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Possible Postsynaptic Action of Aminoglycosides in the Frog Rectus Abdominis

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The present study was undertaken to investigate the postsynaptic effects of aminoglycosides on contractions evoked by acetylcholine (ACh), KCl, electrical field stimulation (EFS) and Na^+- and Ca^{2+}-free Ringer solution with 0.2 mM Na_2 EDTA (Na_FCa_FR) in the isolated frog rectus abdominis. Neomycin inhibited contraction elicited by ACh, Na_FCa_FR, and EFS at the higher frequencies (8 and 10 Hz) but not those elicited by KCl and EFS at the lower frequencies (2, 3 and 5 Hz). D-tubocurarine inhibited ACh-induced contractions in a concentration-dependent manner. In addition, drug reduced EFS-evoked contractions to a limited extent. Lower concentrations (10^{-5}, 5×10^{-5}, 10^{-4}, 2×10^{-4} and 3×10^{-4} M) but not higher concentrations (4×10^{-4} and 5×10^{-4} M) of methoxyverapamil exhibited a concentration-dependent inhibitory action on Na_FCa_FR-induced contractions. Similar inhibitions of the same type of contraction were displayed by aminoglycosides (neomycin, streptomycin, netilmycin, gentamycin and amikacin). These results suggest that in addition to their antagonistic action on nicotinic receptors in the frog rectus abdominis, aminoglycosides may exert stabilizing effects on some functional components contributing to contractions at the membrane.

Key words: aminoglycoside, voltage sensor, sodium and calcium free Ringer solution, frog rectus abdominis, contraction

It is well known that aminoglycosides possess neuromuscular blocking action by inhibiting prejunctional acetylcholine (ACh) release and reducing postsynaptic sensitivity to the transmitter (1, 2). The latter action has been referred to as a Mg^{2+}-like effect, but the mechanism remains to be clarified. On the other hand, the isolated frog rectus abdominis seems to be the most suitable striated muscle preparation for analysis of the postsynaptic effects of various agents, since the response of the tissue to a variety of stimuli is contraction resulting from the activation of receptors or non-receptor mechanism(s). This can be seen, for example, in the cases of ACh administrated exogenously electrical field stimulation (EFS) and exposure of the tissue to Ca^{2+}-free or Ca^{2+}- and Na^+-free Ringer solution with Na_2EDTA (3-7). The aim of the present study was thus to investigate postsynaptic effects of aminoglycosides on contractions evoked by ACh, EFS, high levels of KCl or Na^+- and Ca^{2+}-free Ringer solution with Na_2EDTA (Na_FCa_FR) in the isolated frog rectus abdominis.

Materials and Methods

Frogs (Rana pipiens) of both sexes weighing 15-25 g were used in the experiments. The rectus abdominis was carefully removed after the animal was decapitated and pithed. The muscle was divided into 2 halves by cutting along the linea alba. Thus, 2 preparations were obtained. The preparation was mounted under 0.5 g tension between 2 platinum wires in a jacketed organ bath in a 50 ml volume filled with Ringer solution (in mM: NaCl 111.11, KCl 1.87, $CaCl_2$ 1.08, $NaHCO_3$ 2.38, NaH_2PO_4 0.083, glucose 10.1). The temperature of the bathing medium was maintained at 20 °C and the medium was continuously aerated with 95% O_2 and 5% CO_2. The tissue was equilibrated for 1 h before starting the experiment. Changes in muscle length were recorded on a

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smoked drum *via* an isotonic lever (× 8 - 10 magnification).

