Title

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Subconductance Block of Single Mechanosensitive Ion Channels in Skeletal Muscle Fibers by Aminoglycoside Antibiotics

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ABSTRACT The activity of single mechanosensitive channels was recorded from cell-attached patches on acutely isolated skeletal muscle fibers from the mouse. The experiments were designed to investigate the mechanism of channel block produced by externally applied aminoglycoside antibiotics. Neomycin and other aminoglycosides reduced the amplitude of the single-channel current at negative membrane potentials. The block was concentration-dependent, with a half-maximal concentration of ~200 μM. At high drug concentrations, however, block was incomplete with roughly one third of the current remaining unblocked. Neomycin also caused the channel to fluctuate between the open state and a subconductance level that was also roughly one third the amplitude of the fully open level. An analysis of the kinetics of the subconductance fluctuations was consistent with a bimolecular reaction between an aminoglycoside molecule and the open channel ($k_{on} = \sim1 \times 10^6$ $M^{-1}s^{-1}$ and $k_{off} = \sim400$ s^{-1} at -60 mV). Increasing the external pH reduced both the rapid block of the open channel and the frequency of the subconductance fluctuations, as if both blocking actions were produced by a single active drug species with a $pK_a = \sim7.5$. The results are interpreted in terms of a mechanism in which an aminoglycoside molecule partially occludes ion flow through the channel pore.

INTRODUCTION

The blocking actions of many ions and drugs have been interpreted in terms of a physical occlusion of the channel conduction pathway. This intuitively simple picture has proved useful for explaining blocking phenomena in different types of ionic channels (e.g., Armstrong, 1969; Woodhull, 1973; Adelman and French, 1978; Neher and Steinbach, 1978; Coronado and Miller, 1982). There are now, however, a number of experimental observations that cannot be reconciled with this simple physical picture. These include strongly voltage-dependent blocking kinetics that are not correlated with the charge of the blocker (Moczydlowski et al., 1984) and fluctuations of single-channel currents between the open state and a discrete subconductance level (Pietrobon et al., 1989; Lucchesi and Moczydlowski, 1990; Schild et al., 1991). Subconductance fluctuations have been attributed to a number of mecha-

nisms, including a conformational change associated with partial channel closure, rapid changes in the structure of the ion permeation pathway, and a reduction in the concentration of permanent ion at the entrance to the channel. The contribution of these mechanisms to the subconductance fluctuations that have been observed in biological channels is poorly understood.

Mechanosensitive ion channels are found in a wide variety of cells (reviewed by Morris, 1990; Sackin, 1995). The study of mechanosensitive ion channels has been hampered by the lack of pharmacological probes with which to investigate channel structure and function. The aminoglycoside antibiotics have been shown to block mechanotransduction currents at micromolar concentrations in hair cells, the most well-characterized mechanosensory cell (Ohmori, 1985; Kroese et al., 1989). There is, however, no information at the single-channel level on the mechanism by which these drugs inhibit currents through mechanosensitive ion channels. In this paper, we examined the action of various aminoglycosides on single mechanosensitive ion channels in mouse skeletal muscle. The results show that the aminoglycosides produce a fast, voltage-dependent block of the open channel. In addition, some aminoglycosides cause the channel to fluctuate between the open state and a subconductance level. A preliminary report

of this work has appeared as an abstract (Winegar et al., 1992).

METHODS

Single flexor digitorum brevis (FDB) muscle fibres were isolated from 17-d old wild type mice following the procedures outlined in the previous paper (Haws et al., 1995). Isolated fibers were plated into 35 mm plastic Petri dishes coated with Matrigel™ (Collaborative Research, Inc., Waltham, MA) to promote adhesion. Single-channel activity was recorded from cell-attached patches following the method of Hamill et al. (1981). Patch electrodes were pulled from borosilicate capillary tubes (Dynalab Corp., Rochester, NY), the shanks coated with Sylgard® (Dow Corning, Corning, NY) and the tips heat polished. The patch electrode filling solution was a physiological solution containing (in millimolar) 150 NaCl, 5 KCl, 1 MgCl$_2$, 2.5 CaCl$_2$, 10 HEPES, and 17 glucose. The pH was adjusted to 7.5 by adding tetraethylammonium hydroxide (TEA-OH). The bathing solution contained (in millimolar) 150 potassium aspartate, 5 MgCl$_2$, 10 EGTA, 10 HEPES, and 10 glucose. The pH was adjusted to 7.5 with TEA-OH. The isotonic K$^+$ bathing solution was used to zero the cell's resting potential so that the patch potential was the same as the voltage applied to the electrode. The voltage error introduced by this procedure was <5 mV. All experiments were done at room temperature (~21–23°C).

Currents were measured with a List EPC-7 patch clamp amplifier, filtered at 1 kHz with an eight-pole Bessel filter (−3 dB at 3 kHz), and stored on video tape with a 200T PCM data recorder (A.R. Vetter Co., Rebersburg, PA). The current records were later analyzed on a laboratory computer (LSI 11/73, Indec Systems, Sunnyvale, CA). The subconductance fluctuations produced by the various antibiotics were well resolved within the 1-kHz bandwidth of the recording system. Transitions between the two conductance levels were detected as crossings of more than two sample points of the midpoint between the fully and open and subconductance levels. Idealized records of channel activity were generated and compared with the original records. Records in which more than one channel was open at the same time were eliminated from the analysis. Open times were corrected for missed sojourns to the subconductance level when >20% of the events were missed, following the approach described by Colquhoun and Sigworth (1983) and Blatz and Magleby (1988). Openings separated by a missed sojourn to the subconductance level would be detected as a single prolonged event. The ratio of the number of missed substate transitions to the total number of substate events was calculated as

$$1 - \exp\left(-D/\mu_{sub}\right), \qquad (1)$$

where D is the dead time of the recording system (~20 μs) and μ_{sub} is the mean substate duration obtained from the slower exponential component to the histogram of open times. Mean open times were corrected by multiplying the uncorrected mean open time by the ratio given by Eq. 1 and then subtracting the duration of all missed substate transitions.

