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# Ultrastructural localization of calcium in neuromuscular junctions of smooth and skeletal muscles after aminoglycoside antibiotics treatment

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**Summary.** Aminoglycoside antibiotics are all capable of producing clinically significant neuromuscular paralysis. Since part of the mechanism of action of these antibiotics at neuromuscular junction is a calcium-dependent inhibition of acetylcholine release, so this experiment was carried out in vitro on both somatic (isolated rat phrenic-nerve hemidiaphragm) and autonomic neuro-effector transmission (guinea-pig ileum) using gentamicin and amikacin, to determine the calcium contents at this level.

Electron microscopic observations on gentamycin- and/or amikacin-treated materials, using potassium pyroantimonate method suggest a reduction of internal calcium in nerve terminals of both preparations.

**Key words:** Aminoglycosides, Calcium, Neuromuscular junctions

## Introduction

Although the effects of aminoglycoside antibiotics (AGs) on somatic neuromuscular transmission have been extensively investigated, little consideration has been given to the possible effects of these compounds on autonomic neuro-effector transmission in gastrointestinal tract.

One of the most important adverse effects of AGs is neuromuscular blockage (Pridgen, 1956; Paradelis et al., 1980). Several lines of evidence indicate that this effect involves both an inhibition of the presynaptic release of acetylcholine, and an inhibition of the postsynaptic receptor sites for acetylcholine (Sokoll and Gergis, 1981; Singh et al., 1982). The presynaptic action, i.e. the inhibition of acetylcholine release, has been shown to be dependent on the ability of AGs to inhibit the internalization of calcium into the presynaptic site region of the neural axon (Fiekers, 1983). As a matter of fact the AGs interfere with calcium ion fluxes at the membrane of the motor nerve endings. Since calcium is necessary for the fast axonal transport of the substances (Ochs and Jersild, 1984) and it also modulates neurotransmitter synthesis (Szerb and O'Regan, 1985), the following study was undertaken to investigate the ultrastructural localization of calcium ions after gentamycin and amikacin treatment in nerve terminals of both somatic (rat phrenic-nerve hemidiaphragm) and autonomic (ginea-pig ileum) neurotransmission. In the present study the potassium pyroantimonate (PPA) method has been used for localization of calcium in order to see the relation between calcium concentration and the secondary effects induced by these antibiotics, at the level of neuromuscular junctions (NMJ).

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## Materials and methods

### *Sample preparation*

Section A

Male albino guinea-pigs weighing 260-500 g were killed by cervical fracture. Ileums were removed and segments of approximately 3 cm were suspended under 1 g tension for transmural electrical stimulation (Paton, 1955). The preparations were placed in a double-walled bath, perfused with mammalian Krebs solution and supramaximally stimulated at a rate of 0.1 Hz for 3 msec delivered by a Grass S88 stimulator (Rutten et al., 1980). Muscle responses were recorded on a polygraph (Physiograph MK-IV-Narco Biosystem).

Section B

Female albino rats weighing 150-200 g were used

after decapitation. A triangular-shaped section of the hemidiaphragm was dissected with its phrenic-nerve and was incubated in 50 ml bath containing mammalian Krebs solution. The resting tension of the muscle was adjusted at 5 g. The preparations were supramaximally stimulated via the phrenic-nerve at a rate of 0.1 Hz and 0.2 msec delivered by the same apparatus as stated in section A.

Dose-response curves

The antibiotic concentrations chosen for the two sections were those producing half-maximal (IC50), maximal and supramaximal inhibition of contractions of electrically-stimulated guinea-pig ileum (Table 1) and phrenic-nerve hemidiaphragm (Table 2).

