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PERTURBATION OF GLYCOPROTEIN PROCESSING AFFECTS THE NEUROTOXIN-RESPONSIVE Na^+ CHANNEL IN NEUROBLASTOMA CELLS*

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ABSTRACT

The activity of neurotoxin-responsive Na^+ channels in mouse neuroblastoma cells, N-18, was examined after treating the cells with compounds that are reported to perturb intracellular traffic. The compounds used have been shown to either alter glycoprotein synthesis and processing, (swainsonine, castanospermine, monensin, and retinoic acid) or receptor mediated endocytosis (mevinolin, 7-ketocholesterol, and chloroquine), or both. All of these compounds inhibited the activity of the neurotoxin-responsive Na^+ channel with the exception of retinoic acid which increased the activity. Na^+ channel activity was measured by two methods: (a) *In vivo*, the efflux of ^{86}Rb was measured by use of the cells in monolayer culture, and (b) *in vitro*, the flux of ^{86}Rb was measured from artificial phospholipid vesicles containing the partially purified Na^+ channel. In both cases, ^{86}Rb flux responded to stimulating neurotoxins, veratridine and scorpion venom, and was inhibited by tetrodotoxin as characteristic of excitable membranes. One of the perturbing compounds, swainsonine, was examined in detail. Treatment of N-18 cells with 10μM swainsonine for 24 h markedly reduced the activity of the neurotoxin-responsive Na^+ channel, as shown by the neurotoxin-stimulated efflux of ^{86}Rb *in vivo*. In addition, after reconstitution into phospholipid vesicles of the partially purified Na^+ channel from swainsonine-treated cells, reduced ^{86}Rb flux was observed when compared with that of nontreated cells. Furthermore, the activity was not recovered in other less purified fractions. A comparison of the glycopeptides from the treated and nontreated cells by size, charge, and lectin-binding affinities was consistent with the formation of hybrid oligosaccharides after swainsonine treatment. It is concluded that the oligosaccharide residues of the Na^+ channel glycoprotein must be processed to the mature complex-type for full activity. The stimulation of channel activity by treatment with retinoic acid supported this conclusion.

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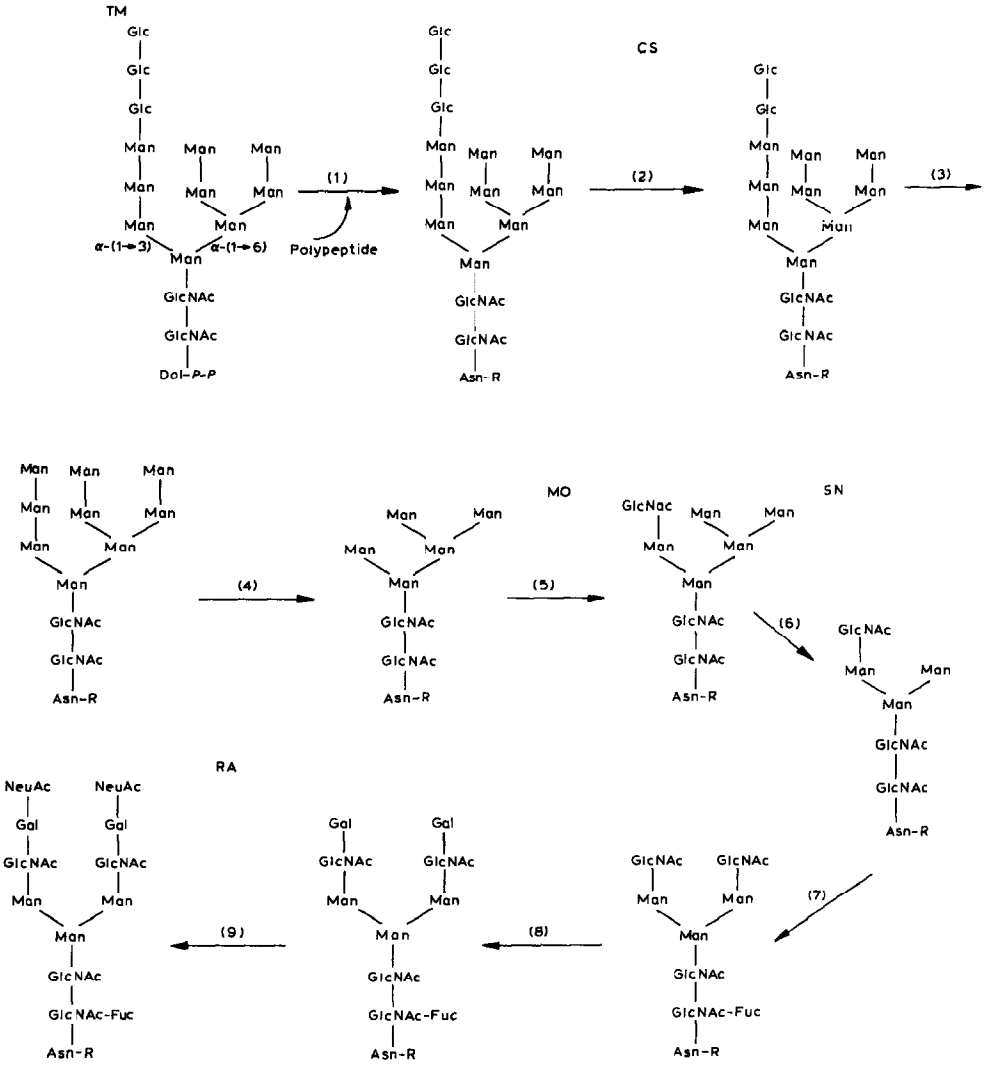
INTRODUCTION