RESULTS

The activity of single mechanosensitive ion channels was recorded from cell-attached patches on flexor digi-

torum brevis (FDB) muscle fibres. Channel activity appeared as short bursts of inward current that had an amplitude of ~−1.8 pA at −60 mV. This activity was due to the opening of single mechanosensitive ion channels as described previously (Haws and Lansman, 1991; Franco-Obrégon and Lansman, 1994). In these experiments, we studied the effects of the aminoglycosides on the bursts of channel activity that appeared in the absence of an applied pressure stimulus. After the electrode had sealed to the membrane, the pressure in the recording electrode was set to 0 mm Hg. Channel activity was recorded after several minutes when it had settled to a steady level.

Fig. 1 illustrates the blocking actions of the aminoglycosides on the single-channel activity recorded at a holding potential of −60 mV. The patch electrode filling solution was a physiological saline solution. Aminoglycosides were added directly to the electrode filling solution. Single-channel activity was recorded from cell-attached membrane patches. In the absence of aminoglycoside (*top record*, control), channel activity appeared as bursts of openings in which there were many unresolved brief closings. When the patch electrode contained 133 μM gentamicin (Fig. 1, *second record*), the amplitude of the single-channel current was reduced compared with control recordings made with saline alone. The reduction of the amplitude of the single-channel current can be seen by comparing the distribution of current amplitudes (shown to the right of each record). The distribution of the open-channel current was measured during a burst of openings. In the presence of gentamicin, the peak of the amplitude distribution was shifted to a smaller value compared to the peak of the distribution measured in the absence of drug. Fig. 1 shows that streptomycin, dihydrostreptomycin, and neomycin also reduced the amplitude of the open-channel level. Unlike gentamicin, however, these drugs produced a second peak in the amplitude distribution that was smaller than the peak corresponding to the fully open state. The second peak in the amplitude distribution corresponds to the partial closing of the channel to a subconductance level. Neomycin produced the most well-resolved transitions to the subconductance level, which appeared as discrete partial closures (Fig. 1, *bottom record*).

Fast Block of the Open Channel

The first set of experiments examined the mechanism underlying the reduction in the amplitude of the current. Fig. 2 (*top*) shows the single-channel currents recorded with a patch electrode filled with either saline alone (*left*) or with saline and 500 μM neomycin (*right*). Single-channel currents were measured at a holding potential of either +60 (*top records*) or −60 mV (*bottom records*). The amplitude of the outward currents through
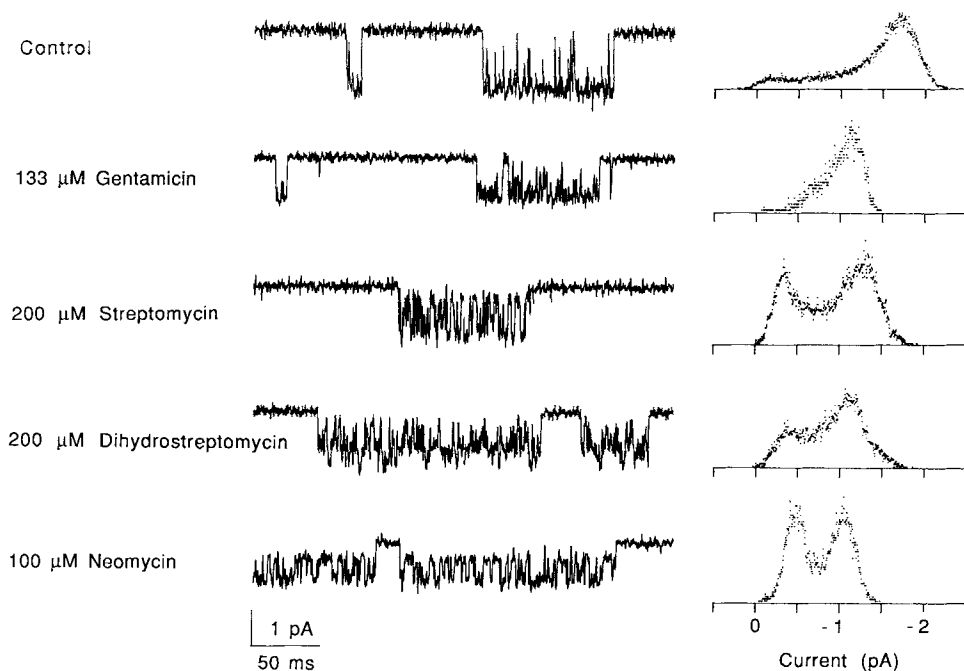
FIGURE 1. Block of single-channel currents through mechanosensitive ion channels by aminoglycoside antibiotics. Each record is from a different cell-attached patch with the indicated concentration of aminoglycoside antibiotic added to the patch electrode filling solution. The holding potential was −60 mV. Records were filtered at 1 kHz and sampled at 5 kHz. The distribution of the amplitude of the open-channel current is shown at the right. There are two peaks corresponding to the full and subconductance levels in the presence of streptomycin, dihydrostreptomycin, and neomycin.

the channel at +60 mV was roughly the same in either the absence or presence of neomycin. At a holding potential of −60 mV, however, neomycin reduced the amplitude of the open channel. Fig. 2 (*bottom*) shows the effect of neomycin on the single-channel current-voltage (*i-V*) relation. The results were obtained in nine experiments with 400 μM neomycin in the patch electrode. In the absence of aminoglycoside (*open symbols*), the single-channel conductance was ∼19 pS and the current reversed at ∼+13 mV. The values of the single-channel conductance and reversal potential are consistent with previous measurements of mechanosensitive channel activity in cultured myoblasts and myotubes (Franco and Lansman, 1990*a,b*) and intact fibers (Haws and Lansman, 1991; Franco and Lansman, 1994). In the presence of 400 μM neomycin, however, the slope conductance for the inward current was reduced to ∼12 pS (*triangles*). In addition, a subconductance level appeared with a conductance of ∼5 pS (*squares*). Fig. 2 also shows that at positive patch potentials, the *i-V* relation in the presence and absence of neomycin were indistinguishable.

