane g: 1 μg VI^3Neu5Ac–*n*LcOse$_6$Cer (C_{16}-fatty acid).

stimulus of about 800 cpm (Fig. 2). These results gave strong evidence of the specific modulatory capacity of gangliosides, since both, G_{M3}(Neu5Ac) and G_{M3}(Neu5Gc), are monosialogangliosides with identical charge, exhibiting only a little difference owing to the acetyl- (CH_3–CO–R) versus glycolylneuraminic acid (CH_2OH–CO–R). The data suggest rather a specific than unspecific alteration of $^{45}Ca^{2+}$-influx.

Modulation of $^{45}Ca^{2+}$-uptake by neolacto-series gangliosides.—The second most abundant gangliosides of skeletal muscle (after G_{M3}) belong to members of the neolacto-family [49]. The aim of the following experiments was to ask for whether (i) oligosaccharides with isomeric sialic acid configuration (IV^3Neu5Ac–*n*LcOse$_4$, IV^6Neu5Ac–*n*LcOse$_4$), (ii) oligosaccharide chain length (IV^3Neu5Ac–*n*LcOse$_4$, VI^3Neu5Ac–*n*LcOse$_6$) and/or fatty acid chain length of the ceramide portion (C_{24}, C_{16}) of neolacto-series gangliosides might be able to elicit specific modulation of Ca^{2+}-channels. For this purpose, individual neolacto-series gangliosides, homogeneous in their respective oligosaccharide and ceramide moieties, were isolated from a G_{M3}-deprived ganglioside fraction of human granulocytes [52] by means of preparative HPTLC [53]. The resorcinol stained thin-layer chromatogram of individual neolacto-series gangliosides used in the following assays is shown in Fig. 3 and their structures are displayed in Table 1. As shown in Fig. 4, IV^3Neu5Ac–*n*LcOse$_4$Cer (C_{16}) considerably enhanced the $^{45}Ca^{2+}$-flux (+162%), whereas the same ganglioside with C_{24}-fatty acid did not change incorporation (−3%). The administration of the equimolar mixture of both gangliosides indicated the dominance of IV^3Neu5Ac–*n*LcOse$_4$Cer (C_{16}) stimulating by +123%. The α2–6-sialylated gangliosides IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{24}) and IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{16}) showed similar effects towards $^{45}Ca^{2+}$-flux compared to their α2–3-sialylated anomeric counterparts (Fig. 5). Weak inhibition of −17% was observed for IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{24}) and considerable enhancement in ion-flux of +120% was found in case of IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{16}). In contrast to the former experiment, the addition of the equimolar mixture of both compounds led to almost complete loss of the stimulus (+11%), indicating in this case the dominance of the ganglioside with C_{24}-fatty acid over that with C_{16}-fatty acid substitution.

The final experiments concerning the modulatory potential of neolacto-series gangliosides are shown in Fig. 6. In contrast to α2–3- and α2–6-sialylated *n*LcOse$_4$Cer, the elongated ganglioside VI^3Neu5Ac–*n*LcOse$_6$Cer with C_{24}-fatty acid showed a large increase in $^{45}Ca^{2+}$-flux of +208%, whereas the same ganglioside with C_{16}-fatty acid as well as the mixture of both revealed a slow decrease of −6 and −11%, respectively. Here, VI^3Neu5Ac–*n*LcOse$_6$Cer (C_{16}) obviously dominates in mixture over the corresponding ganglioside with C_{24}-fatty acid.

In conclusion, neolacto-series gangliosides evoked miscellaneous effects upon $^{45}Ca^{2+}$-flux depending on anomeric sialic acid configuration, oligosaccharide size and fatty acid chain length of the ceramide portion.

Table 1
Structures of neolacto-series monosialogangliosides

Formula	Structure	Abbreviation
IV^3Neu5Ac–*n*LcOse$_4$Cer	Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer 3 \|α 2 Neu5Ac	IV^3*n*Lc4
IV^6Neu5Ac–*n*LcOse$_4$Cer	Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer 6 \|α 2 Neu5Ac	IV^6*n*Lc4
VI^3Neu5Ac–*n*LcOse$_6$Cer	Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer 3 \|α 2 Neu5Ac	VI^3*n*Lc6

