Block of P/Q-Type Calcium Channels by Therapeutic Concentrations of Aminoglycoside Antibiotics†

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ABSTRACT: Aminoglycoside antibiotics can cause neuromuscular block by inhibiting Ca^{2+} influx into motor nerve terminals. P/Q-type Ca^{2+} channels, which are formed by α1A subunits, are mainly responsible for depolarization-dependent presynaptic Ca^{2+} entry in motor neurons. We therefore investigated the possibility that aminoglycosides function as P/Q-type channel blockers. They inhibited [^{125}I]-ω-CTx-MVIIC binding to P/Q-type channels in guinea pig cerebellum membranes with nanomolar IC_{50} values (e.g., 8 nM for neomycin). Divalent cations decreased the apparent affinity of neomycin. Barium inward currents through α1A subunits expressed in *Xenopus* oocytes were partially blocked by therapeutic concentrations of aminoglycosides. This explains that therapeutically relevant concentrations of these drugs decrease the reserve of neuromuscular transmission, which can lead to neuromuscular block. We conclude that micromolar concentrations of aminoglycosides block not only N-type but also P/Q-type channels in mammalian neurons.

Neuromuscular blockade is a rare but potentially harmful toxic reaction of aminoglycoside antibiotics (Chambers & Sande, 1995). It is mainly observed under conditions where neuromuscular transmission is impaired, e.g., in association with the administration of anesthetics or neuromuscular blocking agents (Chambers & Sande, 1995). Patients with myasthenia gravis also have a decreased reserve of neuromuscular transmission and are particularly susceptible to neuromuscular blockade by aminoglycosides.

Although some aminoglycosides reduce the sensitivity of the postsynaptic membrane for acetylcholine (Fiekers, 1983), a presynaptic mechanism of action seems to play the major role for the depression of neuromuscular transmission (Fiekers, 1983; Sokoll & Gergis, 1981; Chambers & Sande, 1995). Aminoglycosides inhibit the Ca^{2+}-dependent release of acetylcholine from motor nerve terminals (Fiekers, 1983; Caputy et al., 1981). Ca^{2+} overcomes their blocking effect and the intravenous administration of Ca^{2+} salt is therefore the preferred treatment of aminoglycoside toxicity (Chambers & Sande, 1995).

At least three different types of Ca^{2+} channels were described in the mammalian motor nerve terminals: L-type channels (Hong & Chang, 1989), ω-CTx-GVIA[1] -sensitive N-type channels (Rossoni et al., 1994; Protti et al., 1991), and ω-CTx-MVIIC/ω-Aga-IVA-sensitive P/Q-type channels (Protti & Uchitel, 1993; Bowersox et al., 1995; Wessler et al., 1995; Hong & Chang, 1995; Sugiura et al., 1995). However, only Ca^{2+} entry through P/Q-type Ca^{2+} channels is coupled to electrically evoked acetylcholine release and muscle contraction (Protti & Uchitel, 1993; Bowersox et al., 1995; Wessler et al., 1995; Hong & Chang, 1995). α1A subunits, which form the channel pore of the P/Q-type Ca^{2+} channel complex (Birnbaumer et al., 1994; Sather et al., 1993), are concentrated within the "active zones" (Sugiura et al., 1995; Ousley & Froehner, 1994), the major sites of presynaptic Ca^{2+} entry. Taken together, P/Q-type channels play a central role for excitation−contraction coupling in skeletal muscle.

It is therefore likely that aminoglycosides, which are known blockers of N-type Ca^{2+} channels (Wagner et al., 1987; Knaus et al., 1987), also inhibit P/Q-type channels, thus decreasing presynaptic Ca^{2+} entry into motor nerve terminals. As effects on P/Q-type Ca^{2+} channels have not yet been studied, we investigated the interaction of aminoglycosides with these channels at the molecular and functional level. We found that aminoglycosides bind to [^{125}I]-ω-CTx-MVIIC-labeled P/Q-type channels with nanomolar affinity and block α1A-mediated channel currents at therapeutically relevant concentrations.