*Electron microscopic demonstration of calcium*

Histochemical procedure

Pices of approximately 1 $mm^3$ of ileum and phrenic-nerve hemidiaphragm were excised and immersed at room temperature in 0.1M sodium cacodylate-buffered 2.5% glutaraldehyde at pH 7.4 for fixation. They were rinsed in the same buffer and postfixed with a mixture of 2.5% of PPA and 1% $OsO_4$ in deionized water for 2 hrs. The excess of PPA was rinsed away and washed for 15 min with distilled water and sections brought to pH 10 with 0.1 N KOH using pH paper. The samples were then dehydrated in cold ethanol series and embedded in Epon 812 (Borgers et al., 1983; Kashiwa and Thiersch, 1984). Ultrathin sections were cut using glass knives on an LKB Nova ultramicrotome, collected from water surface on uncoated copper grids of 400 mesh, doubly stained with uranyl acetate and lead citrate and examined using a Zeiss EM 10C transmission electron microscope (Carl Zeiss, Oberkochen), operated at 60 KV.

Control sections were obtained by: 1.- Omitting the PPA in postfixation. 2.- Treatment of Epon-embedded sections with 5 mM specific chelator EGTA for 1h.

**Table 1.** Doses of gentamycin and amikacin producing inhibition of electrically tensions in the guinea-pig ileum.

| INHIBITION | GENTAMYCIN (mM) | AMIKACIN (mM) |
|---|---|---|
| Half-maximal (IC50) | 0.23 | 0.26 |
| Maximal (2-3 x IC50) | 0.69 | 0.78 |
| Supramaximal (5 x IC50) | 1.15 | 1.30 |

**Table 2.** Doses of gentamycin and amikacin producing neuromuscular inhibition in the rat phrenic-nerve hemidiaphragm.

| INHIBITION | GENTAMYCIN (mM) | AMIKACIN (mM) |
|---|---|---|
| Half-maximal (IC50) | 1.76 | 2 |
| Maximal (2-3 x IC50) | 3.60 | 6 |
| Supramaximal (5 x IC50) | 5.26 | 10 |

## Results

*Localization of calcium*

At electron microscopic level, the electron-dense precipitates, round or oval in shape, were distributed in PPA-treated material almost uniformly in control sections of ileum and phrenic-nerve hemidiaphragm (Figs. 1, 2). The deposits were abolished in sections treated on the absence of PPA and the EGTA treated sections resulted in a complete removal of the precipitates. Treated materials showed calcium deposits which were proportional to the use drug doses, i.e. the increased doses of antibiotics resulted in lower calcium deposition in synaptic areas. The results obtained can be depicted as shown in Figs. 3-7, and summarized in Table 3.

As can be seen gentamycin and amikacin significantly decrease the calcium contents in NMJ, suggesting the inhibition of a dose-dependent calcium uptake in this area. In other words, the highest concentration investigated, i.e. maximal and supra-maximal of either drugs, reduced the neuromuscular calcium content. No major difference is recognized between the two antibiotics.

## Discussion

Calcium plays an essential role in excitation-contraction coupling (Suarez-Kurt, 1982) and is necessary for nerve membrane excitability, coupled at peripheral NMJ (Galvan et al., 1985) in smooth and skeletal muscles. A competitive antagonism between calcium and AGs has been reported by many investigators (Fiekers, 1983; Corrado et al., 1989; Parson et al., 1992). AGs inhibit contractility of various muscles in a dose-dependent manner, including; guinea-pig vas deferens (Papaioannidou et al., 1988), isolated rat uterus (Paradelis et al., 1982), guinea-pig papillary muscles (Hino et al., 1982) and rabbit intestine (Paradelis, 1981). It has been reported that the effect of AGs on contractility of smooth muscles can be attributed to their ability to interfere with calcium entry through cell membranes of the tissue and consequently, reduction in the level of the myoplasmic calcium concentration and less activation of the contractile machinery (Pimenta de

**Table 3.** Qualitative evaluation of calcium deposition in smooth and skeletal muscles after gentamycin and/or amikacin treatment.

| | GENTAMYCIN | | AMIKACIN | |
|---|---|---|---|---|
| | Smooth muscle | Skeletal muscle | Smooth muscle | Skeletal muscle |
| Control | ++++ | ++++ | ++++ | ++++ |
| EGTA | - | - | - | - |
| IC50 | +++ | +++ | +++ | +++ |
| 2-3 x IC50 | + | + | + | + |
| 5 x IC50 | - | - | - | - |

