Neomycin Inhibits Catecholamine Secretion by Blocking Nicotinic Acetylcholine Receptors in Bovine Adrenal Chromaffin Cells

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ABSTRACT

We investigated the effects of neomycin on nicotinic acetylcholine receptor-induced responses in bovine adrenal chromaffin cells. Neomycin inhibited the nicotinic agonist dimethylphenylpiperazinium iodide (DMPP)-induced norepinephrine secretion in a concentration-dependent manner. Neomycin had also an inhibitory effect on the DMPP-induced increase in cytosolic calcium concentration ([Ca$^{2+}$]). This effect was further confirmed by inhibition of the DMPP-induced fluorescence quenching of fura-2 upon Mn$^{2+}$ entry. Under the same conditions, however, neomycin did not change the bradykinin-induced [Ca$^{2+}$], increase, which follows the downstream signal of phospholipase C activation in this cell. The inhibitory effect of neomycin on the DMPP-induced [Ca$^{2+}$], increase was apparent when the neomycin treatment was performed simultaneously with DMPP, suggesting a direct action on the nicotinic receptor. The direct inhibitory action of neomycin on the nicotinic receptor was also evident when neomycin inhibited the DMPP-induced cytosolic calcium increase, which is not affected by nifedipine or ω-conotoxin MVIIC, and the cytosolic Na$^+$ increase, which is not affected by tetrodotoxin. In addition, we observed that neomycin inhibited the binding of nicotine to the acetylcholine receptor in a noncompetitive manner. The data suggest that neomycin inhibits the nicotinic acetylcholine receptor directly, which results in blockage of the nicotinic receptor-mediated signaling without involvement of phospholipase C.

Aminoglycosides are used primarily to treat infectious diseases caused by aerobic Gram-negative bacteria (for review, see Chambers and Sande, 1996). One of the aminoglycosides, neomycin, is an antibiotic and anticancer agent with a wide spectrum and also several side effects including ototoxicity and nephrotoxicity (Siegenthaler et al., 1986).

On the cellular level, neomycin exhibits various actions on several targets. Neomycin is one of the inhibitors along the PLC pathway because of its binding affinity to phosphatidylinositol 4,5-bisphosphate (PIP$_2$) (Orsulakova et al., 1976; Negishi et al., 1990). Therefore, together with 1-[(17β-3-methoxyestra-1,3,5(10)-triene-17-yl)amino]hexyl-1Hpyrrol-2,5-dione (U-73122), neomycin is a widely used tool in the study of PLC-mediated signal transduction (Suh et al., 1996; Stanimirovic et al., 1996; Hildebrandt et al., 1997). Some types of ion channels, including the volume-sensitive Cl$^-$ channel and the voltage-sensitive Na$^+$ channel in Xenopus oocytes, are known to be activated by PLC and inhibited by neomycin (Charpentier et al., 1995; Mitchell et al., 1997). However, it has also been reported that neomycin inhibits voltage-sensitive calcium channels without the involvement of PLC (Duarte et al. 1993; Langton et al., 1996; Pichler et al., 1996). Neomycin also inhibits calcium release-activated channels known to be stimulated by capacitative calcium entry (Sipma et al., 1996) and the mechanosensitive ion channels in skeletal muscle (Winegar et al., 1996).

Early studies revealed that aminoglycoside antibiotics including neomycin could regulate the function of cholinergic receptors and pre- and postsynaptic neurotransmission at neuromuscular junctions (Brown and Taylor, 1983; Fiekers, 1983a,b). However, the exact mechanism of the inhibition of the nicotinic acetylcholine receptor-mediated secretion by neomycin is not yet known. The bovine adrenal chromaffin cell has been a good model for the study of the neuroendocrine system. We investigated the inhibitory effect of neomycin on nicotinic receptor signaling in bovine chromaffin cells. We demonstrate here a direct inhibition of the nicotinic acetylcholine receptor by neomycin and the subsequent inhibition of acetylcholine receptor-mediated norepinephrine release.

