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Induction and inhibition of pinocytosis by aminoglycoside antibiotics

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1 We investigated whether differences in induction or stimulation of pinocytosis by six aminoglycosides reflected reported differences in their nephrotoxicity. Pinocytosis induced by antibiotics, Na^+, K^+ or Ca^{2+} was quantified by the number of pinocytotic channels in *Amoeba proteus*, a cell suitable for the study of the pinocytotic process.

2 The aminoglycosides were potent inducers of pinocytosis. They were effective in the order of their cationic charge: neomycin > gentamicin > netilmicin = tobramycin > kanamycin > streptomycin. Factors which reduced the charge of the molecules, i.e. alkaline pH and combination with carbenicillin or heparin, diminished pinocytosis.

3 Like La^{3+} the antibiotics inhibited Na^+-induced pinocytosis. The order of efficacy was netilmicin > gentamicin > neomycin. A similar rank order, which is the reverse of the order of nephrotoxicity, was observed for inhibition of Ca^{2+}-stimulated, Na^+-induced pinocytosis. Netilmicin was also the most potent inhibitor of the Ca^{2+}-induced pinocytosis in cells treated with concanavalin A. Inhibition of Ca^{2+}-stimulated pinocytosis by netilmicin was reversed by Ca^{2+}, the calcium ionophore A 23187, or 4-aminopyridine.

4 We have shown that several nephrotoxic cations are strong inducers of pinocytosis in the amoeba, that aminoglycosides in Ringer solution induce pinocytosis in the approximate order of their nephrotoxicity and that factors which are known to diminish toxicity reduce pinocytosis. It, therefore, appears that the mechanism of aminoglycoside nephrotoxicity is related to their ability to induce pinocytosis in the amoeba. Low inducing potency and strong Ca^{2+}-antagonism, as for netilmicin, are qualities which may reduce the tendency of polycationic compounds to damage proximal tubular cells.

Introduction

Aminoglycosides are known to be nephrotoxic in man and experimental animals. There is no simple explanation for the mechanism of this side effect, but the first step in the pathogenesis is generally thought to involve the transport of these drugs into proximal tubular cells (Kaloyanides & Pastoriza-Munoz, 1980). Polycations as a group are recognized as potential nephrotoxins (Simmons *et al.*, 1981; Whelton & Solez, 1982) and the cationic charges are essential for the development of the renal lesions (Luft *et al.*, 1975). The degree of nephrotoxicity differs among the aminoglycosides and with a few exceptions it correlates well with their net cationic charge (Josepowitz *et al.*, 1982). These agents bind to anionic sites of the brush border membrane (Just & Habermann, 1977) and become interiorized probably by pinocytosis (Silverblatt & Kuhn, 1979). Aminoglycosides may enter tubular cells through the basolateral membrane (Bennett *et al.*, 1982) but luminal absorption predominates under circumstances of normal glomerular filtration. The drugs accumulate in the lysosomes (Morin *et al.*, 1980), inhibit phospholipases, and so cause overloading of phospholipids (Aubert-Tulkens *et al.*, 1979) and inhibition of several metabolic pathways (Lipsky & Lietman, 1982; Sastrasinh *et al.*, 1982a). It is not known whether these antibiotics also affect the onset of the pinocytotic process. Interference with induction of pinocytosis would influence the subsequent steps of pathogenesis of nephrotoxicity.

We have therefore used *Amoeba proteus* as a model cell to investigate the effect of aminoglycosides on the first step of events in the mechanism of pinocytosis. We have obtained quantitative infor-

mation on the induction-potency of six aminoglycosides and their effects on different types of cation-induced pinocytosis. Some of the results have been reported previously in a brief form (Johansson *et al.*, 1982a).

Methods

The experiments were carried out with *Tetrahymena*-fed *Amoeba proteus*, Bristol strain. They were cultured in Pringsheim's medium according to conditions described previously (Josefsson, 1975). The cells were starved for 3 days and carefully washed in Pringsheim's medium. They were transferred to Chalkley's solution the day before the experiment. Intensity of pinocytosis was measured using a modification of the technique described by Josefsson (1975). The cells were allowed to adhere to microscope slides in moist chambers for 20 min before induction of pinocytosis. The medium was replaced by 2.5 ml of the inducing solution and a coverslip was applied. The number of channels per amoeba was determined continuously during the first 20 min after application of the inducer. Every minute the channels were counted from 3 new amoebae. The mean number of channels per cell during this period was used as an index of the pinocytotic activity.