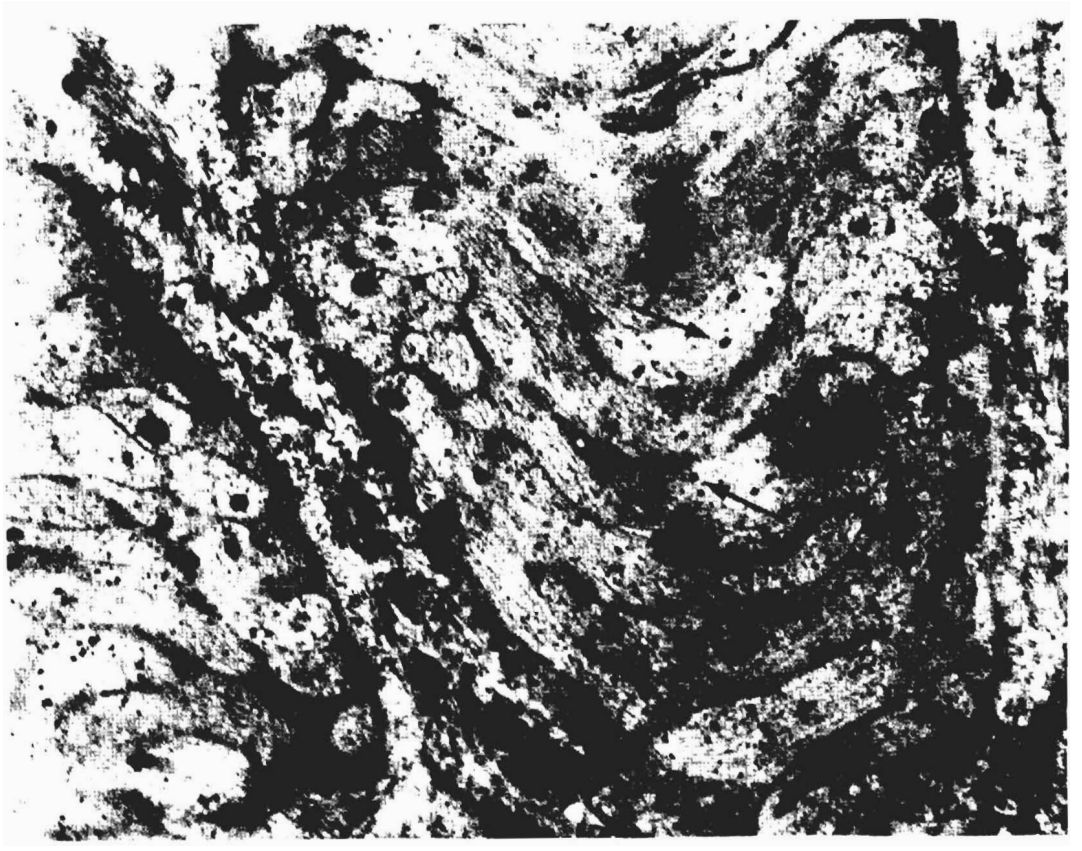

**Fig. 1.** Photomicrograph of nerve endings in untreated guinea-pig ileum smooth muscle section, showing calcium deposition (arrows). x 5,000

