Neurokinin A Depolarizes Neurons of Bullfrog Dorsal Root Ganglia by Suppressing K^+ Conductances

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Summary: The effect of neurokinin A (NKA) on neurons of bullfrog dorsal root ganglia (DRG) in primary culture was examined by using whole-cell patch-clamp methods. Application of NKA ($1\mu\text{M}$) depolarized the DRG neurons, resulting in spontaneous firing of action potentials. Under voltage-clamp condition, NKA (3 nM – $1\mu\text{M}$) caused an inward current (I_{NKA}) associated with decreased membrane conductance. The I_{NKA} reversed its polarity at the equilibrium potential for K^+ . The I_{NKA} was blocked by extracellular Ba^{2+} (1 mM) but not by nominally 0 mM Ca^{2+} , tetraethylammonium (40 mM), 4-aminopyridine (2 mM) or apamin (50 nM). Intracellular Cs^+ blocked the I_{NKA} . NKA depressed a voltage-dependent non-inactivating K^+ current, the M-current (I_{M}), at potentials more positive than -55 mV . NKA reduced the maximum M-conductance (G_{M}) without changing the kinetics of M-channels. NKA also depressed a voltage- and time-independent background K^+ current, $I_{\text{K(B)}}$. It is concluded that the I_{NKA} is produced by suppression of both I_{M} and $I_{\text{K(B)}}$ in bullfrog primary afferent neurons.

Key words: dorsal root ganglia — dissociated neurons — tachykinins — background K^+ current — M-current — whole-cell patch-clamp

Introduction

Mammalian tachykinins, such as substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), have been demonstrated to exist widely in the peripheral and central nervous systems (Ogawa et al. 1985; Leeman et al. 1991; Otsuka and Yoshioka, 1993). NKA-like immunoreactivity co-exists with SP-like immunoreactivity in neurons of rat dorsal root ganglia (DRG) and in the dorsal horn of the spinal cord (Dalsgaard et al. 1985; Ogawa

et al. 1985; Lee et al. 1986; Takano et al. 1986; Helke and Niederer, 1990). Molecular cloning of cDNAs has revealed three distinct types of tachykinin receptors, NK_1 , NK_2 and NK_3 that belong to the GTP binding protein-coupled receptor superfamily (Masu et al. 1987; Hershey and Krause, 1990; Shigemoto et al. 1990). Functional study showed that NKA and NKB depolarized neurons in mammalian sympathetic ganglia (Saria et al. 1985; Konishi et al. 1992). Recently, we also observed that SP produced an inward

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current by suppressing potassium conductances in acutely dissociated neurons of bullfrog DRG (Ishimatsu, 1994). The purpose of the present study is to examine the effect of NKA on neuronal excitability of DRG by using whole-cell patch-clamp methods. The results show that NKA produced depolarizing response by suppressing both a voltage dependent non-inactivating K^+ current, the M-current (I_M) (Brown and Adams, 1980; Adams et al. 1982a,b; Tokimasa and Akasu, 1990b) and a voltage-independent background K^+ current, $I_{K(B)}$ (Ishimatsu, 1994).

Materials and Methods

Tissue culture

The methods for the isolation of single neurons of DRG are essentially the same as described previously (Tokimasa and Akasu, 1990a,b). Bullfrogs (*Rana catesbeiana*) were sacrificed by decapitation and their brains were mechanically destroyed. Isolated DRG were incubated in a Ringer solution containing trypsin (Sigma type XI; 2.5 mg/ml) and collagenase (Sigma type A; 0.5 mg/ml) at 36 °C for 15-30 min. The Ringer solution had the following composition (mM): NaCl, 112; KCl, 2; $CaCl_2$, 1.8; N-[2-hydroxyethyl]piperazine-N'-2-ethanesulphonic acid (HEPES), 4 and tris (hydroxymethyl) aminomethane (Tris), 1. After digestion, DRG cells were dispersed with gentle pipetting and collected by centrifugation at 80 g for 4-5 min. The dissociated cells were stored for 2-4 days at 4 °C in Leibovitz's L-15 medium (GIBCO 320-1415); 10-20% fetal bovine serum (GIBCO 200-6140 AG) was added and the diluted to 80% with water

in 35 mm culture dishes (Falcon 3001).

