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# Decrease in calcium currents induced by aminoglycoside antibiotics in frog motor nerve endings

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1 The effects of the aminoglycoside antibiotics, streptomycin, neomycin and gentamicin were examined on perineural currents and evoked acetylcholine (ACh) release at frog motor nerve endings.

2 In the standard solutions used previously to measure $Ca^{2+}$ currents, streptomycin reduced the peak amplitude of the $Ca^{2+}$ component of the perineural current.

3 In a solution in which changes in both $Ca^{2+}$ currents and evoked ACh release can be recorded simultaneously, both $Ca^{2+}$ currents and evoked ACh release were reduced by aminoglycosides in the potency order neomycin > streptomycin > gentamicin. This potency sequence is similar to that reported previously for these agents as inhibitors of neurally-evoked contractions of mammalian skeletal muscle.

4 These data suggest that the presynaptic inhibitory effects of aminoglycoside antibiotics at the neuromuscular junction occur as a consequence of a reduction in $Ca^{2+}$ currents in the motor nerve terminal.

**Keywords:** Aminoglycosides; calcium channels; calcium currents; motor endplate; myaesthenia gravis; neuromuscular junction; neuromuscular transmission; neurotransmitter release; streptomycin; neomycin; gentamicin

## Introduction

When certain aminoglycoside antibiotics are used clinically under conditions in which the function of the postjunctional endplate membrane is compromised, synergistic blockade of neuromuscular transmission occurs (Pittinger & Adamson, 1972; Burkett *et al.*, 1979). For example, the release of acetylcholine (ACh) is inhibited by streptomycin, neomycin, kanamycin and gentamicin (for review see Mandell & Sande, 1990); such presynaptic effects have led to the paralysis by these drugs of unanesthetized patients afflicted with myaesthenia gravis (Pittinger & Adamson, 1972), a disorder in which the number of post-junctional ACh receptors is reduced (Lopate & Pestronk, 1990).

The neuromuscular blocking effects of these antibiotics, which may be surmounted by increasing the extracellular $Ca^{2+}$ concentrations (see e.g. Singh *et al.*, 1978), are often ascribed to a reduction in $Ca^{2+}$ entry into the nerve ending (Molgo *et al.*, 1979; Atchison *et al.*, 1988). In contrast, it has also been suggested that these drugs could work by competing with $Ca^{2+}$ for ACh release sites (Fieckers, 1983). This latter suggestion provides a viable alternative interpretation of the data, as the presence of spare $Ca^{2+}$ channels would allow a competitive relationship between *intracellular* $Ca^{2+}$ and aminoglycoside drugs to be reflected in the relationship between *extracellular* $Ca^{2+}$ and the drug (Silinsky, 1981). In an attempt to distinguish between these two possibilities, we thus decided to investigate if streptomycin, neomycin, and gentamicin blocked $Ca^{2+}$ channels under conditions in which simultaneous measurements of $Ca^{2+}$ currents and evoked ACh release (i.e. e.p.ps) may be made. The relative potencies of these agents as inhibitors of $Ca^{2+}$ currents were also evaluated and compared with published data on the potencies of these agents in reducing the neurally-evoked twitch of skeletal muscle.

## Methods

Methods for electrophysiological recording and superfusion of solutions were as described previously (Silinsky & Solsona, 1992; Redman & Silinsky, 1993a,b; 1994). Briefly, following immersion anaesthesia, cutaneous pectoris nerve muscle preparations of frog were dissected and superfused with flowing Ringer solution. Neurally-evoked responses ($Ca^{2+}$ currents and e.p.ps) were recorded with conventional electrophysiological equipment (for complete details see Silinsky & Solsona, 1992; Redman & Silinsky, 1993a; 1994). Simultaneous measurements of $Ca^{2+}$ currents and e.p.ps were made after blockade of a proportion of the $K^+$ channels. For the perineural currents, microelectrodes of 5–15 MΩ resistances filled with normal Ringer solution were positioned under visual control near small axon bundles at the termination of the myelin sheaths and within 50 μm of the intracellular recording electrode used for measuring e.p.ps. For further details of the perineural current waveforms and potential sources of contamination of $Ca^{2+}$ currents at frog motor nerve endings, see Silinsky & Solsona (1992), Mallart (1984), Anderson *et al.* (1988) and Molgo *et al.* (1991).

