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Polyamine-like actions of aminoglycosides and aminoglycoside derivatives at NMDA receptors

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Abstract

Recent pharmacological studies indicate that aminoglycoside-induced hearing loss may be an excitotoxic process modulated by a polyamine-like activation of cochlear NMDA receptors. Aminoglycoside antibiotics are constituted by a series of glycosidically linked aminocyclitols and aminosugars. We report here on the actions of these individual aminocyclitols and aminosugars on wild type NMDA receptors from rat brain. Compared to the parent molecules, neither aminocyclitols (e.g., 2-deoxystreptamine, streptamine, and streptidine) nor aminosugars (e.g., D-glucosamine and kanosamine) were effective at enhancing [^{3}H]dizocilpine ([^{3}H]MK-801) binding or inhibiting [^{3}H]ifenprodil at NMDA receptors. Moreover, the appropriate combinations of aminosugars and aminocyclitols did not reconstitute the activity of the parent aminoglycoside at NMDA receptors. These data indicate that the polyamine-like actions of aminoglycosides are attributable to the conformation of the parent molecule rather than a particular amine containing constituent. Thus, it may be possible to synthesize or isolate aminoglycoside antibiotics devoid of ototoxicity. Published by Elsevier Science B.V.

Keywords: NMDA receptor; Polyamine; Aminoglycoside; Aminocyclitol; Aminosugar; Ototoxicity

1. Introduction

Aminoglycoside antibiotics have been in clinical use for over 50 years (Begg and Barclay, 1991). Despite the efficacy of aminoglycosides in treating Gram negative infections as well as multi-drug resistant tuberculosis, the use of these drugs is limited by serious ototoxicity (Begg and Barclay, 1991). This ototoxicity can be manifested as a cochlear damage that can produce permanent hearing loss and/or damage to the vestibular apparatus resulting in dizziness, ataxia, and/or nystagmus (Hodges, 1984). Because it is possible to physiologically compensate for vestibular damage, cochleotoxicity is generally considered to be a far more serious problem (Hodges, 1984). Despite these limiting side effects, aminoglycosides are likely to remain an important component of antibiotic treatment worldwide (Priuska and Schacht, 1997). Thus, a means of limiting the ototoxic effects of these drugs is desirable.

The cellular basis for aminoglycoside-induced hearing loss is a destruction of cochlear hair cells. However, the biochemical and molecular mechanisms underlying this event are poorly understood. A recent study demonstrating that NMDA receptor antagonists attenuate aminoglycoside-induced hearing loss and cochlear hair cell death suggests that aminoglycoside-induced cochleotoxicity is, at least in part, an excitotoxic process involving activation of cochlear NMDA receptors (Basile et al., 1996).

The ability of endogenous polyamines (e.g., spermine and spermidine) to act as positive modulators at NMDA receptors has been recognized for almost a decade (Ransom and Stec, 1988). A structurally diverse group of synthetic polyamines (Reynolds, 1992; Romano and Williams, 1994; Zhou et al., 1996) as well as a variety of naturally occurring compounds, including aminoglycoside antibiotics, (Pullan et al., 1992; Chandler et al., 1993; Basile et al., 1996), can mimic the positive modulatory actions of endogenous polyamines. Polyamine actions at NMDA receptors are complex, and are likely to involve multiple loci (Benveniste and Mayer, 1993; Romano and Williams,

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1994). Nonetheless, aminoglycosides enhance the binding of channel blockers such as 1-[1-(2-thienyl) cyclohexyl][^{3}H]piperidine ([^{3}H]TCP) and [^{3}H]dizocilpine ([^{3}H]MK-801) to wild type NMDA receptors (Pullan et al., 1992; Basile et al., 1996) and this enhancement occurs at the same concentrations that inhibit [^{3}H]ifenprodil binding to polyamine-associated sites (Schoemaker et al., 1990; Hashimoto et al., 1994; Basile et al., 1996). The concentrations of aminoglycosides required to produce these in vitro effects on radioligand binding to NMDA receptors are well within the range found in the cochlear perilymph following ototoxic regimens of these antibiotics (Brummett et al., 1978; Desrochers and Schacht, 1982). Further, the potencies of a series of aminoglycosides to enhance [^{3}H]MK-801 binding are highly correlated with their cochleotoxicities in humans (Basile et al., 1996).