MATERIALS AND METHODS

Materials. [^{125}I]-ω-CTx-MVIIC and [^{125}I]-ω-CTx-GVIA (2200 Ci/mmol) were from New England Nuclear (Vienna, Austria), unlabeled ω-CTx-MVIIC was from Saxon Biochemicals (Hannover, Germany); unlabeled ω-CTx-GVIA and aminoglycoside antibiotics were from Sigma (Vienna, Austria). Netilmicin was a kind gift of AESCA (Traiskirchen, Austria).

Preparation of the Free Base or Acetate Salts of Aminoglycoside Antibiotics. Tobramycin and amikacin were obtained as the free base. All other aminoglycosides were obtained as sulfate salts. As sulfate can form insoluble $BaSO_4$, thus decreasing the effective Ba^{2+} concentration in our electrophysiological experiments, the free base of neomycin and the acetate salt of kanamycin A were prepared.

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[1] Abbreviations: Aga, agatoxin; CTx, conotoxin; I_{Ba}, inward barium currents; IC_{50}, concentration causing half-maximal inhibition; k_{+1} and k_{-1}, association and dissociation rate constant, respectively; PMSF, phenylmethanesulfonyl fluoride.

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Neomycin base was obtained by cation-exchange chromatography on SP-Sepharose (fast-flow, Pharmacia, Vienna, Austria). Neomycin sulfate (30−50 mg) was dissolved in distilled water and loaded onto SP-Sepharose equilibrated in water after being washed in 10% ammonia hydroxide. The resin was washed with distilled water, neomycin was eluted with 10% ammonia solution, and the free base was obtained by lyophilization. The acetate salt of kanamycin A was prepared by exchanging sulfate for acetate by anion-exchange chromatography. Kanamycin A (50 mg) was dissolved in 5 mL of distilled water and incubated overnight with 5 mL of the acetate form of Q-Sepharose (fast-flow, Pharmacia). The resin was removed by centrifugation and the acetate form was recovered from the supernatant by lyophilization. The purity of neomycin and kanamycin A was verified by mass spectroscopy.

[^{125}I]-ω-CTx-MVIIC and [^{125}I]-ω-CTx-GVIA Binding Experiments. Guinea pig cerebellum and cortex membranes were prepared as described (Knaus et al., 1987; Glossmann & Ferry, 1985). [^{125}I]-ω-CTx-MVIIC binding experiments were performed in final assay volumes of 2−8 mL in 25 mM Tris-HCl, pH 7.4, 0.2 mg/mL lysozyme, 0.1 mM PMSF, and 0.05% (v/v) Tween 20 (binding buffer). For binding inhibition studies, 0.2 μg/mL membrane protein was incubated with 0.3−0.6 pM radioligand for 6 h at 22 °C. Nonspecific binding was measured in the presence of 3 nM unlabeled toxin. Serial dilutions of aminoglycosides (or toxins) were made in binding buffer and added in volumes of 200−800 μL to the assay mixture. The concentration of bound ligand was determined by rapid filtration of the incubation mixture over GF/C Whatman filters [pretreated with 0.1% (v/v) polyethylenimine] after dilution in wash buffer (25 mM Tris-HCl, pH 7.4, 160 mM choline chloride, 0.5 mg/mL bovine serum albumin, 1.5 mM $CaCl_2$, and 0.05% Tween 20) followed by extensive washing (5 × 4 mL of wash buffer). Filter-bound radioactivity was determined in a γ-counter (about 80% counting efficiency).

To measure association kinetics, 1.5−3.5 pM radioligand was incubated with 0.5−1.1 pM receptor for various times (up to 31 h) before bound ligand was determined by filtration. Dissociation kinetics were measured by 50-fold dilution of ligand−receptor complexes in assay buffer after equilibrium had been reached.

[^{125}I]-ω-CTx-GVIA binding to N-type Ca^{2+} channels in guinea pig cortex membranes was measured in 25 mM Tris-HCl (pH 7.4), 0.1 mM PMSF, and 0.03 mg/mL bovine serum albumin. For binding inhibition studies, 0.4 pM ligand was incubated for 30−60 min (22 °C) with 0.13 μg/mL membrane protein in the absence and presence of unlabeled inhibitors. Filtration was as described for the [^{125}I]-ω-CTx-MVIIC binding assay.