Inhibitors of glycoprotein processing and intracellular trafficking provide excellent tools to determine the role of particular molecules in cellular activities[1–3]. One component of neuronal cells that has been the subject of much investigation is the neurotoxin-responsive Na^+ channel[4]. The subunits of this channel have been shown to be a high-molecular-weight glycoprotein in neuroblastoma cells[5] and



Scheme 1. Stages in biosynthesis of a biantennary, asparagine-linked oligosaccharide. Enzymes are: (1) Oligosaccharyltransferase; (2) Glucosidase I A/B; (3) Glucosidase II: (4) Mannosidase I; (5) GlcNAc-transferase I; (6) Mannosidase II; (7) GlcNActransferase II; Fucosyltransferase; (8) Galactosyltransferase; and (9) Sialyltransferase. R, polypeptide; Dol-*P*-*P*, dolichol pyrophosphate. Compounds act at the points indicated: TM, tunicamycin; CS, castanospermine; SN, swainsonine; and MO, monensin, which disrupts processing within the Golgi apparatus, and thus, may inhibit at Steps 4, 5, or thereafter; and RA, retinoic acid which stimulates sialyltransferases.

other neuronal tissue[4]. The relationship of glycosylation to the neurotoxin-responsive Na^+ channel was shown previously by suppression of channel activity after growth of human or mouse neuroblastoma cells in low concentrations of specific monosaccharides and by treatment with neuraminidase[6]. Tunicamycin, an agent that blocks the first step of lipid-linked biosynthesis of glycoproteins, was used previously to inhibit the binding of saxitoxin (a neurotoxin known to bind to the Na^+ channel in mouse neuroblastoma cells[7]) and the binding of α-bungarotoxin to the acetylcholine receptor in muscle[8]. Tunicamycin will completely prevent glycosylation of proteins at asparagine residues. An unglycosylated protein may have altered properties, such as greater susceptibility to proteolytic cleavage[9], thus experiments using tunicamycin are hard to interpret in terms of the necessity for glycosylation for these neuronal activities. Therefore, we examined other compounds that alter latter stages of processing of mature oligosaccharides on the polypeptide and monitored their effect on the activity of the excitable membrane.

A schematic representation of the enzymes involved in the biosynthesis of *N*-linked glycoprotein processing[10] is shown in Scheme 1, and the sites of action of some of the compounds used in this study are given. We selected swainsonine[11–13] (Scheme 1, step 6), castanospermine which acts[14] on glycoprotein processing in the endoplastic reticulum[14] (ER) (Scheme 1, step 2), and monensin which exerts a topographical effect in the Golgi apparatus[15] (Scheme 1, step 5 and thereafter). Since monensin is known to also inhibit endocytosis, we used other known inhibitors of endocytosis, mevinolin, 7-ketocholesterol, and chloroquine[16], and observed the activity of the neurotoxin-responsive Na^+ channel.

EXPERIMENTAL

Cell growth. — For measurement of neurotoxin-responsive Na^+ channels, a clonal cell-line of mouse neuroblastoma, N-18 (ref. 17), was grown on 25-mm dishes as described[6] for 3 days. The medium was replaced, the cells were grown for an additional 24 h, and then the medium was replaced with fresh medium with or without the compounds examined in the stated concentrations. After 24 h, the cells were examined for the efflux of ^{86}Rb as described[6]. The perturbating agents were: swainsonine, generously supplied by Dr. Alan Elbein, San Antonio, Texas, and Dr. Peter Dorling, University of Murdoch, Murdoch, Australia; mevinolin, supplied by Dr. A. Alberts, Merck, Sharp & Dohme, Rahway, NJ: monensin, 7-ketocholesterol, and retinoic acid, purchased from Calbiochem–Behring Corp., Schwartz/Mann Div., and Eastman Kodak Co., respectively; castanospermine purchased from Boehringer–Mannheim Biochemicals; and chloroquine purchased from Sigma Chemical Co.

For determination of the effect of swainsonine on the partially purified Na^+ channel and the other cell glycoproteins, N-18 cells were seeded in 75-cm^2 Falcon flasks and grown in a manner similar to that just described for the cells grown on the plates. Fresh medium with or without swainsonine (10μM) was added on day 4,

and 3 h later 185 KBq per flask of D-[6-^{3}H(N)]glucosamine (New England Nuclear: 1.16 TBq/mmol) was added for 24 h.