Most clinically useful aminoglycosides are composed of an aminocyclitol moiety joined by glycosidic linkage to one or more aminosugars (Kirst, 1996; Fig. 1). Based on the structural diversity of compounds that modulate NMDA receptors through polyamine-associated sites, it is likely that the multiple primary and secondary amines distributed across the individual aminosugar and aminocyclitol moieties impart these polyamine-like actions to aminoglycosides. While all clinically useful aminoglycosides studied to date possess polyamine-like actions at NMDA receptors (Pullan et al., 1992; Hashimoto et al., 1994; Basile et al., 1996), the polyamine-like properties of individual aminosugar and aminocyclitol components have not been examined. The high correlation between the potencies of a series of aminoglycosides to enhance [^{3}H]MK-801 binding and their cochleotoxicities in humans (Basile et al., 1996) makes this issue particularly relevant. Thus, by identifying aminocyclitols or aminosugars that activate NMDA receptors, it may be possible to design aminoglycosides with a lower potential for ototoxicity. We report here the effects

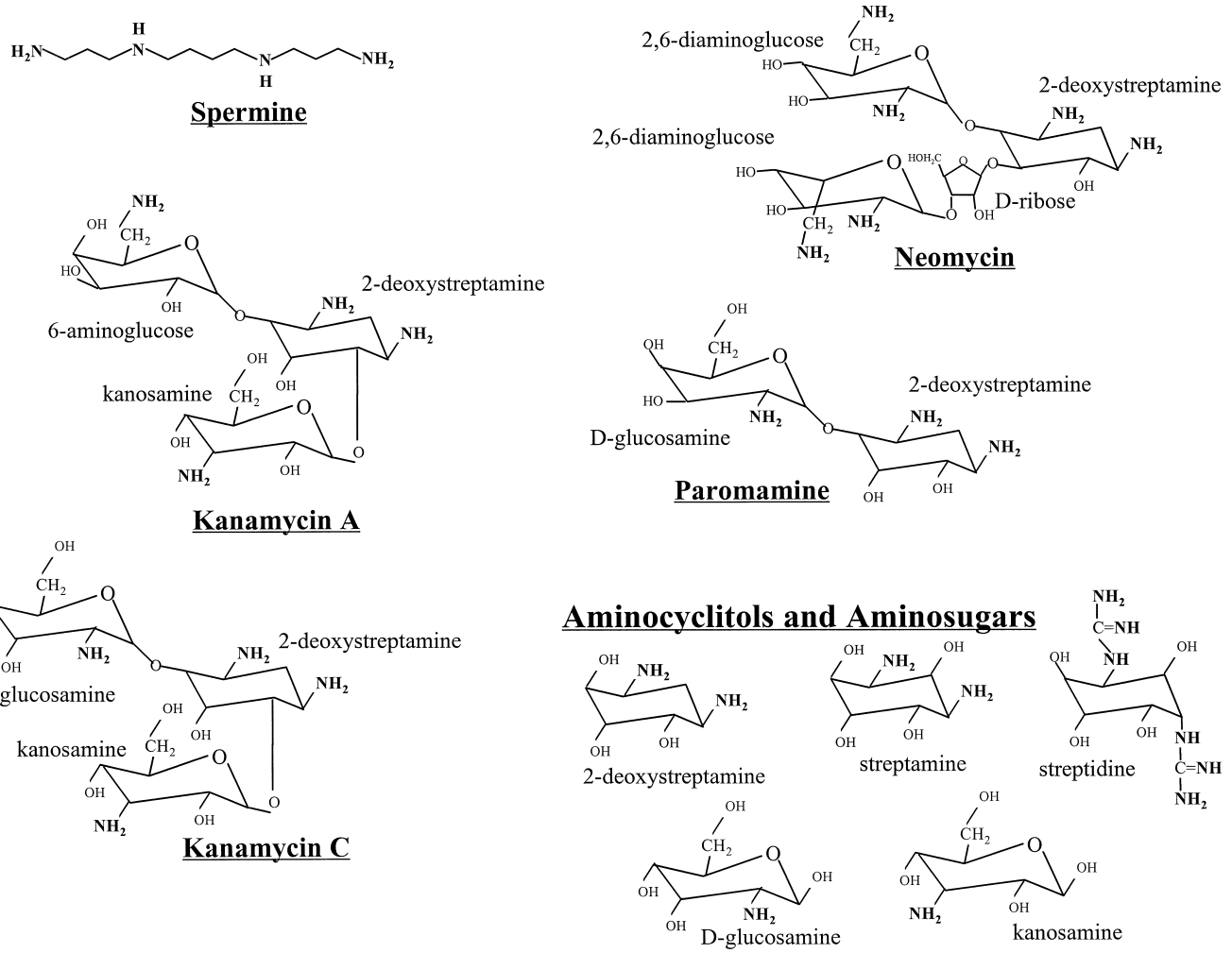

Fig. 1. Structures of aminoglycosides, aminosugars, and aminocyclitols.

of aminocyclitols and aminosugars that are constituents of many aminoglycoside antibiotics on activation of NMDA receptors and interactions with polyamine sites.

2. Materials and methods

2.1. Materials

[^{3}H]MK-801 (Sp. Act. 22.0 Ci/mmol) and [^{3}H]ifenprodil (Sp. Act. 68.1 Ci/mmol) were supplied by DuPont-NEN (Boston, MA). Neomycin sulfate, kanamycin A, D-glucosamine, and spermine were purchased from Sigma Chemical (St. Louis, MO). Ifenprodil was obtained from Research Biochemicals International (Natick, MA). Paromamine was donated by Dr. Joel Berger (Schering-Plough Research Institute, Bloomfield, NJ). 2-Deoxystreptamine, streptamine, and streptidine were gifts from Dr. Herbert A. Kirst (Lilly Research Laboratories, Greenfield, IN). Kanamycin C and kanosamine were donated by Dr. Kuniaki Tatsuta (Waseda University, Tokyo, Japan). All other materials were purchased from standard commercial sources.