Heterologous Expression of Ca^{2+} Channels in Xenopus Oocytes. α1A subunits were expressed in *Xenopus laevis* oocytes together with β1 and α2-δ-subunits as previously described (Grabner et al., 1996). α1A-mediated currents were measured at room temperature using the two-microelectrode voltage-clamp technique with 2 mM Ba^{2+} as the charge carrier. Voltage recording microelectrodes and current injecting electrodes were filled with 2.8 M CsCl, 0.2 M CsOH, 10 mM EGTA, and 10 mM HEPES (pH 7.4) and had resistances of 0.3−1 MΩ. The bath solution contained 2 mM $Ba(OH)_2$, 40 mM *N*-methyl-D-glucamine, 10 mM HEPES, and 10 mM glucose (pH 7.4 adjusted with methanesulfonate). The recording chamber was perfused with control or drug-containing solution at a flow rate of 1 mL/min. Leakage-current correction was performed digitally by using average values of scaled leakage current elicited by hyperpolarizing voltage pulses (P/4 protocol). We have previously demonstrated (Grabner et al., 1995; Döring et al., 1996) that under our experimental conditions α1A-mediated Ba^{2+} inward currents (I_{Ba}) exhibit biophysical and pharmacological (sensitivity to ω-CTx-MVIIC and ω-Aga-IVA) properties described for "Q-type currents" (Sather et al., 1993; Stea et al., 1994).

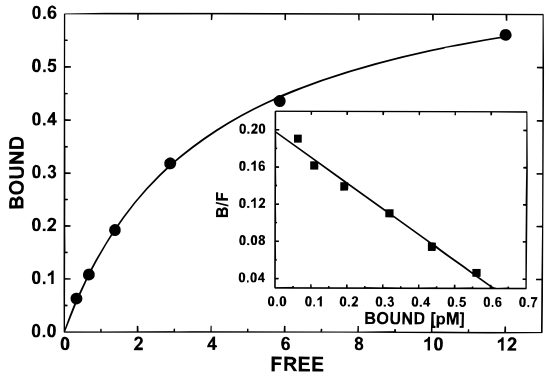

FIGURE 1: Saturation analysis of [^{125}I]-ω-CTx-MVIIC binding to P/Q-type Ca^{2+} channels in guinea pig cerebellum membranes: Guinea pig cerebellum membrane protein (0.29 μg/mL) was incubated with increasing concentrations of [^{125}I]-ω-CTx-MVIIC at 22 °C for 240 min in a final assay volume of 2 mL. Specifically bound ligand was determined as described under Materials and Methods. Specific binding at around K_d concentrations of radioligand (3.2 pM) was 2480 cpm (516 dpm of nonspecific binding). Binding parameters were obtained by linear regression analysis of the data after Scatchard transformation: K_d = 3.6 pM, B_{max} = 2.1 pmol/mg of protein. Almost identical data were obtained in an independent experiment.

Binding Data Analysis. k_{+1} and k_{-1} were calculated by computer-fitting the association data to a second-order association reaction using the software package SCIENTIST (MicroMath Inc., Salt Lake City, UT). k_{-1} was calculated from dissociation data as the slope of the monoexponential decrease of binding activity with time. Binding inhibition data were fitted to the general dose response equation (DeLean et al., 1978) to obtain IC_{50} values and slope factors.

Statistics. Data are given as means ± SD for the indicated number of experiments.

RESULTS

Aminoglycosides Are Potent Blockers of [^{125}I]-ω-CTx-MVIIC Binding to P/Q-Type Ca^{2+} Channels. The peptide toxin [^{125}I]-ω-CTx-MVIIC selectively labels the α1A subunit of P/Q-type Ca^{2+} channels (Hillyard et al., 1992; Martin-Moutot et al., 1995; Sakurai et al., 1995; Lennon et al., 1995; Liu et al., 1996) in mammalian brain. We therefore used this radioligand to investigate whether aminoglycoside antibiotics are able to interact with the P/Q-type channel complex, thereby interfering with toxin binding. Saturation analysis (Figure 1) revealed that [^{125}I]-ω-CTx-MVIIC binding to guinea pig cerebellum membranes occurred with high affinity (K_d = 1.5−3.8 pM; B_{max} = 2.5−3.1 pmol/mg of protein, n = 2) and to a homogenous population of sites (linear Scatchard plot, Figure 1). The high affinity was confirmed by the low IC_{50} for the inhibition of radioligand by unlabeled toxin (IC_{50} = 1.9−2 pM, n = 2). The lower