Efflux of ^{86}Rb. — The activity of the neurotoxin-responsive Na^+ channel was measured *in vivo*, with N-18 cells as described[6,18], by the efflux of ^{86}Rb, in the presence of stimulating (veratridine and scorpion venom) or inhibiting (tetrodotoxin) neurotoxins. Rb^+ flows through the Na^+ channel albeit at a slower rate than sodium[18,19]. Cells treated with the perturbating agents were tested at the same time as cells which were treated similarly but without agents. ^{86}Rb (New England Nuclear; 0.2 GBq/mg) was added to the plates for 80 min; after washing and replacing the assay medium, aliquots of medium were removed to measure the passive efflux. Then, veratridine (100 μM) and *Leiurus quinquestriatus* scorpion venom (5 μg/mL) were added and aliquots removed to measure the active efflux of ^{86}Rb. In some cases, tetrodotoxin (1μM) was added. After radioactive counting, the data were processed and graphed by computer[6].

Extraction and characterization of Na^+ channel-enriched fraction. — N-18 cells were grown, as described earlier, on 75-cm^2 flasks. One half of the cultures were treated with 10μM swainsonine and the other half were not treated. Both treated and nontreated cultures were extracted with 1% Nonidet P-40 (NP-40) and the remaining cell pellets removed. The extracts were centrifuged at 27 000*g* for 30 min, and the supernatant solutions from both the swainsonine treated and nontreated cultures were processed as follows. Each extract was passed over a column (8 × 70 mm) of WGA–Sepharose (Pharmacia). The column was washed with 10mM Tris in 0.15M NaCl (pH 7.5) containing 0.1% of NP-40 (Buffer A), and the bound glycoproteins were eluted with 0.02 and 0.1M GlcNAc in buffer A. The fraction from the nontreated cells that was eluted with 0.02M GlcNAc was enriched (10%) in the glycoprotein which had been shown previously, by reconstitution into phospholipid vesicles, to have the activity of the neurotoxin-responsive Na^+ channel[5]. The fraction was passed over a column (4 × 0.5 cm) of Bio-Beads SM-2 to remove NP-40, lyophilized, and subsequently digested exhaustively with Pronase (Calbiochem–Behring Corp.) as described[20]. After being passed over Bio-Gel P-2 and lyophilized, the fraction was characterized by lectin-affinity columns[21]. The column sizes, eluting buffers, and all other details were as described[22]. Con A– and lentil–Sepharose were from Pharmacia and L-PHA–agarose was from E. Y. Laboratories. Serotonin–Sepharose of high affinity was prepared by Dr. C. P. Stowell and used as described[23,24]. The size of the glycopeptides was determined by passage through a column (1 × 120 cm) of Bio-Gel P-10 in 50mM ammonium acetate buffer[22]. The charge characteristics were examined by passage through a column (1 × 10 cm) of DEAE–cellulose, the glycopeptides being eluted with a gradient of 0.5–30mM KH_2PO_4 buffer (pH 6.8), followed by 100mM buffer as described[20].

Reconstitution of radioactively labeled glycoprotein fractions and demonstration of biological activity. — The [^{3}H]-glycoproteins from the swainsonine treated and nontreated cells, which were eluted from WGA–Sepharose with 0.02M

GlcNAc, were reconstituted into 2-oleoyl-1-palmitoylphosphatidylcholine (Avanti Polar Lipids, Inc.) in a protein-to-phospholipid ratio of 1:10 as described[5]. After removal of the excess detergent on a column of Bio-Beads SM-2, the radioactive vesicles were centrifuged off at 76 000*g* for 2 h. The pellet was resuspended in 20mM K_2HPO_4 buffer, (pH 7.4) with 100mM NaCl and divided into three aliquots. To two aliquots were added 250μM veratridine and scorpion venom (12.5 μg/mL) each, and one of these contained a prior addition of 5μM tetrodotoxin. The third aliquot served as a control without neurotoxins. ^{86}Rb (0.89 MBq) was added to each fraction and, after a 30-min incubation, the vesicles were passed over columns of Dowex 50W-X2 to remove free ^{86}Rb not taken up by the vesicles. The vesicles were collected and counted in a liquid-scintillation counter. The percentage of ^{86}Rb flux that was stimulated by neurotoxins was that amount of radioactivity in the stimulated vesicles *vs.* that of the nonstimulated vesicles. Vesicles treated with tetrodotoxin and then stimulated served as a control for specific stimulation.