Whole-cell recording

The dissociated cells were re-suspended in the Ringer solution 2-3 hs before starting the electrophysiological experiment. DRG neurons were pipetted into the recording chamber (1.5 ml total volume) and continuously superfused (1-3 ml/min) with the Ringer solution. The cells having diameter greater than 50 μ m (A-type cells) were used for whole-cell patch-clamp recording. Pipettes for the whole-cell recording had a tip resistance of 3-6 M Ω , when filled with a solution of the following composition (mM): KCl, 100; $MgCl_2$, 4; adenosine 5'-triphosphate sodium (Na_2 -ATP), 5; 1,2-bis (*o*-aminophenoxy) ethane-N, N, N', N'-tetraacetic acid tetraacetoxymethyl salt (BAPTA), 1; and HEPES (sodium salt), 2.5 (pH adjusted with KOH to 7.0). Gigaohm-seal (≥ 1 G Ω) contact between the recording pipette and cell membrane was established by adding a gentle negative pressure to the recording pipette. A sample-and-hold/voltage-clamp amplifier (Axoclamp-2A; Axon Instruments) was used at the switching frequency of 10-17 kHz. The gain of head-stage current was 0.1, indicating that a clamping current as large as ± 20 nA could be applied to the cell. The output of the amplifier was monitored using an oscilloscope (Toshiba, DM-1562A), and connected to a digital oscilloscope equipped with a disc recorder (Nicolet, 4094 and XF-44) and with a chart recorder (Nihon Kohden, RJG-4124). All data were stored in a digital recorder (TEAC, RD-1100) for later analysis. Axodata and Axograph softwares (Axon Instruments) operating Macintosh Centris 650 computer (Apple Computer, Inc.)

were used to command the membrane potential and to analyze membrane currents.

Isolation of I_M and $I_{K(B)}$

To record the M-current (I_M), DRG neurons were superfused with a Ringer solution containing tetrodotoxin (TTX; 3 μ M), tetraethylammonium (TEA; 40 mM), Cs^+ (2 mM), 4-aminopyridine (4-AP; 2 mM) and apamin (50 nM) (Tokimasa and Akasu, 1990b). NaCl (72 mM) was replaced with equimolar choline chloride. HEPES, 4 mM and tris (hydroxymethyl) aminomethane (Tris, 1 mM) were used as buffer instead of NaHCO_3 . In this modified Ringer solution, called M-solution, voltage-dependent Na^+ current, delayed rectifier K^+ current (I_{DR}), A-current (I_A), H-current (I_H) apamin-sensitive Ca^{2+} dependent K^+ current ($I_{\text{K-Ca}}$) were eliminated (Tokimasa and Akasu, 1990a,b). To isolate a voltage-independent background K^+ current ($I_{\text{K(B)}}$), DRG neurons were superfused with an M-solution containing nominally zero Ca^{2+} and Co^{2+} (300 μ M), and voltage-clamped at a potential more negative than -60 mV. Ca^{2+} -free Ringer solution contained nominally 0 mM Ca^{2+} , 300 μ M Co^{2+} and 11 mM Mg^{2+} .

Drugs

Drugs used in the present study were as follows. Apamin and 4-aminopyridine were purchased from Sigma. Neurokinin A (NKA) was from Peptide Institute Inc. (Osaka). Other drugs were tetrodotoxin (Wako Pure Chemicals Industries, LTD.) and tetraethylammonium chloride (Tokyo Kasei). NKA was applied by either perfusion or a train of 3 pressure pulses (8–20 psi for 30 ms) through a broken-tip pipette utilizing a Picospritzer (General

Valve).

All experiments were carried out at 22–24 °C. The statistics are expressed as mean $\pm$ S.E. of the mean for the cells tested. pH of the superfusate was adjusted to 7.2.

Results

NKA-induced inward current in DRG neurons

Acutely dissociated neurons of bullfrog DRG showed the resting membrane potential of -72.2 ± 1.6 mV ($n=163$) and input resistance of 224 ± 60 M Ω ($n=124$) in the Ringer solution. NKA (1 μ M) was applied by a train of three pressure pulses (20 psi for 30 ms) through a micropipette placed near the recording cells. DRG neurons ($n=8$) exhibited a depolarizing response associated with spontaneous firing of action potentials (Fig. 1A). The DRG neurons were voltage-clamped at a given resting membrane potential in the whole-cell configuration. Pressure application of NKA (1 μ M) produced an inward current (I_{NKA}) associated with decreased membrane conductance in 82 out of 87 neurons (Fig. 1B). The remaining cells showed no significant change in the membrane current. The amplitude of the NKA-induced inward current was concentration-dependent. The minimum effective concentration of NKA (3 nM) produced the inward current of 0.35 ± 0.08 nA ($n=6$). At a concentration of 100 nM, NKA produced the maximum response (1.2 ± 0.4 nA, $n=8$) in the Ringer solution. The EC_{50} for NKA was 3.4 nM (Fig. 1C). In contrast, NKB (10 μ M–1 mM) produced no significant current in DRG neurons.

When DRG neurons were hyperpolarized, the I_{NKA} decreased in amplitude

Fig. 1. Effects of NKA on the membrane potential (A) and current (B) in bullfrog DRG neurons. NKA was applied by a train of 3 pressure pulses (20 psi for 30 ms) through a micropipette filled with 1 μ M NKA. Filled triangles indicate the time of application of NKA. A: current-clamp recording was made from a DRG neuron superfused with Ringer solution. The resting membrane potential was -70 mV. Downward deflections were electrotonic potentials produced by injection of inward current pulses (0.1 nA for 100 ms) to the neuron. B: a sample record of the NKA-induced inward current obtained by whole-cell voltage-clamp methods. Downward deflections indicate membrane currents (lower trace) produced by hyperpolarizing step-commands with duration of 300 ms (upper trace). C: concentration-response curve for NKA (1-100 nM). Ordinate and abscissa denote the amplitude of the I_{NKA} and concentration of NKA. NKA was applied by superfusion for 10 sec.

