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MECHANISM OF NEOMYCIN STIMULATION OF D-GLUCOSE UPTAKE IN RABBIT INTESTINAL BRUSH BORDER MEMBRANE

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In order to study the effect of the antibiotic neomycin on the intestinal epithelium, D-glucose was used as a probe molecule and its transport into rabbit brush border membrane vesicles was measured by a rapid filtration method. Treatment of the epithelium with neomycin sulfate prior to the preparation of the brush border membrane enhanced the D-glucose uptake, whereas neutral *N*-acetylated neomycin did not. This action of neomycin was related to its polycationic character and not to its bactericidal action. No significant difference could be demonstrated between the protein content or disaccharidase-specific activities of the brush border fractions from treated or non-treated intestines. Electrophoretic protein patterns of SDS-solubilized membrane were not significantly different after neomycin treatment. To gain more information on the mechanism involved in the stimulation of D-glucose transport, experiments were conducted on phosphatidyl glycerol artificial membranes and the results compared with those obtained with brush border membrane. At a concentration of 10^{-7} M, neomycin decreased the nonactin-induced K^+ conductance by a factor of approx. 100. The membrane conductance was linearly dependent on the neomycin concentration and the conductance in 10^{-2} M KCl was 10 times that in 10^{-3} M KCl. The valence of neomycin was estimated, from the slope of these curves, to be between 6 and 4. In contrast, acetylated neomycin had no effect on the nonactin-induced K^+ membrane conductance. Therefore, the effect of neomycin on artificial membrane is related to its 4 to 6 positive charges. It is proposed that the stimulation of sugar transport in brush border membrane is related to screening of the membrane negative charges by the positively-charged neomycin. Accumulation of anions at the membrane surface then occurs and their diffusion into the intravesicular space would increase the transmembrane potential which, in turn, stimulates the entry of D-glucose.

Introduction

Neomycin is a weakly-absorbed [1] broad-spectrum antibiotic of the aminoglycoside group, isolated from a strain of *Streptomyces fradiae* [2]. Its bactericidal power derives from the binding of the molecule to protein of the bacterial ribosome subunit 30S [3] which disturbs protein synthesis. The clinical usefulness of this antibiotic is however limited by its auditory toxicity and renal toxicity as well as some other toxic effects.

Oral administration of neomycin disturbs the absorptive and digestive processes in animals and man. Intestinal absorption of glucose in the rat is increased by either oral administration of neomycin or by its addition to the solution bathing the mucosal surface of everted intestinal sacs [4–7]. In vitro transport of glucose, galactose, arginine and histidine in mouse ileum is increased when neomycin (26 mg per day) is added to the drinking

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water of animals for two weeks prior to the transport experiment [8]. In man, oral neomycin administration causes a reversible malabsorption syndrome [9–11] with inhibition of sugar and amino acid absorption. Minor or nonsignificant morphological changes accompany the administration of neomycin [12–15]. Intestinal disaccharidase activity was found to be inhibited by neomycin [16,17] although the presence of nonhydrolyzed disaccharides cannot be demonstrated in the feces of patients treated with neomycin [18]. These effects of neomycin on sugar and amino acid absorption in man have been mainly attributed to a direct toxic effect on the intestinal epithelium.

Recently it has been observed that the intragastric administration of neomycin sulfate causes an increase in amino acid uptake, water permeability, and release of lipoproteins, by the intestinal epithelium [19]. These effects of neomycin appear to be produced at various sites of action on the enterocyte membrane (basolateral and brush border membranes) and are linked to the polycationic character of the antibiotic molecule [19].

In previous work we used this antibiotic to demonstrate that bacterial contamination, which alters D-glucose binding [20], was not responsible for the D-glucose uptake observed in experiments designed to characterize the D-glucose transport system in rabbit intestinal brush border membrane [21]. In the present work, effects on the brush border membrane were distinguished from those on the basolateral membrane or on cellular metabolism by using brush border membrane vesicle preparations. These were obtained rapidly in high yield and purity in the rabbit by a method similar to that reported in man [22]. D-Glucose transport experiments were performed with these membrane vesicles prepared from jejunal segments instilled with a neomycin sulfate solution. Neomycin treatment stimulated, rather than reduced, sugar uptake. In this paper the stimulation of D-glucose transport by neomycin was compared in biological membranes and artificial planar bilayer membranes using the approach reported by McLaughlin et al. [23]. We thought that this approach would increase our understanding of the basic mechanisms of action of neomycin in the intestine. A preliminary report of this work has already been presented [24].