2.2. Animals and membrane preparation

Adult male Sprague–Dawley rats (200–300 g; Taconic Farms, Germantown, NY) were killed by decapitation. Forebrains were rapidly removed and the tissues disrupted using a Polytron (setting 7 for 30 s) in 50 volumes of ice cold 5 mM HEPES/4.5 mM Tris buffer (pH 7.4). The homogenate was centrifuged (4°C) at $20\,000 \times g$ for 20 min and the resulting pellet was resuspended in 50 volumes of buffer and recentrifuged at $20\,000 \times g$ for 20 min. This resuspension/recentrifugation procedure was repeated five more times (for a total of seven 'washes'). The resulting pellet was resuspended in five volumes of the same buffer, quickly frozen on solid CO_2 and stored at −70°C until assayed.

2.3. [^{3}H]MK-801 binding

Tissues were thawed on ice, diluted to 50 volumes with 5 mM HEPES/4.5 mM Tris buffer (pH. 7.4; assay buffer) and centrifuged at $20\,000 \times g$ for 20 min. The pellet was resuspended in 50 volumes assay buffer, recentrifuged at $20\,000 \times g$ for 20 min, and resuspended in 50 volumes assay buffer. Assays were performed in a total volume of 0.5 ml containing membrane suspension (~60 μg of protein), [^{3}H]MK-801 (final concentration 3.5– 5 nM), test compounds, and buffer to final volume. All assays were performed in the nominal absence of endogenous glutamate and glycine; reagents and buffers were prepared in Milli-Q water to exclude exogenous glycine and glutamate from the assay. Assays were incubated at room temperature for 2 h and terminated by rapid filtration (model MB-48R cell harvester; Brandel) over glass fiber filters (Whatman GF/B) presoaked in 0.03% polyethylenimine. This filtration was followed by two 3–5 ml washes with ice-cold assay buffer. Non-specific binding was determined with 100 μM phencyclidine hydrochloride. The radioactivity retained on the filters was measured in a Beckman model LS 6500 liquid scintillation counter. Protein content was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, IL).

