Antinociceptive potency of aminoglycoside antibiotics and magnesium chloride: a comparative study on models of phasic and incisional pain in rats

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Abstract

A close relationship exists between calcium concentration in the central nervous system and nociceptive processing. Aminoglycoside antibiotics and magnesium interact with N- and P/Q-type voltage-operated calcium channels. In the present study we compare the antinociceptive potency of intrathecal administration of aminoglycoside antibiotics and magnesium chloride in the tail-flick test and on incisional pain in rats, taken as models of phasic and persistent postsurgical pain, respectively. The order of potency in the tail-flick test was gentamicin (ED$_{50}$ = 3.34 µg; confidence limits 2.65 and 4.2) > streptomycin (5.68 µg; 3.76 and 8.57) = neomycin (9.22 µg; 6.98 and 12.17) > magnesium (19.49 µg; 11.46 and 33.13). The order of potency to reduce incisional pain was gentamicin (ED$_{50}$ = 2.06 µg; confidence limits 1.46 and 2.9) > streptomycin (47.86 µg; 26.3 and 87.1) = neomycin (83.17 µg; 51.6 and 133.9). The dose-response curves for each test did not deviate significantly from parallelism. We conclude that neomycin and streptomycin are more potent against phasic pain than against persistent pain, whereas gentamicin is equipotent against both types of pain. Magnesium was less potent than the antibiotics and effective in the tail-flick test only.

Key words
- Gentamicin
- Neomycin
- Streptomycin
- Magnesium
- Antinociception
- Aminoglycosides
- Pain

Introduction

Calcium ions (Ca$^{2+}$) play an important role in nociception regulation (for a review, see 1). Elevation of Ca$^{2+}$ concentration in the brain produces hyperalgesia (2,3) and antagonizes opiate analgesia (4,5). In contrast, intracerebral administration of Ca$^{2+}$ chelators causes antinociception (5,6) and potentiates opiate analgesia (3,7,8).

A transient increase in cytosolic Ca$^{2+}$ levels represents a key step for neurotransmitter release (for a review, see 9). Ca$^{2+}$ influx occurs mainly through L-, N-, P/Q-, and T-type voltage-operated calcium channels (VOCC) (for a review, see 1,10). Antagonists of L-type VOCC potentiate opiate analgesia in rodents, but this effect depends on the route of administration and the algesimetric test used (for a review, see 11). Mibeferdin (12), a selective T-type VOCC antagonist, does not alter the response of mice in the tail-flick test. Intrathecal administration of $\alpha$-agatoxin IVA, a selective an-
agonist of P/Q-type VOCC, reduces the first phase and blocks the second phase of the rat response to formalin (13). In contrast, N-type VOCC antagonists are analgesic in several models of pain (for a review, see 11). According to Malmberg and Yaksh (13), N-type, and possibly P/Q-type, but not L-type VOCC antagonists have a selective inhibitory effect on nociceptive transmission at the spinal cord level.

Aminoglycoside antibiotics such as streptomycin, gentamicin, kanamycin, neomycin and amikacin act as competitive antagonists of the cellular influx of Ca^{2+} in several biological processes (for a review, see 14). These drugs act as antagonists on N- (15,16) and P/Q-type (17) VOCC. A marked similarity between the effects of aminoglycoside antibiotics and magnesium ions, as well as a prompt antagonism of their effects when the extracellular Ca^{2+} concentration is increased, have been demonstrated earlier in neuromuscular transmission (for a review, see 11), and confirmed in models of artificial membranes, in rodent cardiac and smooth muscles, and isolated mast cells (for a review, see 18). Antinociception following gentamicin has been demonstrated in rodent models of phasic or persistent pain (19-22). Intrathecal neomycin (22), or subcutaneous or intraperitoneal amikacin (23) also produced antinociception in the mouse writhing test.

The present study compares the ED_{50} of intrathecal administration of streptomycin, neomycin, gentamicin, and magnesium chloride needed to change the nociceptive thresholds of rats in models of phasic (tail-flick test) and persistent (incisinal) pain.