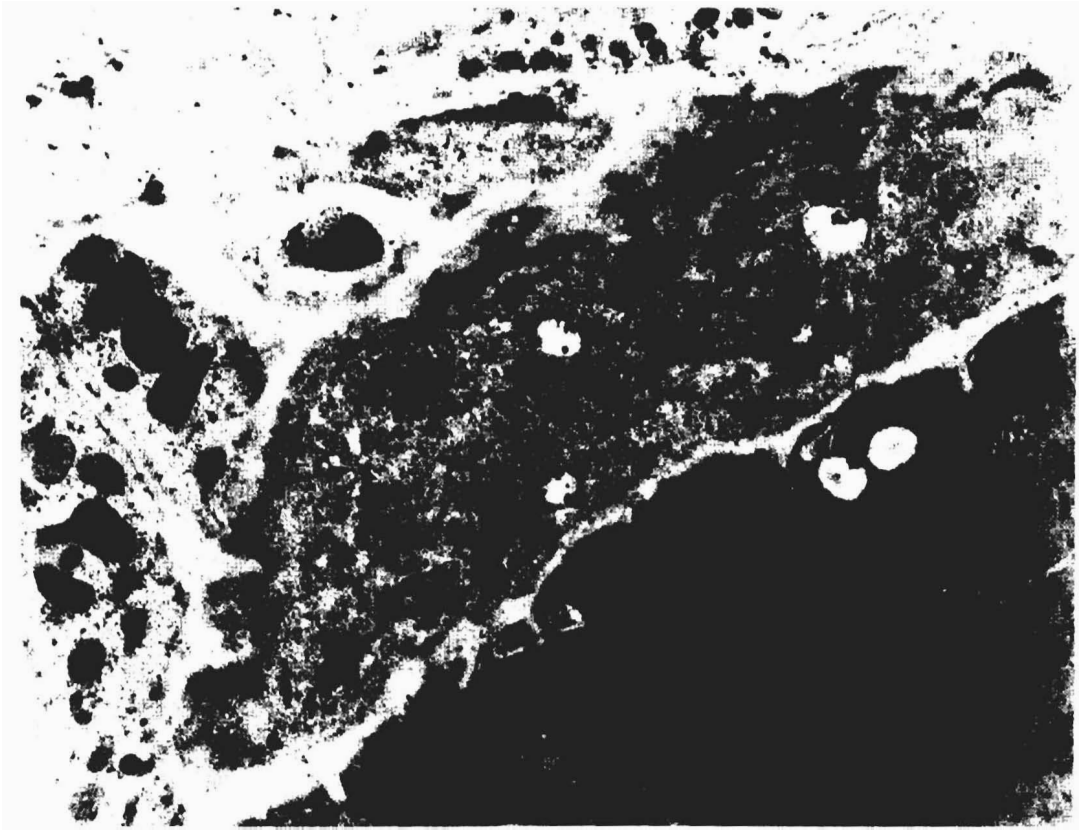

**Fig. 2.** Photomicrograph presenting the endplate containing calcium deposition in untreated rat hemidiaphragm section (arrows). x 6,300