Modulation of $^{45}Ca^{2+}$-uptake by ganglio-series gangliosides.—In the last set of experiments the question should be answered whether degree of sialylation of ganglio-series gangliosides, all with identical GgOse$_4$Cer-backbone and substituted with Neu5Ac, might influence the $^{45}Ca^{2+}$-uptake into liposomes. The resorcinol stained thin-layer chromatogram of individual brain derived ganglio-series gangliosides used in the following assays is shown in Fig. 7 and their structures are displayed in Table 2. As shown in Fig. 8, G_{M1} exhibited the strongest stimulation of $^{45}Ca^{2+}$-flux (+327%) out of all gangliosides tested in this study. G_{D1a} and G_{T1b} gave rise to remarkable flux-stimulation of +283 and +255%, respectively, ranging on the level of G_{M3}(Neu5Ac) as shown above, whereas G_{D1b} exhibited only a slightly positive effect (+38%). It should be stressed, that the anomeric disialogangliosides G_{D1a} and G_{D1b} which are different in their intramolecular linkages of sialic acids, evoked completely distinct responses in $^{45}Ca^{2+}$-flux, a further indication for a rather specific than unspecific charge-dependent change of ion-flux.

3. Discussion

A large body of data indicates that gangliosides, apart from their proposed structural roles in

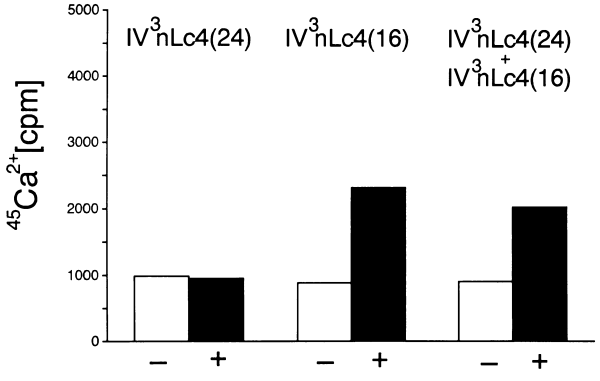

Fig. 4. Modulation of $^{45}Ca^{2+}$-flux by neolacto-series gangliosides IV^3Neu5Ac–*n*LcOse$_4$Cer (C_{24}-fatty acid) and IV^3Neu5Ac–*n*LcOse$_4$Cer (C_{16}-fatty acid). Triad Ca^{2+}-channels were reconstituted into phospholipid vesicles and $^{45}Ca^{2+}$-uptake into liposomes was measured after ganglioside incorporation into the outer lipid layer (+) compared to controls without ganglioside (–). The standard deviation ranged within 8%. IV^3*n*Lc4 = IV^3Neu5Ac–*n*LcOse$_4$Cer.

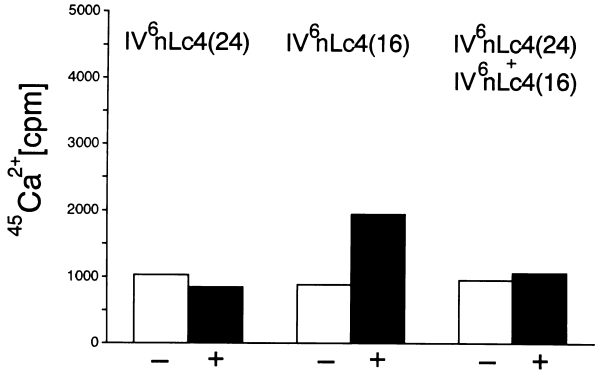

Fig. 5. Modulation of $^{45}Ca^{2+}$-flux by neolacto-series gangliosides IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{24}-fatty acid) and IV^6Neu5Ac–*n*LcOse$_4$Cer (C_{16}-fatty acid). Triad Ca^{2+}-channels were reconstituted into phospholipid vesicles and $^{45}Ca^{2+}$-uptake into liposomes was measured after ganglioside incorporation into the outer lipid layer (+) compared to controls without ganglioside (–). The standard deviation ranged within 15%. IV^6*n*Lc4 = IV^6Neu5Ac–*n*LcOse$_4$Cer.