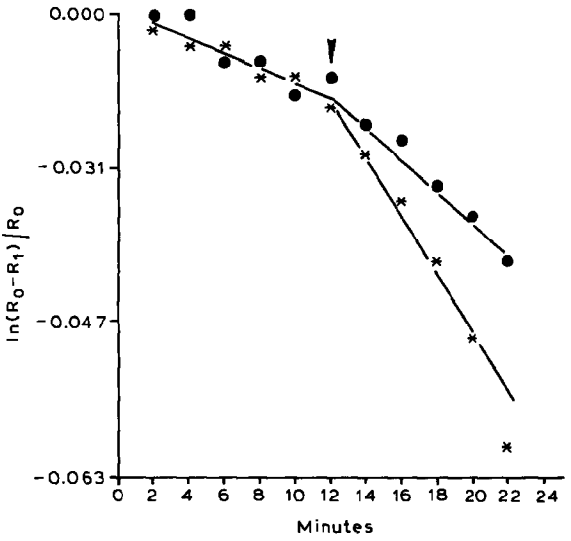


Fig. 1. Efflux of ^{86}Rb from mouse neuroblastoma cells treated (—●—●—) or not (—★—★—) with 20μM swainsonine. The experiment from which these results were obtained was as follows: N-18 cells were grown on 25-mm Petri dishes for four days as described in the Experimental section. On day 4, fresh medium containing 1, 10, 20, and 50μM swainsonine was added to each of four cultures, respectively, and only medium to control cultures. On day 5, each culture of N-18 cells was assayed for the presence of active Na^+ channels by measuring the efflux of ^{86}Rb (ref. 18). After the uptake of ^{86}Rb by N-18 cells and washing, aliquots of the assay medium were removed for 12 min to establish the nonstimulated efflux. At 12 min, 100μM veratridine and *Leiurus quinquestriatus* scorpion venom (5 μg/mL) were added (designated by the arrow) and aliquots (R_1) were removed to measure the neurotoxin-stimulated efflux of ^{86}Rb. After removal of the final aliquot at 22 min, the remaining ^{86}Rb was extracted from the cells with 10% trichloroacetic acid. The radioactivity was measured and used to calculate the original amount of ^{86}Rb (R_0) taken up by the cells and the efflux was plotted as the negative number. Addition of μM tetrodotoxin abolished the veratridine–scorpion venom-stimulated efflux of ^{86}Rb and did not affect the nonstimulated efflux.

RESULTS

Inhibition of neurotoxin-stimulated ^{86}Rb flux by swainsonine. — When cultures of N-18 cells were treated with 1–50μM swainsonine for 24 h, the neurotoxin-stimulated efflux of ^{86}Rb was inhibited up to 75% when compared with that of nontreated cells. Fig. 1 shows an example of the measurement of ^{86}Rb efflux from a culture of the nontreated N-18 cells and that of a parallel culture treated with 20μM swainsonine.

In order to further define the inhibition of Na^+ channel activity with swainsonine, a fraction enriched in a glycoprotein previously shown to contain the neurotoxin-responsive Na^+ channel[5] was reconstituted into artificial phospholipid vesicles. The uptake of ^{86}Rb into the vesicles was measured in the presence of stimulating and inhibiting neurotoxins.

TABLE I

PURIFICATION AND RECONSTITUTION OF THE PARTIALLY PURIFIED Na^+ CHANNEL IN THE PRESENCE AND ABSENCE OF SWAINSONINE

Procedure used and fractions of N-18 cells	*Swainsonine added*	
	None	*10μM*
Purification[a,b]		
NP-40 extract[c] (% of total in cells)	43	38
WGA–Sepharose (% of total in NP-40 extract)		
Unbound	89	94
Eluted with 0.02M GlcNAc	9	4
Eluted with 0.1M GlcNAc	2	2
Reconstitution into PLV[d,b]		
WGA–Sepharose (% of total fraction[e] incorporated into PLV)		
Unbound	9	8
Eluted with 0.02M GlcNAc	23	25
Na^+ channel activity[f]		
WGA–Sepharose (% stimulated)		
Unbound	0	0
Eluted with 0.02M GlcNAc	86	18

[a]Glycoproteins were extracted with 1% NP-40 from N-18 cells metabolically labeled with [^{3}H]GlcN in the presence or absence of swainsonine. The extract was separated on WGA–Sepharose and the bound fractions were eluted with 0.02 and 0.1M GlcNAc. [b]Percentages determined by ^{3}H-radioactivity. [c]Total radioactivity of substances extracted with NP-40 from 1×10^8 cells was 7.2×10^6 c.p.m. and 7.3×10^6 c.p.m. for nontreated and swainsonine treated cells, respectively. [d]PLV, phospholipid vesicles. The [^{3}H]glycoprotein fractions were incorporated into PLV. [e]Percentage of each WGA–Sepharose separated fraction incorporated in PLV. [f]PLV containing glycoproteins were incubated in the presence of ^{86}Rb. The amount of ^{86}Rb in the PLV stimulated with neurotoxins or not stimulated was measured and expressed as the percentage of ^{86}Rb radioactivity found in PLV; stimulated *vs.* nonstimulated. Tetrodotoxin inhibited the stimulated ^{86}Rb flux of the PLV containing glycoproteins from swainsonine treated or nontreated cells by 100 to 70%, respectively. All details are described in the Experimental section.