In a series of experiments, ACh at 10^{-6}, 3×10^{-6}, 5×10^{-6}, 10^{-5} and 5×10^{-5} M concentrations was successively added into the bath for 30 sec in 30 min intervals. The tissue was washed with fresh Ringer solution after each application of ACh. The procedure was repeated after a resting period of 1 h. Responses of the same preparation were therefore recorded as the first and the second series. In some experiments, after the first series of responses were recorded, the tissue was resubmerged in Ringer solution with neomycin (10^{-6}, 10^{-5}, 10^{-4} or 5×10^{-4} M) at the beginning of the resting period. The second series of responses was recorded in the presence of the aminoglycoside. Additional experiments were performed to consider the effects of elevated ACh concentrations (from 5×10^{-5} M to10^{-4}, 5×10^{-4} and 10^{-3} M) on the reduction observed in the contraction amplitude induced by ACh in the presence of neomycin (5×10^{-4} M). A sperate experimental group was used for each neomycin concentration.

In another series of experiments, the same experimental protocol was followed with the exception that EFS (15 V, 1 ms; at frequencies of 2, 3, 5, 8 and 10 Hz) or Ringer solution containing high levels of KCl (20, 30, 40, 60 and 80 mM) was used instead of ACh as a constrictor stimulus. Also, the second series of responses was examined in the presence of neomycin in the same concentrations mentioned above. Ringer solution with high KCl was prepared by substituting equimolar amounts of KCl for NaCl.

In some trials, ACh (5×10^{-6} M), EFS (5 Hz), KCl (40 mM) and Na^+-and Ca^{2+}-free Ringer solution (Na_FCa_FR; in mM sucrose 225.6, KCl 1.87, KH_2PO_4 0.083, $KHCO_3$ 2.38, glucose 10.1, Na_2EDTA 0.2) were successively applied to the tissue in 30 min intervals and the preparation was left to rest for 1 h. Thereafter, the procedure was repeated to obtain a second series of responses. The duration of exposure of the tissue to the Na_FCa_FR was 10 min, as the development of the response was relatively slow. The duration of exposure to other stimuli was 30 sec. In the subgroups of this type of experiment, the tissue was washed with Ringer solution containing d-tubocurarine (10^{-8}, 5×10^{-8}, 10^{-7}, 5×10^{-7} or 10^{-6} M) after the first series of responses were recorded; a second series of responses was monitored in the presence of d-tubocurarine.

Another series of experiments was designed to examine the effects of the major aminoglycosides on the Na_FCa_FR-induced contraction such that, at the end of the equilibrium period (1 h), the tissue was incubated with Na_FCa_FR for 30 min and left to rest for 1 h in a fresh Ringer solution bath. Thereafter, the procedure was repeated. Thus, 2 responses were obtained as controls. In the experimental subgroups, the tissue was resubmerged in Ringer solution with streptomycin, neomycin, netilmycin, gentamycin, amikacin (10^{-5}, 5×10^{-5}, 10^{-4}, 2×10^{-4} or 5×10^{-4} M), or methoxyverapamil (D-600) (10^{-5}, 5×10^{-5}, 10^{-4}, 2×10^{-4}, 3×10^{-4}, 4×10^{-4} or 5×10^{-4} M) after the first response was recorded. The second response was obtained by using Na_FCa_FR which contained the same concentration of the aminoglycosides or methoxyverapamil in the bathing medium. A spare subgroup was established for each drug concentration.