The reduction in the amplitude of the single-channel current by neomycin could be due to either a voltage-dependent reduction in channel opening probability or block of the open channel. We tested the blocking model by comparing the experimental results to the predictions of a simple, one-site model. We made use of the approach introduced by Woodhull (1973) and based on Eyring rate theory (Woodbury, 1971). We assumed that the binding affinity of the positively charged aminoglycoside molecule to a site in the channel is a function of voltage. We then obtained the empirical pa-

rameters that describe the voltage dependence of blocker affinity. Although the one-site model makes several simplifying assumptions about the energy profile of the pore and does not take into account the existence of fixed negative charges at the membrane surface (see below), it provided a starting point for comparing the blocking actions of structurally distinct aminoglycosides.

If an aminoglycoside molecule binds to a site in the pore and obstructs the flow of current, then the ratio of the current in the presence and absence of blocker is

$$i/i_{max} = (1/\{1 + [B]/K_D(V)\}) \qquad (2)$$

where i is the current in the presence of drug, i_{max} is the amplitude of the current in the absence of the drug, $K_D(V)$ is the voltage-dependent equilibrium dissociation constant and $[B]$ is the blocker concentration. Eq. 2 can be linearized to fit the voltage-dependent reduction of the single-channel current (Coronado and Miller, 1982):

$$\ln[(i_{max}/i)-1] = (\ln[B]/K_D(0)-z\partial FV/RT) \qquad (3)$$

where $K_D(0)$ is the equilibrium dissociation constant at 0 mV, z is the valence of the blocking particle, ∂ is the fraction of the membrane field the blocking particle must traverse to reach its binding site, and F/RT has its usual thermodynamic meaning. Fig. 3 shows the data obtained in the presence of 400 μM neomycin plotted according to Eq. 3. The fit of Eq. 3 gave a zero voltage dissociation constant, $K_{D(0)} = \sim650$ μM and $z\delta = 0.10$. If only one of the charged amino groups interacted with the blocking site ($z = +1$), then the equivalent electrical distance $\delta = 0.1$. If more than one amino
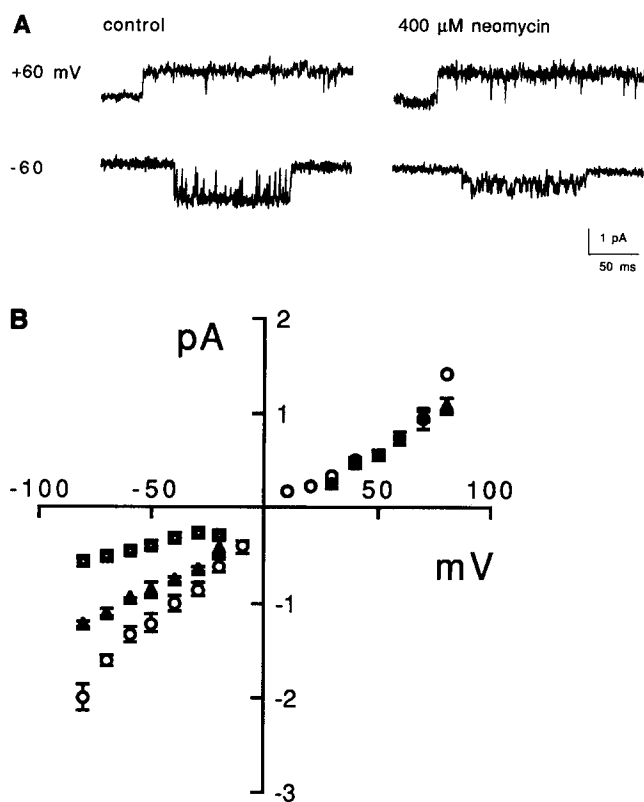
FIGURE 2. Single-channel currents through mechanosensitive channels in the absence and presence of neomycin. (*A*) Records of channel activity at −60 and +60 mV with normal saline in the patch electrode in the absence (control) and presence (400 μM) of neomycin. (*B*) The single-channel *i*-*V* relation in the absence of drug (*open circles*, *n* = 13) and 400 μM neomycin showing the full (*triangles*) and the subconductance level (*squares*). The points are the mean ± SE (*n* = 9). The conductance of the channel in the absence of drug was ∼19 pS (*r*² = 0.99). The conductance of the channel in the presence of 400 μM neomycin was ∼12 pS for the full conductance state and ∼5 pS for the subconductance state (*r*² = 0.99 and 0.97, respectively).

FIGURE 3. Analysis of the voltage dependence of the reduction of the amplitude of the single-channel current by neomycin. Each point is the mean ± SEM (*n* = 9). See text for details.

group interacted with the drug binding site, then the equivalent electrical distance is even smaller. Therefore, the small value of δ indicates that the distance from the external membrane surface to the blocking site must be small.