The glycoprotein fractions that were reconstituted were obtained from ten 75-cm^2 flasks of N-18 cells treated or nontreated for 24 h with 10μM swainsonine in the presence of D-[^{3}H]glucosamine. The cells were extracted with 1% NP-40 and the glycoproteins that bound to WGA–Sepharose and were eluted with 0.02M GlcNAc were used for reconstitution into artificial vesicles. Table I gives the percentage of radioactivity recovered during the purification procedure. After swainsonine treatment, less [^{3}H]-glycoproteins (55%) bound to WGA–Sepharose and were eluted with 0.02M GlcNAc. However, similar percentages of glycoproteins unbound and bound to WGA–Sepharose were reconstituted into artificial vesicles, irrespective of the swainsonine treatment.

In contrast, when ^{86}Rb flux was measured in the reconstituted vesicles, a marked difference was observed after stimulation by the neurotoxins. The phospholipid vesicles containing the [^{3}H]-glycoproteins from the swainsonine-treated cells had less than one fourth the ^{86}Rb flux stimulated by the neurotoxin when compared with those containing the control glycoproteins (Table I). The ^{86}Rb flux of both fractions was inhibited by tetrodotoxin.

In order to examine the possibility that, after swainsonine treatment, the Na^+ channel glycoprotein did not bind to WGA–Sepharose and was sequestered in the unbound fraction, the unbound [^{3}H]-glycoprotein fraction was also reconstituted and the ^{86}Rb flux in the vesicles was examined. Although the unbound glycoproteins from the swainsonine-treated cells were reconstituted into the artificial vesicles, no activity for the neurotoxin-responsive Na^+ channel was demonstrated (Table I).

TABLE II

CHARACTERIZATION OF [^{3}H]-GLYCOPEPTIDES EXTRACTED AND PARTIALLY PURIFIED FROM N-18 CELLS[a]

Immobilized lectin	*Swainsonine added (Percent of total radioactivity)*	
	None	*10μM*
Con A bound	8.2	41.6
unbound		
Lentil bound	53.1	28.6
unbound	38.7	29.8
L-PHA bound	24.0	9.5
unbound	14.7	20.3
Serotonin bound	82.0	65.0
unbound	18.0	35.0

[a]The Pronase-digested glycopeptides from swainsonine-treated or nontreated N-18 cells, metabolically labeled with [^{3}H]GlcN, were passed over Con A–Sepharose and the unbound glycopeptides flowed directly onto lentil–Sepharose as described[22]. Aliquots were removed to determine the radioactivity. The unbound fraction from lentil–Sepharose was passed over L-PHA and eluted with buffer[22]. The Con A and lentil-bound fractions were eluted with 0.01 and 0.2M methyl α-D-mannopyranoside. By use of another aliquot of the [^{3}H]-glycopeptides, the percentage that bound to serotonin–Sepharose was determined after being eluted with 0.1M Tris buffer[23]. The recoveries from all of the columns were 98–100%.

Altered glycosylation of glycoproteins after swainsonine treatment. — Extraction with 1% NP-40 of the glycoproteins from N-18 cells, which were treated or not treated with 10μM swainsonine, removed 38 and 43% of the radioactivity from the cells, respectively (Table I). When the NP-40 extracts were purified by chromatography on WGA–Sepharose, a higher percentage of radioactivity from the nontreated cells bound to WGA–Sepharose (11 as compared with 6%).

To show more definitely that the oligosaccharide chains of the N-18 glycoproteins were altered after treatment for 24 h with 10μM swainsonine, radioactively-labeled glycoproteins that bound to WGA–Sepharose and were eluted with 0.02M GlcNAc were examined. The glycoproteins were digested exhaustively with Pronase and, after removal of the ions on Bio-Gel P-2, were sequentially passed over Con A–Sepharose, lentil–Sepharose, and L-PHA–agarose. Under these conditions, biantennary glycopeptides are separated from core-, fucosylated-, triantennary glycopeptides and nonfucosylated-, tri- and tetra-antennary glycopeptides[22]. The results obtained for the [^{3}H]-glycopeptides from the swainsonine-treated and nontreated cells are given in Table II. Marked differences were noted in the affinity to all three lectins when the [^{3}H]-glycopeptides from the swainsonine-treated and nontreated cells were compared. The most pronounced difference was in binding of the [^{3}H]-glycopeptides from the swainsonine-treated cells to Con A–Sepharose as the percentage of radioactivity that bound was increased. That is, 42% of the [^{3}H]glucosamine-labeled glycopeptides bound, whereas only 8% of the control glycopeptides were bound (Table II). Since a hybrid oligosaccharide, which binds to immobilized Con A, has been reported by others[25,26], the increased binding suggested that similar oligosaccharides were formed in N-18 cells as a result of swainsonine treatment. The percentage of [^{3}H]-glycopeptides from the swainsonine-treated cells which bound to immobilized lentil and L-PHA (the separation columns which followed Con A–Sepharose) was correspondingly decreased. Only 29 and 10%, respectively, of the radioactively-labeled glycopeptides bound as compared with 53 and 24%, respectively, of the control glycopeptides (Table II).