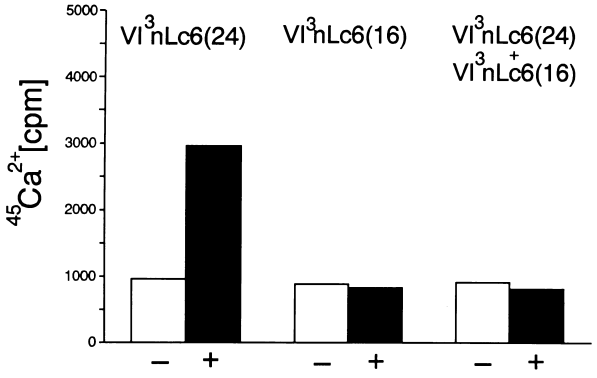

Fig. 6. Modulation of $^{45}Ca^{2+}$-flux by neolacto-series gangliosides VI^{3}Neu5Ac–*n*LcOse$_6$Cer (C_{24}-fatty acid) and VI^3-Neu5Ac–*n*LcOse$_6$Cer (C_{16}-fatty acid). Triad Ca^{2+}-channels were reconstituted into phospholipid vesicles and $^{45}Ca^{2+}$-uptake into liposomes was measured after ganglioside incorporation into the outer lipid layer (+) compared to controls without ganglioside (–). The standard deviation ranged within 15%. VI^3*n*Lc6 = VI^{3}Neu5Ac–*n*LcOse$_6$Cer.

biomembranes, may serve as molecules affecting cell–cell and cell–matrix interactions as outlined in the Introduction. The intracellular mechanisms mediating these effects of gangliosides are poorly understood. Many investigations have focused on G_{M1} the highly specific molecule for the B subunit of cholera toxin. The G_{M1}-mediated neurotrophic properties have been studied by means of administration of exogenous G_{M1} to in vitro grown cells or by manipulation of endogenous G_{M1} with, e.g. cholera toxin B subunit and neuraminidase, the latter increasing G_{M1} on the cell surface at the expense of oligosialogangliosides [54]. The signalling pathway activated by B subunit binding to cell surface G_{M1} involves a rapid rise in intracellular free Ca^{2+}, e.g. in astrocytes [55], sensory neurons [56], N18 neuroblastoma cells [57] and cerebellar granule neurons [58]. Exogenously added G_{M1} can

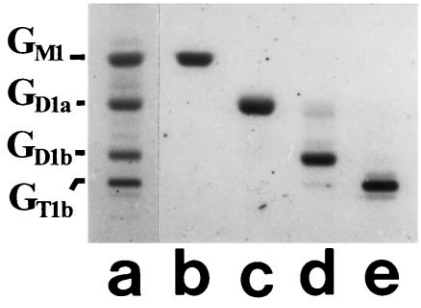

Fig. 7. Resorcinol stained thin-layer chromatogram of individual ganglio-series gangliosides used for $^{45}Ca^{2+}$-flux studies in liposomes. Lane a: 5 μg of a ganglioside mixture from human brain (reference); lane b: 2 μg G_{M1}; lane c: 2 μg G_{D1a}; lane d: 2 μg G_{D1b}; lane e: 2 μg G_{T1b}.

stably insert into the neural cell membranes, raising the intracellular Ca^{2+}-levels, apparently accounting for enhanced neuritogenesis [59,60].

Cell calcium signaling via G_{M1} has also been reported for non-neuronal cells, e.g. quiescent [61] and stimulated Swiss 3T3 cells [62], rat lymphocytes [63], human Jurkat T cell line [64] and human platelets [65,66]. In view of many reports describing ganglioside-induced modulation of Ca^{2+} across the plasma membrane, scarce data are available exploring the phenomenon on subcellular, i.e. intracellular level. The nuclear envelope of neuronal cells possesses G_{M1} along with other gangliosides [67], and its modulatory activity for nuclear calcium homeostasis during neurite outgrowth has been well documented [68]. In skeletal muscle, ganglio-series gangliosides G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} are only minor constituents and contribute to minimum extent to the ganglioside content of the Tr/M fraction [49]. However, out of all gangliosides tested upon their Ca^{2+}-flux modulation in muscle, G_{M1} is the one which caused the strongest stimulation. Its ability to modulate the intracellular ion flux of vesicle-reconstituted calcium channel complex of rat gastric mucosa has been demonstrated by Slomiany et al. [48], but data on the activity of G_{D1a}, G_{D1b} and G_{T1b} towards rat gastric mucosa Ca^{2+}-channels are not available. In muscle, the isomeric disialogangliosides G_{D1a} and G_{D1b} showed clearly distinct modulatory capacity, i.e. considerably reduced flux by G_{D1b} which is distinguished from G_{D1a} by the disialo-(Neu5Ac)$_2$-configuration at the internal galactose of the GgOse$_4$-core (Table 2). Ganglioside G_{T1b}, which comprises the two features of both disialogangliosides, i.e. the Neu5Acα2–3Gal-terminus of G_{D1a} and the internal Neu5Acα2–8Neu5Acα2–3Gal-configuration of G_{D1b} gave rise to intermediate $^{45}Ca^{2+}$-flux, less than G_{D1a} but more than G_{D1b}.