The block produced by the other aminoglycosides was analyzed in a similar manner. These results are also presented in Table I. The values of $K_{D(0)}$ obtained from the analysis gave a rank order of aminoglycoside blocking potency of dihydrostreptomycin, neomycin > gentamicin > amikacin, streptomycin > kanamycin. In the experiments with dihydrostreptomycin and neomycin, the value of $K_{D(0)}$ increased with aminoglycoside concentration. This suggests that the one-site model cannot provide a complete description of the blocking mechanism. As shown below, the ability of the positively charged aminoglycoside to screen membrane surface charge can account for some of the reduction in
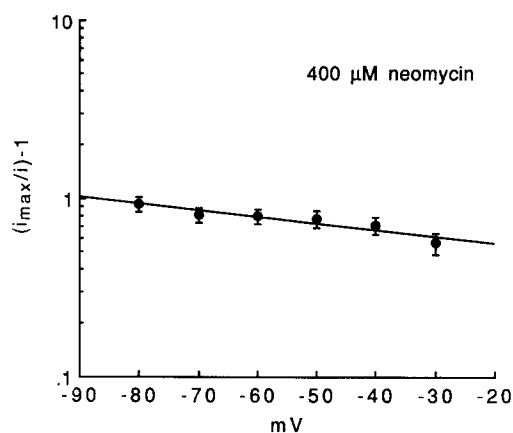
the amplitude of the current. Table I also shows that the products of the valence and the electrical distance, $z\delta$, for each of the antibiotics studied ranged from 0.1–0.3. Since the net charge on these molecules ranges from ∼2-4, the values of δ become too small to compare the locations of the binding site of the various aminoglycosides in any quantitative way. Nonetheless, the results indicate that all the aminoglycosides bind to a blocking site that is located close to the external membrane surface.

To obtain additional information on the mechanism of the fast blocking process, we analyzed the form of the dose-response relation. Fig. 4 shows the effects of the neomycin concentration on the single-channel current. Low concentrations of neomycin (10 μM) caused only a small reduction in the amplitude of the open-channel current and there were few clear excursions to the subconductance level. The effects of neomycin are

TABLE I
Blocking Parameters for Aminoglycosides

Antibiotic	μM	$K_D(0)$ (μM)	$z\delta$	n
Dihydrostreptomycin	50	270	.10	3
	100	505	.20	3
Neomycin	50	35	.15	2
	100	425	.20	2
	400	650	.10	9
Gentamicin	1000	1475	.25	2
	2000	1415	.20	2
Amikacin	100	2675	.20	1
	400	2780	.20	1
Streptomycin	500	2755	.50	2
Kanamycin	800	4010	.30	1
	1000	3490	.25	1

Blocking parameters obtained from the fit to Eq. 3 where $K_D(0)$ is the zero voltage dissociation constant (μM) and $z\delta$ is the blocking valence. The number of experiments (*n*) is indicated in the right-hand column
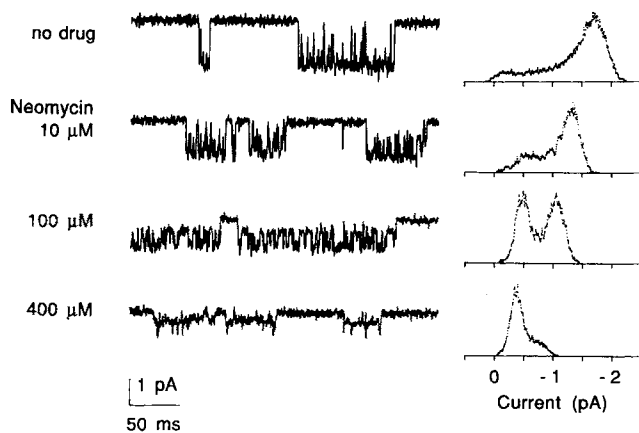
FIGURE 4. The effects of the neomycin concentration on the single-channel activity. The amplitude distribution of the open-channel current is shown next to each record. The patch holding potential was −60 mV. Currents were filtered at 1 kHz and digitized at 5 kHz.
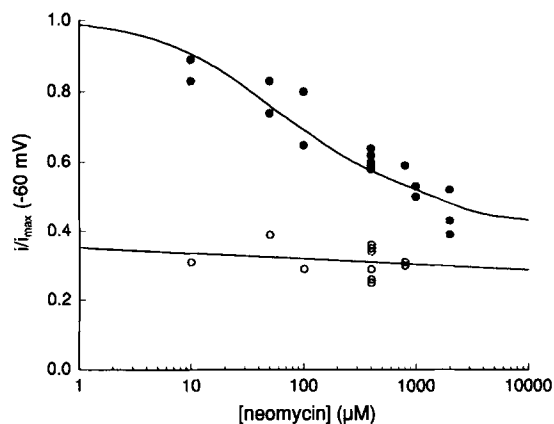

FIGURE 5. Concentration dependence of the reduction of the single-channel current. The amplitude of the single-channel current in the presence of drug normalized to that in the absence of drug (i/i_{max}) for the full conductance state (*filled symbols*) and subconductance state (*open symbols*). Each point represents a measurement from a different patch at the indicated concentration. The smooth curve drawn through the open symbols is the fit to a model in which drug binding to a single site is modified by the presence of a fixed negative surface charge. The straight line through the substate amplitudes (*open symbols*) is the least squares fit to $i/i_{max(substate)} = 0.01 \times \log$ [neomycin] + 0.35. All measurements were made at a holding potential of −60 mV.

best seen in the amplitude distribution of the open-channel current, which is shown at the right of each record. In the presence of 10 μM neomycin, there were far more points at the open-channel level than at the subconductance level. In the presence of 100 μM neomycin, however, the amplitude distribution had two clearly defined peaks, corresponding to the fully open (*right peak*) and subconductance levels (*left peak*). As the neomycin concentration was raised, the peak corresponding to the fully open level shifted to the left towards a smaller value of current. The peak corresponding the subconductance level, however, remained centered near 0.4 pA.