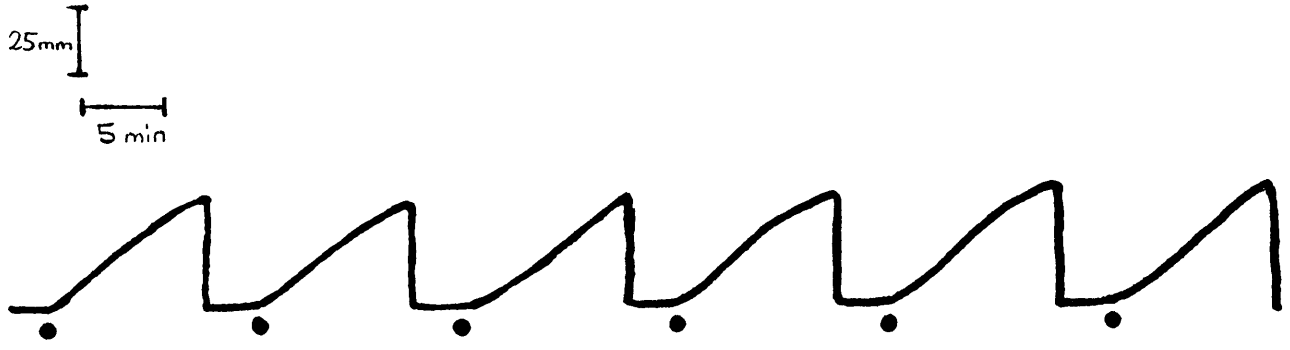
Values were measured in millimeters and expressed as percent of the first response recorded as a control. Data were statistically analyzed according to one-way ANOVA (analysis of variance) by using the computer program SPSS. $P < 0.05$ was assumed to denote a significant difference. Drugs and agents: Acetylcholine chloride (Sigma Chemical Co., St. Louis, MO, USA), neomycin sulphate (Sigma), streptomycin sulphate (Sigma), d-tubocurarine (Sigma) and methoxyverapamil (Sigma) were dissolved in distilled water and added in volume of a 0.1 ml to the bath. Ampoules of netilmycin sulphate (Netromycine, Eczacıbaşı, Istanbul, Turkey), gentamycin sulfate (Garamycin, Eczacıbaşı), amikacin sulphate (Amikozit, Eczacıbaşı) were used as stock solutions. Sucrose was obtained from E Merck, Darmstadt, Germany.

Results

ACh (n = 8), EFS (n = 8) and KCl (n = 8) caused concentration or frequency-dependent contractions. There was no significant difference between the first and second series of responses in control experiments. Exposure of the tissue to Na_FCa_FR for 10 min (n = 8) or 30 min (n = 8) caused reproducible and marked contractions (Table 1). No significant difference was observed between the first and second series contractions.

Effects of neomycin. Neomycin (10^{-6}, 10^{-5}, 10^{-4} and 5×10^{-4} M) significantly inhibited ACh-induced contractions in a concentration-dependent manner and the concentration response curve of ACh was shifted to the right. Elevation of ACh concentrations from 5×10^{-5} M

Table I Mean values of contractil activities evoked by exposure of the tissue to Na^+-and Ca^{2+}-free Ringer solution with 0, 2 mM Na_2EDTA (Na_FCa_FR) for 10 min



I*	II	III	IV	V	VI
36.7 ± 1.7**	38.8 ± 1.8	39.5 ± 1.8	40.6 ± 1.8	41.3 ± 1.8	41.6 ± 1.8

A typical tracing for responses due to Na_FCa_FR is represested on the top.
*Applications of Na_FCa_FR
**Contraction amplitudes are presented in mm (± SE)

to 10^{-4}, 5×10^{-4} and 10^{-3} M completely abolished the reduction in the maximum amplitude of the ACh-induced contraction (Fig. 1). Contractions produced by EFS (15 V, 1 ms) at lower frequencies (2, 3 and 5 Hz) were not affected by neomycin, whereas the contractions due to EFS at higher frequencies (8 and 10 Hz) were significantly $P < 0.05$) reduced by the highest concentration used (5×10^{-4} M) of the drug (Fig. 2). Neomycin did not cause any changes in KCl-induced contractions (Fig. 3). On the other hand, the drug caused concentration-dependent inhibition in the contractions evoked by exposure of the tissue to Na_FCa_FR for 10 min (Fig. 4). Eight preparations were considered in each concentration of the drug (n = 8).

Effects of d-tubocurarine. The drug inhibited ACh (5×10^{-6} M)-induced contractions completely in a concentration-dependent manner. Its inhibitory action on EFS (5 Hz) was weaker; the contractions could only be reduced to 77.6 ± 5.7 and 57.3 ± 6.1% by the agent at higher concentrations (5×10^{-7} and 10^{-6} M, respectively). D-tubocurarine was ineffective at altering the contractions elicited by KCl (40 mM) or by Na_FCa_FR. Results of the experiments are presented in Fig. 5. The number of preparations considered was 5-6 (n = 5-6) for each concentration of the drug.

