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Effects of neomycin on high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents in rat dorsal root ganglion neuron

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Abstract

High-threshold Ca^{2+} channels and tetrodotoxin-resistant Na^+ channels are highly expressed in small dorsal root ganglion neurons. In acutely isolated rat dorsal root ganglion neurons, the effects of neomycin, one of the aminoglycoside antibiotics, on high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents were examined using whole-cell patch recording. We showed for the first time that neomycin dose-dependently inhibited peak high-threshold Ca^{2+} currents and peak tetrodotoxin-resistant Na^+ currents with half-maximal inhibitory concentrations at 3.69 μM ($n=20$) and 1213.44 μM ($n=25$), respectively. Inactivation properties of high-threshold Ca^{2+} currents and activation properties of tetrodotoxin-resistant Na^+ currents were also affected by neomycin with reduction of excitability of small dorsal root ganglion neurons. Half-maximal inactivation voltage of high-threshold Ca^{2+} currents was −45.56 mV before and −50.46 mV after application of neomycin ($n=10$). Half-maximal activation voltage of tetrodotoxin-resistant Na^+ currents was −19.93 mV before and −11.19 mV after administration of neomycin ($n=15$). These results suggest that neomycin can inhibit high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents in small dorsal root ganglion neurons, which may contribute to neomycin-induced peripheral and central analgesia.

Keywords: Ca^{2+} channel, high-threshold; Neomycin; Whole-cell patch recording; Na^+ channel, tetrodotoxin-resistant; Pain

1. Introduction

Small dorsal root ganglion neurons with unmyelinated or thin myelinated fibers conduct the nociceptive information from peripheral tissue to the spinal cord (Harper and Lawson, 1985a,b). Besides low-threshold T-type Ca^{2+} channel, small dorsal root ganglion neurons are known to express multiple types of high-voltage-activated Ca^{2+} channels, including L-, N-, P/Q- and R-type Ca^{2+} channel (Scroggs and Fox, 1992; Moises et al.,1994; Abdulla and Smith, 2001; Luo et al., 2001). Given that blockade of these high-threshold Ca^{2+} channels can prevent/attenuate subjective pain as well as the hyperalgesia and allodynia induced by peripheral lesions (see review by Vanegas and Schaible, 2000), high-threshold Ca^{2+} channels may play important roles in nociceptive processing.

The other important characteristic of small dorsal root ganglion neurons is the high expression of tetrodotoxin-resistant Na^+ currents (Akopian et al., 1996; Elliott and Elliott, 1993; Novakovic et al., 1998; Ogata and Tatebayashi, 1993; Roy and Narahashi, 1992; Rush et al., 1998). The newly cloned two types of tetrodotoxin-resistant Na^+ channels are preferentially expressed in small dorsal root ganglion neurons (Akopian et al., 1996; Dib-Hajj et al., 1998; Sangameswaran et al., 1996; Tate et al., 1998). Therefore, tetrodotoxin-resistant Na^+ channels-positive small dorsal root ganglion neurons are widely used as a model for pain-related study.

Neomycin belongs to the family of aminoglycoside antibiotics. When injected intrathecally, neomycin had an analgesic effect in tail-flick and hot-plate tests in mice (Ocana and Baeyens, 1991) and reduced the nociceptive behavior induced by formalin injection (Coderre, 1992). In addition, Blenk et al. (1997) observed that neomycin locally applied to the transected L_5 spinal nerve could prevent mechanical allodynia in rats. These behavioral studies strongly suggest that both peripheral and central mechanisms are likely involved in neomycin-induced analgesia. To explore the possible mechanisms underlying neomycin-induced analgesia, the present study was designed to deter-