Fig. 5 shows the amplitude of the full and subconductance levels plotted as a function of the concentration of neomycin in the electrode. The current amplitude in the presence of the drug was normalized to the control current in its absence. Increasing the concentration of neomycin in the patch electrode up to ∼1 mM caused a dose-dependent reduction in the current amplitude (*filled symbols*). We found, however, that neomycin did not reduce the current completely at high millimolar concentrations. The dose-response relation reached a nonzero plateau where ∼30–40% of the current remained unblocked. A plateau level in the dose-response relation suggests that binding of an aminoglycoside to the channel might only partially obstruct current flow. Fig. 5 also shows that the amplitude of the subconductance level (*open symbols*) was more or less concentration independent over the range studied.

The dose-response relation for the reduction of the current amplitude was more shallow than would be expected for one-to-one binding. A similar dose dependence was observed for the block of Na+ channels by divalent cations (Green et al., 1987; Ravindran et al., 1991). A dose dependence of this form has been attrib-

uted to the screening of fixed negative charges at the entrance to the channel by the charged blocker. Screening of negative charges would reduce the concentration of permeant ions at the channel entrance, thereby reducing the single-channel conductance. The highly charged aminoglycosides might be expected to screen negative charges, since these drugs bind strongly to negatively charged membranes (Gâbev et al., 1989). We used surface charge theory to predict the effects of reducing the membrane surface charge on the shape of the dose-response relation. The approach follows closely that of Ravindran et al. (1991) and is based on the model of McLaughlin et al. (1981).

Surface charge theory relates the local concentration of blocker, $[B]_s$, to its bulk solution concentration, $[B]_b$, and the membrane surface potential near the blocking site Ψ_s by the Boltzmann relation

$$[B]_s = ([B]_b \exp \{-2F\Psi_s/RT\}. \tag{4}$$

The dose-response relation is obtained by replacing the bulk concentration of the drug, $[B]_b$, in Eq. 2 with its surface concentration, $[B]_s$, obtained from Eq. 4. The surface potential ψ_s is related to the surface charge density s and the concentration of ions in the bulk solution by the Grahame equation

$$s = \pm \{2\epsilon_r\epsilon_o RT \Sigma c_i [\exp(-z_i F\Psi_s/RT) - 1]\}^{0.5} \tag{5}$$

where ϵ_r is the dielectric constant in water, ϵ_o is the permittivity of free space, and the summation is taken over

the concentration, c_i of all ions of valence z. To fit the dose dependence of the fast block, we obtained the best fit for the dissociation constant (K_D) at different values of the surface charge density s. The fit is shown by the solid line in Fig. 5 and was obtained with $s = 1$ $e^-/300$ Å^2 and $K_D = 200$ μM. The value of the surface charge is within the range of values obtained in studies of divalent cation block of Na$^+$ channels (Green et al., 1987; Ravindran et al., 1991). The ability of surface charge theory to account for the form of the dose-response relation suggests that part of the reduction of the current is due to a reduction in permeant ion concentration near the entrance to the channel caused by the screening of fixed negative charges.

Analysis of the Subconductance Fluctuations

The plateau in the dose response relation at high aminoglycoside concentrations suggested that the blocked state has a conductance roughly one-third of the fully open state. Fig. 5 shows that the amplitude of the single-channel current in the presence of neomycin converged to the subconductance level at high concentrations (*open symbols*). Consequently, the subconductance level might also represent the partial occlusion of the channel pore. To investigate the mechanism underlying the subconductance events, we analyzed the kinetics of the transitions between the open and subconductance levels. We assumed that neomycin plugged the open channel, but the plugged channel still allowed current to flow. The mechanism predicts that the distribution of open and substate lifetimes are fit by single exponential functions. Furthermore, the apparent blocking rate, τ_o^{-1}, should increase linearly with the neomycin concentration, while the unblocking rate, τ_{sub}^{-1}, is concentration independent. These predictions were tested by examining the concentration dependence of block.

Fig. 6 A shows the effects of aminoglycoside concentration on the kinetics of the subconductance fluctuations. Fig. 6 A shows the histograms of the open and substate durations that were measured from the currents recorded at -60 mV in the presence of either 50 or 400 μM neomycin. The smooth curves drawn through the histograms show the maximum likelihood fit to the data with the indicated time constants. The histograms of full openings were well fit by a single exponential. The mean open time was $\sim$1.5 and $\sim$0.9 ms in the presence of 50 and 400 μM neomycin, respectively. The histograms of subconductance events were fit best by two exponentials. The faster exponential component fit a small population of very brief subconductance events ($\tau_{sub} = \sim$100 μs). A component with a similar duration appeared in the histograms of closed times that were measured in the absence of drug (data not shown). We did not analyze the distribution of full closures in the presence of aminoglycoside, since they were too infrequent to obtain adequate frequency histograms (e.g., Fig. 4, record with 100 μM neomycin).