The Ringer solution contained (mM) NaCl 115, KCl 2, HEPES 2 (pH 7.2–7.4), tubocurarine chloride (4–30 mg $l^{-1}$), and various concentrations of $Ca^{2+}$, $Mg^{2+}$, and the $K^+$ channel blockers 3,4,diaminopyridine (DAP) and tetraethylammonium (TEA). A modified Ringer solution, which contained $CaCl_2$ 0.9 mM, $MgCl_2$ 10 mM, DAP 100 μM and TEA 250 μM was used for the experiments in which $Ca^{2+}$ currents were measured simultaneously with e.p.ps ($Ca^{2+}$ current Ringer, e.g. Figure 2). These specific concentrations of $K^+$ channel blockers enabled us to measure changes in ACh release (i.e. e.p.ps) and changes in $Ca^{2+}$ currents simultaneously with minimal complications arising from opposing $Ca^{2+}$-activated $K^+$ currents observed in the absence of TEA and without the profound depletion of ACh release seen in the presence of higher concentrations of $K^+$ channel blockers (see e.g. Anderson *et al.*, 1988). These concentrations of $K^+$ channel blockers also allowed for the detection of both increases and decreases in $Ca^{2+}$ currents as the extracellular $Ca^{2+}$ concentrations were changed accordingly (Redman & Silinsky, 1993a,b). The mean number of ACh quanta released in response to a nerve impulse (m) was estimated using the tubocurarine method in accordance with the following equation:

$$m = \frac{\text{e.p.p. amplitude}}{\text{m.e.p.p. amplitude}} (1 + 4\ [\text{tubocurarine}]) \qquad (1)$$

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where the m.e.p.p. amplitude refers to the mean amplitude of the miniature e.p.ps *in the absence of tubocurarine* and the e.p.p. amplitude reflects the mean e.p.p. amplitude *in the presence of tubocurarine*. Concentrations of tubocurarine are in units of $mg\,l^{-1}$, with the number 4 reflecting the equilibrium affinity of tubocurarine for the ACh receptor in $1\,mg^{-1}$ (see Silinsky, 1981 for further details of this method). In $Ca^{2+}$ current Ringer, the number of ACh quanta released by a nerve impulse as calculated by eqn 1 ranged from 344 to 569 with a mean of $457 \pm 23.8$ (mean $\pm$ s.e.mean, $n = 22$). Streptomycin, which was pilfered with permission from Dr Chau Wu, was generally used at a concentration of 50 $\mu$M. This concentration inhibits evoked ACh release by approximately 50% (Farley *et al.*, 1982) with only minor effects on the postjunctional sensitivity to ACh (Farley *et al.*, 1982; Fieckers, 1983). Neomycin and gentamicin were obtained from Sigma (St. Louis, MO, U.S.A.).

## Results

Figure 1 shows perineural recordings of $Ca^{2+}$ currents (upward deflection) under conditions similar to those used by others to measure these currents in frog motor nerve endings (e.g. Figure 4, Molgo *et al.*, 1991). The solution contained TEA 10 mM, DAP 1 mM and $Ca^{2+}$ 8 mM. Note that streptomycin at a concentration found previously to depress evoked ACh release from frog motor nerve by approximately 50% (Farley *et al.*, 1982), reduced the peak of the $Ca^{2+}$ component of the perineural current without changing the $Na^+$ component. The $Ca^{2+}$ channel blocker, $Cd^{2+}$ (500 $\mu$M, Figure 1), further reduced the amplitude of the $Ca^{2+}$ current beyond that produced by streptomycin.