Material and Methods

Chemical products. Reagents for polyacrylamide gel electrophoresis (acrylamide, *N,N'*-methylenebisacrylamide and *N,N,N',N'*-tetramethylethylenediamine) were purchased from Eastman Kodak Co. Tris(hydroxymethyl)aminomethane (Trizma base), ammonium persulfate, bovine serum albumin, horseradish peroxidase (type II), *o*-dianisidine dihydrochloride, Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), phloridzin, neomycin sulfate, and Triton X-100 (octyl phenoxy polyethoxyethanol) were supplied by Sigma. SDS (sodium dodecyl sulfate, purified for electrophoresis) was obtained from Bio-Rad, Coomassie brilliant blue R-250 from Schwarz-Mann, and glucose oxydase from Miles. KCl, NaSCN, toluene (liquid scintillation grade), and the ion exchanger (Rexin 201, hydroxyl form) came from Fisher Scientific Co. Bromophenol blue was purchased from Matheson, Coleman and Bell. Liquifluor, D-[1(n)-^{3}H]mannitol (22.2 Ci/mmol) and D-[U-^{14}C]glucose (239 mCi/mmol) were supplied by New England Nuclear. All other chemicals used were of the highest purity available commercially. For the experiments with artificial membranes, the KCl used (Fisher certified) was dissolved in deionized water without further purification. The nonactin generously supplied by Dr. Hans Bickel of CIBA, was used without purification to make a 10^{-4} M stock solution in absolute ethanol. Phosphatidyl glycerol was purchased from Supelco Inc.

Preparation of the N-acetylated neomycin derivative. The principles laid down by Rinehart et al. [25] for the preparation of *N*-acetylneomycins were followed. The neomycin sulfate salts were converted to free base by use of the anion exchange resin, Rexin 201 (hydroxyl form). Two grams of neomycin sulfate were dissolved in 100 ml of distilled water and the resultant solution passed over 60 ml of Rexin 201 and the column eluates were concentrated to dryness in vacuo. Neomycin free base was dissolved in absolute methanol; anhydrous acetic acid was added, and the resultant solution shaken for 24 h at room temperature. *N*-Acetylneomycin was precipitated from the solution by adding anhydrous ether, after which the precipitate was washed several times with small

aliquots of ether and finally dried in vacuo. The acetylated neomycin derivative thus obtained is a neutral molecule with no antibiotic properties. Before use, the precipitate was dissolved in a 0.154 M NaCl solution.

Preparaton of intestinal brush border membranes. Male New Zealand albino rabbits (4–7 kg), fed with Purina rabbit chow were used in all experiments. They were fasted overnight, and killed by cervical dislocation. The jejunum was rapidly removed and divided into equal segments (starting at the duodeno-jejunal flexure), which were randomly distributed to compensate for the oral-aboral gradients in the absorptive capacity of the intestine. They were rinsed with a cold 0.154 M sodium chloride solution, or a cold 0.154 M sodium chloride solution containing either 1% (w/v) neomycin sulfate or *N*-acetylated neomycin. Mannitol was used to adjust the osmolarity of the NaCl solution. Intestinal segments were gently blotted with absorbent paper and mesenteric fat removed. The intestinal tissue was immediately placed on a glass plate resting on crushed ice and opened longitudinally. The mucosa was gently scraped with a scalpel. Brush border membranes were pepared according to the method of Schmitz et al. [22], starting with a 2.5% (w/v) mucosa homogenate. The brush border fragments obtained were suspended in a pH 7.5 buffer containing 5 mM Tris-Hepes, 1 mM dithiotreitol, 0.1 mM $MgSO_4$, and 100 mM KCl. By successive passes through a syringe needle, the brush border membrane fragments acquired a vesicular form. Purity of the membranous fractions was checked routinely by assaying sucrase and alkaline phosphatase activity.

Assays. Protein determination was done by the method of Lowry et al. [26] using crystalline bovine serum albumin as a standard. Sucrase and lactase activities were assayed according to the method of Dahlqvist [27] as modified by Lloyd and Whelan [28]. Alkaline phosphatase activity was measured by the method of Eichholz [29] using *p*-nitrophenylphosphate as substrate.

Polyacrylamide gel electrophoresis. Brush border membrane proteins were separated by electrophoresis on polyacrylamide gel in the presence of SDS as previously described [30]. Electrophoresis was carried out at pH 9.5 using the buffer system described by Neville [31]; upper and lower gels were 3.2×6.25 and 8.2×1.2, respectively [32]. The gels loaded with 100–115 μg of protein were run at 1.5 mA per tube at room temperature for 90 min. After electrophoresis the gels were removed and fixed in 12.5% trichloroacetic acid for 1 h: protein was stained as previously described [33]. The relative densities of the various protein bands were estimated by scanning the gels at 530 nm using a Gilford 2400 spectrophotometer fitted with a 2410-S Linear Transport mechanism and a 6040 recorder (Gilford Instrument Laboratories Inc.). An auxiliary slit of 0.05 mm was always inserted.