Pringsheim's medium has the following composition (mM): Na^+ 0.22, K^+ 0.35, Ca^{2+} 0.85, Mg^{2+} 0.08, $HPO_4^{2-} + H_2PO_4^-$ 0.11, Cl^- 0.35, NO_3^- 1.7, SO_4^{2-} 0.08 at pH 7.0. As a low Ca^{2+} medium suitable as a vehicle for inducers of pinocytosis we used Chalkley's solution (mM): Na^+ 1.43, K^+ 0.027, Ca^{2+} 0.007, $HPO_4^{2-} + H_2PO_4^-$ 0.014, Cl^- 1.4, HCO_3^- 0.047 at pH 6.9. In some experiments the aminoglycosides were applied to the cells in Ringer solution (mM): Na^+ 147, K^+ 4, Ca^{2+} 1.5, Cl^- 154 at pH 6.8. The drugs were dissolved in Chalkely's solution, or in some experiments in Ringer solution. The pH was adjusted to 6.9 with NaOH unless stated otherwise. A stock solution of 10 mg ml^{-1} of the calcium ionophore A 23187 dissolved in DMSO was used.

EGTA (ethylenglycol-bis(β-aminoethylether)-N_1 N'tetraacetic acid) was purchased from Hopkins and Williams, A 23187 from Upjohn Co., Kalamazoo, Mi, U.S.A., lysozyme and 4-aminopyridine (4-AP) from Sigma Ltd, St Louis and concanavalin A (Con A) from Miles-Yeda Ltd, Rehovot, Israel.

Drugs were gifts from the following firms: gentamicin sulphate and netilmicin sulphate from Schering Corp. Bloomfield, NJ, U.S.A.; tobramycin and cefalothin from Lilly and Co., Indianapolis, In., U.S.A.; neomycin sulphate from Upjohn Co., Kalamazoo, Mi, U.S.A.; kanamycin sulphate from Ferrosan, Malmö, Sweden; polymyxin B sulphate, Novo, Copenhagen, Denmark; cephalexin and cephaloridine from Glaxo, Ulverston, Cumbria; protamine sulphate, streptomycin sulphate and heparin from Kabi, Stockholm, Sweden; and carbenicillin from Astra, Södertälje, Sweden.

Results

Induction of pinocytosis

The aminoglycosides induced a dose-related pinocytotic response in *Amoeba proteus*. Within 2 min thin pinocytotic channels similar to those evoked by inorganic polycations like La^{3+} and lysozyme appeared in the periphery of the cells.

The rank order of inducing potency among the aminoglycosides applied to cells in Chalkley's medium (Figure 1) was: neomycin > gentamicin > netilmicin = tobramycin > kanamycin > streptomycin. The aminoglycoside antibiotics were among the most effective inducers. Thus, neomycin was more potent than protamine, spermine and uranyl ions, and was equipotent to aprotinin, lysozyme and polymyxin B (Table 1). The time course of the pinocytotic cycles induced by these polycations was similar. Channel formation started within one minute after application of the drug and no channels were formed after 30 min. Channel formation was very weak in alkaline media (Figure 2a), indicating that decreased ionization of amino groups reduced interaction with acidic groups in the amoeba membrane. Drugs which interact with the amino groups of the antibiotics also reduced pinocytosis. The polyanion heparin moved the dose-pinocytosis curves to the right and increased their slopes (Figure 2b). The shifts of the curves varied with different aminoglycosides but their slopes always became steeper with increasing concentration of the anion. This dose-

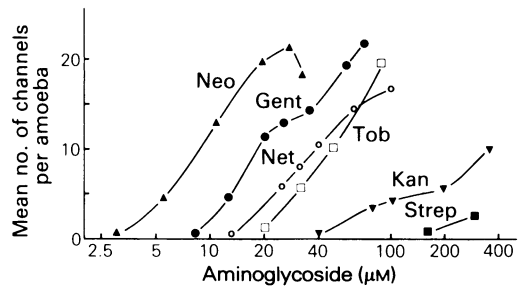

Figure 1 Pinocytosis induced by six aminoglycoside antibiotics: Neomycin (triangles), gentamicin (closed circles), netilmicin (open circles), tobramycin (open squares), kanamycin (triangles, base up), streptomycin (closed squares). All drugs were dissolved in Chalkley's solution at pH 6.9 in this and the following experiments unless otherwise stated.