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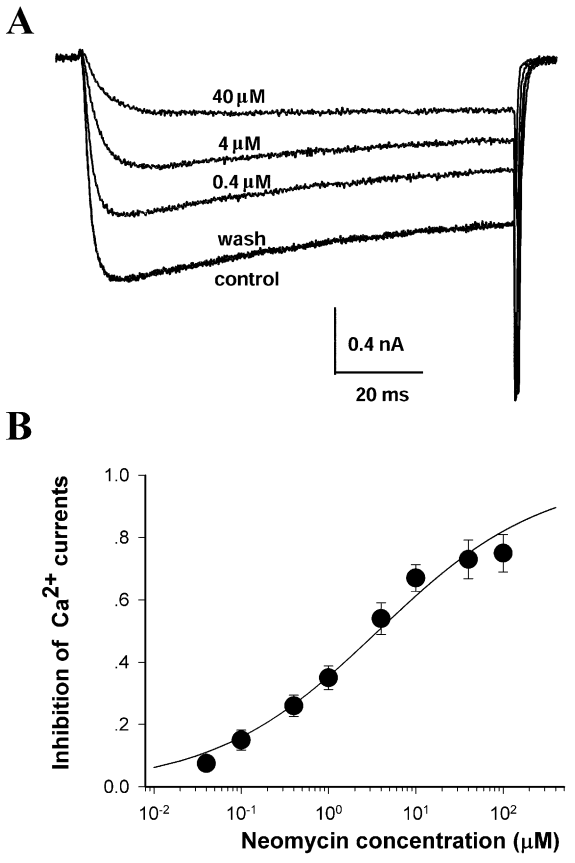

Fig. 1. Neomycin inhibited high-threshold Ca^{2+} currents in a dose-dependent manner. (A) Neomycin inhibited peak high-threshold Ca^{2+} currents in dose-dependent manner. The concentrations of neomycin used were marked in figure. (B) Dose–response curves of neomycin-induced inhibition on high-threshold Ca^{2+} currents. Data were fitted with Hill equation. Half-maximal inhibitory concentration (IC_{50}) was 3.69 μM. Hill coefficient was 0.46. Error bars give S.E.M. Ca^{2+} currents were evoked at 30-s interval by 100 ms voltage step to −10 mV from a holding potential of −80 mV.

mine the effects of neomycin on high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^{+} currents in dissociated rat dorsal root ganglion neurons by means of whole-cell patch–clamp recording technique.

2. Materials and methods

2.1. Preparation of dorsal root ganglion neurons

Dispersed cells were prepared similarly as our previous work (Li and Zhao, 1998). In brief, Sprague–Dawlay male rats weighing 80–140 g were used. After decapitation under ethylether anaethesia, a laminectomy was done to expose the lumbar segments of the spinal cord. The L_{4-6} dorsal root ganglions with nerves were picked out and rinsed in 4 °C 1640 solution (Life Technologies, USA) oxygenated with 95% O_2 and 5% CO_2. The nerve trunks were cut off and dorsal root ganglions were incubated in oxygenated 1640 solution with 2.8 mg/ml collegenase (type IA, Sigma) and 1 mg/ml trypsin (type III, Sigma) at 34.5 °C for 60 min. After the enzymatic digestion, the dorsal root ganglions were washed three times with standard external solution (composition given below). The washed dorsal root ganglions were triturated using progressively fine fire-polished Paster pipettes in 0.5 mg/ml DNase (Sigma) containing standard external solution. The isolated dorsal root ganglion neurons were placed in 3.5-cm plastic dishes perfused with the standard external solution at a speed of 2 ml/min.

2.2. Electrophysiological recordings

Whole-cell patch–clamp recordings were carried out at room temperature (~22 °C) using either an Axonpatch 200A amplifier (Axon Instrument, USA) or an EPC-9 amplifier (Heka Electronic, Germany). Pipettes (GG-17) were fabricated with P801-A Puller (Narishige, Japan). The resistance of micropipettes was 2–6 MΩ. Data acquisition and stimulation protocols were controlled by a 686 computer (Compaq) equipped with software pClamp 6.03, through interface Digidata 1200 (Axon Instrument) or software Pulse + Pulsefit 8.5 (Heka Electronic). Capacitance transients were cancelled and series resistance was compensated (>70%). Leak subtraction was performed using P/4 protocol. Data were low-passed at 10 kHz.