Measurement of glucose transport. The kinetics of D-glucose transport and the initial transport rate were studied. Purified brush border membranes were suspended in a buffer containing 5 mM Tris-Hepes (pH 7.5), 1 mM dithiothreitol, 0.1 mM $MgSO_4$. Sugar transport was measured by adding an aliquot (50 μl) of the suspended membranes (4–8 mg of protein/ml) into appropriately constituted buffers (presence of 0.1 M KCl, 0.1 M NaSCN, or 0.1 M NaSCN and 0.1 mM phloridzin) containing 0.33 mM of D-[^{14}C]glucose in a final volume of 300 μl. The sugar uptake was measured at 22°C. For the kinetic study, aliquots (40 μl) were removed at intervals: for the study of the initial transport rate, a single aliquot (100 μl) was removed at 15 s. These aliquots were immediately diluted in 1 ml of cold stopping solution (0.154 M NaCl + 0.5 mM phloridzin at 4°C) containing tracer amounts of tritiated mannitol which served as extravesicular marker. The mixture was rapidly filtered through 0.22 μM millipore filters and the filters washed with 5 ml of the ice-cold stopping solution. The filters were removed to counting vials and the radioactivity measured in an Intertechnique SL4000 counter.

Artificial membranes. Artificial bilayer membranes were formed from a solution of phosphatidylglycerol in *n*-decane at a concentration of 25 mg/ml. Prior to preparing the decane solution, the lipid, which was in a chloroform-methanol solution, was washed in a diethyl ether-mild sulphuric acid two-phase mixture. The experimental apparatus and procedures used to obtain conductance measurements have already been described [34]. All experiments were conducted at 22.5 ± 0.5°C.

Results

Experiments on brush border membranes

In brush border membrane fragments representing 2.7 ± 0.2% (mean ± S.E.) of the protein of the homogenate, the specific activities of sucrase and alkaline phosphatase were increased approx. 13 times over that of the homogenate. Electron microscopic analysis of these preparations demonstrated the purity of the fraction and the vesicular form of the membranes thus allowing to pursue the in vitro sugar transport studies.

Controls

When brush border membrane vesicles were preincubated in a buffer containing 100 mM KCl and energized with a 100 mM NaSCN gradient (extravesicular > intravesicular at time 0), the D-glucose uptake showed a transitory sugar accumulation beyond the equilibrium value (at 30 min). The maximal uptake (0.65 nmoles of glucose per mg of protein) was reached at 60–90 s. The equilibrium uptake (0.1 nmol of glucose per mg of protein) was identical in the presence of sodium and potassium. A correctly-oriented KCl gradient cannot produce the overshoot of D-glucose uptake observed with NaSCN. Addition of 0.1 mM phloridzin to the incubation medium containing the 100 mM NaSCN gradient abolished the phenomenon of glucose accumulation beyond the equilibrium value, and more than 90% of the D-glucose uptake was inhibited at 15 s.

The amplitude of the Na^+-stimulated D-glucose transport was dependent on the anion accompanying the Na^+ in the gradient and the efficiency for thiocyanate (SCN^-), chloride (Cl^-) and sulfate (SO_4^{2-}) was $SCN^- > Cl^- > SO_4^{2-}$. Maximal uptake was 728.2 ± 123.1, 375.5 ± 43.9, 219.8 ± 8.7 pmol of glucose per mg of protein, respectively. When the salt gradient was dissipated (30 min) the transport reached equal levels for the three anions (approx. 60 pmol/mg of protein).

Effects of neomycin

When compared with saline treatment, perfusion of the intestine with 1% neomycin sulfate solution did not change significantly the protein content of the homogenate and of the brush border membrane vesicles (Table I). Protein recovery in the membrane fraction (BBM/H) was unaffected by the neomycin treatment (neomycin 3.0 ± 0.3%; saline 2.6 ± 0.2%; $P > 0.05$). Furthermore, the specific activities of sucrase and lactase in the homogenate and in the brush border membrane fraction of saline-treated and neomycin-treated intestine were not significantly different.

Sucrase and lactase purification factors (BBM/H) in the neomycin-treated intestine, which were 14.3 ± 1.3 and 7.7 ± 1.1, respectively, were not significantly different ($P > 0.05$) from those found in saline-treated intestine (12.9 ± 1.2 and 7.6 ± 0.8, respectively).