ABBREVIATIONS: DMPP, dimethylphenylpiperazinium iodide; InsP$_3$, inositol 1,4,5-trisphosphate; [Ca$^{2+}$], cytosolic calcium ion concentration; [Na$^+$], cytosolic sodium ion concentration; IC$_{50}$, half-maximal inhibitory concentration; SBFI/AM, sodium binding furan isophthalate tetracetoxy methyl ester; PLC, phospholipase C; PIP$_2$, phosphatidylinositol 4,5-bisphosphate.
Materials and Methods

Materials. Neomycin, DMPP, bradykinin, isoinitol 1,4,5-trisphosphate (InsP3), nicotine, and sulfonpyrazone were purchased from Sigma Chemical Co. (St. Louis, MO). Fura-2 penta-acetoxyethyl ester, sodium binding furan isophthalate tetraacetoxyethyl ester (SBFI/AM), and pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Veratridine and tetrodotoxin were purchased from Research Biochemicals Inc. (Natick, MA). [3H]Norepinephrine, [3H]nicotine, and [3H]InsP3 were purchased from New England Nuclear (Boston, MA). Dulbecco's modified essential medium (DMEM)/Ham's F-12 (F-12) and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY). Bovine calf serum and horse serum were obtained from HyClone (Logan, UT).

Chromaffin Cell Preparation. Chromaffin cells were isolated from bovine adrenal medulla by two-step collagenase digestion as previously described (Kilpatrick et al., 1980). For the measurement of [3H]norepinephrine secretion, cells were plated in 24-well plates at a density of 5 × 10^5 cells per well. Chromaffin cells transferred to 100-mm culture dishes (1 × 10^6 cells per dish) were used to measure cytosolic free calcium and membrane concentrations. The cells were maintained in DMEM/F-12 containing 10% of bovine calf serum and 1% of antibiotics. They were incubated in a humidified atmosphere of 5% CO2/95% air at 37°C for 3 to 7 days before use.

Measurement of Catecholamine Secretion. Catecholamine secretion from chromaffin cells was measured in 24-well plates following the method described by Park et al. (1998). In brief, cells were loaded with [3H]norepinephrine (1 μCi/ml) in DMEM/F-12 for 1 h at 37°C in 5% CO2/95% air. The cells were then washed twice and incubated in Locke’s solution (154 mM NaCl, 5.6 mM KCl, 10 mM glucos, 2.2 mM CaCl2, 1.2 mM MgCl2, and 5 mM HEPES buffer adjusted to pH 7.4) for 15 min to let them stabilize. The cells were then incubated again in fresh Locke’s solution for 15 min and their basal secretion was determined. The cells were subsequently stimulated with the drugs being tested for 15 min. The medium was removed from each well and residual catecholamines were extracted from the cells by adding 10% trichloroacetic acid. The radioactivity was measured with a scintillation counter. The amount of [3H]norepinephrine secreted was calculated as the percentage of total [3H]norepinephrine content.

Endogenous catecholamine secretion from chromaffin cells was measured in a flowing stream as described by Kim and Westhead (1989). A quartz plate (12.5 × 45 × 1 mm) with attached bovine chromaffin cells was placed in a flow chamber with high-performance liquid chromatography. Locke’s solution flowed over the cells at a rate of 1 ml/min. The effluent from this flow chamber went through capillary tubing to an electrochemical detector (Metrom, Westbury, NY) to measure catecholamine released from the cells. Dispersion ratio from the injection valve to the detector was 0.37. The total catecholamine in the cells in the chamber was about 0.13 μmol, as determined after trichloroacetic acid extraction.

The highest level of secretion shown is about 2% of the total secretory capacity. The responses in various cell batches differed from each other, but the relative behavior of different stimulants was reproducible.