**Material and Methods**

**Animals**

Adult male Wistar rats (220-250 g) were individually housed in cages with water and food available *ad libitum*, under controlled light and temperature conditions (12-h light-dark cycle, with the dark cycle beginning at 7:00 h). All tests were performed in the morning. The study was conducted in accordance with the IASP guidelines on the use of laboratory animals. Each rat was used in only one test and on only one occasion.

**Intrathecal catheterization**

Each animal was placed in a closed box containing 3% halothane in oxygen for induction of anesthesia. Anesthesia was then maintained via a loose-fitting, cone-shaped mask through which 1.5% halothane in oxygen was passed. With the rat in the prone position, the back was shaved and a 5.8-cm long, 20-gauge Weiss needle was introduced through the skin into the L5-L6 intervertebral space. The correct subarachnoid positioning of the needle was assured by a typical flick of the tail or hind paw. A 9-cm long catheter (PE tubing, OD = 0.4 mm, dead space = 10 μl) was then introduced through the needle to protrude 1.5 cm into the subarachnoid space in a cranial direction. The needle was carefully removed and the catheter was gently fixed to the paravertebral musculature with a cotton thread ligature. Anesthesia was discontinued and the animal allowed to recover. Only animals showing no sign of motor impairment were utilized for further experimentation. Each experiment was then initiated 2 h after these procedures.

**Tail-flick test**

The animal was placed in a glass tube for about 15 s, with its tail lying across a nichrome wire coil maintained at room temperature (23 ± 2°C). The coil temperature was then raised by the passage of an electric current which was adjusted to ensure a tail withdrawal reflex within 2.5-3.5 s. A cut-off time of 6 s was established to minimize the probability of skin damage. Animals were tested every 5 min until a stable baseline was ob-
tained over three consecutive trials. Only rats showing a stable baseline latency after six trials were used in the experiments. After these procedures, saline or a calcium antagonist was injected intrathecally and tail-flick latency was measured at 5-min intervals for up to 60 min.

**Model of incisional pain**

Soon after the procedures for intrathecal catheterization and still under halothane anesthesia, the plantar aspect of the right hind paw of each rat was sterilized with a 10% povidone-iodine solution. A 1-cm longitudinal incision was made with a surgical blade through the skin and fascia of the plantar region, starting 0.5 cm from the proximal edge of the heel. The plantaris muscle was elevated, but its origin and insertion were left intact. After hemostasis, the skin incision was closed with two 5-0 nylon sutures. After this procedure, anesthesia was discontinued and the animal allowed to recover in its home cage for a period of 2 h. Details of this model have been described elsewhere (24).

**Nociceptive testing of post-incisional mechanical threshold**

Mechanical threshold was measured with von Frey filaments (Stoelting). Rats were placed in an elevated clear plastic cage with a nylon mesh bottom, which allowed easy access to the paw plantar surface. Before the experiment, the animals remained in the cage for approximately 15 min for behavioral accommodation. The area tested was the midplantar right hind paw and the baseline mechanical threshold was determined 2 h after the incision. The paw was touched with one of a series of 15 von Frey filaments with logarithmically incremental stiffness (0.0275 and 75.858 g, lower and upper limit of the test, respectively). Each filament was applied from underneath the nylon mesh floor, through the mesh, vertically to the plantar surface with sufficient force to bend the filament a little. A single trial consisted of six applications of a particular filament, applied once every 3-4 s. Testing was initiated with the 2.041 g filament which is in the middle of the series. A response was defined as a withdrawal of the stimulated paw. In the absence of a response to a particular filament, the next stronger filament was used; in the case of a response, the next weaker filament was applied. The up-down method was used to record the threshold (25). Five minutes later, saline or a calcium antagonist was injected intrathecally and the thresholds were determined shortly after the injection and then at 10-min intervals for up to 40 min.

**Drugs**

The following drugs were used: gentamicin, neomycin, and streptomycin sulfates, kindly supplied by Fontoura-Wyeth (São Paulo, SP, Brazil), and magnesium chloride from Merck (Rio de Janeiro, RJ, Brazil). All doses refer to the salt. Drugs were diluted in saline (10 μl) and injected intrathecally at a constant rate (1 μl/10 s). The catheter was then flushed with 10 μl of drug-free saline delivered at the same rate.