Brush border membrane electrophoretic patterns from intestine treated, or untreated, with

TABLE I

Protein content and disaccharidase activity of the homogenate (H) and the brush border membrane fraction (BBM) obtained from intestine treated with physiological saline and with saline + neomycin 1% (w/v). Protein content (± S.E.) expressed in mg of protein. Specific activities (± S.E.) of sucrase and lactase are expressed as the number of μmol of glucose detected per min per mg of protein. Number of different experiments in parenthesis.

		Protein	Sucrase	Lactase
Saline	H	324.1 ± 43.3 (9)	85.9 ± 12.7 (9)	3.7 ± 1.0 (5)
	BBM	8.3 ± 1.5 (9)	1 100.0 ± 166.9 (9)	28.1 ± 7.4 (5)
	BBM/H	2.6 ± 0.2 (9) [a]	12.9 ± 1.2 (9) [b]	7.6 ± 0.8 (5) [b]
Saline + neomycin	H	313.4 ± 24.8	86.7 ± 9.5 (9)	4.2 ± 1.2 (5)
	BBM	9.5 ± 1.4 (9)	1 243.9 ± 163.9 (9)	32.3 ± 10.9 (5)
	BBM/H	3.0 ± 0.3 (9)	14.3 ± 1.3 (9)	7.7 ± 1.1 (5)

[a] Protein recovery expressed in percentage.
[b] Purification factor for the enzymatic activities.

neomycin, showed minor changes in either the relative mobilities or the relative proportions of the different gel bands (Fig. 1).

In the presence of a 100 mM KCl gradient (medium > intravesicular) no significant difference in D-glucose transport was observed when brush border membranes from untreated and neomycin-treated intestines were compared (Table II). On the contrary, significant differences were observed in the presence of a 100 mM NaSCN gradient, the most significant occurring at 15 s ($P < 0.001$). These differences were abolished after adding 0.1 mM phloridzin.

In the presence of a 100 mM NaSCN gradient, the *N*-acetylated neomycin derivative, contrary to the neomycin base, did not stimulate the initial rate of D-glucose uptake (Table III). The effect of neomycin on D-glucose transport in the brush border membranes seems therefore to be linked to the charges on the molecules of this antibiotic.

Experiments on artificial bilayer membranes

The results obtained on brush border membranes indicate a strong influence of the anion on D-glucose uptake. Since neomycin is positively charged, while the membrane is negatively charged, a correlation between the neomycin-enhanced D-glucose transport and the increase in the local anion concentration in the vicinity of the membrane outer surface can be predicted.

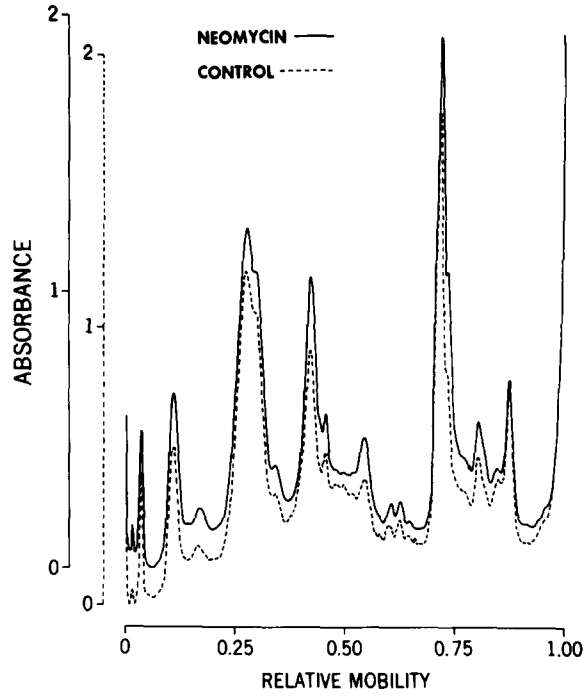

Fig. 1. Densitometric scanning at 530 nm of intestinal brush border membrane proteins stained with Coomassie brilliant blue R-250. Membrane vesicles from intestine treated with neomycin (————) or not treated (- - - - - -) were dissolved in SDS 1% (w/v) and submitted to electrophoresis. Mobility of proteins is relative to that of Bromophenol blue.

TABLE II

D-Glucose uptake as a function of three incubation times (15, 45 and 450 s) in the presence of different salt gradients (100 mM KCl, 100 mM NaSCN, and 100 mM NaSCN + 0.1 mM phloridzin). Values are the mean of six different experiments and were analysed by Student's *t*-tests for paired data. Uptake value expressed as picomol per mg of protein (± S.E.).