Cytosolic Calcium Ion Concentration [Ca^{2+}]i Measurement and Influx Assay by Mn^{2+} Quenching. [Ca^{2+}]i was determined using the fluorescent Ca^{2+} indicator fura-2 as reported previously (Suh et al., 1996). Brieelly, the cell suspension was incubated in Locke’s solution with 3 mM fura-2 penta-acetoxyethyl ester for 50 min at 37°C under continuous stirring. The loaded cells were then washed twice with Locke’s solution. Sulfonpyrazone (250 μM) was added to all solutions to prevent dye leakage. For the fluorimetric measurement of [Ca^{2+}]i, 1 × 10^6 cells/ml were placed into a quartz cuvette in a thermosometrically controlled cell holder at 37°C and continuously stirred. Fluorescence ratios were monitored with dual excitation at 340 and 380 nm and emission at 500 nm. Calibration of the fluorescent signal in terms of [Ca^{2+}]i was performed as described by Grynkiewicz et al. (1985) using the following equation

\[ [Ca^{2+}]_i = K_D (R - R_{min})/(R_{max} - R)(Sf/Sb) \]

where R is the ratio of fluorescence emitted by excitation at 340 and 380 nm. Sf and Sb are the proportionality coefficients at 380 nm excitation of Ca^{2+}-free fura-2 and Ca^{2+}-saturated fura-2, respectively. To obtain R_{min}, the fluorescence ratios of the cell suspension were measured successively at a final concentration of 4 mM EGTA, 30 mM Trizma base, and 0.1% Triton X-100. Then the cell suspension was treated with CaCl2 for a final concentration of 10 mM Ca^{2+} and the fluorescence ratios were measured to obtain R_{max}.

The Mn^{2+} quenching assay was performed as described by Lee et al. (1997) to measure the influx of Ca^{2+} from the extracellular space. Briefly, fura-2-loaded cells (5 × 10^6 cells/ml) were placed into a quartz cuvette in a thermostatically controlled cell holder at 37°C and continuously stirred. Fluorescence ratios were monitored at the isosbestic wavelength of 360 nm and emission wavelength at 500 nm. The potency and slope of change in fluorescence intensity were recorded after applying 2 mM MnCl2 and the drugs to be tested.

Cytosolic-Free Na+ Concentration ([Na+]i) Measurement. [Na+]i, was measured using the fluorescent Na+ indicator SBFI as previously described by Choi and Kim (1996) with some modification. In brief, the chromaffin cell suspension was incubated in fresh DMEM/F-12 medium containing 15 μM SBFI/AM, 10% bovine calf serum, and 0.2% pluronic F-127 for 2 h at 37°C with continuous stirring. The cells were then washed twice with fresh DMEM/F-12 medium and left at room temperature until use. Sulfonpyrazone (250 μM) was added to all solutions to prevent dye leakage. Fluorescence ratios were taken with alternate excitation at 340 and 380 nm and emission at 530 nm. Changes in [Na+]i, are presented as fluorescence ratios.

Measurement of InsP3 Production. InsP3 mobilization was determined by competition assay with [3H]InsP3 as described previously (Lee et al., 1997). In brief, the InsP3 production, 1 × 10^6 cells per well of a 6-well plate were stimulated with the drugs. The reaction was terminated by addition of ice-cold 5% trichloroacetic acid containing 10 mM EGTA. The supernatant of the cell lysate was saved and the trichloroacetic acid was removed by extraction with diethyl ether. The aqueous fraction was neutralized with 200 mM of Trizma base and adjusted to pH 7.4. Twenty microliters of the mixture was incubated for 15 min on ice and then centrifuged at 2000g for 10 min. One hundred microliters of water and 1 ml of liquid scintillation cocktail were added to the pellet to measure the radioactivity. The InsP3 concentration of the sample was determined by comparison to a standard curve and expressed as picomoles per milligram of protein. The total cellular protein concentration was measured by the Bradford method after sonication of 2 × 10^6 cells.