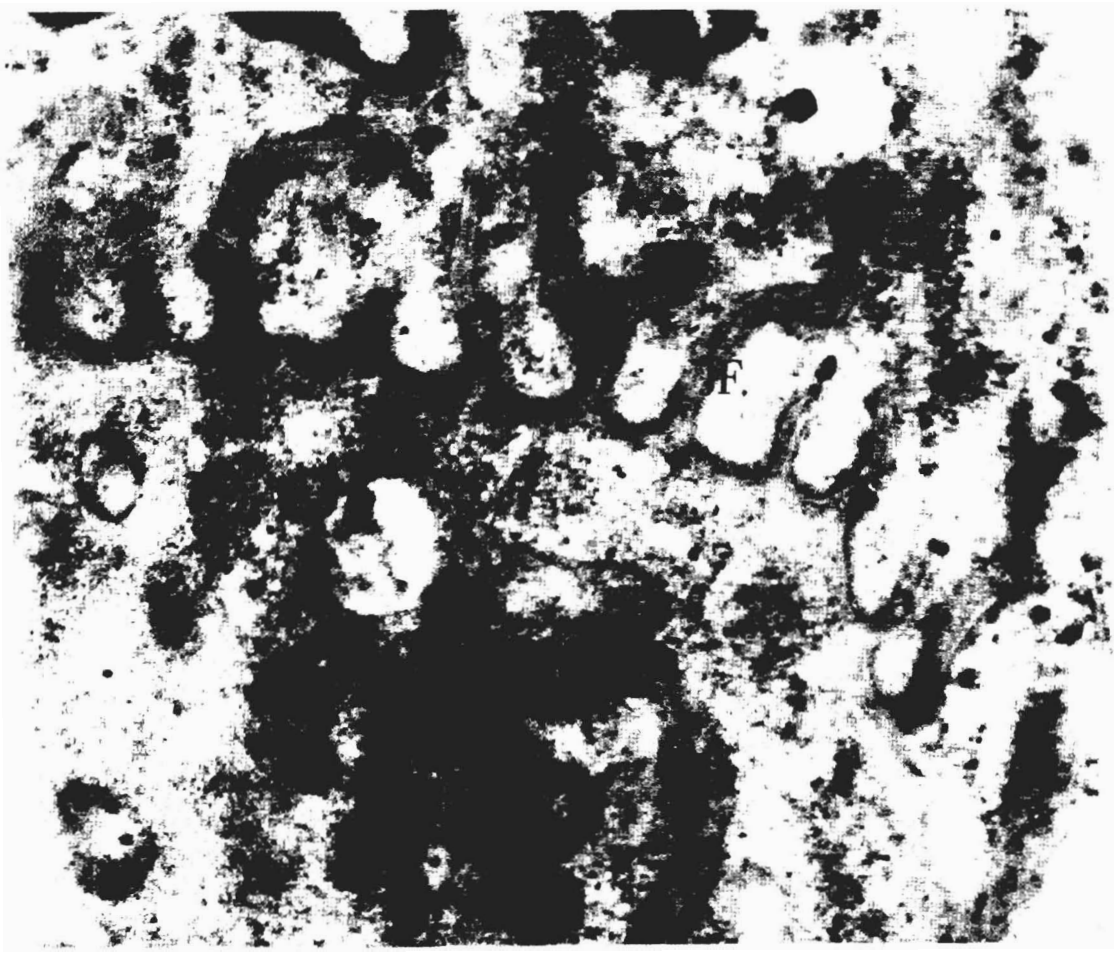


**Fig. 3.** Electron micrograph of the motor endplate of half-maximal gentamycin-treated rat hemidiaphragm section, viewing calcium deposits. Junctional folds (JF) and secondary synaptic cleft (SS) are seen. x 20,000