		D-Glucose uptake		
		15 s	45 s	450 s
Saline	KCl	19.8 ± 4.1	50.7 ± 10.8	60.6 ± 25.9
	NaSCN	398.7 ± 96.3	528.1 ± 101.9	182.2 ± 11.0
	NaSCN + phloridzin	47.0 ± 10.4	79.6 ± 13.2	152.2 ± 17.5
Saline + neomycin	KCl	16.6 ± 5.2	34.7 ± 14.7	40.4 ± 15.9
	NaSCN	507.8 ± 101.4 [a]	754.3 ± 124.3 [b]	248.8 ± 33.8 [c]
	NaSCN + phloridzin	48.6 ± 13.1	90.2 ± 20.6	202.6 ± 39.1

[a] $P < 0.001$.
[b] $P < 0.005$.
[c] $P < 0.05$.

TABLE III

Initial D-glucose uptake rate measured at 15 s for different salt gradients (100 mM KCl, 100 mM NaSCN and 100 mM NaSCN + 0.1 mM phloridzin) in brush border membrane vesicles obtained from intestines treated with saline, saline + neomycin (1%) and saline + *N*-acetylated neomycin (1%). Values are the mean ± S.E. of six different incubation media and were analysed by Student's *t*-tests. Initial uptake rate expressed as picomol per mg of protein per 15 s (± S.E.).

	Initial uptake rate		
	Saline	Saline + neomycin	Saline + *N*-acetylated neomycin
KCl	89.2 ± 33.1	53.0 ± 13.4	91.0 ± 52.2
NaSCN	789.9 ± 116.7	1318.9 ± 23.1 [a]	846.5 ± 24.6
NaSCN + phloridzin	95.9 ± 8.1	106.7 ± 13.1	80.9 ± 7.0

[a] $P < 0.01$.

Experiments were thus conducted on artificial bilayer membranes in which the membrane ionic conductance induced by the nonactin carrier was used as an indicator of the cation concentration at the membrane surface and/or of the state of the membrane structure.

When equilibrium prevails at the interfaces and there is negligible aqueous ion-carrier association (which can be assumed to be the case in our experimental conditions) the membrane conductance of monovalent permeant cations can be expressed [34] as:

$$G_o = \frac{F^2 Z^2}{RT} A^{\star} K k_s C_s C_i \exp \frac{(-F\psi)}{RT} \qquad (1)$$

where Z, F, R and T have conventional meanings ($RT/F = 25$ mV at room temperature); $A^{\star}$ represents the ion-carrier complex mobility in the membrane; K, the equilibrium constant for the reaction between ion and carrier; k_s, the carrier aqueous phase-membrane partition coefficient; C_s, the aqueous carrier concentration; C_i, the permeant ion concentration and ψ, the membrane surface potential.

It is the membrane surface potential (ψ) that interests us most in the presence of neomycin. Indeed, although in principle the parameters $A^{\star}$, K and k_s could change in the presence of neomycin, we believe these changes to be negligible, since we have verified, using relaxation techniques [35], that neomycin does not modify the kinetics of nonactin-induced transport, and that it does not alter significantly the conductance of neutral monooleine membranes.

Taking into account the relation between ψ, the surface charge (η), and the ionic composition of the aqueous solution, we show in the appendix, that for our experimental conditions:

$$\log G_o = A' - \frac{1}{Z_n} \log C_n$$

where

$$A' = \log\left(\frac{F^2 Z^2}{RT} A^{\star} K k_s C_s\right) - \frac{1}{Z_n} \log \frac{\eta^2}{A} + \log C_i$$

Experiments in which the membrane current, generated by a ±5 mV amplitude square wave, was recorded continuously, are illustrated in Fig. 2. Initially the current was very small when measured in a 10^{-3} M KCl aqueous solution without nonactin. Under these conditions in the absence of nonactin, neomycin does not alter the membrane conductance. After adding $5 \cdot 10^{-7}$ M nonactin, the current increased 300-fold and attained a steady level. The addition of neomycin, after this steady level was obtained, drastically reduced the membrane conductance (500-fold).