[3H]Nicotine Binding Assay. Binding of [3H]nicotine to intact cells was measured as previously described by Higgins and Berg (1988). Intact chromaffin cells in 24-well plates (5 × 10^5 cells/well) were washed twice with Locke’s solution and incubated with 20 nM [3H]nicotine and the drugs to be tested for 60 min at 25°C. Then the cells were washed three times with 1 ml of ice-cold Ca2+-free Locke’s solution containing 100 μM EGTA. Finally, the cells were lysed and scraped into 0.5 ml of 5% trichloroacetic acid, and the radioactivity was measured by liquid scintillation counting. Nonspecific binding, determined by coinubation with 1 mM nicotine, amounted to less than 20% of the total binding and was routinely subtracted from the total binding. The binding data were analyzed and expressed as percentage of total binding. Binding of [3H]nicotine to cell membranes was measured as previously described by Lee et al. (1995). Brieelly, the cultured cells were washed and sonicated in the homogenization buffer (100 mM NaHCO3, 5 mM EDTA, 5 μg/ml leupeptin, 10% sucrose, 1 mM phenylmethyloxysulfon formide, ad-
justed to pH 8.3). The membrane fraction was harvested and its concentration was determined with the Bradford protein assay. The membranes (50 µg of protein) were incubated with [3H]nicotine in the absence or presence of neomycin for 60 min at 25°C. The cells were then washed, and their radioactivity was measured by liquid scintillation counting. Nonspecific binding was also determined by coinubcation with 1 mM nicotine for each point.

**Analysis of Data.** All quantitative data are expressed as means ± S.E.M. The results were analyzed using the unpaired Student’s *t* test. We calculated the half-maximal inhibitory concentration (IC50) with the AllFit for Windows program (De Lean et al., 1978). Results were considered significant only for *P* < .05.

**Results**

In bovine adrenal chromaffin cells, stimulation of nicotinic acetylcholine receptor causes the influx of Na⁺ and Ca²⁺ through the receptor leading to membrane depolarization and subsequent influx of Ca²⁺ through voltage-sensitive Ca²⁺ channels (Fenwick et al., 1982). The elevation of the cytosolic Ca²⁺ level triggers catecholamine secretion. To test the effect of neomycin on the nicotinic acetylcholine receptor-mediated response, bovine chromaffin cells were stimulated with a specific nicotinic agonist, DMPP, in the presence of neomycin. DMPP evoked an increase in [Ca²⁺], in fura-2-loaded bovine adrenal chromaffin cells. In addition, pretreatment with 100 µM d-tubocurarine, an inhibitor of nicotinic acetylcholine receptors, completely inhibited the 20 µM DMPP-evoked [Ca²⁺], increase (data not shown). With these results we confirmed that nicotinic acetylcholine receptors were expressed on our cells and that DMPP acts on the nicotinic receptors. We found that neomycin inhibited the norepinephrine secretion caused by DMPP in a concentration-dependent manner with an IC50 of 273 ± 9 µM (Fig. 1). We confirmed this result by measuring endogenous catecholamine secretion. As shown in Fig. 2, 5 µM DMPP induced the catecholamine secretion by these cells, whereas 100 µM neomycin decreased the DMPP-induced secretion. After washing, the inhibitory effect of neomycin became completely reversed.

In addition, pretreatment with neomycin for 3 min also inhibited the DMPP-induced [Ca²⁺], increase in fura-2-loaded cells (Fig. 3). The concentration-dependent inhibitory effect of neomycin on the Ca²⁺ elevation had a similar IC50 value of 278 ± 74 µM. However, DMPP did not trigger a Ca²⁺ increase when extracellular Ca²⁺ was removed; thus a neomycin effect on the DMPP-induced response in the absence of external calcium was not detected (data not shown). Furthermore, we tested fluorescence quenching in the presence of Mn²⁺ to confirm that neomycin inhibits the influx of Ca²⁺ upon nicotine stimulation. In fura-2-loaded cells, DMPP stimulation accelerated the fura-2 fluorescence quenching in the presence of extracellular Mn²⁺. Treatment with neomycin dramatically reduced the rate of fluorescence quenching caused by DMPP (Fig. 4). The results suggest that neomycin inhibits nicotinic receptor-mediated Ca²⁺ influx and catecholamine secretion.