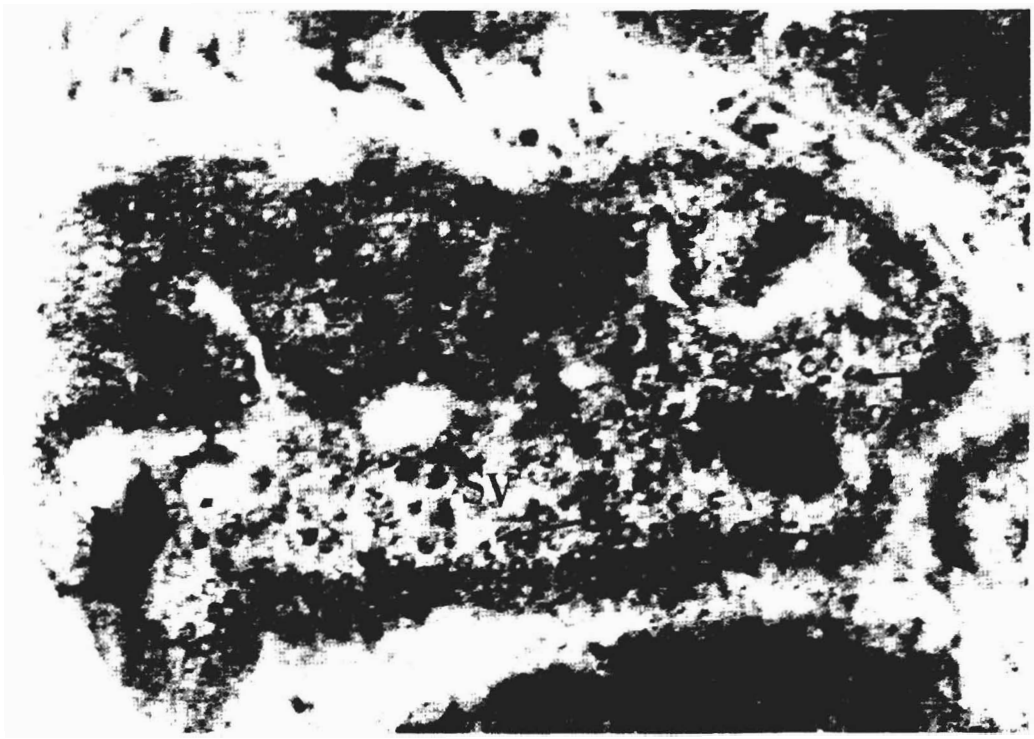


**Fig. 4.** Survey picture of the motor endplate of half-maximal amikacin-treated material, showing calcium deposition (arrows). Numerous synaptic vesicles (SV) are present, but junctional folds are absent. x 16,000

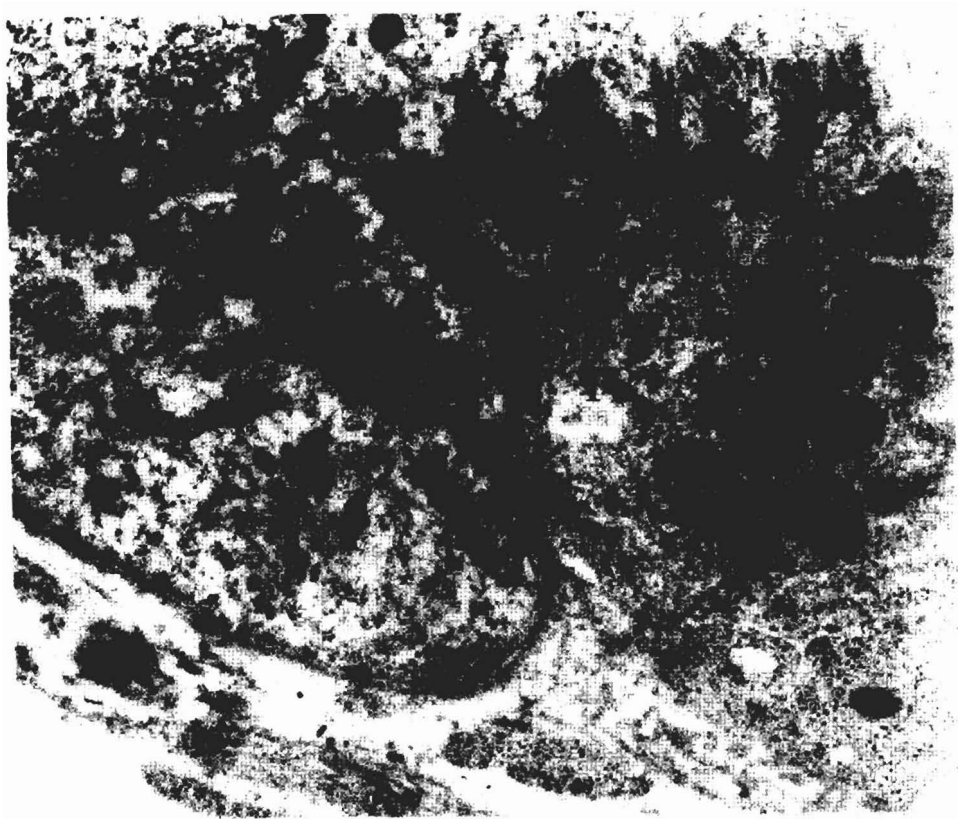

**Fig. 5.** Electron micrograph of the endplate of maximal gentamycin-treated material presenting a calcium decrease in pre- and postsynaptic areas. Large mitochondria (M) in a terminal branchlet (TB) and junctional folds (JF) are seen. x 8,000