Fig. 6 B shows the inverses of the mean open (*filled circles*) and subconductance times (*open circles*) plotted as a function of the neomycin concentration. The inverse of the mean open time varied linearly with concentration and the slope of the least-squares fit gave a second order rate coefficient of $k_{on} = \sim$1 × 10^6 M^{-1}
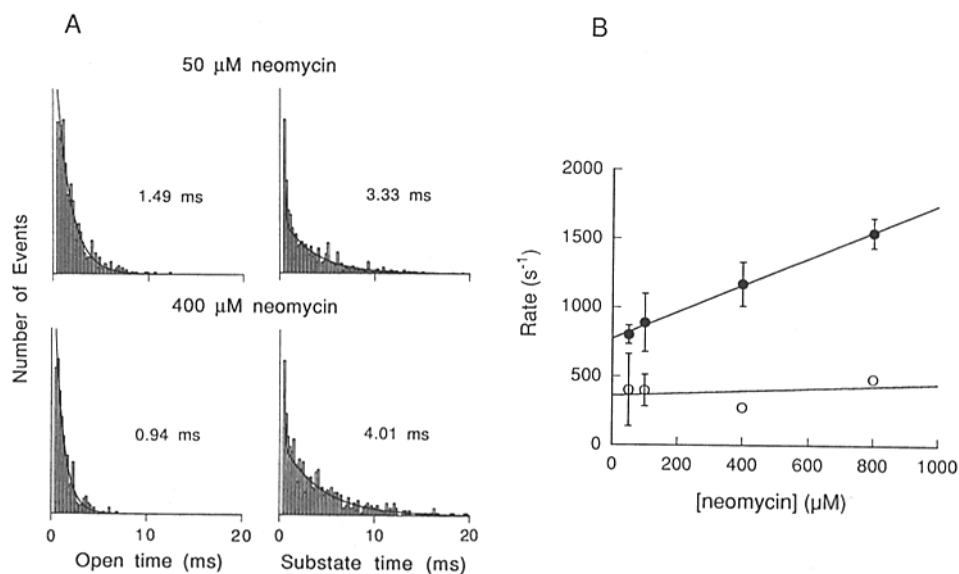

FIGURE 6. Concentration dependence of the durations of the discrete fluctuations between the full and subconductance levels. (A) Histograms of the duration of the full and subconductance states at -60 mV in the presence of 50 μM neomycin (*top*) and 400 μM neomycin (*bottom*). The smooth curves through the histograms represent the maximum likelihood fit to single exponentials for full openings and two exponentials for the subconductance events. The fast component of the histogram of substate durations is likely to represent fast gating transitions which appear in the absence of neomycin. The indicated time constants are the slower component of the histogram of channel dwell times in the subconductance state. (B) The inverse of the time constants measured at -60 mV for full and subconductance states. Each point represents the mean ± SE of two to three experiments. The straight line through the experimental points (*filled circles*) is a least-squares fit to $y = 774 + 0.97$ [neomycin] ($r^2 = 0.99$). The line through the inverse of the substate dwell times is the least squares fit to $y = 0.09$ [neomycin] + 360.
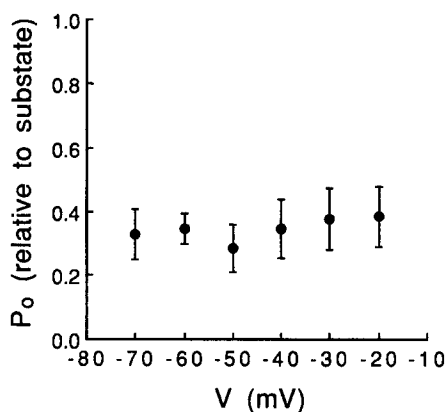
FIGURE 7. Voltage independence of the subconductance fluctuations. Fraction of time spent in the full relative to the subconductance state in the presence of 400 μM neomycin plotted as a function of holding potential. The data points are mean $\pm$ SE ($n = 6$).

S^{-1}. As expected for bimolecular kinetics, substate durations were independent of the neomycin concentration. The slower exponential components that fit the histogram of subconductance times were averaged and the inverse gave an apparent unblocking rate constant $k_{off} = \sim 400 \ s^{-1}$. The apparent dissociation constant K_D obtained from k_{off}/k_{on} was $\sim 350 \ \mu$M at -60 mV. This value is slightly larger than the affinity that was measured from the reduction in the single-channel current ($K_{D(0)} = \sim 200 \ \mu$M).

To determine the location of the aminoglycoside binding site in the membrane field, we examined the voltage dependence of the subconductance fluctuations. We were unable to measure the effects of voltage on the individual subconductance and open state dwell times, because they were poorly resolved at negative potentials at the recording bandwidth. Instead, we measured the steady-state probability the channel was in the fully open state relative to the subconductance level. The open probability was measured within bursts of openings. The open probability measured in this way only reflected the transitions between the fully open and subconductance levels. Fig. 7 shows that the fluctuations between the open and subconductance level showed little sensitivity to membrane potential over the range -20 to -70 mV. The probability of being in the open state (relative to the subconductance state) was $\sim 0.37 \pm .08$ ($n = 6$) in the presence of 400 μM neomycin. This find indicates that there is little or no voltage sensitivity to the subconductance fluctuations.

Effects of External pH

Neomycin can hold up to six positive charges at acidic pH. At physiological pH, neomycin has a net charge of $\sim +4$ (Gâbev et al., 1989). According to the simple blocking model, a change in the net charge of the blocker might be expected to produce a change in the

voltage dependence of the blocking process. We investigated the pH dependence of the block by neomycin to determine whether the reduction of the single-channel current and subconductance fluctuations are sensitive to the net charge on the aminoglycoside molecule.

Fig. 8 shows the single-channel activity recorded with 100 μM neomycin in the patch electrode at three different external pHs. At pH 6.1, the channel spent most of the time at the subconductance level. This can be seen in both the records and the corresponding amplitude distributions. At pH 8.5, by contrast, the channel spent most of the time at the full conductance level and channel activity resembled that recorded in the absence of neomycin. In control experiments, changing the external pH over the range 6.1–8.5 did not produce subconductance fluctuations in the absence of drug (data not shown). Evidently, the blocking potency of neomycin is reduced at high pH. The reduced blocking potency at the higher pH could be due either to the reduced net charge of the blocker, which reduces the energy available for drug binding, or to a reduction in the concentration of a single active drug species. Subsequent experiments tested these mechanisms.

