Aminoglycoside Antibiotics Block Voltage-dependent Calcium Channels in Intact Vertebrate Nerve Terminals

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ABSTRACT Intrinsic and extrinsic optical signals recorded from the intact nerve terminals of vertebrate neurohypophyses were used to investigate the anatomical site and physiological mechanism of the antagonistic effects of aminoglycoside antibiotics on neurotransmission. Aminoglycoside antibiotics blocked the intrinsic light scattering signal closely associated with neurosecretion in the mouse neurohypophysis in a concentration-dependent manner with an IC50 of ~ 60 μM and the block was relieved by increasing [Ca2+]o. The rank order potency of different aminoglycoside antibiotics for blocking neurosecretion in this preparation was determined to be: neomycin > gentamicin = kanamycin > streptomycin. Optical recordings of rapid changes in membrane potential using voltage-sensitive dyes revealed that aminoglycoside antibiotics decreased the Ca2+-dependent after-hyperpolarization of the normal action potential and both the magnitude and after-hyperpolarization of the regenerative Ca2+ spike. The after-hyperpolarization results from a Ca-activated potassium conductance whose block by aminoglycoside antibiotics was also reversed by increased [Ca2+]o. These studies demonstrate that the capacity of aminoglycoside antibiotics to antagonize neurotransmission can be attributed to the block of Ca channels in the nerve terminal.

INTRODUCTION

Aminoglycoside antibiotics are a family of therapeutic agents whose antimicrobial action is mediated by high affinity binding to bacterial ribosomes, and consequent altered protein synthesis. While these compounds are common and very effective drugs, they are not without serious clinical side effects such as muscle weakness, paralysis, respiratory distress, and ototoxicity. Numerous studies have demonstrated the ability of aminoglycosides to depress synaptic transmission at the neuromuscular junction (Vital Brazil and Corrado, 1957; Pittinger, Long, and Miller, 1958; Timmer-
man, Long, and Pittinger, 1959; Elmqvist and Josefsson, 1962; Vital Brazil and
Prado-Franceschi, 1969; Dretchen, Gergis, Sokoll, and Long, 1972; Dretchen, Sokoll,
Gergis, and Long, 1973; Dunkeley, Sanghui, and Goldstein, 1973; Lee, Chen,
Barnes, and Katz, 1976; Wright and Collier, 1977; Prado, Corrado, and Marseillan,
1978; Lee and DeSilva, 1979; Singh, Marshall, and Harvey, 1979; Caputy, Kim, and
Sanders, 1981; Enomoto and Maeno, 1981; Fiekers, 1983a, b; Del Pozo and Baeyens,
1986). This reduction in synaptic transmission can be antagonized by increased
[Ca²⁺]₀, and this observation has led to the suggestion that aminoglycosides inhibit
the influx of Ca ions into presynaptic nerve terminals (Vital Brazil and Prado-
Franceschi, 1969; Wright and Collier, 1977; Prado et al., 1978; Singh et al., 1979;
Fiekers, 1983a, b). In the past, attempts to ascertain the anatomical site and
physiological mechanism of action of aminoglycoside antibiotics on neurotransmis-
sion have often been thwarted by the technical limitations of the neuromuscular
junction preparation. For example, to prevent muscle contraction, synaptic transmis-
sion is frequently reduced, either by lowering [Ca²⁺]₀ or by lowering postsynaptic
responsiveness. These manipulations may alter the effects of aminoglycoside antibi-
otics and render the results difficult to interpret (see Fiekers, 1983a).