Table 1 Threshold concentrations for cations inducing pinocytosis in *Amoeba proteus* in Chalkley's medium pH 6.7–6.9

Inducer	*Concentration* (mol l^{-1})	*Inducer*	*Concentration* (mol l^{-1})
Polymyxin B	0.5×10^{-6}	Protamine	5×10^{-6}
Lysozyme	1×10^{-6}	UO^{2+}	10×10^{-6}
Aprotinin	1×10^{-6}	Spermine	20×10^{-6}
Neomycin	2×10^{-6}	K^+	3×10^{-3}

The concentrations which elicit the formation of 2 channels per amoeba during a 20 min pinocytotic cycle are shown.

effect relationship is typical for an interaction between compounds by chemical antagonism (Ariens *et al.*, 1956) and supports the concept that heparin inhibits pinocytosis by forming a complex with the aminoglycosides.

It is known that penicillins may inactivate aminoglycosides by forming conjugates with their amino groups. We investigated carbenicillin, which inhibited pinocytosis induced by the aminoglycosides but not that induced by other cations (Na^+, K^+, lysozyme). The effect of the interaction between 1 mg ml^{-1} carbenicillin and neomycin is shown in Figure 2b. The cephalosporins did not inhibit pinocytosis; instead they stimulated aminoglycoside-induced pinocytosis. Figure 2b shows stimulation of neomycin-induced pinocytosis by cephalexin 80 μg ml^{-1}. Similar results were obtained with cephalothin and cephaloridine and with other cations (Na^+, K^+, spermine, lysozyme) as inducers. The effect of the cephalosporins was abolished by the calcium-binding agent EGTA (data not shown) and so they may enhance cation induced pinocytosis by reducing the concentration of Ca^{2+} at the cell surface.

Effects of Ca^{2+} and Na^+ on aminoglycoside-induced pinocytosis

Induction by aminoglycosides was inhibited by Ca^{2+} and enhanced by calcium binding agents. This is illustrated for netilmicin in Figure 2c where induction of pinocytosis was inhibited by 20 μM and 100 μM $CaCl_2$ while addition of 20 μM EGTA to the inducing solution facilitated pinocytosis induced by netilmicin. Higher concentrations of EGTA (100 μM not shown) diminished the intensity of pinocytosis indicating that a minimal calcium concentration was required for channel formation.

Pinocytosis induced by aminoglycosides was less sensitive to variations in the extracellular calcium concentration than pinocytosis induced by monoval-

