Published in final edited form as:

Neuropharmacology. 2009 ; 57(5-6): 502–505. doi:10.1016/j.neuropharm.2009.07.031.

Calcium-sensing receptor: a high-affinity presynaptic target for aminoglycoside-induced weakness

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Abstract

Administration of aminoglycoside antibiotics can precipitate sudden, profound bouts of weakness that have been attributed to block of presynaptic voltage-activated calcium channels (VACC) and failure of neuromuscular transmission. This serious adverse drug reaction is more likely in neuromuscular diseases such as myasthenia gravis. The relatively low affinity of VACC for aminoglycosides prompted us to explore alternative mechanisms. We hypothesized that the presynaptic Ca^{2+} -sensing receptor (CaSR) may contribute to aminoglycoside-induced weakness due to its role in modulating synaptic transmission and its sensitivity to aminoglycosides in heterologous expression systems. We have previously shown that presynaptic CaSR controls a non-specific cation channel (NSCC) that regulates nerve terminal excitability and transmitter release. Using direct, electrophysiological recording, we report that neuronal VACCs are inhibited by neomycin (IC_{50} $830 \pm 110 \mu\text{M}$) at a much lower affinity than CaSR-modulated NSCC currents recorded from acutely isolated presynaptic terminals (synaptosomes; IC_{50} $20 \pm 1 \mu\text{M}$). Thus, at clinically relevant concentrations, aminoglycoside-induced weakness is likely precipitated by enhanced CaSR activation and subsequent decrease in terminal excitability rather than through direct inhibition of VACCs themselves.

Introduction

Sudden, severe potentially lethal episodes of weakness can be precipitated by antibiotics, including aminoglycosides, and the likelihood of this is substantially increased in the setting of underlying neuromuscular disease (Adams et al., 1984; Pridgen, 1956). Aminoglycosides decrease neuromuscular transmission via both pre- and postsynaptic mechanisms, although since presynaptic mechanisms dominate at lower concentrations, they are thought to be the more clinically relevant (Fiekers, 1983). It has been proposed that weakness develops when aminoglycosides block presynaptic voltage-activated Ca^{2+} channels (VACCs), thereby reducing Ca^{2+} entry and the probability of acetylcholine release (Molgo et al., 1979). Indirect measurements indicated that neuronal VACC currents were inhibited by aminoglycosides supporting this mechanism of action (Dobrev and Ravens, 2003; Redman and Silinsky, 1994). However, the aminoglycoside concentrations used to inhibit VACCs were ~20 times higher

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than those required to block neurotransmission (Fiekers, 1983; Parsons et al., 1992) suggesting that other mechanisms may also be involved.

A potential presynaptic substrate for aminoglycoside-induced inhibition of synaptic transmission is the Ca^{2+} -sensing receptor (CaSR), a G-protein coupled receptor that is localized to central and peripheral axons and terminals (Chen et al., 2006; Ruat et al., 1995; Wang and Bukoski, 1998) and is activated by aminoglycosides (Hammerland et al., 1998; Sakwe et al., 2005). However the small size and poor accessibility of mammalian nerve terminals prevented the direct examination of aminoglycoside action at terminals. Using a modified patch-clamp technique we previously performed direct neocortical terminal recordings and determined that activation of CaSR inhibits a voltage-dependent non-selective cation channel (NSCC) (Chen et al., 2006; Smith et al., 2004). Furthermore, activation of presynaptic CaSR reduces the probability of release of neurotransmitter at excitatory synapses (Phillips et al., 2008). These data support a model in which activation of CaSR inhibits the NSCC current, shortening action potential duration and reducing time for Ca^{2+} entry (Phillips et al., 2008). At the neuromuscular junction such a reduction in Ca^{2+} entry would decrease the probability of transmitter release. Consequently, we hypothesized that CaSR may mediate aminoglycoside-induced weakness in myasthenia gravis. Here, we addressed this hypothesis by comparing the action of the aminoglycoside neomycin on CaSR function in single small nerve terminals and on VACC currents in neuronal soma. By performing a concentration-effect analysis we tested the relative potency of the CaSR-NSCC pathway and VACC to neomycin.

Materials and methods

Synaptosomes

Six to eight week old Sprague-Dawley rats (Sasco, Inc.) were deeply anesthetized with isoflurane and decapitated in accordance with the OHSU Animal Use Committee guidelines. The neocortex was submerged in ice-cold ($\sim 0^{\circ}\text{C}$) 320 mM sucrose solution in a Teflon-glass tissue homogenizer driven at 400–500 RPM, then centrifuged at 3000 g for 3 minutes. The supernatant was then centrifuged at 14600 g for 12 minutes and the upper layer of the resulting pellet resuspended in approximately 2 ml ice-cold sucrose. Before use the synaptosomes were washed with Tyrode solution.