In this work we have recorded both intrinsic and extrinsic optical signals from
isolated vertebrate neurohypophyses (Salzberg, Obaid, Senseman, and Gainer, 1983;
Obaid, Orkand, Gainer, and Salzberg, 1985; Salzberg, Obaid, and Gainer, 1985;
Terakawa and Nagano, 1986; Obaid, Flores, and Salzberg, 1989a) in order to
examine unambiguously the effects of aminoglycoside antibiotics on excitation–
secretion coupling at intact nerve terminals. The posterior pituitary (neurohypophy-
sis) is a neurosecretory organ and is thus devoid of any excitable post-synaptic
structures. This large population of synchronously stimulable nerve terminals, which
arises from hypothalamic magnocellular neurons, provides an ideal preparation for
the study of mechanisms of neurotransmitter release (Salzberg and Obaid, 1988). We
report that aminoglycoside antibiotics block the intrinsic light scattering signal
associated with neurosecretion, and reduce the magnitude of certain extrinsic optical
signals recorded from the plasma membrane, namely, the Ca-dependent after-
hyperpolarizations of both the normal action potential and the Ca spike, and the Ca
spike itself, in the posterior pituitary. The inhibitory effects are all relieved by
increased [Ca²⁺]₀. The data demonstrate the capacity of aminoglycoside antibiotics to
block Ca channels required for normal secretion from intact nerve terminals of
vertebrates.

Preliminary reports of this work have appeared (Parsons, Obaid, and Salzberg,
1985; Parsons, Obaid, and Salzberg, 1987).

METHODS

The experimental procedures for recording of both extrinsic and intrinsic optical signals from
neurohypophyses were the same as those described previously (Salzberg et al., 1983, 1985;
Obaid et al., 1985, 1989a). Aminoglycoside antibiotics (Sigma Chemical Co., St. Louis, MO)
were added to the bath solutions to achieve the final concentrations described in the text.
RESULTS

Aminoglycoside Antibiotics Depress Light Scattering Change Associated with Secretion

Optical changes exhibited by the neurohypophysis of the mouse allow us to study neurosecretion with millisecond temporal resolution in the absence of a post-synaptic monitor. These intrinsic optical signals, arising from the neurosecretory terminals, reflect variations in large angle light scattering that occur during stimulus paradigms known to trigger neurosecretion (Salzberg et al., 1985). The intrinsic optical signal, which is measured as a change in transparency, has been shown to be well correlated with the release of neuropeptides from the nerve terminals (Gainer, Wolfe, Obaid, and Salzberg, 1986). The large and rapid changes in light scattering were recorded without averaging during stimulation of the infundibular stalk of the neurohypophysis at 16 Hz for 400 ms (Fig. 1a). The optical response to an individual stimulus consists of at least three separable components. A rapid upstroke (increase in large angle light scattering and decrease in transmitted light intensity), termed the E-wave (Salzberg et al., 1985), signals the arrival of excitation in the terminals, while a large, long-lasting decrease in scattered intensity, the S-wave, reflects some aspect of the secretory event itself. The E-wave appears to include components that depend on both current and voltage (Cohen, Keynes, and Landowne, 1972a, b; Salzberg et al., 1985) and is unrelated to secretion. A third late phase, very much slower than E or S (Obaid, Staley, Shammas, and Salzberg, 1989b) appears to be related to chloride movement across the terminal membrane.

The S-wave is intimately associated with the release of neuropeptides from these terminals. This change in the intrinsic optical properties of vertebrate nerve terminals has already been shown (Salzberg et al., 1985) to exhibit features that are characteristic of neurosecretory systems in general, and the release of neurohypophysial peptides in particular, that is, dependence on stimulation frequency (with marked facilitation), dependence on [Ca^{2+}]_{o}, and sensitivity to Ca^{2+} antagonists and to various interventions (e.g., D_{2}O substitution for water) known to influence secretion.

Fig. 1b shows the apparent inhibition of neuropeptide secretion from the mouse neurohypophysis by 190 μM gentamicin. A marked decrease in the magnitude of the intrinsic optical signal is observed, and is reversed upon wash-out of the drug (Fig. 1c). The amplitude of the E-wave of the intrinsic optical signal is roughly proportional to the number of terminals excited and is probably related to the degree of invasion of the terminal aborization by stimulatory action potentials (Salzberg et al., 1985). Thus, when the records are normalized to the height of this upstroke, the effects of pharmacological agents on secretion per se can be studied. Fig. 1d shows that at this concentration of gentamicin, the downstroke or S-wave is reversibly inhibited by ~40%.