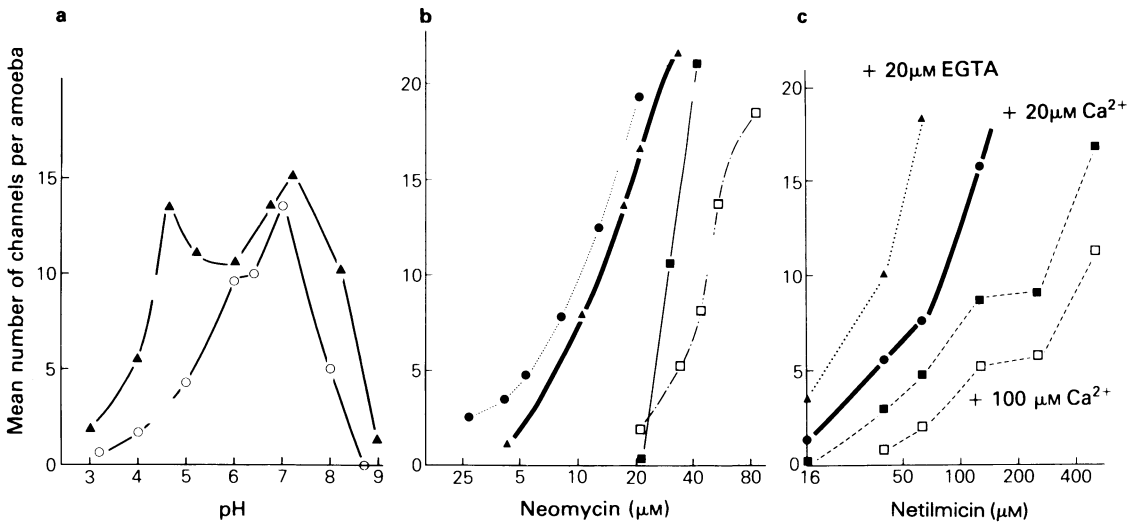

Figure 2 (a) Effect of pH on aminoglycoside-induced pinocytosis. Pinocytosis was induced by 16 μM neomycin (triangles) and 160 μM netilmicin (circles) at varying pH. All six aminoglycosides had pH optimum at about 7. Neomycin and gentamicin (not shown) had an additional optimum at acid pH. (b) Effect of heparin (25 μg ml^{-1}, closed squares), carbenicillin (1 mg ml^{-1}, open squares) and cephalexin (80 mg ml^{-1}, closed circles) on pinocytosis induced by neomycin (triangles). Note the steep dose-response curve in the presence of heparin. (c) Dose-response-curves for netilmicin-induced pinocytosis in the presence of Ca^{2+} and the calcium chelating agent Na_2EGTA. The salts were dissolved in distilled water and pH adjusted to 6.5 with NaOH. Control (closed circles), plus Ca^{2+} 20 μM (closed squares), 100 μM (open squares), EGTA 20 μM (triangles).

Table 2 Effect of $CaCl_2$ 100 μM on pinocytosis induced by four different aminoglycosides at 65 and 200 μM in Chalkley's solution, pH 6.8

Aminoglycoside	% pinocytotic activity 65 μM	200 μM
Neomycin	66 ± 6	95 ± 17
Gentamicin	42 ± 2	45 ± 7
Netilmicin	17 ± 4	27 ± 5

The pinocytotic activity is given as % of controls where no calcium was added. Mean ± s.e. of five experiments with each drug are shown.

ent cations. In both this respect and in its inhibition by Na^+ it resembled pinocytosis induced by La^{3+} (Josefsson & Hansson, 1976). Thus, the intense pinocytotic activity exhibited by cells in media containing 160 μM netilmicin was reduced by 90% in the presence of 16 mM NaCl. The same concentration of Na^+ inhibited pinocytosis induced by gentamicin (160 μM) and neomycin (16 μM) by 40 and 20%, respectively. Like Na^+, Ca^{2+} was more effective in reducing pinocytosis induced by netilmicin than that induced by gentamicin or neomycin (Table 2).

Inhibition of Na^+-induced pinocytosis

Na^+-induced pinocytosis, which is distinguished by its high sensitivity to Ca^{2+}, La^{3+} and EGTA (Josefsson, 1975) was inhibited by the aminoglycosides (Figure 3a). The inhibition was observed at any inducing concentration of Na^+ and especially at 50–150 mM. The efficacy of inhibition varied among the drugs irrespective of their cationic charge. Netilmicin was the most potent inhibitor followed by gentamicin and neomycin (Figure 3a). Although pinocytosis induced by Na^+ is inhibited by extracellular Ca^{2+} in a competitive manner, there is a narrow range, 20–40 μM, of Ca^{2+}-concentrations which stimulate pinocytosis (see Josefsson, 1975 and Figure 3b). The aminoglycosides also reduced this kind of pinocytosis in a similar rank order. Ca^{2+}-stimulated, Na^+-induced pinocytosis is probably caused by the influx of Ca^{2+}. This is suggested from the fact that low concentrations of the calcium entry blockers verapamil, methoxyverapamil and nifedipine preferentially block this kind of Na^+-induced pinocytosis. In the presence of these drugs or netilmicin the sensitivity of the amoeba to Ca^{2+} was reduced, so that the maximum number of channels occurred at higher concentrations of Ca^{2+} (Figure 3b). The potency of