A similar effect was noted when the glycopeptides were analyzed on a column of serotonin–Sepharose as 50% more radioactivity from the treated cells was unbound (Table II). Serotonin–Sepharose has been shown to bind glycopeptides containing sialic acid[23,24]. The charge classes, as separated on DEAE-cellulose, also differed; that is, the glycopeptides obtained from the nontreated cells had more charges. There was approximately a 70% increase in the noncharged and slightly charged glycopeptides after swainsonine treatment of the cells, supporting the observation of decreased binding of these glycopeptides to serotonin–Sepharose, when compared with those from the nontreated cells.

Size properties as analyzed on Bio-Gel P-10 revealed that the glycopeptides from the swainsonine-treated cells were smaller in size and appeared more heterogeneous than the glycopeptides from the nontreated cells.

By all of the aforementioned criteria, the glycopeptides of the swainsonine-treated cells were markedly different from those of the nontreated cells. Further,

TABLE III

PERTURBATION OF THE NEUROTOXIN-RESPONSIVE Na^+ CHANNEL[a] WITH VARIOUS AGENTS

Agent[b]	*Time (h)*	*Maximal effect*		*Half-maximal effect*	
		Concentration (M)	*Percent of control*	*Concentration* (M)	*Percent of control*
Swainsonine	24	5×10^{-5}	25	1×10^{-6}	63
Swainsonine	0.5[c]	4×10^{-5}	90		
Castanospermine	24	5×10^{-5}	44	2.6×10^{-6}	72
Monensin	24	1×10^{-6}	41	1.8×10^{-8}	71
Chloroquine	0.5[c]	4×10^{-4}	40	4×10^{-5}	70
Mevinolin	24	2.5×10^{-7}	38	4×10^{-6}	69
7-Ketocholesterol	24	7×10^{-7}	10	7×10^{-8}	45
Retinoic acid	96	1×10^{-6}	188		

[a]Measured by the efflux of ^{86}Rb in response to neurotoxins[18]. [b]Added to the cells for the designated time prior to the assay and again in the assay medium. [c]Added during assay 30 min prior to the addition of neurotoxins.

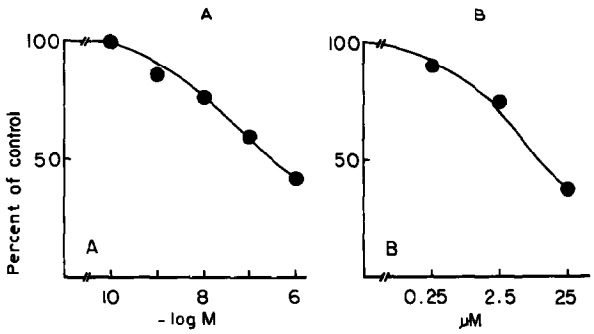


Fig. 2. In hibition of neurotoxin-stimulated ^{86}Rb efflux from mouse neuroblastoma cells N-18 by (A) monensin and (B) mevinolin. N-18 cells were grown for 4 days and fresh medium was added containing monensin or mevinolin in the concentrations designated in the graph. Control cells received fresh medium. Data as shown in Fig. 1 were used to calculate an efflux rate constant[18] for each culture treated with the agents and for the controls. The efflux rate constants are given as percentage of the control (100%).

the characteristics of the glycopeptides were similar to incompletely processed oligosaccharides found in other cell types as a result of swainsonine treatment (see Scheme 1).

Inhibition of ^{86}Rb efflux by other inhibitors of glycoprotein processing. — In order to determine whether other compounds that inhibit glycoprotein processing also affect the activity of the neurotoxin-responsive Na^+ channel, castanospermine and monensin were examined (Scheme 1). Castanospermine (0.5–50μM) inhibited the efflux of ^{86}Rb from N-18 cells in monolayer culture up to 54% after a 24-h treatment (Table III). The passive efflux of ^{86}Rb was not inhibited.

Monensin has also been shown to inhibit glycoprotein processing (Scheme 1) and secretion since it causes a major topographical effect on the Golgi apparatus[3,15].

Monensin (10^{-9} to 10^{-6}M) inhibited ^{86}Rb efflux in response to neurotoxins (Fig. 2A) when added to the cultures for 24 h. At concentrations greater than μM, monensin has been reported to completely disrupt the Golgi apparatus[15]. Monensin (1μM) had no effect on the passive-rate constant and, therefore, did not affect Na^+ channel activity as an ionophore.