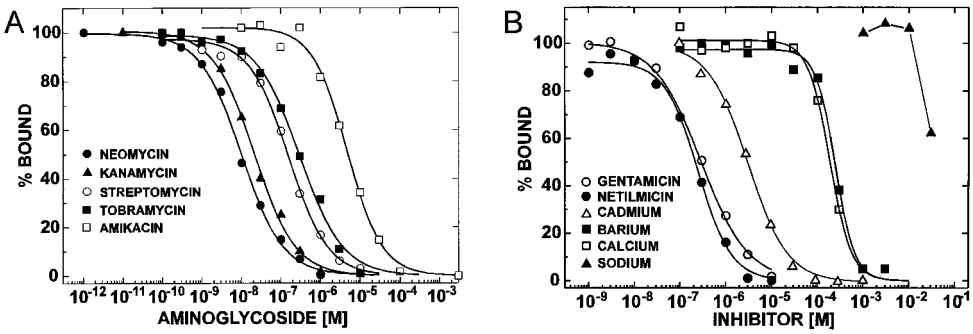

FIGURE 2: Inhibition of [^{125}I]-ω-CTx-MVIIC binding by aminoglycoside antibiotics and cations: Binding experiments were performed as described under Materials and Methods. The following binding parameters were obtained by nonlinear curve-fitting of the data. IC_{50} values and slope factors are given. Panel A (IC_{50} values in nanomolar): neomycin, 9.9, 0.83; kanamycin A, 21.7, 0.81; streptomycin, 142, 0.81; tobramycin, 280, 0.79; amikacin, 4400, 1.00. Panel B (IC_{50} values in micromolar): netilmicin, 0.195, 0.99; gentamicin, 0.294, 0.86; Ca^{2+}, 185, 1.80; Ba^{2+}, 244, 2.10; Cd^{2+}, 4.9, 1.23; Na, >30 000. Statistics for aminoglycoside block are given in Table 1. Similar binding parameters for the inhibition by mono- and divalent cations were obtained in an independent experiment.

Table 1: Inhibition of [^{125}I]-ω-CTx-MVIIC and [^{125}I]-ω-CTx-GVIA Binding to Neuronal Ca^{2+} Channels by Aminoglycosides[a]

	[^{125}I]-ω-CTx-MVIIC			[^{125}I]-ω-CTx-GVIA		
aminoglycoside	IC_{50} (nM)	slope	*n*	IC_{50} (nM)	slope	*n*
neomycin	8.5 ± 3.4	0.84 ± 0.10	7	26 ± 3	1.07 ± 0.01	2
kanamycin A	16 ± 5	0.79 ± 0.03	3	41 ± 7	1.19 ± 0.01	2
netilmicin	177 ± 27	0.84 ± 0.11	6	205 ± 23	1.15 ± 0.10	2
streptomycin	192 ± 62	0.88 ± 0.08	4	603 ± 205	1.24 ± 0.10	2
gentamicin	211 ± 93	0.88 ± 0.10	4	533 ± 44	1.04 ± 0.05	2
tobramycin	224 ± 102	0.87 ± 0.20	3	n.d.	n.d.	2
amikacin	4300 ± 1300	0.99 ± 0.20	3	1971 ± 755	1.03 ± 0.02	2

[a] Experimental conditions were as described under Materials and Methods. The binding parameters for [^{125}I]-ω-CTx-MVIIC are given in the text. For N-type channel labeling with [^{125}I]-ω-CTx-GVIA we measured a subpicomolar K_d in saturation and kinetic experiments (K_d = 0.1−0.2 pM; n = 2), in good agreement with an earlier study (Feigenbaum et al., 1988).

affinity for the N-type selective toxin ω-CTx-GVIA (IC_{50} > 20 nM, n = 2) was also in accordance with the binding profile observed previously for [^{125}I]-ω-CTx-MVIIC labeling (Hillyard et al., 1992; Liu et al., 1996; Hammond et al., 1987). From association experiments an association rate constant (k_{+1}) of (1.96−4.06) × 10^7 M^{-1} s^{-1} and a dissociation rate constant (k_{-1}) of (1.07−1.17) × 10^{-4} s^{-1}, n = 2) were calculated. The K_d derived from the mean of these kinetic constants ($K_d = k_{-1}/k_{+1}$ = 3.7 pM) was in good agreement with the K_d measured in equilibrium binding studies.