Effects of major aminoglycosides and methoxyverapamil (D-600) on Na_FCa_FR-induced contractions. All aminoglycosides used (streptomycin, neomycin, netilmycin, gentamicin, and amikacin) exhibited similar inhibitory actions on contractions evoked by exposure of the tissue to Na_FCa_FR for 30 min (Fig. 6). Methoxyverapamil at lower concentrations (up to 3×10^{-4} M) displayed concentration-dependent inhibitory action on contractions; the inhibitory effect of the drug was progressively decreased and completely abolished when the concentrations continued to be ele-

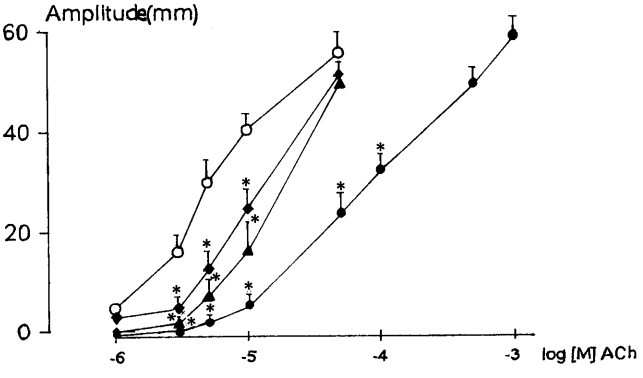


Fig. 1 Concentration-response curve for acetylcholine (ACh) in the absence (○) and presence of neomycin at concentrations of 10^{-5} M (◆), 10^{-4} M (▲) and 5×10^{-4} M (●). Each dot presents the mean of results obtained from 8-10 experiments (n = 8-10). *indicates statistical significance.