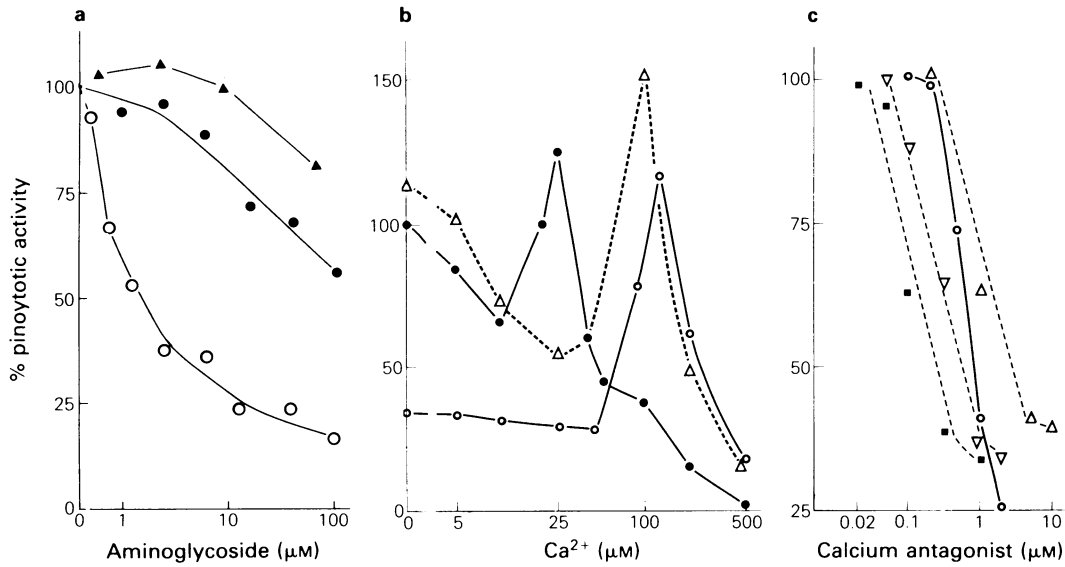

Figure 3 (a) Inhibition of Na^+-induced pinocytosis by neomycin (triangles), gentamicin (closed circles) and netilmicin (open circles). The inducing solution was 100 mM NaCl at pH 6.9. Pinocytotic activity is given as % of controls without aminoglycosides. (b) Inhibition of Ca^{2+}-stimulated Na^+-induced pinocytosis by 1 μM verapamil (open triangles) and 3.2 μM netilmicin (open circles). Pinocytosis was induced by 100 mM NaCl in distilled water at pH 6.5 and by this solution containing 5–500 μM $CaCl_2$ (closed circles). Pinocytotic activity is given as % of controls induced by 100 mM NaCl. (c) Calcium antagonistic drugs inhibiting pinocytosis induced by 100 mM NaCl plus 25 μM $CaCl_2$ in distilled water at pH 6.5. The following drugs were added to the inducer: nifedipine (squares), methoxyverapamil (triangles, base up), netilmicin (circles), verapamil (triangles, base down). Pinocytotic activity is given as % of controls.

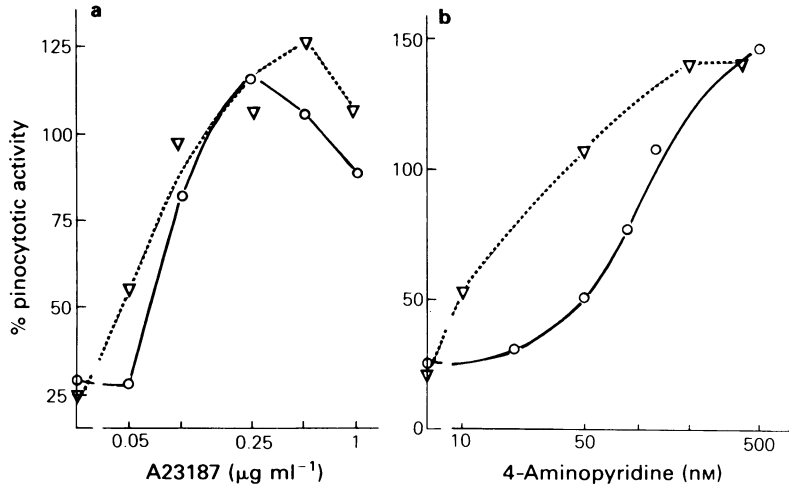

Figure 4 Inhibition of pinocytosis by 2 μM methoxyverapamil (triangles) and 3.2 μM netilmicin (circles) and its reversal by (a) the calcium ionophore A 23187 and (b) 4-aminopyridine. The inducer was 100 mM NaCl plus 25 μM Ca^{2+} pH 6.5 dissolved in distilled water. The vehicle of the ionophore DMSO, dilution factor 1:10000, had no effect.