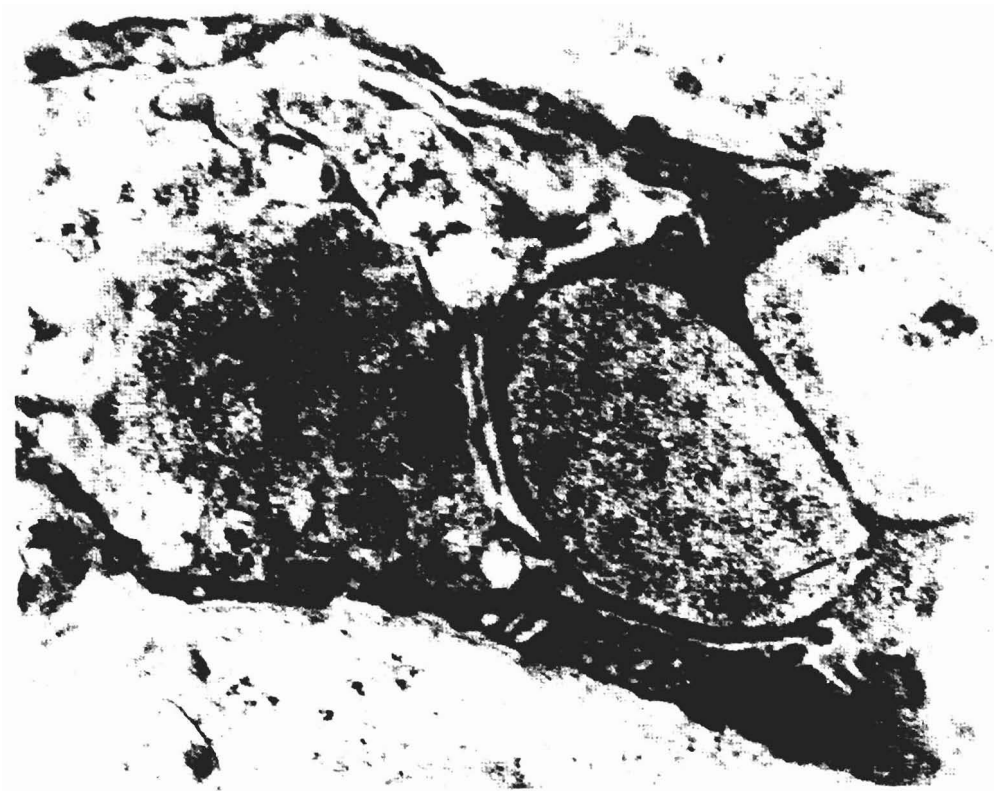

**Fig. 6.** Electron micrograph of the motor endplate of supramaximal amikacin-treated material, almost resulting in an absence of calcium precipitates. A calcium deposit is pointed out (arrows). x 16,000