Neomycin, another aminoglycoside antibiotic, has similar effects on the light scattering change associated with secretion at the mouse neurohypophysis. The rapid and reversible inhibition of the intrinsic optical signal can be seen in Fig. 1, f and g. A ~60% depression of the light scattering signal was observed in this experiment (Fig. 1h). Neomycin inhibited the light scattering signal in a concentration-dependent
FIGURE 1.
manner with an apparent IC₅₀ of ~ 60 μM, as determined by a Marquardt fit to the normalized data from two experiments (not shown). At similar concentrations of the antibiotic, neomycin was observed to provide more complete block of the intrinsic optical signal than gentamicin (cf. Fig. 1, d and h). Several additional aminoglycoside antibiotics were also tested for their ability to inhibit the intrinsic optical signal associated with secretion from the mouse neurohypophysis. The following rank order of potency was determined: neomycin > gentamicin = kanamycin > streptomycin.

**Aminoglycoside Antibiotic Reduction of the Light Scattering Change Is Reversed by Ca²⁺**

Fig. 2 demonstrates the capacity of increased [Ca²⁺]₀ to reverse the inhibition by neomycin of the light scattering signal associated with secretion from the mouse neurohypophysis. At physiological concentrations of [Ca²⁺]₀, exposure to 220 μM neomycin reduced the intrinsic optical signal by ~ 40% (Fig. 2 A, traces a and b). This block was completely reversed by raising [Ca²⁺]₀ to 7 mM (Fig. 2 A, trace c). The concentration-dependent reversal of the neomycin block of the intrinsic light scattering by [Ca²⁺]₀ from 1 to 10 mM is shown in Fig. 2 B. Similar antagonism between Ca²⁺ and gentamicin was also observed (data not shown). These findings suggested the presence of a competitive interaction between aminoglycoside antibiotics and [Ca²⁺]₀ in the process of secretion at the intact nerve terminal.

**FIGURE 1. (opposite)** Intrinsic optical signals that accompany secretion from intact nerve terminals in the mouse neurohypophysis are blocked by the aminoglycoside antibiotics gentamicin (A) and neomycin (B). (a) Light-scattering signal resulting from stimulation of the infundibulum of an unstained mouse (CD-1) neurohypophysis at 16 Hz for 400 ms in control Ringer’s solution. A downward deflection of the trace represents an increase in the transparency of the tissue. (b) The effect of an exposure to a Ringer’s solution containing 190 μM gentamicin. (c) Washout of gentamicin after return to control Ringer’s solution. (d) The superimposed, temporal expansion of the light-scattering signal corresponding to the three experimental conditions described above (denoted a, b, and c). These records are the averages of 16 sweeps normalized to the height of the first upstroke (E-wave) of the light-scattering signal. The light-scattering signal closely associated with secretion (S-wave) is reversibly blocked by 190 μM gentamicin. (e) Light-scattering signal resulting from stimulation of the infundibulum of another unstained mouse neurohypophysis at 16 Hz for 400 ms in control Ringer’s solution. (f) The effect of an exposure to a Ringer’s solution containing 220 μM neomycin. (g) Washout of neomycin after return to control Ringer’s solution. (h) The superimposed, temporal expansion of the light-scattering signal corresponding to the three experimental conditions described above (denoted e, f, and g). These records are again the averages of 16 sweeps normalized to the height of the first upstroke (E-wave) of the light-scattering signal. The light-scattering signal associated with secretion (S-wave) is reversibly blocked by 220 μM neomycin to a greater extent than by comparable concentrations of gentamicin. All traces are the analog output of a single representative element of the photodiode array: single sweep (except where noted); 10×; 0.4 n.a.; 675 ± 25 nm; infundibular stimulation; AC coupling time constant, 3 s. In these and all of the following records the response time constant of the optical recording system, (10–90%) was 1.1 ms. Experiments MPP103 (a–d) and MPP096 (e–h).
FIGURE 2. The inhibition by aminoglycoside antibiotics of the intrinsic optical signals that accompany secretion from intact nerve terminals in the mouse neurohypophysis is reversed by increasing [Ca\(^{2+}\)]. (A) Trace a, Light-scattering signal resulting from stimulation of the infundibulum of an unstained mouse (CD-1) neurohypophysis at 16 Hz for 400 ms in control Ringer's solution. Trace b, The effect of an exposure to a Ringer's solution containing 220 \(\mu\)M neomycin. Trace c, Inhibition of the light-scattering signal by 220 \(\mu\)M neomycin is completely reversed by exposure to a 220-\(\mu\)M neomycin solution with a [Ca\(^{2+}\)]\(_{\text{o}}\) of 7 mM. Digital outputs are the sums of nine contiguous elements of the photodiode array, normalized (Salzberg et al., 1985) to the size of the first E-wave in control Ringer's solution: single sweep; 10\(\times\); 0.4 n.a.; 675 \(\pm\) 25 nm; infundibular stimulation; AC coupling time constant, 9 s. Experiment MP276. (B) Concentration-dependent reversal of the aminoglycoside inhibition of light scattering by increasing [Ca\(^{2+}\)]. Block of light-scattering signal is expressed with respect to the control light-scattering signal recorded in normal Ringer's solution ([Ca\(^{2+}\)]\(_{\text{o}}\) = 2.2 mM). Experiment MPP099.
Aminoglycoside Antibiotics Block the Ca-dependent After-Hyperpolarization