All experimental solutions were adjusted to pH 7.4. The standard external solution contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and 10 D-glucose. The standard pipette solution was made up as follows (in mM): 140 KCl, 0.5 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 3 ATP, 5 EGTA. To eliminate K^{+} currents, TEA^{+} was used extracellularly and Cs^{+} was used intracellularly. To isolate Ca^{2+} currents in rat

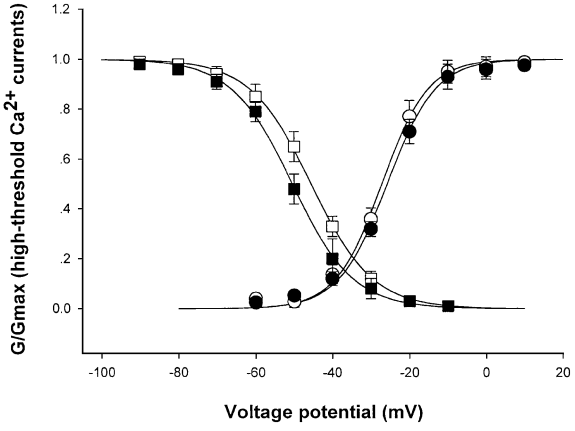

Fig. 2. Activation (circles) and inactivation (squares) curves of high-threshold Ca^{2+} currents before (open symbols) and after (solid symbols) the application of 1 μM neomycin ($n=10$). Data were fitted with Boltzmann function. Activation curves were evoked from −80 mV holding potential by 100 ms command potentials ranging from −60 to 10 mV in the increasing 10 mV step. The protocol of inactivation curves consisted of a 3-s prepulse to a potential in the range of −90 to −10 mV, followed by a 100-ms depolarization to −10 mV.

dorsal root ganglion neurons, the following solutions were employed. The external solution contained (in mM): 160 tetraethylammonium chloride, 2 $BaCl_2$, 0.5 tetrodotoxin, 10 HEPES, 5 D-glucose. The internal solution contained (in mM): 140 CsCl, 1 $MgCl_2$, 5 Na_2ATP, 0.4 Na_2GTP, 10 HEPES, 10 EGTA. Inward Ba^{2+} current through Ca^{2+} channels was referred to as Ca^{2+} currents. Ca^{2+} currents were evoked at 30 s intervals by 100 ms voltage step to −10 mV from a holding potential of −80 mV. At this potential, low-threshold T-type current was at least partially inactivated (Moises et al., 1994). To reduce the influence of the decline in Ca^{2+} current (rundown) on calculation, the magnitude of neomycin-induced current inhibition was expressed as a percentage change in the averaged value of the predrug current and stabilized recovery current amplitude.

For total Na^+ current recording, the external solution of the following composition was used (in mM): 80 NaCl, 78 tetraethylammonium chloride, 1 $MgCl_2$, 10 HEPES, 10 D-glucose and 10 μM $CaCl_2$. The low concentration of 10 μM Ca^{2+} was enough to block Ca^{2+} channels and prevent Ca^{2+} channels from becoming Na^+ conducting (Elliott and Elliott, 1993). The composition of the intracellular solution was as follows (in mM): 140 CsCl, 5 Na_2ATP, 0.4 Na_2GTP, 10 EGTA, 1 $MgCl_2$ and 10 HEPES. For tetrodotoxin-resistant Na^+ current recording, 1 μM tetrodotoxin (Sigma) was used to block tetrodotoxin-sensitive Na^+ channel. Tetrodotoxin-sensitive Na^+ current was obtained by subtracting tetrodotoxin-resistant Na^+ current from the total Na^+ current.

Throughout the experiment, drugs were applied using a gravity-driven perfusion system. Complete exchange of the solutions was reached within 100 ms. Chemicals were prepared as concentrated stock solutions in either distilled water or ethanol and diluted to the final concentration using superfusate solution as indicated in the text. All chemicals used were purchased from Sigma.