Neomycin is known to block PLC-mediated processes. We therefore tested whether neomycin inhibits PLC-mediated Ca²⁺ release from intracellular stores under the above experimental condition. Bradykinin is one of the neuromodulators whose signal is mediated by PLC in bovine adrenal chromaffin cells (Kim and Westhead, 1989). Bradykinin elevated the cytosolic Ca²⁺ even in the absence of extracellular Ca²⁺, indicating Ca²⁺ release from intracellular Ca²⁺ pools. However, neomycin did not affect the bradykinin-induced Ca²⁺ increase (Fig. 5, A and B). Even a 10-min preincubation at 1 and 5 mM DMPP in the presence or absence of 100 µM neomycin, after which the eluant of high-performance liquid chromatography was analyzed with an electrochemical detector. The experiments were performed three times independently, and the results were reproducible.

![Fig. 1. The effect of neomycin on [3H]norepinephrine secretion in bovine adrenal chromaffin cells.](image1)

![Fig. 2. Neomycin effect on exocytosis of endogenous catecholamine in bovine adrenal chromaffin cells.](image2)
mycin might inhibit the bradykinin-induced InsP$_3$ production under the above conditions (Fig. 5C). Neomycin did not affect the bradykinin-induced production of InsP$_3$ after 3 min of preincubation. However, neomycin did dramatically inhibit the bradykinin-induced InsP$_3$ production after a preincubation for 60 min, which is the typical time period necessary for the inhibition of PLC-mediated processes. The results, therefore, suggest that neomycin has an inhibitory effect on the nicotinic acetylcholine receptor-mediated signaling that is distinct from the effect on the PLC-mediated process.

When we preincubated the cells with neomycin for various time periods to determine the time needed to inhibit the DMPP-evoked [Ca$^{2+}$]$_i$ rise response, the inhibition occurred maximally with the 3-min preincubation. However, simultaneous treatment with neomycin and DMPP also decreased the DMPP-evoked [Ca$^{2+}$]$_i$ rise with similar potency (Fig. 6). This result suggests that the inhibitory effect of neomycin is not required during the preincubation time and that it must directly inhibit nicotinic stimulation.

Because possible inhibition of voltage-sensitive Ca$^{2+}$ channels by neomycin may result in the inhibition of the nicotinic receptor, we tested the neomycin effect on the DMPP-induced Ca$^{2+}$ increase in the presence of voltage-sensitive Ca$^{2+}$ channel antagonists to preclude this possibility. As shown in Fig. 7A, 5 μM nifedipine partially inhibited the DMPP-induced Ca$^{2+}$ rise, which means that L-type voltage-sensitive Ca$^{2+}$ channels are involved in the [Ca$^{2+}$]$_i$ elevation induced by nicotinic stimulation. However, neomycin in the presence of nifedipine had an additive inhibitory effect. This suggests that neomycin directly inhibits the nicotinic receptor while also inhibiting the voltage-sensitive Ca$^{2+}$ channel. We obtained similar results with 1 μM ω-conotoxin MVIIC, the Q-type voltage-sensitive Ca$^{2+}$ channel antagonist, as seen in Fig. 7B.

Next, we tested the characteristics of the neomycin-induced inhibition in comparison to lidocaine. Lidocaine also inhibited the DMPP-induced Ca$^{2+}$ increase in a concentration-dependent manner (data not shown) as has also been reported previously (Purifoy and Holz, 1984). In addition, neomycin had an additive inhibitory effect on the DMPP-induced calcium increase by 100 μM lidocaine, but it failed to show increased inhibition in the presence of 300 μM lidocaine (data not shown). The results indicate that neomycin and lidocaine share the inhibition target, the nicotinic receptor.