Fig. 9 shows that the inhibition of both the fast block and subconductance fluctuations at high external pH can be overcome simply by raising the neomycin concentration. The pH of the external solution was set to 8.5 in this experiment. 100 μM neomycin produced virtually no block of the single-channel current (Fig. 9, *top record*), even though this concentration of neomycin produced substantial block at physiological pH (Fig. 8). Fig. 9 shows, however, that as the concentration of neomycin is increased, the single-channel current decreased and subconductance fluctuations reappeared. The results show that roughly a 10-fold increase in the neomycin concentration at pH 8.5 is required to produce the
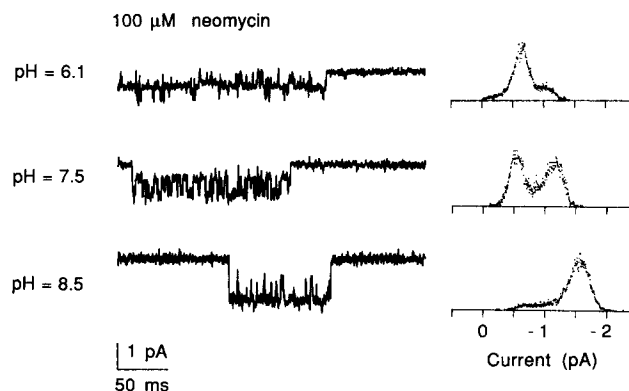

FIGURE 8. pH-dependence of neomycin block of currents through single mechanosensitive channels. The patch electrode contained normal saline with 100 μM neomycin at the indicated pH. The holding potential was -60 mV. The distribution of the amplitude of the open-channel current is shown next to each current record.
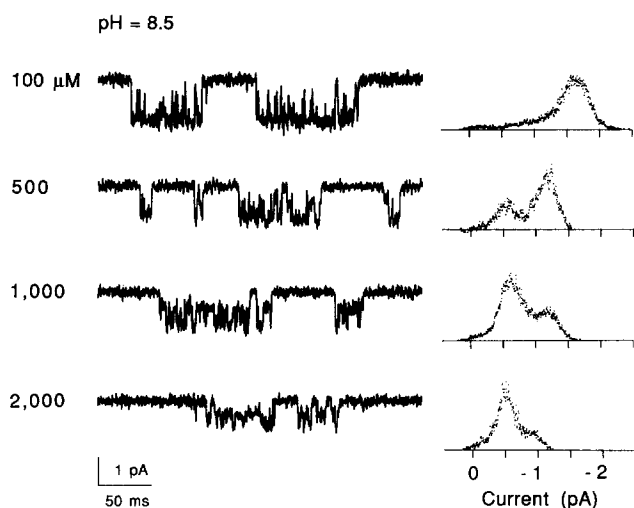
FIGURE 9. Effects of increasing the concentration of neomycin at pH 8.5. The concentration of neomycin is indicated next to each record. The holding potential was −60 mV.
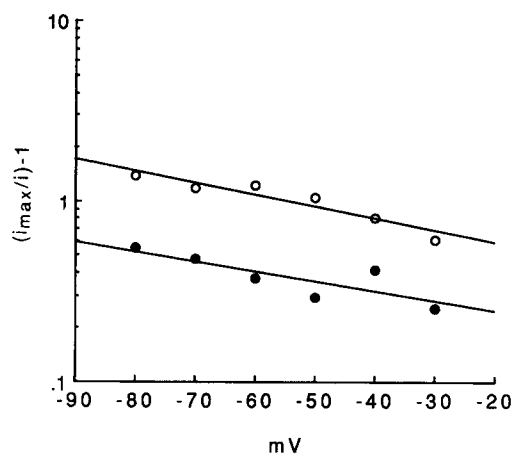

FIGURE 10. Voltage dependence of the fast blocking process at low (pH 6.15) and high (pH 8.5) pH. Block at pH 6.15 (*filled circles*) was measured in the presence of 100 μM neomycin; block at pH 8.5 (*open circles*) was measured in the presence of 2,000 μM. At pH 6.15 and 8.5 the blocking parameters were $K_D(0)$ = 527 and 4,594 μM, respectively, and $z\partial$ = 0.32 and 0.39, respectively.

same amount of block that is observed at pH 7.5. This observation is consistent with the idea that the effect of raising the pH is to reduce the concentration of a single reactive drug species.

If the effect of pH were simply to alter the concentration of active drug species, then the voltage dependence of block would not be expected to vary with pH. On the other hand, a change in the valence of the blocking particle would be expected to produce a large change in the voltage dependence of the blocking process. Fig. 10 shows an experiment in which the voltage-dependence of the reduction of the single-channel current was measured at pH 6.1 (*filled circles*) and 8.5 (*open circles*). The concentration of neomycin was adjusted at each pH so that the current amplitude was reduced by half and the channel spent half the time at the subconductance level. Fig. 10 shows that the voltage dependence of the fast blocking process did not depend strongly on the external pH. Thus, we conclude that the effect of external pH is likely to involve the reduction of the concentration of an active drug species by the titration of a single group with a pKa near 7.5.

DISCUSSION

The results presented in this paper show that aminoglycoside antibiotics produce a rapid and reversible block of single mechanosensitive ion channels. We have provided evidence showing that the inhibitory mechanism consists of both a fast block of the open channel and slower fluctuations of the current between the open state and a subconductance level. These inhibitory effects are produced by the binding of an aminoglycoside to a low affinity site that is located close to the external membrane surface. The concentration of aminoglyco-

side required to block the mechanosensitive channels in skeletal muscle is similar to that reported for the block of the mechanotransduction channels in chick hair cells (Ohmori, 1985). However, the effective concentration is two to three orders of magnitude greater than that required to block mechanotransduction channels in bullfrog saccular hair cells (Kroese et al., 1989). The low aminoglycoside blocking potency may reflect the relatively high concentrations of divalent cations used in this study, which would shift the dose-response relation to higher drug concentrations (Kroese et al., 1989). Despite this difference, the block of mechanosensitive channels in skeletal muscle shows voltage-dependent behavior that is generally similar to the block of the hair channels (Ohmori, 1985; Kroese et al., 1989). Below, the processes that might underlie the fast block and subconductance fluctuations are considered.