Among various rabbit tissues, G_{M3}(Neu5Gc) was found in thymus, lung, kidney, and intestine in addition to G_{M3}(Neu5Ac), but G_{M3}(Neu5Ac) was the sole G_{M3}-species in brain, liver, stomach, kidney, testis, erythrocytes, and muscle [69]. The latter represents the rabbit tissue with the lowest concentration of lipid-bound sialic acid, amounting to 1.4% of that in brain [70]. G_{M3}(Neu5Ac), the major ganglioside of rabbit skeletal muscle, gave rise to a +285% elevation of Ca^{2+}-flux. Most excitingly, G_{M3}(Neu5Gc), which is not expressed in rabbit muscle, reduced the Ca^{2+}-uptake down to –61% relative to the basic level of BAY K 8644

Table 2
Structures of ganglio-series gangliosides

Formula	Structure	Abbreviation
$II^3Neu5Ac–GgOse_4Cer$	Galβ1–3Ga1NAcβ1–4Galβ1–4Glcβ1–1Cer 3 $\|\alpha$ 2 Neu5Ac	G_{M1}
$IV^3Neu5Ac,II^3Neu5Ac–GgOse_4Cer$	Galβ1–3Ga1NAcβ1–4Galβ1–4Glcβ1–1Cer 3 3 $\|\alpha$ $\|\alpha$ 2 2 Neu5Ac Neu5Ac	G_{D1a}
$II^3(Neu5Ac)_2–GgOse_4Cer$	Galβ1–3Ga1NAcβ1–4Galβ1–4Glcβ1–1Cer 3 $\|\alpha$ 2 Neu5Ac 8 $\|\alpha$ 2 Neu5Ac	G_{D1b}
$IV^3Neu5Ac,II^3(Neu5Ac)_2–GgOse_4Cer$	Galβ1–3Ga1NAcβ1–4Galβ1–4Glcβ1–1Cer 3 3 $\|\alpha$ $\|\alpha$ 2 2 Neu5Ac Neu5Ac 8 $\|\alpha$ 2 Neu5Ac	G_{T1b}

derived background stimulus. Hydroxylation of the *N*-acetyl chain of Neu5Ac by the enzyme CMP–Neu5Ac–hydroxylase [71], obviously inactive in muscle but working in several other rabbit tissues, generates a ''channel-blocker'' with exceptional inhibitory potential compared to all the other gangliosides investigated in this study. Gangliosides carrying Neu5Gc have not been studied in ion flux assays so far, but should be considered for their modulatory potency especially in cells of human origin normally lacking these compounds. The neolacto-series gangliosides showed to some extent tremendous stimulatory but on the other hand inhibitory effects as well, depending on oligosaccharide constitution and ceramide fatty acid chain length. The importance of the carbohydrate portion of intact gangliosides, excerting a variety of specific biological events, has been documented in a large number of publications. However, a simple difference in ceramide structure of the intact ganglioside—the length of the fatty acyl chain—considerably influences ganglioside activity as shown by six individual neolacto-series gangliosides in this study. These findings coincide with recent data from the group of Ladisch and colleagues, who reported on the importance of ganglioside fatty acyl chain length with respect to ganglioside mediated immunosuppressive activity [72,73].

Until today, little is known about the physiological function of GSLs in muscle. Examples are the involvement of gangliosides in kinases dependent protein phosphorylation in guinea pig muscle [74], the identification of several ganglioside-binding proteins in the cytosolic fractions of skeletal and cardiac muscle [75], and effects of gangliosides on the electronic (Na^+/K^+) pump of muscle fibres [44] and of G_{M3} on the activity and conformation of Ca^{2+}-ATPase of rabbit skeletal muscle sarcoplasmic reticulum [45]. In summary, our data suggest a functional role of gangliosides in subcellular muscle membrane and thus for muscle in general. Muscle relevant gangliosides G_{M3}, neolacto- and ganglio-series gangliosides, are capable of modulating the cytosolic calcium level, which per se regulates muscle contraction. The results obtained provide the basis for further investigations of gangliosides which might influence membrane protein function in skeletal muscle.