As shown in Figure 2 and Table 1, all aminoglycosides tested were potent blockers of [^{125}I]-ω-CTx-MVIIC binding to P/Q-type channels. Neomycin and kanamycin A were the most potent inhibitors, with low nanomolar IC_{50} values. Streptomycin, tobramycin, gentamicin, and netilmicin also blocked with submicromolar IC_{50} values (177−224 nM). Amikacin was the least potent inhibitor. Inhibition by all aminoglycosides was complete and occurred with slope factors close to unity (Table 1). No significant binding inhibition was found for other antibiotics (1 μM concentrations of penicillin G, cephalothin, lincomycin, tetracycline, and chloramphenicol; data not shown).

Aminoglycosides are known modulators of N-type Ca^{2+} channels (Wagner et al., 1987; Knaus et al., 1987). To compare their apparent binding affinities for N- and P/Q-type channels, we also measured the IC_{50} for the inhibition of [^{125}I]-ω-CTx-GVIA binding to guinea pig brain membranes. Published data were not used for comparison because of the inadequate assay conditions (high ligand and receptor concentrations with respect to K_d) used in earlier studies (Cruz & Olivera, 1986; Knaus et al., 1987; Wagner et al., 1987). As shown in Table 1, most aminoglycosides were also potent inhibitors of [^{125}I]-ω-CTx-GVIA binding with IC_{50} values in the submicromolar concentration range.

Decrease of Apparent Aminoglycoside Affinity for P/Q-Type Channels by Ba^{2+}. Divalent cations block binding of [^{125}I]-ω-CTx-MVIIC, an organic cation, with submillimolar IC_{50} values (Figure 2B). Monovalent cations (e.g., sodium; Figure 2B) were much less potent, suggesting that inhibition of toxin binding by Ba^{2+} and Ca^{2+} occurs by their interaction with a divalent cation selective site rather than by an increase in ionic strength alone. The presence of millimolar concentrations of Ba^{2+} (or Ca^{2+}) in functional experiments may therefore explain why this toxin blocks P/Q-type channels only with nanomolar to micromolar IC_{50} values (Stea et al., 1994; Sather et al., 1993; Grabner et al., 1996; Lennon et al., 1995) despite picomolar K_d values. Aminoglycosides represent organic cations as well, and their apparent potency as channel blockers may also be affected by the presence of millimolar concentrations of divalent cations. To investigate if divalent cations also decrease the apparent affinity of aminoglycosides for P/Q-type channels at micromolar concentrations, we determined the inhibition of toxin binding by neomycin in the absence and presence of 1 mM Ba^{2+} (Figure 3A). Ba^{2+} was used instead of Ca^{2+} because it was used as a charge carrier in our functional studies (see below). Ba^{2+} (1 mM) increased the IC_{50} value for neomycin by more than 10-fold. To determine the apparent K_i of Ba^{2+} for inhibition of aminoglycoside binding, we measured the effect of different Ba^{2+} concentrations (0.1−1 mM) on the IC_{50} value of neomycin. A Schild plot analysis is illustrated in Figure 3B. The slope of the regression line was close to unity. From the x-axis intercept a K_i for Ba^{2+} of 79 μM was calculated. Interaction of Ba^{2+} with a high-affinity binding site therefore decreases aminoglycoside affinity for P/Q-type Ca^{2+} channels in an apparently competitive manner. This K_i is similar to the IC_{50} values of Ba^{2+} and Ca^{2+} for [^{125}I]-ω-CTx-MVIIC binding inhibition. Therefore, the decrease of neomycin potency is most likely also the result of Ba^{2+} binding to the channel rather than just an ionic strength effect.