2.4. [^{3}H]Ifenprodil binding

Tissues were thawed on ice, diluted to 20 volumes with 50 mM Tris–HCl buffer (pH. 7.4; assay buffer), and centrifuged at $20\,000 \times g$ for 20 min. The pellet was resuspended in 20 volumes of assay buffer and recentrifuged. The resulting pellet was resuspended in 20 volumes assay buffer. Assays were performed in a total volume of 0.5 ml containing membrane suspension (~100 μg of protein), [^{3}H]ifenprodil (final concentration ~8 nM), (+)pentazocine (10 μM; to block σ sites), test compounds, and buffer to final volume. Assays were incubated at 4°C for 2 h and terminated by rapid filtration as described above. Non-specific binding was defined by ifenprodil (10 μM).

3. Results

3.1. Effects of aminoglycosides, aminocyclitols, and aminosugars on [^{3}H]MK-801 binding

The effects of polyamines, aminoglycosides, aminocyclitols and aminosugars (Fig. 1) on [^{3}H]MK-801 binding were examined under non-equilibrium conditions in the nominal absence of glutamate and glycine. These assay

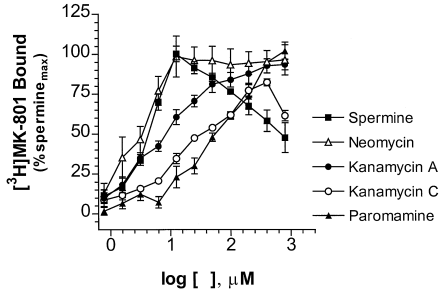

Fig. 2. Enhancement of [^{3}H]MK-801 binding by aminoglycosides: comparison with spermine. Rat forebrain membranes were extensively 'washed' as described in Section 2 to reduce the concentrations of endogenous compounds (e.g., glycine, glutamate, and polyamines) known to affect [^{3}H]MK-801 binding. Data are expressed as a percent of the maximum enhancement achieved with spermine. Values represent the mean ± S.E.M. of ≥3 assays. Basal binding in the nominal absence of added modulatory agents was 42.8 ± 2.6 fmol/assay. The maximal binding in the presence of spermine was 100.7 ± 4.9 fmol/assay. Potency and efficacy values are presented in Table 1.

Table 1
Potencies and efficacies of spermine and aminoglycosides to enhance [^{3}H]MK-801 binding

Compound	EC_{50} (μM)	Efficacy (% max. spermine)
Spermine	4.2 ± 0.47	101.2 ± 3.5
Neomycin	2.7 ± 0.69	96.6 ± 8.7
Kanamycin A	7.3 ± 0.92	94.9 ± 4.3
Kanamycin C	19.0 ± 2.24	82.3 ± 2.2
Paromamine	96.1 ± 16.5	118.0 ± 5.8

Values are mean ± S.E.M. for ≥ 3 assays.
Parameter values were derived using GraphPad Prism. Only the ascending portions of the spermine and kanamycin C concentration response curves were used for curve fitting.

conditions provide a sensitive and specific means of examining the positive modulatory actions of compounds such as polyamines at NMDA receptors (Reynolds et al., 1987; Kemp et al., 1988; Sircar and Zukin, 1991; Zhou et al., 1996). Consistent with previous reports (Reynolds and Miller, 1989; Williams et al., 1989; Romano and Williams, 1994), spermine has a biphasic effect on [^{3}H]MK-801 binding (Fig. 2) with the descending arm of the concentration effect curve likely corresponding to a low affinity channel block (Romano and Williams, 1994) that is observed in electrophysiological studies (Benveniste and Mayer, 1993). The aminoglycosides examined here (Fig. 1) all enhance [^{3}H]MK-801 binding in a concentration dependent fashion (Fig. 2). However, within the concentration range employed, only kanamycin C produces a biphasic effect on [^{3}H]MK-801 binding. While there are no remarkable differences in efficacies between these aminoglycosides and spermine, the EC_{50} (potency) values varied by ~30-fold (Table 1) with a rank order: spermine = neomycin > kanamycin A > kanamycin C > paromamine.