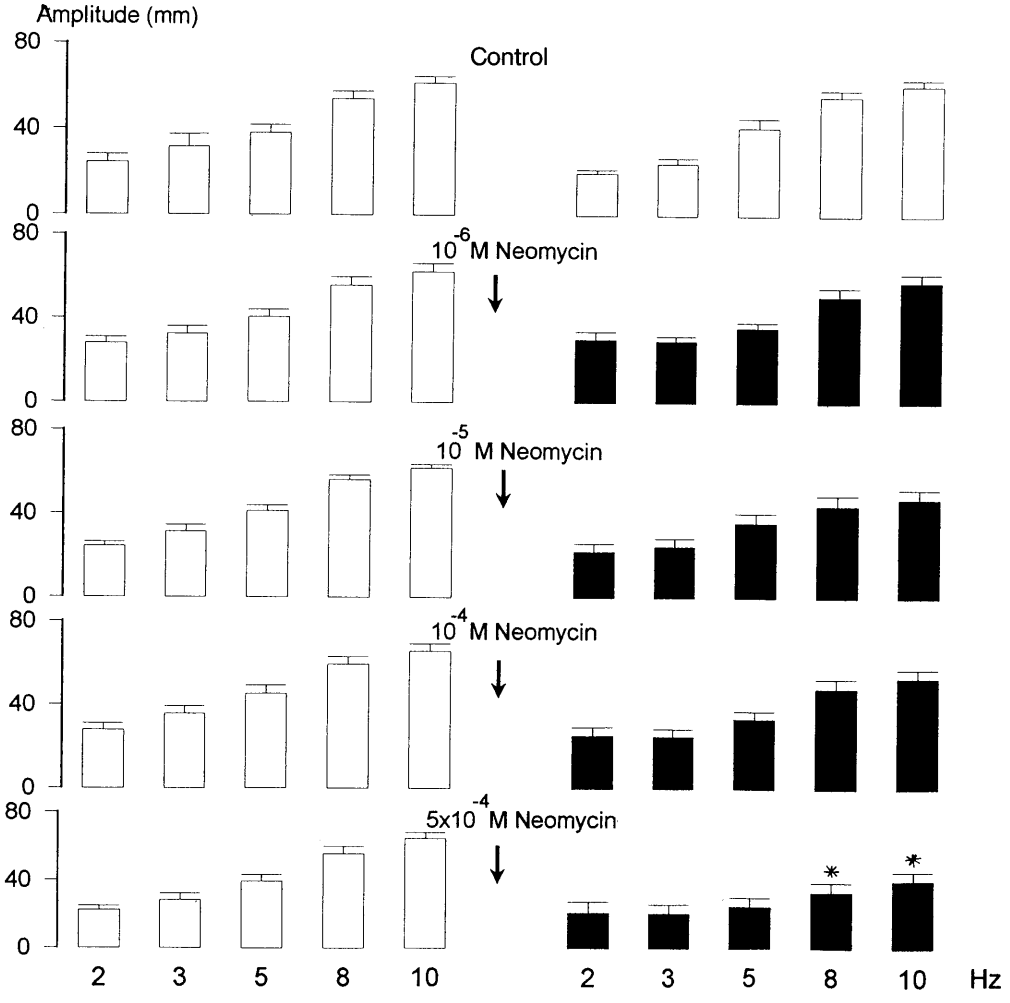


Fig. 2 Effects of neomycin on contractions induced by electrical field stimulation (EFS) (n = 8-10). *indicates statistical significance ($P < 0.05$).

vated. A concentration of 5×10^{-4} M, the Ca^{2+}-channel blocker caused a tendency to increase in the contraction amplitude (Fig. 7).

Discussion

The results of the present study suggest that in addition to their antagonistic action on nicotinic receptors, aminoglycosides may exert an inhibitory effect on some of the functional membranous components that play a role in the initiation of striated muscle contractions. The ACh-induced contractile response was inhibited by d-tubocurarine in a concentration-dependent manner. A similar antagonism was observed with neomycin. The antagonistic effect of the aminoglycoside seemed to be competitive, suggesting that the interaction site may be the nicotinic receptors. This finding further suggests that nicotinic receptor blockage may play an important role in increasing the insensitivity of the tissue to the neurotransmitter. However, it does not exclude the possibility that receptor-operated Ca^{2+} channels may contribute to contractions.

EFS-induced contractions were reduced to some extent by d-tubocurarine, suggesting Na^{+}-channels may play a role in this kind of contraction. This finding suggest that high concentrations of the drug can block the channel directly (8). Neomycin administered in lower concentrations (10^{-6} and 10^{-5} M) did not cause any