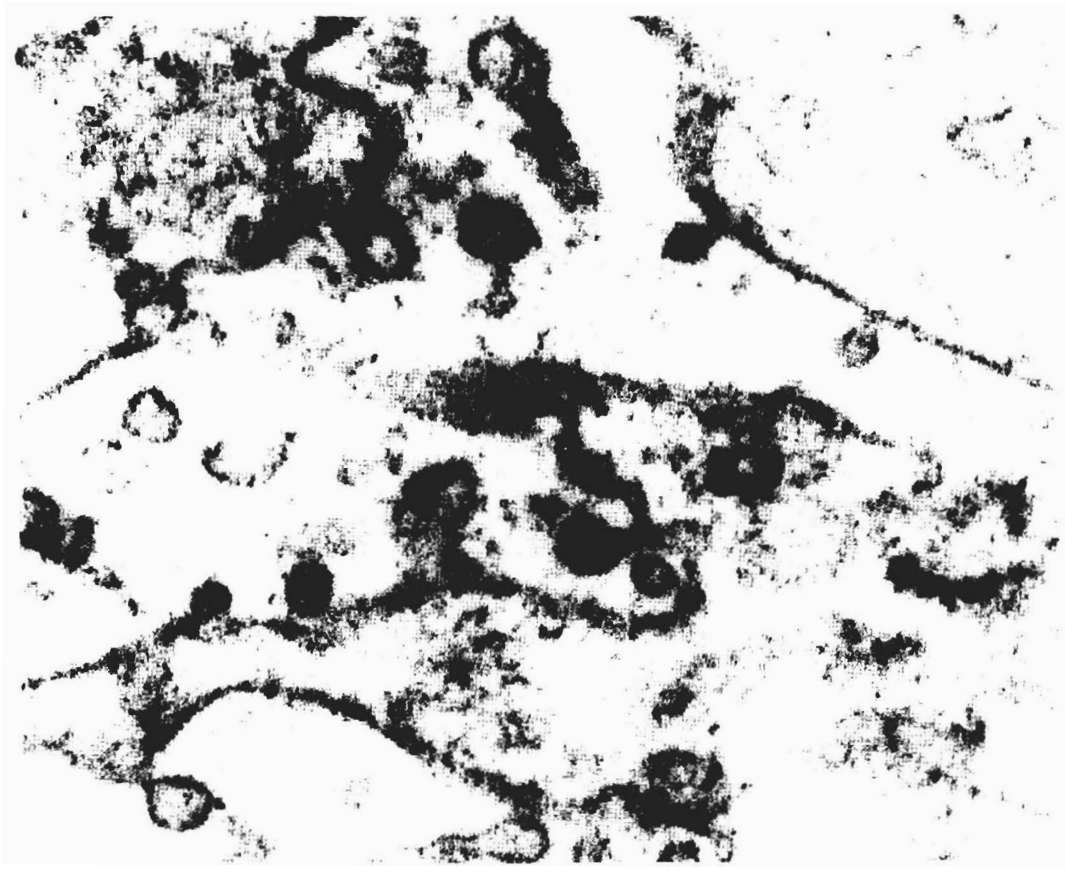

**Fig. 7.** High magnification photomicrograph of the pre- and postsynaptic membrane of supramaximal gentamycin-treated guinea-pig ileum smooth muscle section. No calcium deposition is observed in synaptic areas. x 40,000

Marais et al., 1978). AGs also replace calcium from lipid monolayers on the superficially-bound calcium of isolated beating left atria from guinea-pigs (Lullmann and Schwartz, 1985). Moreover, AGs interfere with calcium ion movements through the calcium channels of the membrane of the motor nerve-endings and displace calcium from binding sites which is necessary for the release of acetylcholine at the synaptic cleft (Suarez-Kurtz and Reuben, 1987; Paradelis et al., 1988). In fact AGs produce neuromusular blockage by a combination of both pre- and postjunctional actions (Sokoll and Gergis, 1981; Singh et al., 1982). Calcium not only has the ability to restore the neuromuscular transmission but also exerts a protective action against the neuromuscular blocking activity of AGs (Paradelis et al., 1980; Singh et al., 1982).

According to the above data we have investigated the calcium contents by histochemical localization of calcium in NMJ of smooth and skeletal muscles after treatment with AGs.

The PPA method is a valid measurement of intracellular calcium localization and translocation. Even though the precipitates contain cations other than calcium ions (Suzuki and Sugi, 1989). The ultrastructural localization of calcium in the articular cartilage was examined by using PPA (Ohira and Ishikawa, 1991). We used the PPA method as a primary fixative to demonstrate the calcium deposits in nerve terminals. Our results suggest that AGs reduce calcium content in NMJ evidenced by a decrease of calcium deposits. No significant difference is observed between gentamycin and amikacin at ultrastructural level. We cannot determine the threshold of the technical sensitivity of the method used, as it can be combined by analytical studies (Singh et al., 1979). The micrographs are analysed qualitatively, but it is interesting to note that calcium ions are markedly decreased and almost disappeared in NMJ after AG treatment in a dose-dependent manner.

Therefore, it is concluded that gentamycin and amikacin reduce the calcium content in nerve terminals of both preparations. This effect may be due to inhibition of calcium influx through nervus terminals in treated materials.

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