Aminoglycosides Block α1A-Mediated Ca^{2+} Channel Currents. When expressed together with auxiliary Ca^{2+} channel subunits (β1 and α2-δ) in *Xenopus* oocytes, α1A subunits

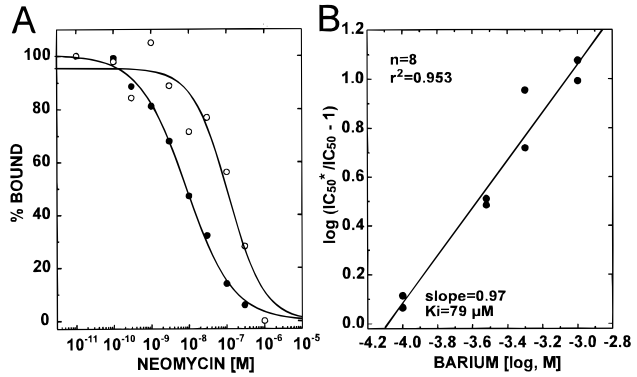

FIGURE 3: Decrease of apparent aminoglycoside affinity by Ba^{2+}. The inhibition of [^{125}I]-ω-CTx-MVIIC binding by neomycin was measured in the absence (control) or presence of increasing concentrations of Ba^{2+} in a final assay volume of 4−8 mL. (A) Control specific binding was 1954 cpm in the absence and 217 cpm in the presence of 1 mM Ba^{2+}. The IC_{50} for neomycin was 8.5 nM in the absence and 110 nM in the presence of 1 mM Ba^{2+}. (B) Schild plot analysis: IC_{50} values for neomycin were obtained in the presence of 0.1, 0.3, 0.5, and 1 mM Ba^{2+}. IC_{50}* is the IC_{50} measured at the respective Ba^{2+} concentration. Two independent experiments were performed at each Ba^{2+} concentration. A Schild plot analysis of the data is shown. The slope of the regression line was 0.97 and the pK_i value for Ba^{2+} was −4.1 (79 μM).

form high-voltage-activated Ca^{2+} channels (Sather et al., 1993; Stea et al., 1994; Grabner et al., 1995) sensitive to micromolar concentrations of ω-Aga-IVA and ω-CTx-MVIIC in low Ba^{2+} solutions. Ba^{2+} inward currents elicited by depolarizations from a holding potential of −80 mV to 0 mV are shown in Figure 4A. Superfusion with solution containing 300 μM neomycin reduced peak inward currents to 33% ± 7% (n = 6) of control current within 1 min. Inhibition was reversible and current returned almost to control levels within about 4 min. The same concentrations of streptomycin, tobramycin (Figure 4A) and kanamycin reversibly blocked to 54% ± 7% (n = 5), 61% ± 2% (n = 4), and 66% ± 3% (n = 3) of control current values, respectively. Amikacin (300 μM) was less effective, inhibiting only 28% ± 9% (n = 3) of peak I_{Ba}. As shown for neomycin (100 μM) in Figure 4B, block was observed over the whole voltage range. The neomycin effect was absent in the presence of 40 mM Ba^{2+} in the extracellular solution (not shown), suggesting that apparent aminoglycoside affinity was decreased by divalent cations.

Therapeutically relevant concentrations of aminoglycosides, i.e., concentrations reached in patients' plasma during drug therapy (peak plasma concentrations; Chambers & Sande, 1995) also produced block of α1A-mediated currents (Figure 4C). These concentrations ranged from 5 μM for gentamicin to 70 μM for amikacin. Inhibition of I_{Ba} was found for all tested aminoglycosides and was most pronounced for neomycin and netilmicin.

DISCUSSION

Aminoglycoside antibiotics are known blockers of N-type Ca^{2+} channels in mammalian neurons. This could explain their depression of autonomic nervous transmission (Said et al., 1995), the inhibition of depolarization-induced $^{45}Ca^{2+}$ uptake into synaptosomes (Atchison et al., 1988; Reynolds et al., 1986) and their analgesic effects after injection into the central nervous system (Ocana & Baeyens, 1991; Robles et al., 1992). However, in mammalians the presynaptic inhibition of neuromuscular transmission by aminoglycosides cannot be explained by the block of neuronal N-type channels. Although N-type channels exist in motor neuron nerve terminals (Protti et al., 1991), they are not involved