The experimental conditions of Figure 1 are associated with such high levels of ACh output that rapid depletion of available ACh quanta precludes simultaneous measurements of $Ca^{2+}$ currents and evoked ACh release (Anderson *et al.*, 1988). We have found that Ringer solution containing reduced concentrations of TEA, DAP, $Ca^{2+}$ 0.9 mM and $Mg^{2+}$ 10 mM ($Ca^{2+}$ current Ringer, see Methods) permits changes in $Ca^{2+}$ currents to be measured simultaneously with changes in e.p.ps (Redman & Silinsky, 1993a,b); however, this solution produced repetitive firing of the nerves. Figure 2 shows the typical experimental result. Note that streptomycin reduced both the average $Ca^{2+}$ current (a) and e.p.p. (b) measured simultaneously in $Ca^{2+}$ current Ringer. The prejunctional inhibitory effect of streptomycin on ACh release is responsible for the reduction in e.p.p. amplitude by 50% (Farley *et al.*, 1982). (For a discussion of repetitive firing in such solutions, see Silinsky & Solsona, 1992.) Similar results

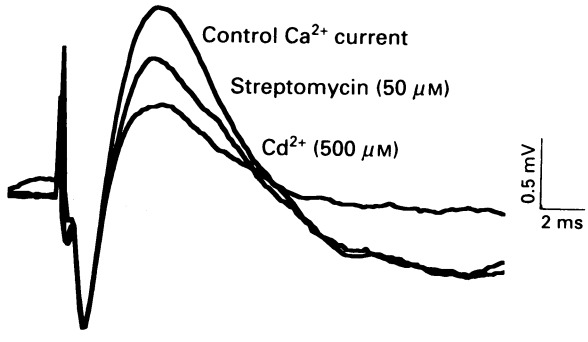


**Figure 1** Effects of streptomycin (50 $\mu$M) on perineural $Ca^{2+}$ currents in a solution containing $Ca^{2+}$ 8 mM, TEA 10 mM and DAP 1 mM. Streptomycin reduced the $Ca^{2+}$ component of the averaged perineural current ($n = 5$ stimuli) to 66% of the control level. $Cd^{2+}$ further reduced the amplitude of the $Ca^{2+}$ current to 25% of the control level. Stimulation frequency 0.015 Hz. Failure of two traces to return to baseline is due to repetitive firing. For abbreviations, see text.