**Data analysis and statistics**

The tail-flick latencies were plotted as means ± SEM. The time course of the effects of different drug doses was analyzed by multivariate analysis of variance (MANOVA). The factors analyzed were treatment, time, and treatment x time interactions. In the case of significant treatment x time interactions, one-way ANOVA followed by the Duncan test was performed for each time point. The level of significance was set at P<0.05.

The mechanical thresholds (in g) are reported as medians and corresponding confidence limits (95%) and are presented in graphs as log of mean ± SEM. The experi-
mental groups were compared by the non-parametric Kruskal-Wallis test and differences between the data at each time point were compared by the Mann-Whitney test. The level of significance was set at P<0.05.

ED_{50}, i.e., the dose of drug producing an antinociceptive effect in 50% of the animals in an experimental group, was estimated by the method of Litchfield Jr. and Wilcoxon (26). For calculation, antinociception was arbitrarily considered to occur whenever latency ≥4.0 s (mean baseline tail-flick latency + 7 SD) or mechanical threshold ≥5.72 g (mean baseline threshold + 7 SD; incisional pain test) was obtained. Dose-response curves were constructed for each drug in each experimental model, and the slope function ratio and corresponding factor for the slope ratio for each curve were calculated by the method of Litchfield Jr. and Wilcoxon (26). The level of significance was also set at P<0.05.

Results

The intrathecal administration of increasing doses of gentamicin (Figure 1A), streptomycin (Figure 1B), neomycin (Figure 1C), or magnesium chloride (Figure 1D) produced a slow onset and dose-dependent increase in the latency for tail withdrawal due to noxious heat on the skin. The peak effect was
observed 10 min after drug administration in all cases. MANOVA applied to the data in Figure 1 revealed significant differences between treatments ($F_{3,25} = 7.7$, $P = 0.001$, in A; $F_{3,27} = 2.98$, $P = 0.049$, in B; $F_{3,27} = 3.02$, $P = 0.04$, in C; $F_{3,24} = 6.44$, $P = 0.002$, in D) and significant time x treatment interaction ($F_{45,375} = 5.21$, $P<0.001$, in A; $F_{45,405} = 2.52$, $P<0.001$, in B; $F_{45,405} = 3.39$, $P<0.001$, in C; $F_{45,360} = 5.39$, $P<0.001$, in D).

The intrathecal administration of increasing doses of gentamicin (Figure 2A), streptomycin (Figure 2B), or neomycin (Figure 2C) reduced incisinal allodynia in a dose-dependent manner. The onset of this effect was slower than in the tail-flick test so that peak effects were obtained 20 min after drug administration. The Kruskal-Wallis test revealed significant differences between treatments and the corresponding control (saline) in all cases. The incisinal allodynia was not modified significantly by the intrathecal administration of magnesium chloride (50 to 400 µg). For this reason, a time-course curve for the effects of this salt in the model of incisinal pain was not constructed. A discrete motor deficit was observed only following the higher dose of magnesium.

The antibiotics and magnesium chloride also dose-dependently increased the percentage of rats showing antinociception in the tail-flick test at 10 min after drug administration (Figure 3A). The ED$_{50}$ (corresponding confidence limits in parentheses) was 3.34 µg (2.65 and 4.2) for gentamicin, 9.22 µg (6.98 and 12.17) for neomycin, 5.68 µg (3.76

**Figure 2.** Time course of the effects of intrathecal administration (vertical arrow) of gentamicin (A), streptomycin (B) and neomycin (C) on the incisinal paw withdrawal threshold to mechanical stimuli in rats. The upper limit (horizontal arrow) representing the maximum force (75.858 g) used in the test is indicated in the upper left corner. Points are means ± SEM. Doses and number of rats are indicated in the upper right corner. *P<0.05 compared to saline (Mann-Whitney test).