netilmicin as an inhibitor of Ca^{2+}-stimulated pinocytosis was intermediate between that of verapamil and methoxyverapamil (Figure 3c) but thirty times more effective than neomycin (not shown). Separate experiments with combinations of methoxyverapamil and netilmicin, not reported here, revealed that these drugs were synergic inhibitors indicating different modes of action on pinocytosis.

The calcium ionophore A 23187 and 4-aminopyridine which enhance calcium entry in nerve terminals (Thesleff, 1980) increase the capacity for Na^+-induced pinocytosis in amoebae inactivated by the Ca^+-binding agent EGTA (Johansson & Josefsson, unpublished). These compounds also reversed blockade of pinocytosis caused by the calcium entry blockers or netilmicin. As shown in Figure 4a and b, normal pinocytotic activity of cells in the presence of 2 μM methoxyverapamil, or 4.2 μM netilimicin was restored in the presence of either 0.25 μg ml^{-1} of the ionophore or 50–100 nM 4-aminopyridine.

Table 3 Effects of aminoglycosides on calcium-induced pinocytosis in cells treated for 1 min in Chalkley's solution containing 30 μg ml^{-1} concanavalin A

Inducer	*Aminoglycoside*	*% pinocytotic activity*
$CaCl_2$ 10 mM	Neomycin	78 ± 12
$CaCl_2$ 10 mM	Gentamicin	75 ± 6
$CaCl_2$ 10 mM	Netilmicin	44 ± 6

20 μM of the aminoglycosides were added to the inducing solution (10 mM $CaCl_2$ in Chalkley's solution pH 6.8). Mean ± s.e. of five experiments with each drug are shown. Controls = 100%.

Inhibition of Ca^{2+}-induced pinocytosis in concanavalin A treated cells

The interaction between the aminoglycosides and Ca^{2+} was further examined in cells capable of Ca^{2+}-induced pinocytosis. Ca^{2+} is a very weak inducer, but a potent inhibitor of cation-induced pinocytosis in the amoeba. However, treatment with concanavalin A (30 μg ml^{-1} for 1 min) alters, their response to Ca^{2+} completely (Johansson *et al.*, 1982b). $CaCl_2$ (1–10 mM) no longer inhibits pinocytosis but induces a dose-related pinocytotic cycle of an intensity and duration comparable to that induced by optimal concentrations of NaCl and KCl. Such Ca^{2+}-induced pinocytosis was reduced by the aminoglycoside antibiotics, of which netilmicin was the most potent inhibitor (Table 3).

Induction of pinocytosis in Ringer solution by aminoglycosides

It is evident from the preceding results that interaction between the aminoglycosides, Na^+, and Ca^{2+} reduced not only the pinocytosis induced or stimulated by the inorganic cations but also the pinocytosis induced by the antibiotics, especially by netilmicin. This was further illustrated in experiments where amoebae in a Ringer solution were used for the study of pinocytosis. In this medium, where the concentrations of inducing (Na^+) and blocking ions (Ca^{2+})

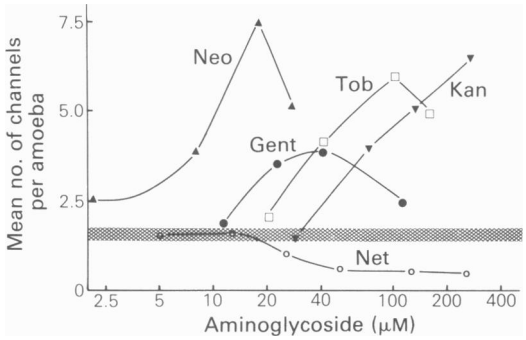

Figure 5 Pinocytosis induced by aminoglycosides dissolved in Ringer solution at pH 6.8. The horizontal bar indicates pinocytotic activity induced by Ringer solution. The following aminoglycosides were investigated: neomycin (triangles), gentamicin (closed circles), tobramycin (squares), kanamycin (triangles, base up) and netilmicin (open circles).