Three aminocyclitols (Fig. 1) that are common constituents of aminoglycoside antibiotics were tested for their ability to enhance [^{3}H]MK-801 binding (Fig. 3A). Neither streptamine nor streptidine enhanced [^{3}H]MK-801 binding above ~25% of the maximal enhancement produced by spermine. In contrast, at the highest concentration tested (800 μM), 2-deoxystreptamine enhanced [^{3}H]MK-801 binding to a maximum of ~75% of that produced by spermine. Although it was not possible to accurately fit these data, it is apparent that the concentration–response curves are all right shifted relative to that of paromamine, the lowest potency aminoglycoside examined. Neither of the aminosugars examined (kanosamine and D-glucosamine; Fig. 1) enhanced [^{3}H]MK-801 binding at concentrations up to 800 μM (Fig. 3B).

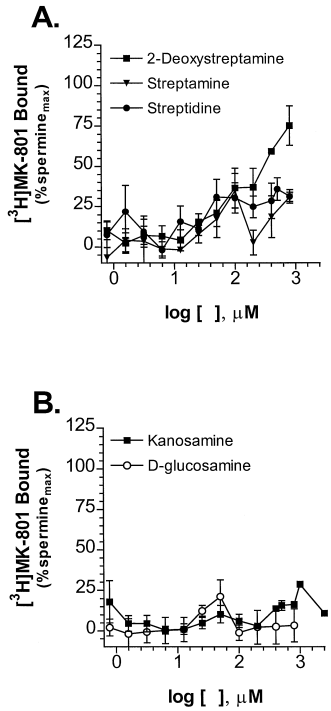

Fig. 3. Effects of aminocyclitols (Panel A) and aminosugars (Panel B) on [^{3}H]MK-801 binding to rat forebrain membranes. Data are expressed as a percent of the maximum enhancement produced by spermine (see Fig. 2). Values represent the mean ± S.E.M. of ≥ 3 assays. The low potencies and efficacies of these compounds did not permit an accurate estimate of parameter values.

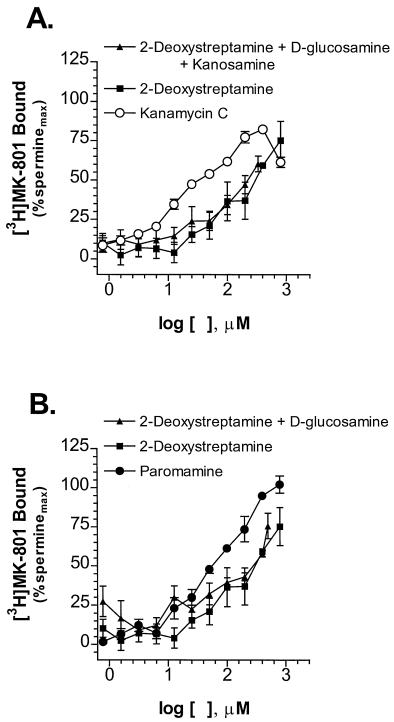

Fig. 4. Effects of kanamycin C and its constituent molecules (Panel A) and paromamine and its constituent molecules (Panel B) on enhancement of [^{3}H]MK-801 binding to rat forebrain membranes. For the curves representing additive effects, each data point is the response obtained by adding equimolar concentrations of each constituent molecule (e.g., 10 μM indicates the presence of this concentration of each of the constituent molecules). Data are expressed as the percent maximum enhancement produced by spermine (see Fig. 2). Values represent the mean ± S.E.M. of ≥ 3 assays.

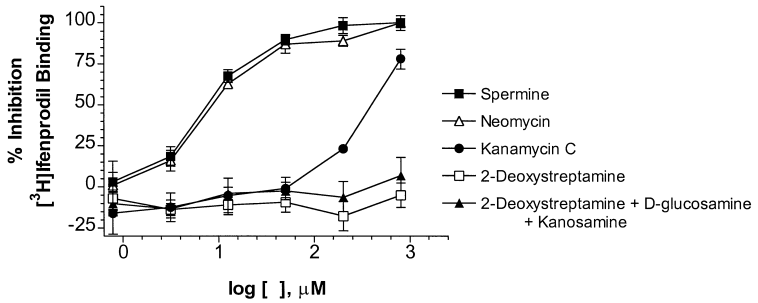

Fig. 5. Inhibition of [^{3}H]ifenprodil binding to rat forebrain membranes. Data are expressed as percent of maximal inhibition achieved with spermine. Values represent the mean ± S.E.M. of ≥ 3 assays. Basal binding in the nominal absence of added inhibitory agents was 75.8 ± 11.0 fmol/assay. The maximal binding in the presence of spermine was 36.0 ± 6.1 fmol/assay.