**Figure 3.** Dose-response regression lines obtained from the experiments in the tail-flick test (A) and incisinal pain model (B). Responses are given as percentage of rats showing antinociception recorded 10 and 20 min after drug administration in the tail-flick test and incisinal pain model, respectively.
and 8.57) for streptomycin, and 19.49 µg (11.46 and 33.13) for magnesium chloride. The straight lines obtained for gentamicin and neomycin had a smaller slope function ratio (S.R. = 1.1) than the corresponding factor for slope ratio (f_{S.R.} = 1.48), thus indicating that they do not deviate significantly from parallelism. A similar conclusion was reached when comparing gentamicin with streptomycin (S.R. = 1.42, f_{S.R.} = 2.2) or magnesium chloride (S.R. = 1.63, f_{S.R.} = 2.5), when comparing neomycin with streptomycin (S.R. = 1.28, f_{S.R.} = 2.25) or magnesium chloride (S.R. = 1.47, f_{S.R.} = 3.7), or when comparing streptomycin with magnesium chloride (S.R. = 1.14, f_{S.R.} = 3.2). Gentamicin was 2.76 times more potent than neomycin (1.9 and 4.0), 1.7 times more potent than streptomycin (1.06 and 2.72), and 5.83 times more potent than magnesium chloride (3.23 and 10.5). Magnesium chloride was 2.11 times less potent than neomycin (1.14 and 3.9) and 3.43 times less potent than streptomycin (1.75 and 6.7). Streptomycin was more potent than neomycin but the difference was not statistically significant (potency ratio = 1.62; confidence limits 0.98 and 2.67).

The antibiotics also increased in a dose-dependent manner the percent of rats showing antinociception in the model of incisional pain at 20 min after drug administration (Figure 3B). The ED_{50} (confidence limits in parentheses) was 2.06 µg (1.46 and 2.9) for gentamicin, 83.17 µg (51.6 and 133.9) for neomycin, and 47.86 µg (26.3 and 87.1) for streptomycin. The straight lines obtained for gentamicin and neomycin had a smaller slope function ratio (S.R. = 1.13) than the corresponding factor for the slope ratio (f_{S.R.} = 1.95), thus indicating that the lines did not deviate significantly from parallelism. A similar conclusion was reached when comparing gentamicin with streptomycin (S.R. = 1.04, f_{S.R.} = 2.48) or when comparing neomycin with streptomycin (S.R. = 1.1, f_{S.R.} = 3.1). Gentamicin was 40.37 times more potent than neomycin (confidence limits 22.42 and 72.6) and 23.23 times more potent than streptomycin (11.73 and 45.99). Streptomycin was more potent than neomycin but the difference did not reach statistical significance (potency ratio = 1.73; confidence limits 0.8 and 3.72).

Discussion

The present results confirm the antinociceptive effect of spinal administration of gentamicin in the rat tail-flick test and demonstrate that neomycin and streptomycin have similar properties. The study also demonstrates that the antibiotics reduce the mechanical allodynia in a rat model of incisional pain. Incisional pain is characterized by a rapid and long-lasting mechanical allodynia and thermal hyperalgesia that correspond to those obtained during postsurgical states in humans (24). Therefore, the spinal administration of aminoglycoside antibiotics is effective against both phasic thermal and persistent incisional types of pain.

The present study (using lumbar puncture) differs from a previous report (using cisterna magna puncture) on the antinociceptive effects of gentamicin regarding the technique of spinal catheterization (20). In spite of this difference, the ED_{50} for gentamicin obtained on that occasion (3.98 µg; confidence limits 2.55 and 6.2) was within the range of that obtained in the present experiment (3.34 µg; 2.65 and 4.2).