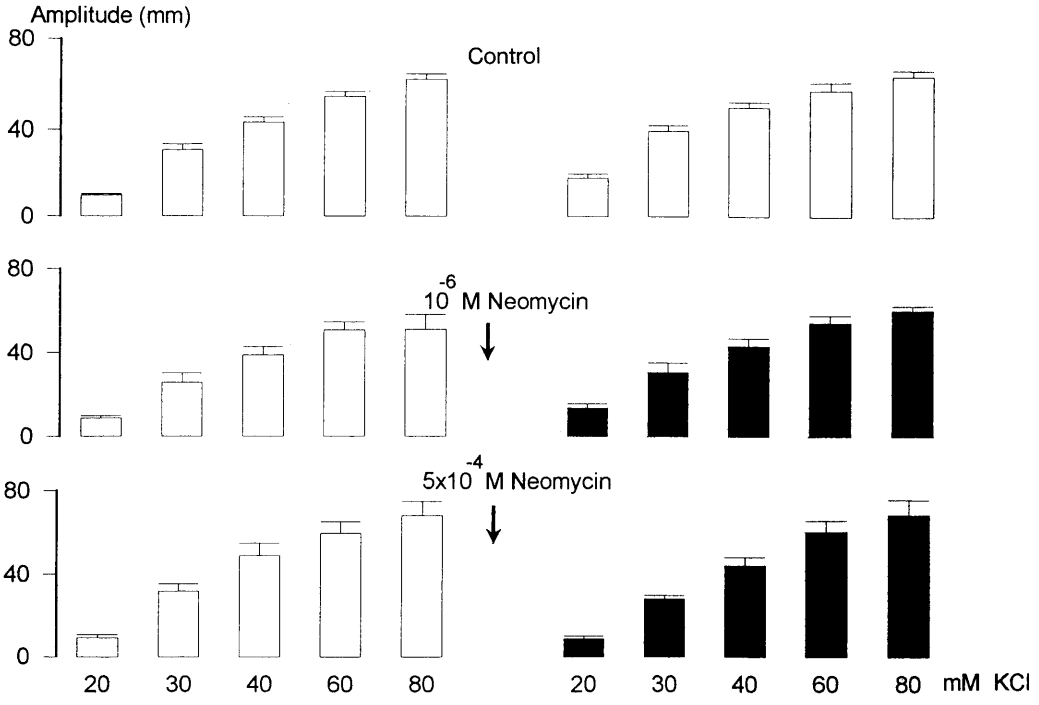


Fig. 3 Ineffectiveness of neomycin on contractions evoked by a high concentration of KCl (n = 8).

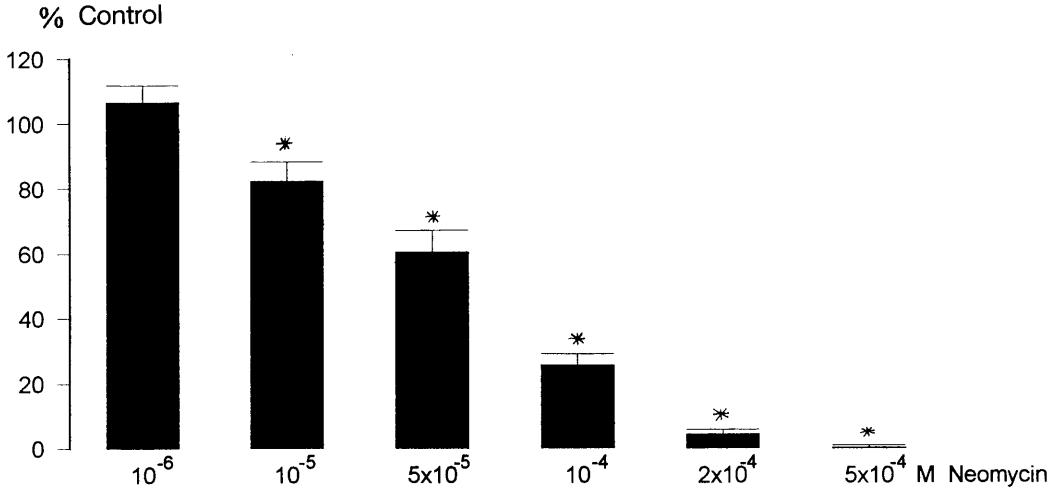


Fig. 4 Effects of neomycin on contractions evoked by exposure of tissue to Na_FCa_FR for 10 min (n = 8). *indicates statistical significance ($P < 0.05$).

significant change in the contraction amplitude. However, higher concentrations (10^{-4} and 5×10^{-4} M) of the aminoglycoside reduced contractions elicited by only high frequencies of EFS (8 and 10 Hz). This finding indicates that the drug may not block Na^+-channels otherwise, the reductions would also be observed in contractions evoked by EFS at lower frequencies (2–5 Hz). On the other hand, this effect may reflect the action of neomycin on Ca^{2+}-channel activity. It is well known that striated muscle fiber possesses Ca^{2+}-channels in the membrane