Superior cervical ganglion neuron preparation

Superior cervical ganglia (SCG) were excised from decapitated rats (see above) stripped of connective tissue and the peri-neuronal sheath, and sectioned into pieces. As described previously (Namkung et al., 1998) the pieces were incubated for 15 minutes in 1.5 mg/ml Collagenase in HBSS plus 6 mg/ml BSA, washed, and then incubated for a further 30 minutes in 1 mg/ml Trypsin in HBSS plus 6 mg/ml BSA. The digested ganglia were then gently dissociated by trituration, plated on glass cover slips, and incubated at 37°C in 5% CO_2 for up to two days. Acutely isolated SCG neurons thus prepared have minimal, short processes ensuring accurate voltage control during low resistance whole-cell recording.

Electrophysiology

Recordings were made from single nerve terminals or SCG somata visualized using an inverted microscope (IX70, Olympus). We employed electrodes with resistances 15–40 $\text{M}\Omega$ and 1–3 $\text{M}\Omega$ to make cell-attached recordings from cortical terminals and whole-cell recordings from SCG somata, respectively. Test solutions were continuously applied from a nearby capillary tube and manifold at 1–3 ml/min ($23\text{--}25^{\circ}\text{C}$). In cell-attached recordings from synaptosomes the membrane potential stated is relative to the unknown resting membrane potential (described voltages are pipette potential multiplied by negative one). In synaptosome recordings we employed a Tyrode solution (in mM; 150 NaCl, 4 KCl, 2 CaCl_2 , 2 MgCl_2 10 HEPES, and 10

glucose, at pH 7.35 with NaOH) in the bath and electrode unless otherwise stated. To isolate VACC currents in SCG recordings external CaCl_2 was increased to 10 mM, MgCl_2 removed, NaCl decreased to 140 mM and tetrodotoxin (TTX: Alamaone, Israel) added at 0.5 μM . Pipette solution was (in mM): 120 N-methy-D-glucamine, 20 TEAOH, 11 EGTA, 10 HEPES, 4 MgCl_2 , 1 CaCl_2 , 4 NaATP, 0.3 NaGTP, 14 creatinine phosphate (pH 7.2 with methanesulfonic acid). Voltage-clamp recordings were obtained using Pulse software with an Axopatch 200B (Axon Instruments, Foster City, CA) or EPC-9 (Heka Instruments Inc, MA) amplifier. Leak currents were subtracted with a -p/4 protocol. In SCG neurons series resistance was compensated by 70–85% and membrane potential corrected for measured liquid junction potential. Currents were prefiltered with a 2 or 5 kHz Bessel filter and digitized at 20–100 μs per point.

Analysis

Data were analyzed using custom macros written in Igor Pro (Wavemetrics, Lake Oswego, OR). Dose-response data were fit with:

$$I/I_{\max} = 1 / \{1 + ([A]/IC_{50})^n\} \quad (1)$$

Where I represents current at the end of the voltage step, $I_{\max}$ is the current in the absence of neomycin, A is neomycin concentration, and n represents the Hill coefficient. Mean $\pm$ S.D. values are reported unless otherwise stated.

Results

Inhibition of VACCs by neomycin

We first tested the effects of neomycin on isolated VACC currents in whole-cell voltage-clamp recordings from acutely-dissociated rat SCG somata. Step depolarization from -70 to 0 mV (40 ms) activated an inward VACC current (Fig. 1A) which remained stable over several minutes when repetitively activated every 10 seconds (Fig. 1B). The currents were reversibly inhibited by bath application of the VACC blocker Cd^{2+} (400 μM). Application of a therapeutically relevant concentration of neomycin (0.01 mM) (Dobrev and Ravens, 2003) reduced the inward current at the end of the depolarizing step by $\sim 5\%$ but had little effect on the rising phase of the VACC current. Increasing the neomycin concentration up to 10 mM produced dose-dependent and reversible decreases in the VACC amplitude (Fig. 1). The rate of VACC inhibition was relatively rapid compared with the neomycin application and new steady states were achieved in less than 10 seconds (Fig. 1B). Neomycin reduced VACC current amplitudes with an average IC_{50} of 0.83 ± 0.11 mM ($n = 3$, Fig. 1C).