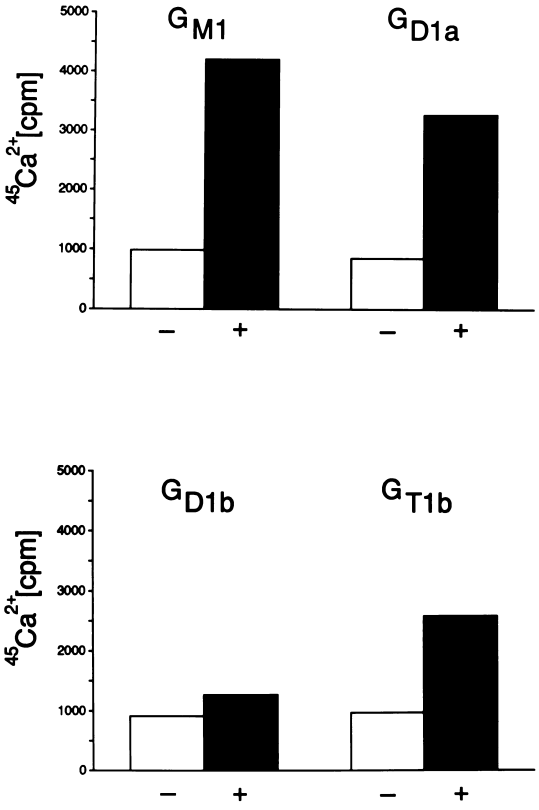

Fig. 8. Modulation of $^{45}Ca^{2+}$-flux by ganglio-series ganglioside G_{M1}, G_{D1a}, G_{D1b} and G_{T1b}. Triad Ca^{2+}-channels were reconstituted into phospholipid vesicles and $^{45}Ca^{2+}$-uptake into liposomes was measured after ganglioside incorporation into the outer lipid layer (+) compared to controls without ganglioside (−). The standard deviation ranged within 20%.

4. Experimental

Preparation of membrane vesicles.—Fractions of membrane vesicles were prepared from 380 g fresh skeletal muscle tissue (back and hindlegs) of a specific pathogen free inbred rabbit (Hoechst, Frankfurt, Germany) according to the method of Mitchell et al. [76], modified in some parts by Weber-Schürholz et al. [77]. The fractions obtained after sucrose density gradient centrifugation contained vesicles, enriched in the following membrane systems (in the order of increasing density): sarcolemma (SL), T-tubules (TT), sarcoplasmlc reticulum (SR), subdivided into SR1 and SR2 (containing smaller and larger vesicles, respectively), and triads and mitochondria (Tr/M). Membrane fractions were monitored by the distribution of marker enzymes, ligand binding and flux activities as reported by Weber-Schürholz et al. [77]. All the membrane characteristics are summarized in Table 1 of the preceding paper by Müthing et al. [49] where all the details are provided.

Reconstitution of calcium channels into liposomes.—The triads containing fraction Tr/M with the highest calcium channel content, determined by its characteristically high binding capacity of the DHP-receptor ligand [^{3}H]PN200-110 [77] (see also Table 1 in the preceding paper of Müthing et al. [49], was used for reconstitution of calcium channels into phospholipid vesicles and Ca^{2+}-flux studies. Liposomes were prepared in sucrose medium (0.34 M sucrose, 0.05 M Tris(hydroxymethyl)-aminomethane, 5 mM $CaCl_2$, 5 μM BAY K 8644 (calcium channel activator [78,79], RBI, Natick, MA, USA, pH 7.4 with HCl) at a final concentration of 5.4 mg liposomes/ml medium, with a lipid matrix consisting of L-α-phosphatidylcholine, L-α-phosphatidylethanolamine, L-α-phosphatidyl-L-serine and cholesterol (all from Sigma, Deisenhofen, Germany) in a 4:2:2:3 ratio (each by weight) and a protein/lipid ratio of 1:4.4 (w/w). This solution was sonified in intervals of 2 min (Sonifier 250, Branson Sonic Power Company, Danbury, CT, USA; 50% duty cycle, output "5") and then extruded 10 times through a polycarbonate membrane (Extruder LiposoFast, Avestin, Ottawa, Canada; 400 nm pores) to form unilamellar vesicles of defined size [80]. Liposomes were now ready to use.