The order of potency calculated here for the antibiotics was gentamicin > neomycin = streptomycin in both models of pain. Magnesium chloride was much less potent than the antibiotics in the tail-flick test and ineffective in the model of incisional pain. Aminoglycoside antibiotics displace the $^{125}$I-¿-conotoxin GVIA, but not trinitiated L-type VOCC antagonists from their binding sites in rat brain homogenates (16) or guinea pig brain cortex membranes (15), and inhibit synaptosomal [$^{45}$Ca] uptake (16). The IC_{50} of the antibiotics for both effects was within
the micromolar range and the order of potency found was neomycin > gentamicin > streptomycin > kanamycin. Also, the antibiotics were all more potent than magnesium (IC_{50} = 3.8 mM) and less potent than o-conotoxin GVIA (IC_{50} = 21 pM) (15). More recently, Pichler et al. (17) found that nanomolar concentrations of antibiotics were effective in inhibiting the binding of [^{125}I]-o-conotoxin GVIA to guinea pig cerebellum membranes. In this case, the order of potency was neomycin > kanamycin > gentamicin > streptomycin. A different order of potency (neomycin > kanamycin > streptomycin > gentamicin) was demonstrated at the nanomolar level for concentrations of antibiotics in displacing the binding of [^{125}I]-o-conotoxin MVIIIC, a P/Q-type VOCC antagonist, in guinea pig cerebellum membranes.

The relative potency of aminoglycoside antibiotics as calcium antagonists in smooth muscles and artificial membranes was similar to that obtained in the present study (for a review, see 18). However, a neomycin > gentamicin > kanamycin order was found in the tail-flick and hot-plate tests following intracerebroventricular administration to mice (21). A similar order was obtained for the antibiotics as antagonists of naloxone-precipitated withdrawal in mice acutely dependent on morphine (27), or as antagonists against the calcium-dependent release of histamine from isolated rat mast cells (28). The differences between the potencies of neomycin, gentamicin and streptomycin were very narrow in all cases, probably because in vitro models allow better control of the final drug concentration than in vivo experiments. The presence of divalent cations in the medium decreases the apparent affinity of antibiotics (17) and may somehow be the reason for the discrepancies.

Another result of the present study was the observation that the ED_{50} for gentamicin in the tail-flick test (3.34 µg; confidence limits 2.65 and 4.2) did not differ from that obtained in the model of incisional pain (2.06 µg; 1.46 and 2.9). In contrast, neomycin and streptomycin were much weaker in reducing the incisional allodynia. Our experiments, however, do not allow us to speculate on the cause(s) for such difference.

Takano et al. (29) have reported that intrathecal magnesium sulfate reduces the second, but not the first phase of the behavioral responses of rats to formalin, and does not change the nociceptive threshold in the rat paw pressure and hot-plate tests. The antinociceptive effect was assumed to be dependent on an inhibitory activity of magnesium ions on NMDA receptors, normally activated in hyperalgesic states. In contrast to the present study, the effectiveness of magnesium sulfate was demonstrated for a dose of 300 µg/rat, but not confirmed for smaller doses. Even very high doses of magnesium chloride were ineffective in reducing the mechanical incisional allodynia, a model of persistent pain in which NMDA receptor activation is expected (30). Therefore, interference with NMDA receptors is unlikely to be the reason for the antinociceptive efficacy of magnesium ions in the tail-flick test, a model of phasic pain in which NMDA receptors are not supposed to be activated.

Aminoglycoside antibiotics act primarily at the presynaptic level to decrease the release of acetylcholine at the neuromuscular junction (31-33), but higher doses may also act postjunctionally, blocking cholinergic receptors or interacting with ionic channels of the cholinergic receptors (34). Therefore, the possibility of higher doses of neomycin or streptomycin acting postsynaptically on the spinal cord to reduce incisional pain cannot be ruled out by the present experiments.

Aminoglycoside antibiotics have long been known to act as inhibitors of phospholipase C (35), but this effect requires very high concentrations of antibiotics and depends on the tissue studied. In the rat kidney proximal tubule brush border membrane,
gentamicin (1.5 mM) inhibits the activity of phospholipase C, an effect independent of the Ca^{2+} concentration but reduced when the pH of the medium is increased (36). The activity of phospholipase C in human amnion tissue may be increased or inhibited by aminoglycoside antibiotics depending on the presence of a high or low Ca^{2+} concentration in the medium, respectively (37). Therefore, aminoglycoside antibiotics acting as inhibitors of phospholipase C are unlikely to be the reason for the antinociceptive effects shown here.

Acknowledgments

We thank Mr. P.R. Castania and Mr. M.A. de Carvalho for valuable technical assistance.

References