The membrane conductance, plotted as a function of neomycin-sulfate concentration (two lower curves) for two KCl concentrations (10^{-3} M and 10^{-2} M), is shown in Fig. 3. It can be observed that neomycin is extremely potent in reducing the nonactin-induced conductance. Indeed, at a concentration as low as 10^{-7} M it decreases the conductance by a factor close to 100 corresponding to a reduction of the surface potential of nearly 100

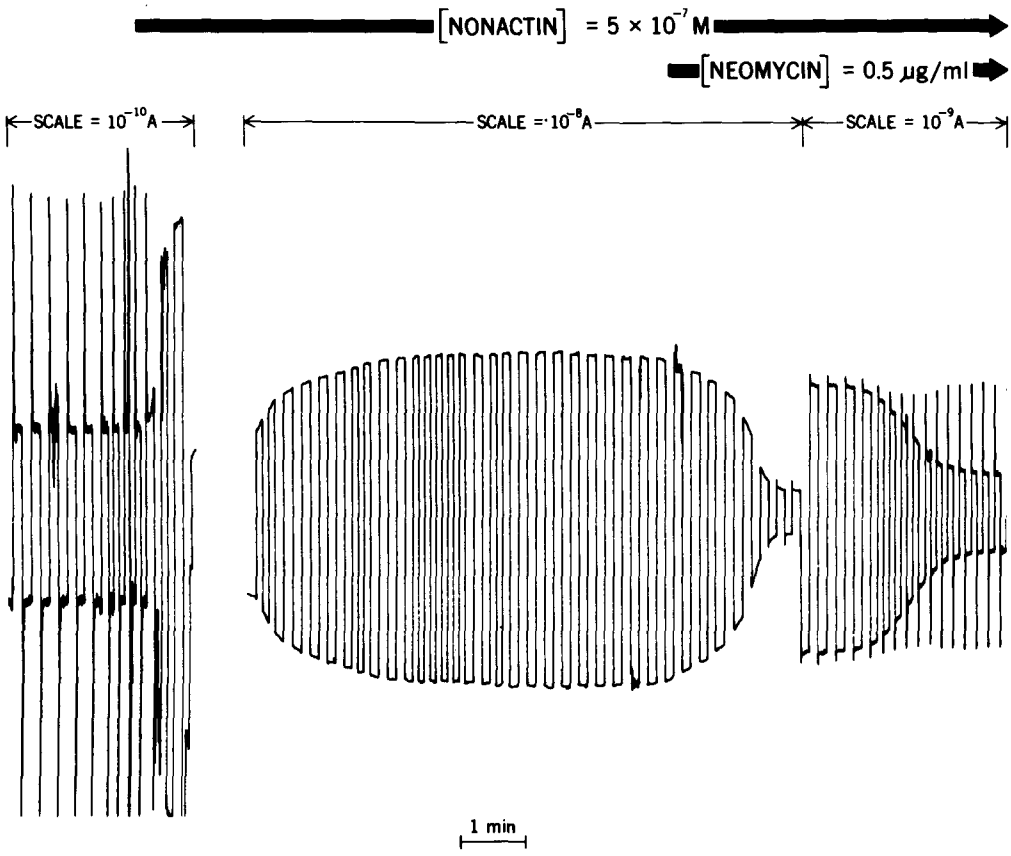

Fig. 2. Typical membrane ionic conductance recording showing the effect of a single dose of nonactin ($5.0 \cdot 10^{-7}$ M) and of neomycin (0.5 µg/ml).

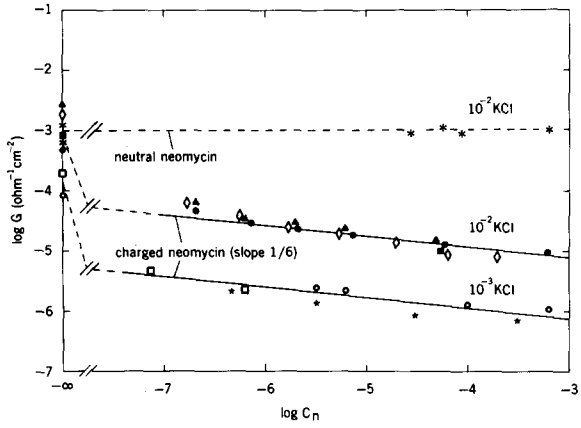

Fig. 3. Effect of neomycin sulfate (charged neomycin) and *N*-acetylated neomycin (neutral neomycin) on the nonactin-induced membrane conductance at two KCl concentrations. Membrane were formed with phosphatidylglycerol dissolved in decane (25 mg/ml). The concentration of nonactin was $5.0 \cdot 10^{-7}$ M.

mV. It can also be seen (Appendix) that the predictions of Eqns. 6 and 7 are verified because log G_o is linearly dependent on log C_n (Eqn. 6) and the conductance in 10^{-2} M KCl is 10-times that in 10^{-3} M KCl (Eqn. 7). Although the two straight lines in the lower part of Fig. 3 have a slope of 1/6, the variability is such that the actual slope could lie between 1/4 and 1/6. The dashed curve in Fig. 3 shows that acetylated neomycin, which is consequently neutral, has no effect on the nonactin-induced membrane conductance.