2.3. Data process and analysis

Data were measured in Clampfit 6.03 or Pulse + Pulsefit 8.5, the results were transferred to SigmaPlot 4.0 or Igor pro 4.0 for curve fitting. Conductance–voltage (G–V) relationships were calculated from current–voltage relationships according to $G = I/(V - V_{rev})$, in which I is the peak current at voltage (V), and V_{rev} is the reversal potential extrapolated

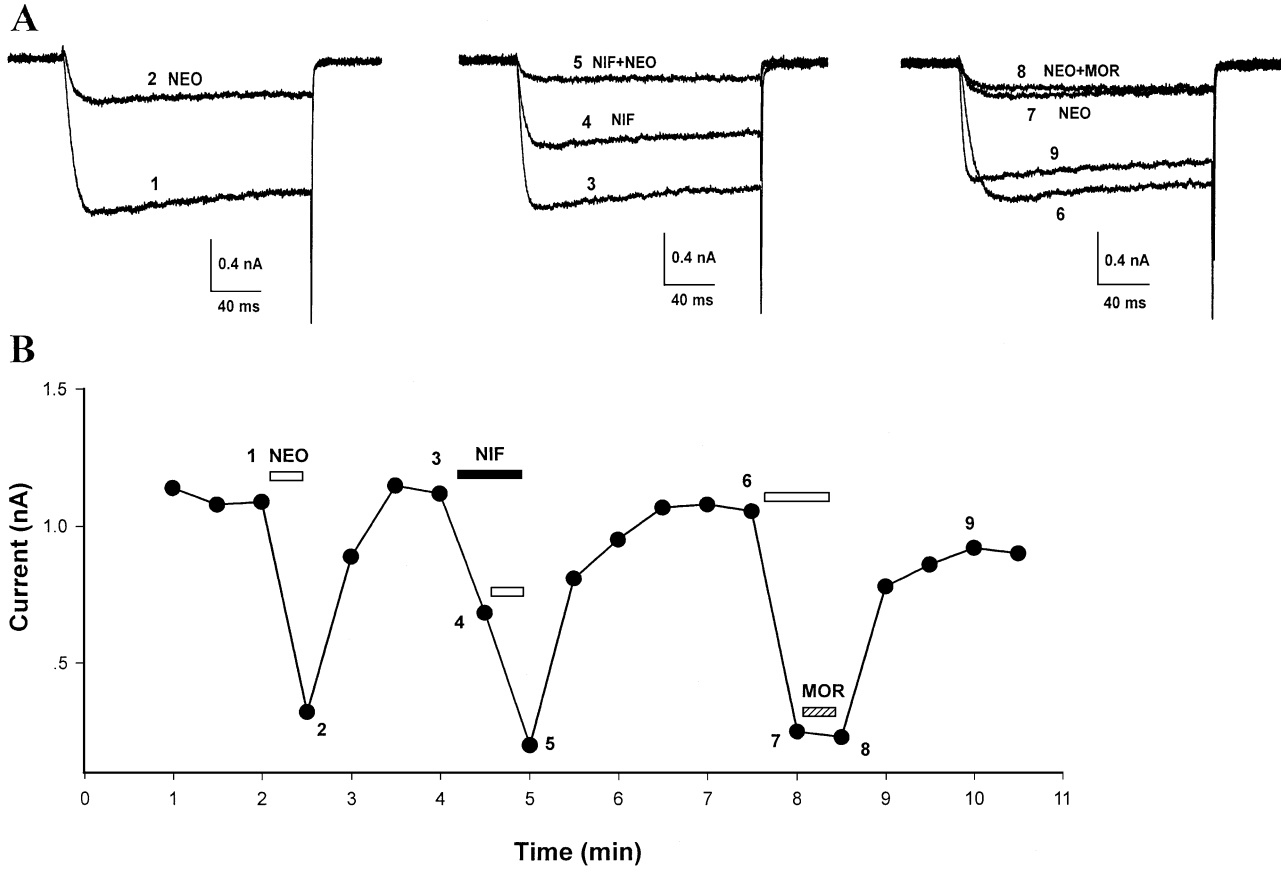

Fig. 3. Time course of an experiment testing the effects of neomycin, nifedipine and morphine on high-threshold Ca^{2+} currents. A, Ca^{2+} currents evoked by 100 ms steps to −10 mV from a holding potential of −80 mV at the times indicated in the graph (B) of current versus time after patch rupture. Application of 20 μM neomycin (open dash) for 30 s reversibly reduced Ca^{2+} currents. Subsequent application of 20 μM neomycin continued to suppress the remaining currents after L-type channel was reversible blockade by 10 μM nifedipine (solid dash). In the presence of 20 μM neomycin, administration of 0.5 μM morphine (striped dash) had no further effect on remained currents.