Fast Block of the Open Channel

The voltage-dependent block of the single-channel current could be described by the binding of the positively charged aminoglycoside molecule to a site within the membrane field. Although the model assumed an intrapore location for the binding site, direct evidence for such a location is lacking.

The dose-dependent reduction of the current deviated substantially from the expectations of one-to-one binding. Some of the deviation could be explained by the ability of the polycationic aminoglycosides to screen negative charges located close to the entrance of the channel. The fit of the dose-dependence of block to an expression derived from surface charge theory indi-

cated that the screening of a surface charge density equivalent to ~ 1 $e^-/300$ Å^2 contributed to the inhibition of the current. The effects of surface charge, however, do not account for the substantial fraction of the single-channel current that remained unblocked in the presence of high aminoglycoside concentrations. We have attributed the incomplete block to partial obstruction of the pore by an aminoglycoside molecule. Evidence for partial pore obstruction can be obtained from the single-channel i-V relation at negative potentials (e.g., Lane et al., 1991). If an aminoglycoside molecule completely obstructed the flow of current through the channel, the single-channel current would approach zero current at very negative potentials. There was, however, little change in the slope conductance with hyperpolarization over the voltage range studied. As discussed by Lane et al. (1991), a partial occlusion model also predicts that the i-V relations measured in the presence of different drug concentrations will converge to a concentration-independent slope conductance. Further experiments are required to test these predictions.

Mechanism of the Subconductance Fluctuations

We have shown that the subconductance fluctuations can be described as a bimolecular reaction between an aminoglycoside molecule and the open channel. The blocking rate increased linearly with aminoglycoside concentration, giving a second-order rate coefficient $k_{on} = \sim 1 \times 10^6$ M^{-1}s^{-1}. In addition, substate durations were concentration-independent. Subconductance fluctuations have been attributed to a number of different mechanisms (e.g., Dani and Fox, 1991). For example, subconductance transitions may be produced by a purely electrostatic mechanism in which the binding of an aminoglycoside reduces the concentration of permeant ion near the entrance to the channel. According to an electrostatic mechanism, an increase in effectiveness of the blocker in reducing the membrane surface potential would appear as a reduction in the amplitude of the subconductance state. The results showed, however, that the amplitude of the subconductance level is relatively insensitive to external pH. Since the charge of an aminoglycoside and, therefore, its effectiveness in screening surface charge varies exponentially with pH, a purely electrostatic mechanism does not account for the appearance of subconductance events.

An alternative mechanism is that the binding of an aminoglycoside molecule to the channel causes a rapid conformational change in which the single-channel conductance is reduced. Pietrobon et al. (1989) proposed a conformational model to explain the subconductance fluctuations produced by protons in single L-type Ca^{2+} channels. According to their model, both the open state and the substate conformations undergo protonation-deprotonation reactions, with protonation favoring substate formation. The conformational model predicted that the duration of the subconductance events increased with blocker concentration. The results of Pietrobon et al. (1989) showed that the duration of the subconductance events depended on concentration as predicted by the conformational model. Schild et al. (1991) subsequently showed that the substates induced by Zn^{2+} in batrachotoxin-modified Na$^+$ channels were also concentration dependent and adopted a conformational model similar to the one proposed by Pietrobon et al. (1989). In contrast to the results of Pietrobon et al. (1989) and Schild et al. (1991), however, we found that substate durations are independent of drug concentration. This last observation together with the finding that the fast block of the open channel does not reduce the single-channel current below the subconductance level led us to reject the simple conformational model in favor of a partial occlusion model as described below.

A Mechanism for Aminoglycoside Block of Mechanosensitive Channels

The results showed that aminoglycosides rapidly enter the open channel. If it is assumed that the blocked state has a nonzero conductance, then the incomplete block at high concentrations of aminoglycoside would simply reflect the conductance of the partially blocked channel. Because the fast blocking kinetics are not resolved, raising the aminoglycoside concentration causes a graded reduction of the single-channel current from the fully open to the partially occluded level. We suggest that the substate transitions involve a slow conformational change in which the low affinity blocked state converts to a high affinity blocked state. In this sequential model, the rate of substate formation is proportional to the aminoglycoside concentration only at low drug concentrations. At high concentrations, the rate of substate formation saturates as the fast block site becomes fully occupied. We have not observed saturation of the apparent association rate at concentrations of neomycin up to ~ 1 mM, although additional experiments are required to look for saturation at higher concentrations. Because both blocked states have identical conductances, the amplitude of the single-channel current will appear to converge to the substate level at high concentrations.

The block of mechanosensitive channels by aminoglycosides can be compared with the block produced by amiloride. Amiloride produces a voltage-dependent block of the channel by binding to a site that is accessible from the external membrane (Jørgensen and Ohmori, 1988; Lane et al., 1991; Rüsch et al., 1994). At negative potentials, however, block by amiloride is incomplete. Lane et al. (1991) and Rusch

et al. (1994) proposed a model in which hyperpolarization causes a conformational change that allows the subsequent voltage-independent binding of amiloride to the open channel. Because the binding of amiloride is voltage-independent, block becomes independent of membrane potential at very negative potentials. The block produced by amiloride differs from aminoglyco-side block in that amiloride completely inhibits the current at high concentrations (Jørgensen and Ohmori, 1988; Rüsch et al., 1994). The absence of complete block by high concentrations of aminoglycoside, is not easily reconciled with the conformational model of Lane et al. (1991) and Rüsch et al. (1994), unless the blocked state also has a finite conductance.

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