The most parsimonious interpretation of the inhibition of neurosecretion in the posterior pituitary by aminoglycoside antibiotics is that the drug acts by antagonizing the entry of Ca\(^{2+}\) ions into the nerve terminal through voltage-dependent Ca channels. Extrinsic optical signals recorded after vital staining of the frog (Xenopus laevis) neurohypophysis with potentiometric probes (Salzberg et al., 1983, Obaid et al., 1985, 1989a) were used to test this hypothesis directly. In Xenopus, the nerve terminal action potential displays a prominent after-hyperpolarization (see Fig. 3a). This undershoot is reduced or eliminated by Ca channel antagonists, is completely blocked by nanomolar concentrations of charybdotoxin, and results from the activation of Ca-mediated potassium channels (Obaid and Salzberg, 1985; Obaid et al., 1989a). These experiments support the notion that the after-hyperpolarization is a very sensitive assay of Ca\(^{2+}\) flux via voltage-dependent Ca channels, especially in the subcellular compartment immediately under the plasma membrane (Obaid et al., 1989a; Roberts, Jacobs, and Hudspeth, 1990). The prominent after-hyperpolarization was also observed in the Ca spike, where it is often exaggerated in comparison to control action potentials (see below, and Fig. 4a). Exposure to 220 \(\mu\)M neomycin resulted in a 90% reduction in the amplitude of the undershoot (Fig. 3b), which

![Figure 3](image-url)
recovered after return to control solutions (Fig. 3 c). In addition, a near doubling in the spike duration was also observed in the presence of the antibiotic. These findings are attributable to a slowing of the repolarizing phase of the action potential due to the reduction in the Ca-activated K conductance(s), and illustrate dramatically the ability of aminoglycoside antibiotics to block the entry of Ca\textsuperscript{2+} into the nerve terminal.

**Aminoglycoside Antibiotics Decrease the Height of the Ca\textsuperscript{2+} Spike**

Under appropriate conditions (Katz and Miledi, 1967), a Ca-dependent action potential, whose depolarizing phase depends on [Ca\textsuperscript{2+}]\textsubscript{o}, and is blocked by several Ca channel antagonists, can be elicited in the frog neurohypophysis (Obaid et al., 1985, 1989a). After the block of voltage-dependent Na channels by tetrodotoxin (TTX) and voltage-dependent K channels by tetraethylammonium (TEA), direct electrical field stimulation of the nerve terminals elicits large active responses of the type recorded in Fig. 4 a. The record shown is the analog output of a single representative element of the photodiode matrix array, photographed directly from the oscilloscope screen without signal averaging. The height of the Ca spike was reduced by nearly 30\% after equilibration of the preparation with 190 \( \mu \)M gentamicin (Fig. 4 b), demonstrating directly the ability of aminoglycoside antibiotics to block Ca channels in these vertebrate nerve terminals. Notice that > 50\% of the Ca-dependent after-hyperpolarization was also blocked by 190 \( \mu \)M gentamicin.