The direct inhibition of the nicotinic receptor by neomycin was analyzed by measuring the Na$^+$ influx through the receptor in the presence of neomycin. To exclude the possible involvement of voltage-sensitive Na$^+$ channels, we incorporated tetrodotoxin, a voltage-sensitive Na$^+$ channel blocker. A voltage-sensitive Na$^+$ channel opener veratridine triggered a rise in the Na$^+$ concentration in SBFI-loaded chromaffin cells, whereas 5 μM tetrodotoxin completely inhibited the veratridine-induced [Na$^+$]$_i$ rise (Fig. 8A). In the presence of 5 μM tetrodotoxin, however, the addition of neomycin still inhibited the DMPP-induced Na$^+$ increase (Fig. 8B). In ad-
dition, we examined the direct inhibition of nicotine binding to the nicotinic receptor by neomycin in a binding competition assay. Neomycin affected the binding property of the nicotinic receptor in a noncompetitive inhibitory manner with a decrease in both the $B_{\text{max}}$ and apparent dissociation constant ($K_D$) (Fig. 9A). In the absence or presence of neomycin, apparent dissociation constant values were 49.95 and 23.10 nM, respectively, and the maximum number of binding sites values was 55.07 and 28.62 fmol/mg protein, respectively. Figure 9B shows that neomycin inhibited $[3H]$nicotine binding to the nicotinic receptor in a concentration-dependent manner. The IC$_{50}$ value was 351 ± 9 μM, which is similar to the IC$_{50}$ values obtained for inhibition of the Ca$^{2+}$ rise and catecholamine secretion. The results, therefore, indicate that neomycin competes with the agonist for binding to the nicotinic acetylcholine receptor and thus inhibits the receptor’s function.

Fig. 5. Effect of neomycin on bradykinin-induced [Ca$^{2+}$], increase and production of InsP$_3$ in bovine adrenal chromaffin cells. A, fura-2-loaded cells were treated with 5 μM bradykinin (BK) with (b) or without (a) 300 μM neomycin (Neo). B, cells were challenged with bradykinin (BK) with (b) or without (a) 300 μM neomycin (Neo) in the absence of extracellular Ca$^{2+}$. Typical Ca$^{2+}$ transients from more than three separate experiments are presented in A and B. The results were reproducible in both cases. C, cells were pretreated with or without 300 μM neomycin for 3 or 60 min and then stimulated with 5 μM bradykinin as indicated. Three separate reproducible experiments were done and each point is the mean ± S.E.M. of triplicate results. Statistical analysis of the data was done by the unpaired Student’s $t$ test in comparison between two experimental groups. **$P<.01$, compared with the result of 3 min incubation with neomycin (column 4).

Fig. 6. Time course of neomycin-induced inhibition of DMPP-evoked [Ca$^{2+}$], rise. Fura-2-loaded cells were treated with 20 μM DMPP with (●) or without (■) preincubation with 300 μM neomycin for the indicated times. Each point is the mean ± S.E.M. of triplicate assays. The experiments were performed three times independently and the results were reproducible.