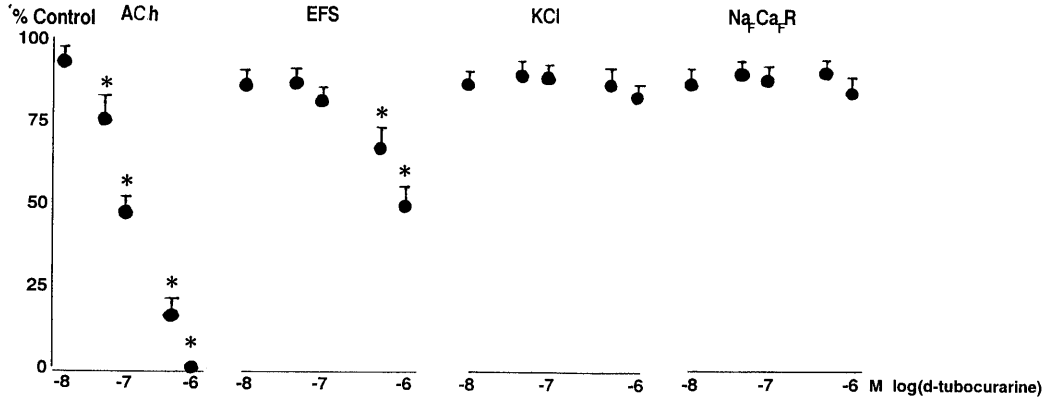


Fig. 5 Effects of d-tubocurarine on contractions produced by ACh (5×10^{-6} M), EFS (5 Hz), KCl (40 mM), and Na_FCa_FR (n = 5–6). *indicates statistical significance ($P < 0.05$).
ACh; EFS, see legends to Fig. 1 and Fig. 2.

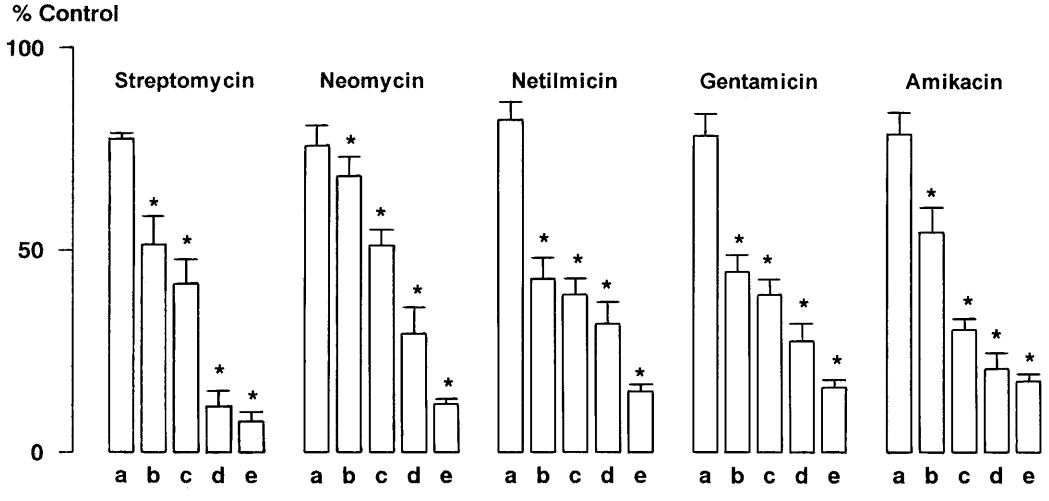


Fig. 6 Effects of major aminoglycosides on contractions evoked by the exposure of tissue to Na_FCa_FR for 30 min (n = 8). a-10^{-5}, b-5×10^{-5}, c-10^{-4}, d-2×10^{-4} and e-5×10^{-4} M. *indicates statistical significance ($P < 0.05$).

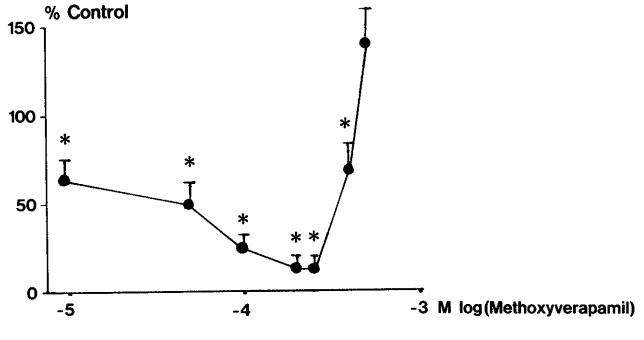


Fig. 7 Effects of methoxyverapamil (D-600) on contractions induced by exposure of tissue to Na_FCa_FR for 30 min. (n = 8–10).