Neomycin inhibits CaSR-coupled NSCC in synaptosomes

CaSR is activated by aminoglycosides, such as neomycin, in heterologous expression systems (Brown et al., 1993; Quinn et al., 1997). To test if neomycin affected the CaSR in nerve terminals and to determine the concentration-effect relationship we examined its effects on the CaSR-coupled outward current which is present in the vast majority of neocortical terminals (Chen et al., 2006; Smith et al., 2004). The CaSR-coupled outward current was shown to be mediated by NSCCs by virtue of the sensitivity of the reversal potential to $[\text{Na}^+]$ and $[\text{K}^+]$ at the intracellular surface (Chen et al., 2006; Smith et al., 2004). In the illustrated experiment, the outward current activated by a 200 ms depolarization in low bath $[\text{Ca}^{2+}]$ (60 μM) was decreased by 85% by co-application of 300 μM neomycin (Fig. 2A). In the same recording, voltage steps applied every 5 s revealed that the action of neomycin was reversible and dose-dependent over the range 3–300 μM (solid line, Fig. 2B) with kinetics that were relatively fast compared to the duty cycle. Averaged data from 9 nerve terminal recordings exhibited an

IC₅₀ of 20 ± 1 μM for the action of neomycin on NSCC amplitude (Fig. 2C). This IC₅₀ was substantially lower (~40 times) than that observed for the block of VACC in rat SCG neurons (Fig. 1C).

Discussion

Aminoglycosides are clinically important antibiotics that impair synaptic transmission and may lead to sudden, potentially lethal deteriorations in patients with myasthenia gravis (Adams et al., 1984; Argov and Mastaglia, 1979) and in patients with normal neuromuscular function at higher concentrations (Pridgen, 1956). It has been proposed that these aminoglycoside actions are mediated via direct presynaptic VACC block at the motor endplate (Dobrev and Ravens, 2003; Fiekers, 1983; Molgo et al., 1979; Pichler et al., 1996; Redman and Silinsky, 1994). However, the findings that heterologously expressed CaSR is activated by aminoglycosides (Hammerland et al., 1998; Sakwe et al., 2005) prompted us to test their action on CaSR-modulated NSCC in nerve terminals. Using direct, patch-clamp recording from neuronal somata and presynaptic terminals, we demonstrate that neomycin does indeed inhibit VACC currents, but at a much higher concentration than that which blocks the presynaptic CaSR-modulated NSCC. We have previously shown that CaSR activation reduces synaptic transmission (Phillips et al., 2008), and our observation that neomycin affinity for the CaSR-NSCC pathway is forty times greater than for VACC (see Results), leads us to suggest that aminoglycosides may contribute to muscle fatigue and weakness via CaSR activation. Giga-seal formation and the cell-attached configuration of our recordings prevented the neomycin in the recording chamber from directly reaching the NSCC within the membrane patch. This made direct block of the NSCC by neomycin extremely unlikely as a mechanism of action and strongly favoring CaSR activation as the major pathway. Consistent with our findings, others found that 100 μM neomycin blocked VACC currents by ~50% (Pichler et al., 1996) which is substantially higher than the therapeutically relevant concentration (10 μM).

The sensitivity of neurotransmitter release to decreases in external Ca^{2+} (Dodge and Rahamimoff, 1967) arises from reduced Ca^{2+} entry through VACC (Jun et al., 1999; Wheeler et al., 1994) (Fig. 3A). Thus aminoglycoside block of VACC will reduce Ca^{2+} entry during presynaptic action potentials, attenuating synaptic transmission at the neuromuscular junction (Fig. 3C). The mechanism by which CaSR modulates neuromuscular transmission is less straightforward. In central nerve terminals the CaSR-coupled NSCC current is activated by action potential waveforms (Smith et al., 2004). Activation of CaSR by neomycin will inhibit such channels (Fig. 3B), shorten action potential duration (Fatt and Katz, 1951), and thereby reduce the activation of VACC, limiting the amount of Ca^{2+} entering the nerve terminal, and ultimately decreasing vesicular fusion and neurotransmitter release (Phillips et al., 2008). Based on the higher affinity of CaSR for neomycin we hypothesize that at low neomycin concentration CaSR activation and NSCC block will be the major pathway mediating aminoglycoside-induced weakness (Fig. 3B). However at higher neomycin concentrations effects on VACC and CaSR will combine to attenuate synaptic transmission (Fig. 3C).

Aminoglycoside mediated inhibition of synaptic transmission has been shown to increase with repetitive nerve stimulation in animal models of myasthenia gravis (Changqin and Fang, 2005). Similarly, we have reported other CaSR agonists have increasing effects upon repetitive stimulation at central synapses (Phillips et al., 2008). Magnesium, which both blocks VACC (Carbone et al., 1997) and activates CaSR (Brown et al., 1993), has also been shown to inhibit synaptic transmission and precipitate weakness in patients with myasthenia gravis (Lee et al., 1996).