Discussion

When neomycin is administered orally or instilled in isolated intestinal segments the first stage of its action occurs at the enterocyte brush border membrane. It has been reported that in vitro

neomycin and phosphoinositides, particularly polyphosphoinositides form strong complexes that are not antagonized by Ca^{2+} [36]. Therefore Ca^{2+} used for the purification of intestinal brush border membrane [22] would not interfere with the formation of the complex. Phosphoinositides are relatively abundant in the small intestine [37] and could serve as a physiological receptor for the aminoglycosides. It has been suggested that they act in this way in kidney [38] and inner ear tissues [39], where neomycin has toxic effects and where phosphoinositides metabolism is important. It has been shown, that alkaline phosphatase is attached to membranes by a strong interaction with phosphatidylinositol [40] which is the site assumed to bind neomycin [36,37]. Since phosphatidylinositol represents 8.3% of total lipid phosphorus of rabbit brush border membrane [41], the possibility should be considered that displacement of alkaline phosphate occurred in brush border membranes exposed to neomycin. Alkaline phosphatase is released by phosphatidylinositol-specific phospholipase C, but is not released by high ionic strength, high and low pH, divalent cations (especially in presence of 100 mM $CaCl_2$) and phospholipase A_2 [40]. Brush border is rich in phospholipase A_2, whereas phosphoinositide inositol phosphohydrolase activity (phospholipase C) was localized in the supernatant fraction after subcellular fractionation of guinea-pig intestinal mucosal cell [42–44]. Therefore it is unlikely that alkaline phosphatase is released in brush border membranes prepared by Ca^{2+} precipitation, since the brush border membrane vesicles obtained are essentially free of contamination by non-brush border material [22]. Moreover, the relative mobility of alkaline phosphatase in brush border membrane electrophoretic patterns from intestine treated or untreated with neomycin was the same. Therefore, it is unlikely that displacement of alkaline phosphatase in the brush border membrane is at the root of the transport increase observed in membrane vesicles obtained from intestine exposed to neomycin.

Enterocyte microsomes, mitochondria and basolateral plasma membranes are aggregated by calcium while microvillus membranes are nearly unaffected, indicating that, despite the negatively-charged glycocalyx, the brush border membrane surface, at or near neutrality, is more positively charged than microsomal, mitochondrial and basolateral plasma membrane surfaces [22]. We elected to perfuse intestine with neomycin and then to isolate membranes to reproduce the conditions of oral neomycin administration rather than to look at membranes treated with neomycin. Therefore the brush border membrane surface charge may be already substantially neutralized by neomycin and the addition of calcium may not influence the degree of neutralization. The transport properties of brush border membrane vesicles isolated by calcium precipitation are essentially the same as those of brush border vesicles isolated by free-flow electrophoresis [45], indicating that the transport properties are not importantly affected by the calcium used in purification. In fact, during purification of rabbit brush border, Ca^{2+} is barely assayable in the homogenate, and after addition of 10 mM calcium (20 mequiv./l) and centrifugation, less than 0.45% of the added calcium is found in the brush border membrane fraction (0.89 mequiv./l, per mg of brush border protein) (Le Grimellec, C., personal communication).

Generally, the various neurotoxic effects of neomycin have been attributed to alterations of electrical events at the membrane level [46]. Based on the characteristics of the sugar transport system, we suggest that the stimulation of D-glucose uptake in brush border membrane vesicles is related to the electrical events at the membrane. Brush border membrane, like other cellular membranes, bears a net negative charge which generates a surface potential that attracts cations and repels anions, creating concentration gradients from the membrane-solution interface to the bulk phase. These local ion concentrations are important parameters that influence the kinetics of the Na^+-coupled D-glucose transport.

The results of the present work support the view that Na^+ and D-glucose are electrogenically co-transported across the brush border membrane. SCN^- has a greater conductivity through the brush border membrane than Cl^- and, when used in the salt gradient, produced a more negative intravesicular potential, resulting in an enhancement of the Na^+-coupled D-glucose transport. Since SO_4^{2-} is probably poorly conducted through the mem-