Fig. 7. The effect of voltage-sensitive Ca$^{2+}$ channel antagonists on the neomycin-induced inhibition of DMPP-evoked [Ca$^{2+}$], rise. A, fura-2-loaded cells were treated with 20 μM DMPP with or without 300 μM neomycin (Neo) and 5 μM nifedipine (Nif) indicated as follows: vehicle (a), nifedipine (b), neomycin (c), and nifedipine plus neomycin (d). B, Cells were treated with 20 μM DMPP with or without 300 μM neomycin (Neo) and 1 μM α-conotoxin MVIIIC indicated as follows: vehicle (a), nifedipine (b), α-conotoxin MVIIIC (c), nifedipine plus α-conotoxin MVIIIC (d). Typical Ca$^{2+}$ transients from more than three separate experiments are presented. The results were reproducible.
Pittinger and Adamson (1972) reported that, as one of the acute effects of aminoglycoside antibiotics in clinical trials, high doses of neomycin induced a neuromuscular blockade. Subsequent reports suggested that the neuromuscular blockade is caused by a neomycin-mediated inhibition of acetylcholine release at the neuromuscular junction (Wright and Collier, 1977; Fiekers, 1983a; Redman and Silinsky, 1994). The effect was thought to be caused by a neomycin-induced inhibition of voltage-sensitive Ca\textsuperscript{2+} channels (Duarte et al., 1993). In addition, several publications reported that treatment with excess Ca\textsuperscript{2+} or a choline esterase inhibitor such as neostigmine could reverse the neomycin-induced neuromuscular blockade (Singh et al., 1978). Fiekers (1983b) suggested an alternative mechanism in which neomycin might directly act on the postsynaptic acetylcholine receptors and thus induce the neuromuscular blockade. Brown and Taylor (1983) reported that polymyxin and neomycin noncompetitively inhibited \textsuperscript{22}Na\textsuperscript{+} permeability and thus augmented receptor desensitization upon cholinergic stimulation in clonal muscle cells. In spite of this evidence, the mechanism of the neomycin action in the neuronal system still needed to be confirmed in cells other than in the muscle cells the authors had studied. In the present study, we found that neomycin can inhibit catecholamine secretion in chromaffin cells by binding to the nicotinic acetylcholine receptor. At present, the target subtype of acetylcholine receptors in chromaffin cells is still controversial, because electrophysiological data in guinea pig chromaffin cells demonstrate that neomycin inhibits muscarinic receptors, which, in turn, activate the phosphatidylinositol turnover and Ca\textsuperscript{2+} influx (Inoue et al., 1995). However, our study suggests that the nicotinic receptor can be inhibited by neomycin without the involvement of PLC. The actions of neomycin can be classified into two distinct mechanisms: one is the blockage of a PLC-mediated signal flow (Winegar et al., 1996) and the other is the direct inhibition of channels without the involvement of PLC (Langton et al., 1996). It is generally thought that the positive charge of neomycin helps it to form a complex with the negatively charged membrane lipids, including PIP\textsubscript{2}, when it inhibits the action of PLC (Orsulakova et al., 1976). Channel inhibition mediated by PLC activation is thought to be reversed by neomycin in this manner. In bovine adrenal chromaffin cells, neomycin has been reported to block PLC signaling (Negishi et al., 1990). Because protein kinase C inhibits the voltage-sensitive Na\textsuperscript{+} channel in chromaffin cells (Yanagita et al., 1996), it is also possible that neomycin reverses the Na\textsuperscript{+} channel inhibition by blocking PLC and a subsequent
activation of protein kinase C. However, direct inhibition of voltage-sensitive Ca\(^{2+}\) channels was also reported for these cells (Duarte et al., 1993). Thus it remains a challenge to understand which type of neomycin action is involved in the inhibition of the nicotinic receptor-mediated responses.

In our study, we tried to elucidate the mechanism of the neomycin effect on catecholamine secretion following stimulation of the nicotinic receptor. We demonstrated that neomycin inhibits the nicotinic acetylcholine receptor-mediated norepinephrine secretion and cytosolic Ca\(^{2+}\) increase in bovine chromaffin cells. The similar IC\(_{50}\) values of neomycin in catecholamine secretion, cytosolic Ca\(^{2+}\) rise, and nicotine binding to the receptor suggest that neomycin does not involve multiple sites during the secretory process evoked by nicotinic stimulation. Neomycin inhibits the nicotinic acetylcholine receptor-mediated response, which results in a subsequent inhibition of downstream signals such as Ca\(^{2+}\) influx or of molecules involved in the secretory pathway. However, the present study demonstrates that neomycin can directly interact with the acetylcholine receptor without involvement of other factors. The following points support a direct action by neomycin: 1) neomycin's inhibition of the cytosolic Ca\(^{2+}\) rise upon simultaneous treatment with DMPP, 2) neomycin's inhibition of the Ca\(^{2+}\) entry in the presence of voltage-sensitive Ca\(^{2+}\) channel antagonists, 3) neomycin's inhibition of the Na\(^{+}\) entry through the nicotinic receptor, and 4) neomycin's interference with nicotine binding to nicotinic receptors. It is generally assumed that neomycin-induced PLC inhibition requires a preincubation time (several minutes to hours) to allow the drug to penetrate the cellular membrane and bind to PI(4,5)P\(_2\). Furthermore, PLC-mediated channel modulation often requires the subsequent activation of protein kinase C (Charpentier et al., 1995). Our study of the time course of the neomycin-induced inhibition shows that there is little correlation between the extent of inhibition and the time of incubation with neomycin. Neomycin did not affect the bradykinin-induced InsP\(_3\) production and Ca\(^{2+}\) elevation after 3 min of preincubation, but it dramatically inhibited the InsP\(_3\) production after a 1-h preincubation. The results reveal that neomycin does indeed inhibit PLC, but it requires a longer time of incubation. Neomycin did not affect the bradykinin-induced Ca\(^{2+}\) increase, when the incubation time was not long enough, whereas it did successfully inhibit the DMPP-induced Ca\(^{2+}\) increase. Therefore we suggest that the neomycin-induced inhibition of the nicotinic receptor is not due to the inhibition of PLC.