(9). Such channels can only be opened by stimuli of a high frequency (10–12). Thus, it is entirely possible that an inhibitory effect on Ca^{2+}-channel activity will cause a decrease in the contribution of the channel to contractions when tissue is stimulated by EFS at high frequencies. This may explain the observed reduction of contractions evoked by EFS at high frequencies in the presence of neomycin.

On the other hand, neomycin and d-tubocurarine did not affect KCl-induced contractions. This finding suggests that contractions may develop as a result of a

mechanism that takes place at a location beyond Na^+-and neomycin-sensitive Ca^{2+} channels in the cytoplasmic membrane in the analyzed tissue. Additionally, it suggests that neomycin may not affect the ryanodine receptors. The latter conclusion is the currently favored hypothesis, the positive charge of aminoglycosides renders them too polar to diffuse into cells.

An interesting finding of the present study is that all aminoglycosides exhibited a similar inhibitory action on contractions elicited by Na_FCa_FR in a concentration-dependent manner. It is well known that aminoglycosides possess molecules with cationic amino groups. Nephrotoxicity has been attributed to these groups because their cationic charges facilitate the binding of filtered drugs to renal tubular epithelial cell luminal membranes where intracellular transportation occurs; this process leads to an accumulation of the drug in lysozomes (13). It has also been suggested that Ca^{2+} supplementation may decrease nephrotoxicity by causing a reduction in the binding of aminoglycosides to the luminal surface of the membrane (14). These findings indicate that aminoglycosides may bind to Ca^{2+} binding sites at the membrane. Similar interaction may have occurred in the tissue used in the present study. Decreasing the cationic charges below a critical level in the external medium (to make it Na^+-and Ca^{2+}-free) may cause an impairment in the stability of some functional membrane components leading to Ca^{2+} release from the sarcoplasmic reticulum. Replacement of cationic charges with aminoglycosides may suppress this process. This view is supported by previous studies of the same tissue. Such studies have found that Ca^{2+}, Mn^{2+}, Cd^{2+} and Sr^{2+} caused a concentration-dependent inhibition in contractions induced by Ca^{2+}-free Ringer solution with Na_2EDTA or by Na_FCa_FR (15, 16). These findings suggest that rather than Ca^{2+} antagonistic or Ca^{2+}-like properties of these cations, their ionic positive charges may be more important for the inhibitory action on such contractions. A strong possible cause of Na_FCa_F R-induced contractions is that the Ca^{2+}-channels acting voltage sensor play a role in the responsive mechanism, since methoxyverapamil (D-600) inhibited Na_FCa_FR-evoked contractions in a concentration-dependent manner in the present study. It is well known that D-600 inhibits L-type Ca^{2+}-channel activity. In addition, its binding sites are on voltage sensors in frog skeletal muscle (17). Therefore, it is plausible that aminoglycosides directly or indirectly affect these channels by binding to particular components of the membrane. However, in the present study, the inhibitory effect of D-600 was observed at lower concentrations (up to 3×10^{-4}). At the highest concentration used (5×10^{-4}), D-600 caused a tendency to increase the amplitude of the contractions. Similar dual action of the drug has been observed in previous studies of guinea pig and cat hearts as well as of frog skeletal muscle; these findings are in support of our results (18, 19).

In conclusion, postsynaptic inhibitory effects of aminoglycosides may consist of at least 2 mechanisms, antagonism of nicotinic receptors and stabilization of some functional components, including the L-typed Ca^{2+}-channels acting voltage sensor in the membrane of the frog rectus abdominis. Both mechanisms may affect the inhibition of contractions. However, the degree of their inhibitory action on non-receptor-mediated contractions may be proportional to the contribution of the latter mechanism to the contractions in the tissue used.

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