brane, it cannot elicit an overshoot phenomena identical to those of SCN^- or Cl^-. Furthermore, these results indicate that the transporter alone has no affinity for the anions, since the uptake levels determined at 30 min were equal, and thus point out the importance of the ionic concentration near the brush border membrane-solution interface in establishing the membrane potential. The local concentrations of cations and anions are, in turn, dependent on the surface potential of the membrane. Consequently, the enhancement of sugar transport by the polycationic neomycin may be related to the fact that anions have accumulated at the membrane interface and have neutralized the membrane negative charges. The diffusion of anions into the intravesicular space increases the membrane potential and stimulates the uptake of D-glucose. That neomycin acts on electrical phenomena and not on the passive permeability of the brush border membrane is supported by the fact that neomycin does not alter the K^+-stimulated D-glucose uptake. The fact that bivalent cations stimulate the Na^+-dependent phosphate uptake by an effect on the surface potential [47] also supports our interpretation of D-glucose stimulation uptake by neomycin. In those experiments, the apparent affinity of phosphate for the transport system was increased, but the Na^+ affinity was decreased. Similarly screening of the membrane negative charges by neomycin, in our system, should decrease the Na^+ concentration near the membrane. The fact that D-glucose uptake is increased means that the driving force is mainly due to the membrane potential (the concentration of the ions accompanying the Na^+) and not the concentration of Na^+ itself. Stimulation of glucose transport by oral administration of neomycin, or by its addition to the solution bathing the mucosal surface of everted intestinal sacs, was observed in the rat and the mouse [4–7], but not in man [9–11] although, the mechanism of this stimulation was not studied. The discrepancy between the results obtained in animals and man may be explained by variations in the doses of neomycin and the experimental procedure used to measure glucose transport.

The results of this work clearly show that intestinal disaccharidase activity is not inhibited by a single dose of 1% neomycin in a saline vehicle administered into the lumen of the intestine. Furthermore, the comparison of the membrane electrophoretic patterns from treated and untreated intestine show that the membrane composition is not altered by neomycin treatment. It is not possible to demonstrate significant changes in the amount of actin-like protein (band with a relative mobility of 0.72) associated with brush border membrane by SDS-dissociation of membrane components, followed by electrophoresis. However, neomycin, in vitro, has been reported [48] to be the most efficient aminoglycoside antibiotic polymerizer of skeletal muscle actin. More precise studies are needed to investigate the interaction of neomycin on intestinal brush border mechanism and to determine their biological significance.

Appendix

The relation between ψ, the surface charge (η) and the ionic composition of the aqueous solution can be summarized in an equation derived by Grahame [49] in his treatment of the diffuse double layer theory:

$$\eta^2 = A \sum C_i (e^{-Z_i F\psi/RT} - 1) \tag{2}$$

where η is the surface charge (assumed constant), A is a constant and Z_i, the valence of the various ion species in the aqueous solution. Under our experimental conditions, KCl and neomycin sulfate are in aqueous solutions, so that the cations are K (valence + 1) and neomycin (n, valence Z_n) and the anions are Cl (valence − 1) and SO_4 (valence − 2), so that Eqn. 2 reads:

$$\eta^2 = A\left[C_n(e^{-Z_n F\psi/RT} - 1) + C_K(e^{-F\psi/RT} - 1) + C_{Cl}(e^{F\psi/RT} - 1) + C_{SO_4}(e^{2F\psi/RT} - 1)\right] \tag{3}$$

Since the surface charge of our phosphatidyl glycerol is very high (about 0.32 C/m^2, McLaughlin et al. [23]), and the total ionic strength is always low in our experiments, we expect $F\psi/RT$ to be negative and $F\psi/RT \gg 1$; under such conditions, we can neglect completely the two last terms in parenthesis. In addition, due to the expected polycationic nature of neomycin ($Z_n > 1$) and the high value of ψ, the second term in parenthesis will

always be smaller than the first. Accordingly, Eqn. 3 can be approximated under such conditions by:

$$\eta^2 \simeq AC_n \exp(-Z_n F\psi/RT) \quad (4)$$

or

$$-\frac{F\psi}{RT} = -\frac{1}{Z_n}\left(nC_n - \ln(\eta^2/A)\right) \quad (5)$$

If we now replace $-F\psi/RT$ of Eqn. 1 by its expression given in Eqn. 5 and taking the logarithm base 10, we find:

$$\log G_o = A' - \frac{1}{Z_n} \log C_n \quad (6)$$

where

$$A' = \log\left(\frac{F^2Z^2}{RT} A^* K k_s C_s\right) + \frac{1}{Z_n} \log\frac{\eta^2}{A} + \log C_i \quad (7)$$

is a constant for a given C_i concentration. We see that for a given KCl concentration (C_i), the membrane conductance should decrease with increasing neomycin concentration. In addition, the slope of the curve of log G_o vs. log C_n gives $1/Z_n$, i.e. the inverse of the valence of neomycin. Therefore, we have in principle a way of assessing the charge of a neomycin molecule.

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