Measurement of ganglioside-modulated $^{45}Ca^{2+}$-uptake into liposomes.—A slightly modified method of Slomiany et al. [48] was used for the determination of $^{45}Ca^{2+}$-uptake into phospholipid vesicles. Samples of 1 ml of the liposome suspension (see above) were applied to a 5 ml Sephadex G-25 column (Pharmacia LKB Biotechnology, Uppsala, Sweden), which had been equilibrated with buffer 1 (0.34 M sucrose, 10 mM MOPS (3-[N-morpholino]propanesulfonic acid), 5 μM, BAY K 8644, pH 7.0 with triethylamine) to remove external divalent cations. For the ganglioside incubation 250 μg of each ganglioside were evaporated in a glass vial to which then the liposome solution was added. Controls of reconstituted vesicles without gangliosides and controls of protein-free liposomes with and without ganglioside supplementation were also prepared in order to be able to subtract the background uptake. Now the liposome samples were incubated for 18 h at +4°C under slightly stirring. $^{45}Ca^{2+}$-uptake into liposomes was started by adding 0.5 ml of sucrose medium containing 0.1 M $CaCl_2$ and 20 μCi (0.74 MBq) of $^{45}Ca^{2+}Cl_2$ (1.85 GBq/mg Ca; Amersham Buchler, Braunschweig, Germany) to each 1 ml sample. The suspension was incubated at room temperature for 2 h;

the uptake reaction was terminated by addition of 0.75 ml stop buffer (150 mM $MgCl_2$, 10 mM HEPES, 10 mM Tris, pH 7.4 with HCl). For removal of externally bound $^{45}Ca^{2+}$ the samples were applied to a 5 ml Chelex 100 (100–200 mesh) column (Bio-Rad Laboratories GmbH, Munich, Germany) equilibrated with buffer 2 (0.34 M sucrose, 50 mM Tris, 1 mg/ml bovine serum albumin, pH 7.4 with HCl) which was also used for the elution of the samples. In each experiment the final eluate of the Chelex column (vol. 3 ml) was split into four identical portions of 750 μl (each corresponding to 250 μg of protein) which were then precipitated to glass fibre membranes with 1.5 μm pore size (Schleicher & Schuell, Dassel, Germany). $^{45}Ca^{2+}$ incorporated into liposomes was measured by liquid scintillation spectrometry in a 1980 CA TRI-CARB liquid scintillation analyzer (Packard Instruments Co., Downers Grove, IL, USA), calculated as counts per min (cpm) for each approach. Each experiment was carried out twice.

Thin layer chromatography; reference gangliosides.—Glass-backed silica gel 60 precoated high-performance thin-layer chromatography plates (HPTLC plates, size 10×10 cm, thickness 0.2 mm, Merck, Darmstadt, Germany) were used [81]. GSLs were applied to the plates with the automatic TLC applicator AS 30 (Desaga, Heidelberg, Germany). Gangliosides were chromatographed in chloroform/methanol/water (120:85:20) containing 2 mM $CaCl_2$ and visualized with resorcinol [82]. A G_{M3}(Neu5Ac) deprived ganglioside mixture composed of IV^{3}Neu5Ac–*n*LcOse$_4$Cer, IV^{6}Neu5Ac–*n*LcOse$_4$Cer, and VI^{3}Neu5Ac–*n*LcOse$_6$Cer was prepared from whole ganglioside fraction of human granulocytes [83,84], as recently described [52]. Human brain gangliosides were purchased from Supelco Inc. (Bellefonte, PA, USA).

Gangliosides used for vesicle reconstitution and Ca^{2+}-flux modulation studies.—G_{M3}(Neu5Ac) was prepared from a G_{M3}(Neu5Ac) enriched ganglioside fraction obtained from whole ganglioside mixture of human granulocytes [50]. G_{M3}(Neu5Gc) was isolated from mouse-mouse hybridoma cells [51]. Individual neolacto-series gangliosides IV^{3}Neu5Ac–*n*LcOse$_4$Cer, IV^{6}Neu5Ac–*n*LcOse$_4$Cer, and VI^{3}Neu5Ac–*n*LcOse$_6$Cer were isolated from a G_{M3}(Neu5Ac) deprived ganglioside mixture of human granulocytes [52] by preparative HPTLC essentially as described earlier [53]. Gangliosides were quantified by densitometry with a CD60 scanner (Desaga) equipped with an IBM compatible personal computer and densitometric software. Intensities of bands were measured in reflectance mode at 580 nm with a light beam slit of 0.1×2 mm. Pure bovine brain derived ganglioseries gangliosides G_{M1}, G_{D1a}, G_{D1b}, and G_{T1b} were from Dr. Pallmann GmbH (Munich, Germany). Structures of neolacto- and ganglio-series gangliosides are listed in Tables 1 and 2, respectively.

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