balanced each other, no pinocytosis was induced by netilmicin (Figure 5). Instead, the low pinocytotic activity elicited by Ringer solution was further depressed by the drug. The other aminoglycosides induced pinocytosis of moderate intensity in the following rank order: neomycin > gentamicin > tobramycin > kanamycin. In comparison to their pinocytotic activity in Chalkley's medium the efficacy of kanamycin was increased and the response to high concentrations of gentamicin was reduced in Ringer solution (compare Figures 1 and 5). The cells were not damaged by these media so the weak pinocytotic activity and the negative slopes of the dose-response curves of the aminoglycosides reflect interaction with the mechanisms which regulate the intensity of pinocytosis.

Discussion

We have found that aminoglycosides are potent inducers of pinocytosis in the amoeba. Like other inducing cations they presumably start formation of pinocytotic channels by displacing Ca^{2+} from 'stabilizing' sites in the membrane and so trigger a membrane reaction which increases influx and release of Ca^{2+} into a contractile matrix beneath the membrane (Josefsson, 1975; Klein & Stockem, 1979; Prusch & Hannafin; 1979). At pH 6.9 in Chalkley's solution they induced pinocytosis in the following order: neomycin > gentamicin > netilmicin = tobramycin > kanamycin > streptomycin. This ranking approximates the order of their net cationic charge (cf. Josepowitz *et al.*, 1982). Reduced protonation of the amino groups at alkaline pH or chemical reactions at these sites with heparin and carbenicillin reduced pinocytosis. Compared to pinocytosis induced by monovalent cations that induced by the aminoglycosides was less inhibited by Ca^{2+}. In this respect and in their inhibition of Na^+-induced pinocytosis the antibiotics resembled La^{3+} (Josefsson & Hansson, 1976). Furthermore, pinocytosis induced by the antibiotics, like that induced by La^{3+}, was inhibited by Na^+. These cations, Na^+, La^{3+} and the aminoglycosides may therefore induce pinocytosis by displacing Ca^{2+} from the same anionic sites in the membrane.

Interaction between the aminoglycosides, Na^+, and Ca^{2+} was particularly obvious with netilmicin. This drug was an effective inducer in the absence of these cations, but traces of Ca^{2+} and low concentrations of Na^+ inhibited pinocytosis. Furthermore, netilmicin was a potent inhibitor of the kinds of pinocytosis which are induced or stimulated by Ca^{2+} and Na^+. This should explain why netilmicin, in contrast to the other aminoglycosides, reduced the pinocytotic activity in amoeba immersed in mammalian physiological salt solutions. The order of induction-potency in Ringer solution was neomycin > gentamicin > tobramycin > kanamycin. Netilmicin had no inducing effect. Instead it reduced the weak Na^+-induced pinocytosis caused by the Ringer solution.

The anomalous behaviour of netilmicin on pinocytosis in Ringer solution is interesting for two reasons. Firstly, studies in animals and humans indicate that netilmicin is less nephrotoxic than gentamicin, kanamycin, and tobramycin (c.f. Szot & Tabachnick, 1980; Kahlmeter & Dahlager, 1984). Secondly, netilmicin differed from other aminoglycosides in an *in vitro* test for binding to phospholipids. Its interaction with phosphatidylinositol liposomes was weaker than expected from the number of amino groups of the molecule (Langford *et al.*, 1982). Uptake of the aminoglycosides in the proximal tubular cells is supposed to occur after binding to acidic phospholipids in the renal brush border membrane (Feldman *et al.*, 1982) but surface binding of the drugs correlates with cationic charge and not with nephrotoxicity (i.e. netilmicin > gentamicin; Sastrasinh *et al.*, 1982b). Therefore, it is an interesting speculation that membrane reactions elicited by the drug bound to the receptor, for example the induction of pinocytosis, are the important determinants of nephrotoxicity.