Effect of inhibitors of endocytosis on ^{86}Rb efflux. — Monensin has been shown to inhibit endocytosis[3,27]. For this reason, several other known inhibitors of endocytosis were used to perturbate the neurotoxin-responsive Na^+ channel. Mevinolin[28] and 7-ketocholesterol[29] dramatically inhibited the neurotoxin-stimulated ^{86}Rb efflux when low amounts of the compounds were incubated with the cells for 24 h (Table III). Inhibition of the neurotoxin-stimulated efflux of ^{86}Rb with increasing concentration of mevinolin is shown in Fig. 2B and is expressed as percentage of control. 7-Ketocholesterol (0.7μM) inhibited channel activity (90%) after a 24-h

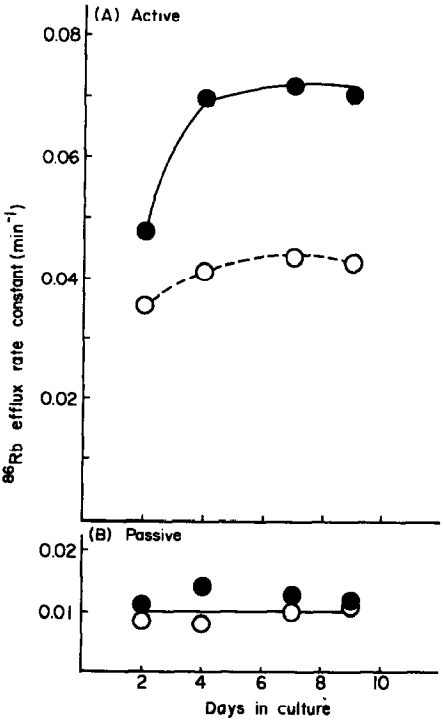


Fig. 3. Efflux rate constants of mouse neuroblastoma cells treated or not treated with retinoic acid. N-18 cells were grown for three days and μM retinoic acid was added in fresh medium to each of four cultures. Four control cultures received fresh medium only. Two days later, one treated and one control culture were assayed for the neurotoxin-stimulated efflux of ^{86}Rb, and the efflux rate constant was calculated by computer (See legend to Fig. 1). Fresh medium containing μM retinoic acid was added to the remaining cultures. A similar procedure was used 4, 7, and 9 days later. The neurotoxin stimulated (A, active) efflux rate constants of the retinoic acid treated (—●—●—) cells were higher than that of the nontreated (---O---O---) cells. The nonstimulated (B, passive) efflux rate constants did not change with this treatment.

treatment of N-18 cells and the inhibition was dependent on the concentration of 7-ketocholesterol.

Both mevinolin and 7-ketocholesterol have been shown to inhibit hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) and, thus, may exert their effect on receptor-mediated endocytosis by a number of pathways of which this enzyme is a key intermediate[30]. Therefore, another compound, chloroquine, which has been reported to inhibit endocytosis[31] by lowering the pH of lysosomes[32], was examined. Treatment of N-18 cells with 100μM chloroquine for 30 min prior to the measurement of the nonstimulated efflux brought about 60% inhibition of the neurotoxin-stimulated efflux of ^{86}Rb (Table III). These conditions have been shown to inhibit endocytosis in other cell types[16,31].

Effect of retinoic acid on ^{86}Rb efflux. — In contrast to other agents used in this study which inhibited glycoprotein processing, retinoic acid has been reported to increase the activity of sialyltransferases[33], enzymes which complete the synthesis of mature oligosaccharides and are found in the trans portion of the Golgi apparatus[34]. Retinoic acid, which has been reported to cause morphological[35] and Na^+ channel[36] differentiation of human neuroblastoma cells, brought about the morphological differentiation of the mouse N-18 cells after 48 h of treatment at a low concentration (1μM). At the same time, the activity of the neurotoxin-responsive Na^+ channel was also increased over that of the nontreated cells (Fig. 3). The enhanced activity was inhibited by μM tetrodotoxin, reducing the efflux rate constant to 0.01 min^{-1}, similar to that of the tetrodotoxin-inhibited, nontreated cells.

Summary of agents examined. — Table III summarizes the results of all the perturbating agents used on the cultures of N-18 cells and gives the concentration of each compound that caused half-maximal inhibition or, in the case of retinoic acid, stimulation. The action of all of the compounds on the activity of the neurotoxin-responsive Na^+ channel were concentration dependent. It should be noted that all of the agents in the concentrations used had no effect on the ability of N-18 cells to adhere to the culture dish since this is a requirement for the efflux assay[18]. In addition, the compounds had no apparent effect on the viability and growth of the N-18 cells in the amounts used.