In the next series of experiments, we attempted to reconstitute the effects of two aminoglycosides on [^{3}H]MK-801 binding by combining equimolar quantities of their individual constituent molecules. Kanamycin C is composed of the aminocyclitol 2-deoxystreptamine glycosidically linked to the aminosugars kanosamine and D-glucosamine (Fig. 1). Paromamine consists of 2-deoxystreptamine glycosidically linked to the aminosugar, D-glucosamine (Fig. 1). The combination of 2-deoxystreptamine, kanosamine, and D-glucosamine did not enhance [^{3}H]MK-801 binding to the same extent as the linked parent molecule, kanamycin C (Fig. 4A). Similarly, the combination of 2-deoxystreptamine and D-glucosamine did not enhance [^{3}H]MK-801 binding to the same extent as paromamine (Fig. 4B). Moreover, the combinations of aminocyclitols and aminosugars were no more effective than 2-deoxystreptamine alone.

3.2. Effects of aminoglycosides, aminocyclitols, and aminosugars on [^{3}H]ifenprodil binding

The effects of spermine, aminoglycosides, aminocyclitols and aminosugars on a polyamine-associated site on NMDA receptors were studied using [^{3}H]ifenprodil. Consistent with previous reports (Schoemaker et al., 1990; Hashimoto et al., 1994), spermine inhibits [^{3}H]ifenprodil binding with an EC_{50} of ~7.5 μM (Fig. 5). The potency of neomycin was equal to that of spermine (Fig. 5). Kanamycin C was considerably less potent (EC_{50} ~316 μM) than either spermine or neomycin. At the highest concentration employed (800 μM) kanamycin C inhibited [^{3}H]ifenprodil binding to ~75% of the maximum achieved with spermine (Fig. 5). Neither 2-deoxystreptamine nor the combination of 2-deoxystreptamine, kanosamine, and D-glucosamine (the component molecules of kanamycin C) inhibited [^{3}H]ifenprodil binding (Fig. 5).

4. Discussion

The data presented here demonstrate that several common constituent moieties of aminoglycoside antibiotics are neither potent nor efficacious positive modulators at NMDA receptors. Based on the recent report that there is a high correlation between the potencies of a series of aminoglycoside antibiotics to enhance [^{3}H]MK-801 binding to rat brain membranes and ototoxicity in humans (Basile et al., 1996), the individual aminocyclitols and aminosugars examined are unlikely to produce ototoxicity. Even in the case of the most active moiety examined (2-deoxystreptamine), the concentrations (> 100 μM) required to enhance [^{3}H]MK-801 binding (to a maximum of ~75% of that produced by spermine) are significantly higher than would be found in the cochlear perilymph (assuming complete hydrolysis of the parent molecule) following ototoxic regimens of commonly used aminoglycosides (Brummett et al., 1978). Thus, if aminoglycoside-induced damage to the cochlea is an excitotoxic phenomenon resulting from a polyamine-like activation of NMDA receptors (Basile et al., 1996), then this toxicity is unlikely to be due to a particular aminocyclitol or aminosugar but instead depends upon the conformation assumed by the parent molecule. Consistent with this hypothesis, we were unable to reconstitute the enhancement of [^{3}H]MK-801 binding produced by either kanamycin C or paromamine by adding equimolar concentrations of the individual aminosugar and aminocyclitol components of these aminoglycosides. These data also support previous studies demonstrating that the number of amino groups in a molecule does not correlate with the polyamine-like actions at NMDA receptors (Sacaan and Johnson, 1990; Romano et al., 1992). Thus, neomycin and the aminocyclitol streptidine each have six amino groups yet differ greatly in their ability to enhance [^{3}H]MK-801 binding (neomycin ⋙ streptidine). Moreover, spermine, kanamycin A, and kanamycin C each have four amino groups yet also differ in their potencies to enhance [^{3}H]MK-801 binding (spermine > kanamycin A > kanamycin C) and displace [^{3}H]ifenprodil (spermine ≫ kanamycin C).