**Aminoglycoside Antibiotic Block of the After-Hyperpolarization Is Relieved by Ca\textsuperscript{2+}**

Fig. 5 shows the capacity of elevated [Ca\textsuperscript{2+}]\textsubscript{o} to reverse the inhibition by neomycin of the after-hyperpolarization recorded optically from the frog neurohypophysis. At
Figure 5. The inhibition by aminoglycoside antibiotics of the after-hyperpolarization of the normal action potential in the intact nerve terminals of the frog neurohypophysis is reversed by increasing [Ca\(^{2+}\)]\(_{o}\). Trace a, The optical recording of the action potential in control Ringer's solution, after staining with NK 2761. Trace b, The action potential recorded after exposure to a Ringer's solution containing 220 μM neomycin. The after-hyperpolarization is almost completely abolished by the aminoglycoside antibiotic. Trace c, Block of the after-hyperpolarization by 220 μM neomycin is partially reversed by increasing [Ca\(^{2+}\)]\(_{o}\) to 5 mM. Trace d, Block of the after-hyperpolarization by 220 μM neomycin is fully reversed by increasing [Ca\(^{2+}\)]\(_{o}\) to 10 mM. Trace e, Upon return to control Ringer's solution, there is an increase in the overall amplitude of the optical signal and a relative decrease in the size of the after-hyperpolarization (see Results). The bar graph summarizes this concentration-dependent reversal of the aminoglycoside block of the after-hyperpolarization by increasing [Ca\(^{2+}\)]\(_{o}\). The ordinate shows the remaining after-hyperpolarization compared with that in normal Ringer's solution ([Ca\(^{2+}\)]\(_{o}\) = 2 mM). This is expressed as a percentage of the ratio of the magnitude of the undershoot to the height of the action potential, in order to compensate for variations in threshold, as well as for dye bleaching, washout, and other nonspecific changes in the signal size over the course of a lengthy experiment. The traces are the analog outputs of a single representative element of the photodiode array; single sweep; infundibular stimulation; 10x; 0.4 n.a.; 700 ± 35 nm, AC coupling time constant, 400 ms. Experiment PPGI83.

Physiological concentrations of [Ca\(^{2+}\)]\(_{o}\), exposure to 220 μM neomycin reduced the undershoot of the extrinsic optical signal by >90% (Fig. 5A, traces a and b). This block was completely reversed by raising [Ca\(^{2+}\)]\(_{o}\) to 10 mM (Fig. 5A, trace d). (Note that variations in the amplitude of the optical signal with [Ca\(^{2+}\)]\(_{o}\) reflect changes in the size of the participating population of terminals, since the action potential recorded optically is a population signal and [Ca\(^{2+}\)]\(_{o}\) modulates threshold [Obaid et al., 1985; Salzberg et al., 1985].) The concentration-dependent reversal of the
neomycin block of the after-hyperpolarization by $[\text{Ca}^{2+}]_o$ from 2 to 10 mM is summarized in the bar graph shown in Fig. 5 B.

**DISCUSSION**

The results presented here demonstrate clearly that aminoglycoside antibiotics impair neurosecretion by blocking the entry of Ca through voltage-dependent Ca channels in vertebrate nerve terminals. Several voltage-clamp studies have shown that aminoglycoside antibiotics can block Ca channels in many nonneuronal cells (Hino, Ochi, and Yanagisawa, 1982; Suarez-Kurtz and Reuben, 1987; Parsons, Lagrutta, White, and Hartzell, 1991 [see their Fig. 9 B]), including skeletal muscle (Suarez-Kurtz, 1974). We have shown here that in the nerve terminals of the neurohypophysis, the effects of aminoglycoside antibiotics are very sensitive to changes in $[\text{Ca}^{2+}]_o$ (Figs. 2 and 5). These results are consistent with observations at the snake neuromuscular junction (Fiekers, 1983a), where some of the complicating factors were controlled.