Our study directly examined function of VACC in neuronal somata and CaSR-NSCC in small nerve terminals. A limitation of our study was the assumption that the CaSR-NSCC signaling

pathway that is present in 80% of central terminals (Chen et al., 2006; Smith et al., 2004) is also present at motor neuron terminals. CaSR has been identified in ventral horn and motor neurons by immunochemistry and *in situ* hybridization (Ingleton et al., 2002; Mitsuma et al., 1999) supporting this conjecture. Another important action of the aminoglycosides is the stabilization of phosphatidylinositol 4,5-bisphosphate (Downes and Michell, 1981). However since this effect occurs slowly, requires high concentrations of millimolar neomycin (Goodyear et al., 2008) and *enhances* neurosecretion (Eberhard et al., 1990), it seems unlikely that it would account for aminoglycoside-mediated weakness.

In conclusion, acute, severe muscle weakness is well recognized to arise with excessive aminoglycoside use especially in patients with neuromuscular disorders like myasthenia gravis. Our data indicate that aminoglycosides activate CaSR at low concentrations and inhibit VACC at higher concentrations. These effects may combine to dramatically weaken synaptic transmission and lead to aminoglycoside-induced weakness in myasthenia gravis.

Acknowledgments

This work was supported by supported by OHSU-HHMI Biomedical Support Program and NINDS (R01-NS 43444). The authors report no conflicts of interest.

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Figure 1. Neomycin blocks voltage-activated calcium channels in rat SCG neurons

A, VACC currents recorded at steady state neomycin concentrations (0.01–10 mM) were decreased by increasing external neomycin concentrations and almost completely blocked by cadmium (400 μ M). The 40 ms depolarizing step (–70 to 0 mV) is indicated in the upper panel. Current amplitude and time are indicated by the calibration bars. **B**, diary plot of action of neomycin on VACC current amplitude in the same voltage-clamped neuron as A, shows neomycin and Cd^{2+} reversibly inhibit VACC. Depolarizing steps were applied every 10 seconds and VACC current amplitude at end of step plotted against time. The concentration of neomycin is indicated by the horizontal bars. **C**, concentration-effect relationship for neomycin on VACC currents normalized against current in absence of neomycin. The data

represents mean $\pm$ standard error from 3 neurons. The curve was drawn using equation 1 (Phillips et al., 2008) employing the mean values and gave IC_{50} of 0.83 ± 0.11 mM and Hill coefficient of 0.7 ± 0.1 .

Figure 2. Neomycin and calcium inhibit the CaSR-modulated outward current in single neocortical nerve terminals

A, outward currents activated with 60 μ M bath Ca^{2+} and 150 mV depolarizing step in a cell-attached synaptosome recording were decreased by bath application of neomycin (300 μ M). **B**, diary plot of NSCC amplitude versus time in same recording as **A**. Currents activated by 150 mV depolarization every 5 sec were reversibly decreased by increases in bath $[Ca]$ (60 μ M–6 mM; upper broken trace) and bath neomycin concentration (3–300 μ M; indicated by middle unbroken trace) reaching new steady states in 5–10 s. The Ca^{2+} and neomycin concentration axes are logarithmic and the absence of the unbroken trace indicates neomycin concentration is zero. **C**, concentration-effect relationship for neomycin on CaSR-modulated

NSCC currents normalized against current in absence of neomycin. The data represents mean $\pm$ standard error from 9 nerve terminals. The curve was drawn using equation 1(Phillips et al., 2008) employing the mean values and gave IC_{50} of $20 \pm 1 \mu M$ and Hill coefficient of 0.67 ± 0.02 .

Figure 3. Diagram of proposed model for neomycin modulation of neurotransmitter release via CaSR and VACC

A, model depicts nerve terminal containing CaSR (yellow oval) which is activated by extracellular Ca (red). A proportion of the NSCC are closed (gray) by an unidentified CaSR-mediated signal (I; yellow circle). At physiological $[Ca]_o$, CaSR is not fully activated and during an action potential some active NSCC (yellow) facilitate vesicle fusion with the membrane and transmitter release. **B**, application of a low concentration of neomycin (green circles) and Ca increases CaSR activation reducing NSCC activity and transmitter release. Unlike Ca, neomycin does not enter the terminal and trigger exocytosis directly. **C**, application of neomycin at a higher concentration also blocks VACC, further reducing Ca entry and attenuating transmitter release. Active molecules are colored yellow and inactive molecules are colored gray. The second messenger mediating signaling between NSCC and CaSR has not been identified and is denoted by a small, yellow, intracellular circle.