Several studies examining the ototoxicity of aminoglycoside derivatives support the hypothesis that toxicity is dependent on the intact parent molecule. For example, the neomycin fragment neamine is not ototoxic in vivo (Weiner

and Schacht, 1981) or in cochlear cultures (Kotecha and Richardson, 1994). Further, Owada (1962) reported that the ototoxicity of kanamycin A was not mimicked by either 2-deoxystreptamine or 6-glucosamine (two of the three components of kanamycin A). However, kanosamine (the third component of kanamycin A) was determined to be cochleotoxic. Because even very high (800 μM) concentrations of kanosamine do not enhance [^{3}H]MK-801 or inhibit [^{3}H]ifenprodil binding (Figs. 3 and 5), the observation of Owada (1962) may be due to a non-NMDA mediated mechanism. In this regard, it is interesting to note that the toxicity of kanosamine was dependent on its aldehyde as well as its amino group (Owada, 1962). Because not all aminosugar and aminocyclitol derivatives that are constituents of ototoxic aminoglycosides (e.g., 2,6 diaminoglucose) were available, it is possible that one or more of these compounds could activate NMDA receptors. However, the aforementioned studies (Owada, 1962; Weiner and Schacht, 1981; Kotecha and Richardson, 1994) demonstrating lack of ototoxicity of aminoglycoside derivatives, when taken together with the data presented here, makes this unlikely.

The apparent requirement for an intact aminoglycoside molecule to modulate NMDA receptors is also reflected in measures of [^{3}H]ifenprodil binding. Ifenprodil is a non-competitive NMDA receptor antagonist that appears to act at or near a polyamine-associated site on NMDA receptors (Reynolds and Miller, 1989; Romano and Williams, 1994; Scatton et al., 1994). While there is some debate concerning the exact nature of the interaction (Romano and Williams, 1994; Scatton et al., 1994), it is clear that structurally diverse molecules containing a polyamine motif inhibit [^{3}H]ifenprodil binding (Schoemaker et al., 1990; Hashimoto et al., 1994; Nicolas and Carter, 1994). Furthermore, the polyamine spermine reverses the NMDA receptor-associated antagonistic effects of ifenprodil (Carter et al., 1990). The observation that spermine and neomycin inhibit [^{3}H]ifenprodil binding is consistent with previous data (Hashimoto et al., 1994; Basile et al., 1996), and supports the notion that [^{3}H]ifenprodil binds to a polyamine-associated locus on NMDA receptors. We demonstrate that kanamycin C inhibits [^{3}H]ifenprodil binding with a much lower potency than spermine or neomycin, although its potency in this measure is about an order of magnitude lower than required to enhance [^{3}H]MK-801 binding. Further, the inhibition of [^{3}H]ifenprodil binding produced by kanamycin C could not be reconstituted by the addition of equimolar concentrations of its component molecules (2-deoxystreptamine, kanosamine, and D-glucosamine) either individually or in combination. This finding is consistent with the failure of the constituent molecules of kanamycin C to enhance [^{3}H]MK-801 binding.

In conclusion, the individual aminosugars and aminocyclitols that are constituents of most clinically useful aminoglycosides have very modest effects on radioligand binding to NMDA receptors compared to the parent molecules. Moreover, the appropriate combinations of aminosugars and aminocyclitols cannot reconstitute the ability of the parent aminoglycoside to either enhance [^{3}H]MK-801 binding or inhibit [^{3}H]ifenprodil binding. Basile et al. (1996) have proposed that aminoglycoside-induced activation of NMDA receptors is responsible for the cochelotoxic actions of these antibiotics. This proposal, taken together with our demonstration that the secondary structure of a parent aminoglycoside molecule (as opposed to any individual constituent) is responsible for its actions at the NMDA receptor indicates that it may be possible to either synthesize or isolate aminoglycoside antibiotics devoid of this limiting side effect.

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