DISCUSSION

It has been shown herein that mature, complex-oligosaccharide residues on glycoproteins are necessary for a Na^+ flux responsive to neurotoxins. To do this, the neurotoxin-responsive Na^+ channel of mouse neuroblastoma cells, N-18, was perturbated by agents, swainsonine, castanospermine, monensin, and retinoic acid, that affect the Golgi processing of glycoproteins (Scheme 1). Swainsonine[11–13] inhibited the activity of the neurotoxin-responsive Na^+ channel as measured by the efflux of ^{86}Rb from the cells in culture (Fig. 1). In further studies, a glycoprotein fraction that bound to WGA–Sepharose and derived from swainsonine-treated

neuroblastoma cells lost the activity of the neurotoxin-responsive Na^+ channel, as measured by ^{86}Rb flux in artificial phospholipid vesicles. Without swainsonine treatment, this fraction was highly active after reconstitution into artificial vesicles[5] (Table I). Moreover, after swainsonine treatment, reconstituted glycoproteins that were unbound to WGA–agarose had no activity (Table I). This later result eliminated the problem of fractionation differences between the treated and nontreated cells and showed that the activity was not sequestered elsewhere. It was then demonstrated that the oligosaccharide composition of the glycoproteins was altered, after swainsonine treatment (Table II), in a manner characteristic of hybrid oligosaccharides that have been shown in other cell types after swainsonine treatment[25,26].

The small amount of neurotoxin-responsive Na^+ channel activity that was observed after reconstitution of the glycoproteins from the swainsonine-treated cells may indicate that the formation of not all of the *N*-linked oligosaccharide residues was inhibited or that the completion of one branch of bi- or tri-antennary oligosaccharides is sufficient to bring about a low level of biological activity. Others have found that the secretion of such proteins as fibronectin[37] or α_1-antitrypsin[38] was not impaired by swainsonine treatment. However, the polypeptides contained a minimum number of oligosaccharide residues having at least one sialic acid residue. The observation that swainsonine inhibition of neurotoxin-responsive ^{86}Rb efflux was never greater than 75% in the cell-culture assay also suggested a "leakiness" of the swainsonine treatment. In addition, similarly to other cells[37], not all of the radioactive-labeled glycopeptides from the treated cells had characteristics that showed a change to hybrid oligosaccharide. It should be noted that higher concentrations of swainsonine were not used because of cell damage.

As shown in Table III, castanospermine also inhibited the neurotoxin-responsive Na^+ channel and, thus, provided supportive evidence for the swainsonine inhibition. Additional evidence was obtained by a similar inhibition of Na^+ channel activity with monensin and stimulation by retinoic acid. Monensin inhibited endocytosis in other cell types[39]. Since chloroquine, 7-ketocholesterol, and mevinolin were also effective in inhibiting ^{86}Rb efflux (Table III), it appears that agents which affect intracellular sorting and trafficking affect also the activity of the neurotoxin-responsive Na^+ channel. Interestingly, a report[40] that 25μM mevinolin caused the differentiation of neuroblastoma cells (N2-A) was not confirmed in these studies with N-18 cells, either by morphological examination or the presence of excitable membranes.

A number of mechanisms may be proposed for the action of these compounds (Table III) on the neurotoxin-responsive Na^+ channel; however, we favor the hypothesis that all of these agents affect glycosylation and, thus, owing to an improperly glycosylated glycoprotein, the ion flux is reduced. This was shown to be the case for swainsonine inhibition and, by analogy, the perturbating effects of castanospermine, monensin, and retinoic acid could be deduced. It remains to be demonstrated for the inhibitors of hydroxymethylglutaryl-CoA reductase and for

chloroquine. Regardless, we unequivocally demonstrated an inhibiting effect of aberrant glycosylation on the activity of the neurotoxin-responsive Na^+ channel in mouse neuroblastoma cells.

All characteristics of the glycopeptides that were examined indicated that less mature, *N*-linked oligosaccharides were formed by N-18 cells in the presence of swainsonine. The increase in binding to Con A was suggestive of an increase in mannosyl residues, which is characteristic of the hybrid oligosaccharides that are formed[25] (Scheme 1). The reduced binding to immobilized lentil and L-PHA (Fig. 2) were also supportive of perturbation at the (1→6)-α-D-mannosyl branch, since galactosylation of the branch is required for binding to these lectins[21,22]. Further support came from the observation of a decreased binding to serotonin–Sepharose (Fig. 2), which has been shown in other cell types to be decreased after swainsonine treatment[23].

Previous experiments had shown a decrease in neurotoxin-responsive ^{86}Rb efflux after neuraminidase treatment of the cells in culture[6] or of the highly purified glycoprotein incorporated into artificial phospholipid vesicles (unpublished observations). As sialic acid-containing glycopeptides bind to serotonin–Sepharose[23,24], thus, decreased binding after swainsonine treatment (Table II) denoted a lack of sialic acid residues. These observations, combined with the increase in activity in the presence of retinoic acid (Fig. 3), led to the suggestion that sialic acid is necessary for full expression of the active Na^+ channel. Lehninger[41] had suggested earlier that a charged molecule, such as sialic acid, could be responsible for the flow of ions through the membrane. We favor a structural feature requiring that sialylation must complete the *N*-linked oligosaccharides to provide a critical number of charges. It has been shown recently, with the use of human neuroblastoma–mouse fibroblast hybrids, that the genes responsible for the expression of Na^+ channel activity are located on more than one chromosome[42]. Therefore, it may be possible that sialyltransferase is a gene product whose activity controls the full biological function of the neurotoxin-responsive Na^+ channel.

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