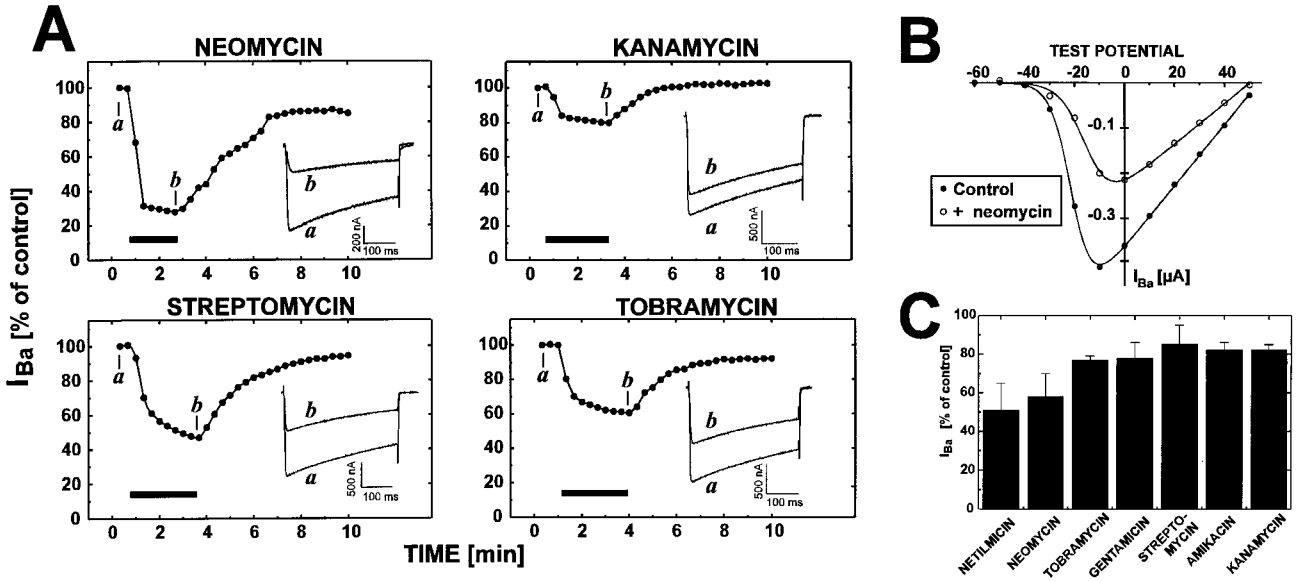

FIGURE 4: Inhibition of α1A-mediated Ca^{2+} channel currents by aminoglycosides. (A) I_{Ba} was elicited by 350-ms depolarizations from a holding potential of −80 mV to 0 mV. Peak inward currents were plotted against time. The bars indicate superfusion of the oocytes with 300 μM of the indicated aminoglycosides dissolved in the bath solution. Drug dilutions were prepared from the free bases of neomycin, kanamycin, and tobramycin. The insets show current traces recorded before starting superfusion with drug (a) and after development of maximal block (b). One of three experiments yielding similar results is shown. (B) $I-V$ curve of α1A-mediated I_{Ba} in the absence and presence of 100 μM neomycin. I_{Ba} were elicited by 300-ms pulses to the indicated test potentials from a holding potential of −80 mV. (C) Inhibition of I_{Ba} by different aminoglycosides at therapeutically relevant concentrations. Note that aminoglycosides do not significantly bind to plasma proteins. Therefore, the tested concentrations reflect pharmacologically relevant (peak plasma) concentrations. These concentrations were as follows (Chambers & Sande, 1995): neomycin, 10 μM; netilmicin, 20 μM; tobramycin, 30 μM; gentamicin, 5 μM; streptomycin, 50 μM; kanamycin A, 60 μM; amikacin, 70 μM. Means ± SD of 3−8 experiments are shown. Inhibition by all drugs (except for streptomycin) was statistically significant (p < 0.05).

in triggering electrically evoked acetylcholine release (Bowersox et al., 1995; Wessler et al., 1990). Instead, recent studies provided convincing evidence that this Ca^{2+} entry is mediated by ω-CTx-MVIIC- and ω-Aga-IVA-sensitive (P- or Q-type) Ca^{2+} channels (Bowersox et al., 1995; Wessler et al., 1995; Hong & Chang, 1995; Sugiura et al., 1995). Here we demonstrate on the molecular and functional level that aminoglycosides interact with P/Q-type Ca^{2+} channels. This explains their presynaptic inhibitory effect on neuromuscular transmission observed in the above studies. Aminoglycosides can bind to [^{125}I]-ω-CTx-MVIIC-labeled α1A subunits with very high affinity and were able to reduce depolarization-induced Ca^{2+} entry through α1A subunits at therapeutically relevant concentrations.