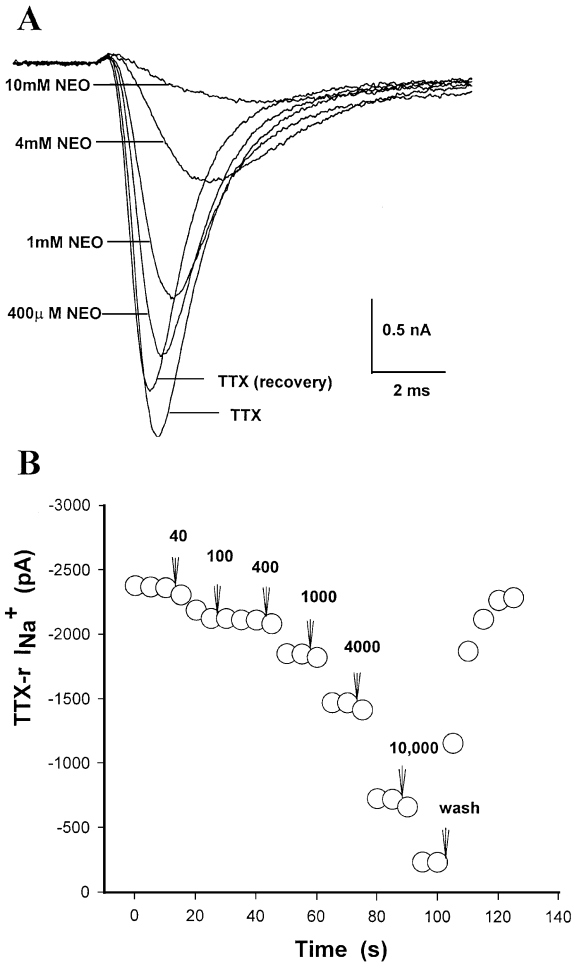

Fig. 4. Neomycin inhibited tetrodotoxin-resistant Na^+ currents in a dose-dependent manner. (A) Neomycin inhibited peak tetrodotoxin-resistant Na^+ currents in a dose-dependent manner. The concentrations of neomycin used were marked in figure. (B) Time course of neomycin-induced dose-dependent inhibition of tetrodotoxin-resistant Na^+ currents in a 19 μm dorsal root ganglion neuron. Command potential for tetrodotoxin-resistant Na^+ currents: 35 ms, 5 mV at a holding potential of −70 mV.

from the $I-V$ relationship. Activation and inactivation curves were fitted with Boltzman equation: $G/G_{max} = 1/(1+\exp((V-V_{1/2})/k))$, where G_{max} is the calculated maximum conductance and $V_{1/2}$ is the voltage at 50% conductance activation or inactivation. Dose–response curves were fitted with Hill equation. Pooled data are expressed as means ± S.E.M. The statistical analysis for comparison between means was performed using a Student's two-tailed t-test. A significant difference was considered for P values <0.05.