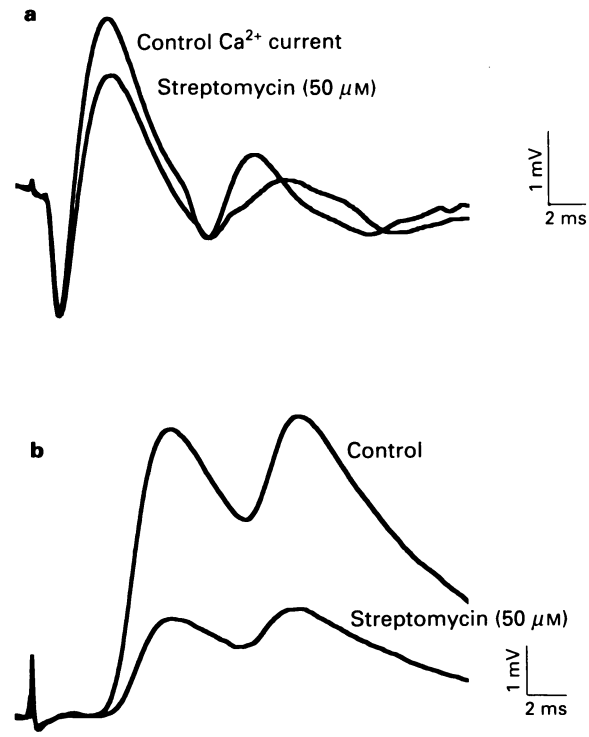


**Figure 2** Effects of streptomycin (50 $\mu$M) on perineural $Ca^{2+}$ currents (a) and evoked acetylcholine release (e.p.ps, b) as measured simultaneously in $Ca^{2+}$ current Ringer (see Methods). Streptomycin reduced the peak $Ca^{2+}$ component of the averaged perineural current ($n = 3$ stimuli) to 67% of the control and the averaged e.p.p. to 34% of the control. For a discussion of the repetitive firing in the presence of $K^+$ channel blockade, see Silinsky & Solsona (1992). Stimulation frequency 0.05 Hz.

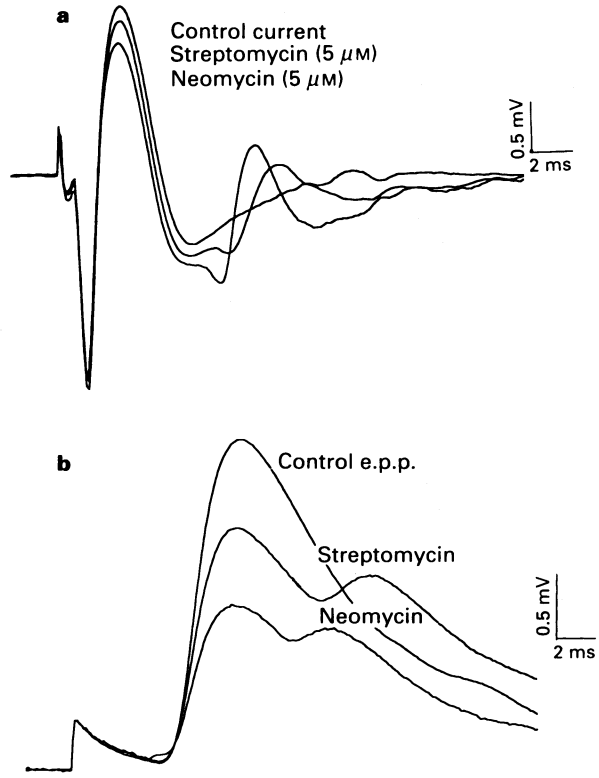


**Figure 3** Simultaneous measurements of the effects of streptomycin and neomycin (5 $\mu$M) on perineural $Ca^{2+}$ currents (a) and evoked acetylcholine release (b). Streptomycin ($n = 14$ stimuli) was applied first, washed off after a maximal response was obtained (approximately 6 min), followed by the application of neomycin ($n = 12$ stimuli).