Although the dimensions of the invaginations are different, the mechanisms of pinocytosis in the proximal tubular cells (Maunsbach, 1976; Christensen *et al.*, 1981) and in *Amoeba proteus* (Chapman-Andresen, 1962) appear to have several characteristics in common. Thus, both processes start by the binding of cations to the membrane, both are dependent on Ca^{2+}-influx (Josefsson, 1968; Prusch & Hannafin, 1979; Goldstone *et al.*, 1983; Lu *et al.*, 1983),

and both are sensitive to hormones (Josefsson *et al.*, 1972; Josefsson & Johansson, 1979; Koenig *et al.*, 1982). Netilmicin, which inhibits Ca^+-dependent pinocytosis in the amoeba, may have a similar effect on tubular cells. The weak pinocytotic activity in the presence of netilmicin may then be related to its relatively low nephrotoxicity. The many strong nephrotoxins among the potent inducers (Table 1) also suggest an association between pinocytosis and toxicity in the kidney. Furthermore, all the measures which are shown here to decrease induction of pinocytosis by the aminoglycosides also reduce their toxicity, i.e. treatment with heparin (Higginbotham, 1960), carbenicillin (Block *et al.*, 1979), Ca^{2+} (Quarum *et al.*, 1984), Na^+ (Sastrasinh *et al.*, 1983), and alkalinization of the urine (Chiu *et al.*, 1979).

Induction of the pinocytotic process may be linked to the toxicity of aminoglycosides in several ways. (1) Large inducing cations interacting with Ca^{2+}-specific membrane sites may cause damage by impairing the barrier function of the plasmalemma and the membranes of pinosomes and lysosomes. The protective effect of Ca^{2+} may be due to competition with the aminoglycosides at these binding sites in the membranes. (2) Extensive pinocytotic activity *per se* would disturb normal recycling of membranes and so contribute to the cytopathic changes. (3) Cell damage may ensue because of the rapid internalization of aminoglycosides inhibiting the normal degradative process in lysosomes (Morin *et al.*, 1980; Hostelter & Hall, 1982). Aminoglycosides alter the phospholipid metabolism in proximal tubular cells and induce phospholipidosis (Aubert-Tulkens *et al.*, 1979) which might represent an early event in the pathogenesis of nephrotoxicity (Feldman *et al.*, 1982). If the transport function of pinocytosis is the important factor in development of the lesions then the renal concentration of individual aminoglycosides should correlate with their nephrotoxic potential. Evidence supporting this view comes from an investigation of renal cortical drug concentrations in dogs infused with aminoglycosides (Whelton, 1982), and from a study of gentamicin and tobramycin uptake in rat kidneys (Aronoff *et al.*, 1983). On the other hand, several authors report poor correlation between renal cortical concentration and nephrotoxicity of aminoglycosides (c.f. Parker *et al.*, 1982). Although experiments of this type face a number of difficulties as discussed by Aronoff *et al.* (1983) they could indicate that the potency of a drug to induce pinocytosis rather than its effect after internalization determines toxicity.

According to the above discussion netilmicin, which depresses pinocytosis by inhibiting the initial Ca^{2+}-dependent events of the pinocytotic process, would accumulate at a low rate, be less harmful to membranes, and consequently less nephrotoxic than potent inducers. Because of its calcium antagonistic effect it may, however, enhance another important side effect of the aminoglycosides, that being neuromuscular blockade. The latter is probably caused by competition between aminoglycosides and Ca^{2+} in the prejunctional process of transmitter release (Elmquist & Josefsson., 1962; Vital Brazil & Prado-Franceschi, 1969). It is therefore interesting to note that netilmicin is a very effective inhibitor of neuromuscular transmission (Albiero *et al.*, 1978). Aminopyridines, which increase Ca^{2+}-entry into presynaptic terminals (Thesleff, 1980), and the Ca^{2+}-ionophore, A 23187, reversed the effect of aminoglycosides on transmission (Burkett *et al.*, 1979; Fiekers, 1983) as well as the netilmicin block of pinocytosis (Figures 3b, 4a and b). Netilmicin may therefore decrease the activity of intracellular Ca^{2+} at sites critical for transmitter release and for invagination of the membrane in the amoeba.

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