3. Results

3.1. Effects of neomycin on high-threshold Ca^{2+} currents

Most of the currents recorded belonged to high-threshold Ca^{2+} currents. Fig. 1A illustrates the concentration-dependent inhibition of peak Ca^{2+} currents produced by 30 s administration of neomycin. The inhibition in currents occurred rapidly and recovered quickly after washout of the drug. A dose–response curve of resting block is shown in Fig. 1B. IC_{50} was 3.69 μM ($n=20$) and Hill coefficent was 0.46. In addition, neomycin affected gating kinetics of the high-threshold Ca^{2+} currents. Fig. 2 shows the gating properties of the high-threshold Ca^{2+} currents before and after the application of 1 μM neomycin. Neomycin obviously shifted half-maximal inactivation voltage ($V_{1/2}$) of high-threshold Ca^{2+} currents, from -45.56 ± 1.25 to -50.46 ± 1.97 mV ($n=10$, $P<0.05$). There was no significant change in slope factor (from 7.988 to 7.9). Neomycin had no significant effect on activation curve of high-threshold Ca^{2+} currents ($n=10$, $P>0.05$). Half-maximal activation voltage ($V_{1/2}$) was -27.19 ± 0.95 mV before and -25.82 ± 0.74 mV after administration of neomycin. V_{rev} calculated from $I-V$ relationship was 43 mV.

Given that μ-opioid receptors agonists selectively reduced three types of Ca^{2+} channels in rat dorsal root ganglion neurons, including N-, P- and Q-type channels, while sparing L-type and T-type components (Rusin and Moises, 1995; Randall and Tsien, 1995), the L-type voltage-dependent Ca^{2+} channel antagonist nifedipine (10 μM) and μ-opioid preferring agonist morphine (0.5 μM) were used to identify the types of high-threshold Ca^{2+} currents that were regulated by neomycin at 20 μM, a near maximal concentration. In this experiment, 0.5 μM morphine administrated alone inhibited part of Ca^{2+} currents and its effect was completely reversed by 1 μM naloxone (data no shown). The traces shown in Fig. 3A illustrate the inhibitory effects of neomycin, nifedipine and morphine on Ca^{2+} currents at the times and under the conditions indicated in the current versus time graph depicted in Fig. 3B. Application of 20 μM neomycin reversibly reduced the peak amplitude of the high-threshold Ca^{2+} currents by 70.5 ± 5.1% of the control ($n=8$). When nifedipine was applied to block the L-type

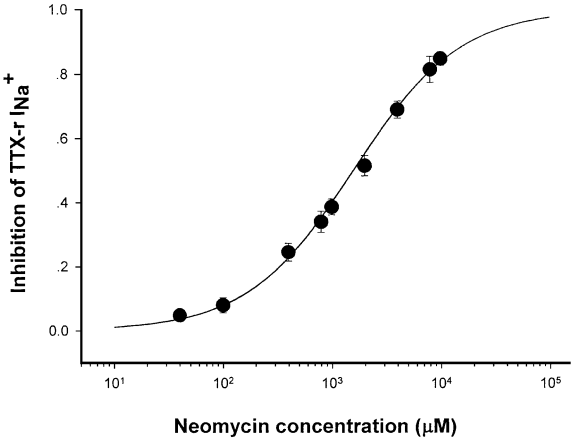

Fig. 5. Dose–response curves of neomycin-induced inhibition on tetrodotoxin-resistant Na^+ currents. Stimulus protocol was the same of Fig. 4. Data were fitted with Hill equation. Half-maximal inhibitory concentration (IC_{50}) for tetrodotoxin-resistant Na^+ currents was 1213.44 μM. Hill coefficient was 0.94. Error bars give S.E.M.

current (38.9 ± 3.7% of the control), subsequent application of neomycin continued to suppress a large fraction (74.7 ± 4.5%) of the remaining nifedipine-insensitive currents. However, in the presence of neomycin, administration of morphine had no additional inhibitory response. These data strongly suggested that neomycin mainly inhibited μ-opioid receptors negatively coupled Ca^{2+} channels.