Since the initial descriptions of multiple types of voltage-gated Ca channels in vertebrate neurons and endocrine cells (Llinas and Sugimori, 1980; Llinas and Yarom, 1981; Carbone and Lux, 1984a, b; Matteson and Armstrong, 1984, 1986; Armstrong and Matteson, 1985; Nowycky, Fox, and Tsien, 1985; Fedulova, Kostyuk, and Veslovsky, 1985; Bossu, Feltz, and Thomann, 1985), there has been great interest in determining if these different types of Ca channels subserve specific cellular functions, e.g., excitation–secretion coupling (Miller, 1987). Our laboratory has recently reported that the Ca channels that dominate the secretory behavior of intact nerve terminals from vertebrate neurohypophyses are blocked, at least partially, by $\omega$-conotoxin and are insensitive, under physiological conditions, to several dihydropyridines. We show here that aminoglycoside antibiotics block both the light scattering signal (Figs. 1 and 2) and the after-hyperpolarization (Figs. 5–5) in a manner similar to $\omega$-conotoxin and funnel web spider toxin (Obaid et al., 1989a; Salzberg, Obaid, Staley, Lin, Sugimori, Cherksey, and Llinas, 1990; Obaid, Komuro, Kumar, Sugimori, Lin, Cherksey, Llinas, and Salzberg, 1990) (cf. Figs. 2 and 4 of Obaid et al., 1989a), except at 10- to 100-fold higher concentrations. In nonneuronal preparations aminoglycosides have been reported to block dihydropyridine-sensitive (Suarez-Kurtz and Reuben, 1987; Parsons et al., 1991) as well as dihydropyridine-insensitive (Suarez-Kurtz and Reuben, 1987) Ca channels. Thus, our findings here are consistent with the observation that, under physiological conditions, the Ca channels that dominate the secretory behavior of intact vertebrate nerve terminals are relatively insensitive to dihydropyridines (Nifedipine, Bay-K 8644).

The polyamine aminoglycoside antibiotics have net positive charges at physiological pH which allow them to bind to membrane phosphoinositides, particularly phosphatidylinositol 4,5-bisphosphate (PIP$_2$), and inhibit the activity of phospholipase C (Rock and Jackowski, 1987). Unpublished experiments from our laboratory do not suggest an important role for either inositol trisphosphate (IP$_3$) or diacylglycerol (DAG) in the evoked release of neuropeptides from the neurohypophysis. Phorbol esters that mimic the effect of DAG by activating protein kinase C had no effect on either the magnitude of the light scattering signal or the waveform of the action potential recorded from the neurohypophysis. Similarly, caffeine, which
mimics the effect of IP₃ by depleting intracellular stores of Ca²⁺, had no effect on the magnitude of the light-scattering signal. Thus, we believe that the effects of aminoglycoside antibiotics on secretion from the neurohypophysis are mediated through a mechanism that does not primarily involve phosphoinositide turnover.

A second consequence of the polycationic nature of the aminoglycosides is their ability to compete with and displace Ca ions at binding sites on lipid monolayers and cell membranes (Sastrasinh, Weinberg, and Humes, 1982; Chung, Kaloyanides, McDaniel, McLaughlin, and McLaughlin, 1985). It has been suggested that this general property of the antibiotics underlies the mechanism of aminoglycoside block of Ca channels (Suarez-Kurtz, 1974; Hino et al., 1982) and that these membrane binding sites are distinct from the high-affinity transition sites within the Ca channel pathway (Suarez-Kurtz and Reuben, 1987). The relatively high IC₅₀ of aminoglycosides and the reversal of the block of the Ca²⁺-dependent optical signals in the neurohypophysis (Figs. 2 and 5) by increased [Ca²⁺]ₐ are at least consistent with the idea that a similar mechanism is responsible for the aminoglycoside block of neurosecretion at vertebrate nerve terminals.

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