Without added divalent cations, neomycin blocked [^{125}I]-ω-CTx-MVIIC binding to the channel complex in guinea pig cerebellum with an IC_{50} value around 10 nM. With the exception of amikacin, all other aminoglycosides were also potent inhibitors of toxin binding with submicromolar IC_{50} values. Similar apparent affinities were also found for [^{125}I]-ω-CTx-GVIA-labeled N-type channels. These data suggest that the aminoglycoside binding domains formed by α1B (N-type) and α1A subunits are pharmacologically very similar. They are therefore most likely located within structurally conserved regions accessible for these hydrophilic drugs from the extracellular side. Amino acid sequence comparison of α1A and α1B reveals that high sequence homology in extracellularly oriented stretches exists within putative pore-forming regions. This supports earlier assumptions (Suarez-Kurtz & Reuben, 1987) that the aminoglycoside binding site must be located close to the extracellular pore entrance.

A comparison of the IC_{50} values of kanamycin A, tobramycin, and amikacin reveals that minor changes of the ring substitutents lead to pronounced changes in affinity. Replacing two hydroxyl groups in kanamycin A by a hydrogen and an amino group in tobramycin reduces apparent binding affinity for P/Q- as well as N-type channels by about 10-fold. An even more dramatic decrease (about 2 orders of magnitude) was observed by replacing an amino group in kanamycin A by a 4-amino-2-hydroxy-1-oxobutyl side chain, yielding amikacin. This clearly shows that the affinity of these compounds is not only determined by the number of basic nitrogens and reveals a more complex structure−activity relationship.

As observed for N-type channels (Wagner et al., 1987), we found that the block of P/Q-type channel currents requires micromolar concentrations of aminoglycosides. The observed discrepancy between the apparent binding affinities (Table 1) and functionally effective concentrations has also been found for peptide toxins. Although picomolar K_d values were measured for [^{125}I]-ω-CTx-GVIA (N-type) (Feigenbaum et al., 1988; Knaus et al., 1987) and [^{125}I]-ω-CTx-MVIIC (Hillyard et al., 1992; Lennon et al., 1995; Sakurai et al., 1995; this paper) binding in isolated membranes, channel block by these toxins requires at least 100−1000-fold higher concentrations (i.e., nanomolar concentrations; Birnbaumer et al., 1994). As shown here for aminoglycosides, this can, at least in part, be explained by the presence of divalent cations. We found half-maximal reduction of the apparent aminoglycoside affinity at Ba^{2+} concentrations of 79 μM. Thus Ba^{2+} binding to a high-affinity divalent cation binding site must be responsible for this effect. So far such high-affinity sites have only been shown to exist within pore-forming regions of the channel (Yang et al., 1993; Peterson & Catterall, 1995; Mitterdorfer et al., 1995). This further supports the assumption that aminoglycoside binding occurs close to or within the channel pore.

We have found a correlation between the potency for the inhibition of toxin binding and current inhibition for most (high potency for neomycin; intermediate potency for streptomycin and tobramycin, low potency for amikacin) but not all aminoglycosides. For example, kanamycin A was a very potent inhibitor of binding but not of current inhibition (300 μM concentration only blocked about one-third of the current). This could be due either to a differential effect of divalent cations on the apparent potency of different aminoglycosides or to slight differences in the pharmacological properties between [^{125}I]-ω-CTx-MVIIC-labeled channel complexes expressed in guinea pig brain and the channel complex expressed in *Xenopus* oocytes.

Our data demonstrate that aminoglycosides, in addition to N-type channels, are also blockers of P/Q-type channels. Our finding that therapeutic concentrations cause a partial inhibition of P/Q-type Ca^{2+} channels is in accord with the finding that these drugs can decrease the reserve of neurotransmission at the neuromuscular junction. This may lead to neuromuscular block under conditions where transmission is already compromised (e.g., myasthenia gravis). Our findings are also relevant for the interpretation of pharmacologial experiments using aminoglycosides as neuronal Ca^{2+} channel blockers (Ocana & Baeyens, 1991; Robles et al., 1992).

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