3.2. Effect of neomycin on TTX-r I_{Na^+}

Tetrodotoxin-resistant Na^+ channels predominantly express in small dorsal root ganglion neurons (< 25 μM in diameter). Neomycin significantly inhibited peak tetrodotoxin-resistant Na^+ currents in a dose-dependent manner (Fig. 4A). Fig. 4B shows the time course and dose dependency of neomycin-induced inhibition of tetrodotoxin-resistant Na^+ currents in a small dorsal root ganglion neuron. The inhibition developed quickly after application of neomycin, and recovered immediately after washing out neomycin, showing the "fast dissociative" property of neomycin. A dose–response curve of resting block is shown in Fig. 5. IC_{50} was 1213.44 μM ($n = 25$) and Hill coefficient was 0.94. Neomycin also affected gating kinetics of tetrodotoxin-resistant Na^+ currents. Fig. 6 shows the gating properties of tetrodotoxin-resistant Na^+ currents after the application of neomycin. Half-maximal activation voltage ($V_{1/2}$) of tetrodotoxin-resistant Na^+ currents was − 19.93 ± 0.86 mV before and − 11.19 ± 0.77 mV after administration of 800 μM neomycin ($n = 15$). Slope factors (k) were 8.47 and 7.91 before and after the application of neomycin, respectively. Statistical analysis showed significant difference of $V_{1/2}$ before and after the application of neomycin ($P < 0.05$).

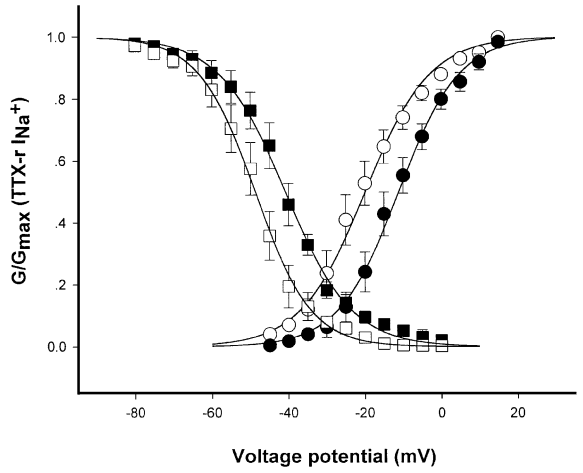

Fig. 6. Activation (circles) and inactivation (squares) curves of tetrodotoxin-resistant Na^+ currents before (open symbols) and after (solid symbols) the application of 800 μM neomycin ($n = 20$). Data were fit with Boltzmann function. Under activation condition, currents was evoked from − 70 mV holding potential by 35 ms command potentials ranging from − 45 to 15 mV in the increasing 5-mV step. Under inactivation condition, currents was evoked by 30 ms, 0 mV command potential prepulsed by 800 ms holding potential ranging from − 80 to − 10 mV in the increasing 5-mV step.

Slope factor k did not change significantly. Neomycin obviously shifted by 7.92 mV the inactivation curve of tetrodotoxin-resistant Na^+ currents, from − 48.75 ± 2.41 to − 40.83 ± 2.06 mV ($n = 15$, $P < 0.05$), but slope factor k did not show significant change (from 7.26 to 8.64). V_{rev} calculated from the I–V relationship was 55 mV.

4. Discussion

The present study indicated for the first time that neomycin significantly inhibited peak high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents in small dorsal root ganglion neurons. Besides the inhibition of peak currents, neomycin also affected gating kinetics of high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents. First, neomycin apparently shifted the inactivation curve of Ca^{2+} currents to more negative potentials. Next, neomycin significantly shifted activation curve of tetrodotoxin-resistant Na^+ currents to the positive direction. Although neomycin also shifted steady-state inactivation curves of tetrodotoxin-resistant Na^+ currents to the positive direction, it in fact had little effect on inactivation of tetrodotoxin-resistant Na^+ currents when holding potential was − 70mV. Taken together, these results suggested that neomycin-induced blockade of high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents resulted in reduction of excitability of small dorsal root ganglion neurons. Hence, our study provides a reasonable explanation for previous behavioral studies indicating that neomycin produce central and peripheral analgesia (Ocana and Baeyens, 1991; Coderre, 1992; Blenk et al., 1997).