In addition, it has also been reported that neomycin inhibits voltage-sensitive Ca\(^{2+}\) channels in chromaffin cells (Duarte et al., 1993; Pichler et al., 1996). Because the activation of nicotinic receptors causes membrane depolarization and activates voltage-sensitive Ca\(^{2+}\) channels, it is possible that neomycin inhibits voltage-sensitive Ca\(^{2+}\) channels, which is shown by the inhibition of the nicotinic receptors. It has been reported previously that there are several different types of voltage-sensitive Ca\(^{2+}\) channels including L, N, P, and Q (Artalejo et al., 1994; Lomax et al., 1997), and that the L- and Q-types are critical for the secretion of neurotransmitters (Lomax et al., 1997) in bovine adrenal chromaffin cells. We tested this with nifedipine and \(\omega\)-conotoxin MVIIIC, which are L-type and Q-type voltage-sensitive Ca\(^{2+}\) channel antagonists, respectively. In our experiments, neomycin exhibited an additive inhibitory effect in the presence of nifedipine or \(\omega\)-conotoxin MVIIIC, whereas they partially inhibited the DMPP-induced Ca\(^{2+}\) increase (Fig. 7). This suggests that neomycin inhibits the nicotinic receptor directly, whereas it also inhibits voltage-sensitive Ca\(^{2+}\) channels, and that the inhibition of the DMPP effect is not an event that follows the inhibition of the voltage-sensitive Ca\(^{2+}\) channels. Another effect of the nicotinic activation, the Na\(^{+}\) influx, was also inhibited by neomycin under conditions in which voltage-sensitive Na\(^{+}\) influx was blocked by tetrodotoxin (Fig. 8).

It has been reported that neomycin has an effect mimicking local anesthetics (Bruckner et al., 1980). This is interesting, because local anesthetics also inhibit not only voltage-sensitive Na\(^{+}\) channels but also nicotinic acetylcholine receptors (Purifoy and Holz, 1984). Our results that neomycin and lidocaine commonly inhibit the nicotinic receptor (data not shown) suggest that neomycin may inhibit the nicotinic receptor in a manner similar to lidocaine and thus have the effect of a local anesthetic.

Recently, it was reported that neomycin inhibits calcium release from the sarcoplasmic reticulum in skeletal muscle, which was elucidated by noncompetitive inhibition of the binding of ryanodine to Ca\(^{2+}\) release channels (Wang et al., 1996). Possible mechanisms of ion channel inhibition include blocking of the channel in the open or the closed state, modulation of the open probability of the channel, or competitive interference in the interaction between stimulant and channel. It has been reported that neomycin blocks mechanosensitive channels in the open state (Winegar et al., 1996). The mode of the interaction between neomycin and the nicotinic receptors still needs to be explored in more detail by electrophysiological studies.

In conclusion, the results of our study suggest a mechanism for the neomycin-induced inhibition of the nicotinic acetylcholine receptor-mediated Ca\(^{2+}\) and Na\(^{+}\) influx and norepinephrine secretion, and also suggests that neomycin interacts directly with the nicotinic receptor, thus interfering with its signaling without the involvement of PLC.

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