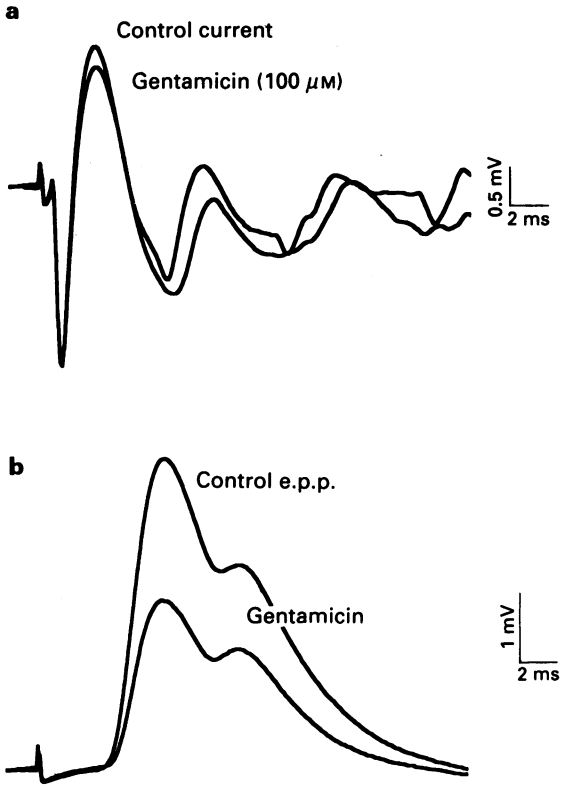


**Figure 4** Effects of gentamicin (100 μM) on perineural $Ca^{2+}$ currents (a) and evoked acetylcholine release (b). Gentamicin reduced the $Ca^{2+}$ component of the averaged current ($n = 6$ stimuli) to 85% of the control and the averaged e.p.p. ($n = 12$ stimuli) to 54% of the control.

were observed in 14 other experiments, in which the inhibitory effects of 50 μM streptomycin on ACh release were associated with decreases in $Ca^{2+}$ currents ranging from 66% to 81% of control. This effect of streptomycin on both ACh release and $Ca^{2+}$ currents can be roughly approximated by a reduction in the $Ca^{2+}$ concentration from 0.9 mM to 0.45 mM in the $Ca^{2+}$ current Ringer.

It would appear of interest to compare the relative potencies of different aminoglycoside antibiotics that impair the twitch of the neuromuscular junction with the potencies of these antibiotics in reducing $Ca^{2+}$ currents. Figure 3 compares the effects of 5 μM neomycin and 5 μM streptomycin on both the $Ca^{2+}$ component of the perineural current and in e.p.p. amplitude. Note that 5 μM neomycin produced a 21% reduction in the $Ca^{2+}$ current and a 52% reduction in the e.p.p. amplitude, whereas, streptomycin at the same concentration produced only an 8% reduction in the $Ca^{2+}$ current and a 30% decrease in e.p.p. A similar high potency of neomycin was seen in three other experiments.

Gentamicin, was the least potent of the tested aminoglycoside antibiotics. It began to inhibit neuromuscular transmission only at concentrations $\geqslant 50$ μM. As Figure 4 shows, a concentration of 100 μM was required to produce a 15% reduction in the $Ca^{2+}$ current and a 45% reduction in the e.p.p. amplitude. A similar low potency of gentamicin was observed in four other experiments.

## Discussion

These results demonstrate a reduction in $Ca^{2+}$ currents by the aminoglycoside antibiotics, streptomycin, neomycin, and gentamicin. The order of potency of these agents, neomycin > streptomycin > gentamicin on $Ca^{2+}$ currents is the same order as that found for these agents to reduce the neurally-evoked twitches of mammalian muscle (Singh *et al.*, 1978). The results thus suggest that the presynaptic neuromuscular blocking effects of aminoglycoside antibiotics are attributable to a reduction in $Ca^{2+}$ entry through voltage-sensitive $Ca^{2+}$ channels. Preliminary experiments in which streptomycin (50 μM) produces reductions of $Ca^{2+}$ currents and e.p.ps in mouse phrenic nerve-hemidiaphragm preparations in a manner similar to the effects shown in Figure 2 in frog provide support for this suggestion.

The $Ca^{2+}$ current studied in this work is also inhibited by ω-conotoxin and by $Cd^{2+}$ and thus represents the $Ca^{2+}$ current flowing through N-type channels that is responsible for evoked ACh release in frog (Molgo *et al.*, 1991; Silinsky & Solsona, 1992; Redman & Silinsky, 1993a). It is of interest that aminoglycoside antibiotics have been shown previously to inhibit the binding of the $Ca^{2+}$ channel blocker, ω-conotoxin to neuronal membranes (Knaus *et al.*, 1987).

In conclusion, this paper provides the first direct electrophysiological evidence in support of the hypothesis that aminoglycoside antibiotics at therapeutic concentrations, inhibit ACh release from vertebrate motor nerve endings by reducing $Ca^{2+}$ entry through voltage-gated $Ca^{2+}$ channels.

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