Consistent with previous studies indicating that neomycin is N-type (Perrier et al., 1992; Wagner et al., 1987) or P-type (Duarte et al., 1993) Ca^{2+} channel blocker in the central nervous system, the present study showed for the first time that neomycin produced a selective inhibition of high-threshold Ca^{2+} currents like morphine in small nociceptive dorsal root ganglion neurons. High-threshold Ca^{2+} currents are often classified as L-, N-, P/Q- and R-type according to their electrophysiological properties and their sensitivity to specific antagonists (Tsien et al., 1988; Dunlap et al., 1995). N-, P/Q- and R-type channels are mainly found at synaptic sites and are involved in the release of transmitters (Bertolino and Llinas, 1992; Dunlap et al., 1995). Furthermore, it has been demonstrated that activation of N-type Ca^{2+} channels produced release of substance P and glutamate from nociceptive afferent terminals in the spinal dorsal horn (Bertrand et al., 2000; Martire et al., 2000; Matthews and Dickenson, 2001). So, the inhibitory effects of neomycin on high-threshold Ca^{2+} currents in dorsal root ganglion neurons may contribute mainly to its presynaptic antinociception in the spinal cord.

Previous studies have indicated that tetrodotoxin-resistant Na^+ channels activated and inactivated at more positive voltage than tetrodotoxin-sensitive Na^+ channels (Elliott

and Elliott, 1993; Ogata and Tatebayashi, 1993), suggesting that tetrodotoxin-resistant Na^+ channels might mediate nociceptive information of the small dorsal root ganglion neurons triggered by high-threshold afferents. Agents producing hyperalgesia increased the rate of activation and inactivation of tetrodotoxin-resistant Na^+ currents and the magnitude of tetrodotoxin-resistant Na^+ currents (Gold et al., 1996). On the basis of these results, we believe that neomycin-induced blockade of tetrodotoxin-resistant Na^+ currents may lead to reduction of excitability of small dorsal root ganglion neurons, which is implicated in the mechanisms underlying peripheral analgesia of neomycin (Ocana and Baeyens, 1991; Coderre, 1992).

Although at least three types of tetrodotoxin-resistant Na^+ currents have been distinguished electrophysiologically in dorsal root ganglion neurons (Rush et al., 1998; Scholz et al., 1998), the previous studies focused on tetrodotoxin-resistant Na^+ currents without distinguishing subtypes (Gold et al., 1996, 1998; Novakovic et al., 1998; Zhou and Zhao, 2000). Moreover, the molecular identity of the persistent tetrodotoxin-resistant Na^+ currents cannot be positively determined at present (Cummins et al., 1990). In the present study, we also emphasized the effects of neomycin on tetrodotoxin-resistant Na^+ currents without separating them, despite that different tetrodotoxin-resistant Na^+ currents might have different sensitivity to neomycin. Further study needs to explore the effects of neomycin on different types of tetrodotoxin-resistant Na^+ currents in dorsal root ganglion neurons.

Altogether, we reported here firstly that neomycin significantly inhibited high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents in small dorsal root ganglion neurons, which may contribute to neomycin-induced analgesia. However, this study was not enough to elucidate the mechanism of neomycin to block high-threshold Ca^{2+} currents and tetrodotoxin-resistant Na^+ currents in small dorsal root ganglion neurons. A non-specific effect of neomycin could not be excluded at present. Perhaps, neomycin acted just as a plug to block high-threshold Ca^{2+} channels and tetrodotoxin-resistant Na^+ channels through an open channel blocking mechanism or an close channel blocking mechanism, like the effect of capsaicin on both voltage dependent potassium currents and the calcium current (Kuenzi and Dale, 1996).

Acknowledgements

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