and finally reversed its polarity at a membrane potential between -70 and -105 mV in the normal K^+ (2 mM) Ringer solution (Fig. 2A). The relationship between hold-

Fig. 2. The reversal potentials of the NKA-induced inward current. A: sample records of the I_{NKA} obtained from a DRG cell superfused with Ringer solution containing 2 mM K^+ . Downward deflections indicate inward currents produced by hyperpolarizing voltage-command pulses with duration of 300 ms. Holding membrane potentials are shown between traces. B: relationship between the amplitude of the NKA-induced response and the holding membrane potential. Data were obtained from (A).

ing voltage and amplitude of the I_{NKA} showed that the reversal potential of the I_{NKA} was -100 mV in 2 mM K^+ Ringer solution (Fig. 2B). From 6 cells, the reversal potentials of the I_{NKA} were -98.2 ± 4.0 mV in Ringer solutions.

Effects of K^+ channel blockers

Intracellular Cs^+ , a K^+ channel blocker in neuronal cells (Rudy, 1988; Hille, 1992) was applied through a patch-pipette containing 96 mM $CsCl$. The holding current (a -30 mV) gradually shifted to the inward direction after rupturing the patch membrane. The inward current produced by 1 μ M NKA was strongly reduced in neurons treated with Cs^+ ($n=3$) (Fig. 3A). Extracellular Ba^{2+} has been reported to block several types of K^+ channels in excitable cells (Rudy, 1988; Hille, 1992).

Fig. 3. Effect of $[Cs^+]_i$ and $[Ba^{2+}]_o$ on $I_{K(B)}$. A: a consecutive recording of the membrane current recorded by a patch-pipette filled with 96 mM CsCl. After rupturing the patch membrane, the ganglion cell was initially held at -30 mV and subjected to hyperpolarizing voltage-step (300 ms in duration) to -60 mV. NKA-induced inward currents were taken 5 and 40 min after rupturing the patch membrane. The recordings were interrupted for 15 min between NKA applications (lower traces). B: effect of Ba^{2+} (1 mM) on the I_{NKA} obtained at -60 mV. Horizontal bar indicates the period of bath-application of Ba^{2+} . Lower traces (a) and (b) are expanded records of the I_{NKA} taken at the times marked with the sauce letters in the trace immediately above.

Bath-application of Ba^{2+} (1 mM) produced an inward current at -60 mV and depressed the amplitude of the I_{NKA} (Fig. 3B). Ba^{2+} depressed the membrane conductance during the inward current at holding potentials more negative than -60 mV. The reversal potential of Ba^{2+} -

induced current was -91.2 ± 5.3 mV ($n=6$). It has been demonstrated that SP depolarizes neurons in submucous ganglia by suppressing a Ca^{2+} -dependent K^+ current, $I_{K(Ca)}$ (Akasu and Tokimasa, 1989; Morita and Katayama, 1992). However, the I_{NKA} in DRG neurons was not inhibited in nominally 0 mM Ca^{2+} Ringer solution containing 300 μ M Co^{2+} and 11 mM Mg^{2+} ($n=5$).

Effect of NKA on the I_M

A voltage-dependent non-inactivating K^+ current, the M-current (I_M) (Brown and Adams, 1980; Adams et al. 1982a,b), has been shown to occur at relatively depolarized membrane potential in neurons of bullfrog DRG (Tokimasa and Akasu, 1990b). To obtain the I_M , DRG neurons were superfused with a modified Ringer solution (M-solution) containing TTX (3 μ M), TEA (40 mM), Cs^+ (1 mM), 4-AP (1 mM) and apamin (30 nM) (Tokimasa and Akasu, 1990b). A hyperpolarizing step command from -30 to -60 mV produced an instantaneous (ohmic) current followed by an inward relaxation that is due to deactivation of the M-channel (Fig. 4). Upon stepping back to the initial holding potential (-30 mV), DRG cells showed an instantaneous current at -60 mV followed by an inward tail current due to re-activation of I_M at -30 mV (Adams et al. 1982a,b; Tokimasa and Akasu, 1990b). Application of NKA (1 μ M) to the superfusing solution (M-solution) produced the inward current. The amplitude of the I_{NKA} was 0.8-1.7 nA ($n=43$) at -30 mV (Fig. 4A). During the inward current the I_M was depressed (Fig. 4A). NKA, however, did not change the time course and the reversal potential of the I_M . NKA also depressed the instantaneous current recorded at the end of

Fig. 4. Effect of NKA on the I_M . The I_M was obtained from a ganglion cell superfused with M-solution which contains tetrodotoxin (TTX, $3 \mu\text{M}$), tetraethylammonium (TEA, 40 mM), Cs^+ (1 mM), 4-aminopyridine (4-AP, 1 mM) and apamin (50 nM). The DRG neuron was initially held at -30 mV and subjected to hyperpolarizing voltage-commands to -60 mV with duration of 200 ms . Horizontal bar indicates period for the bath-application of NKA ($1 \mu\text{M}$). B: records (a), (b) and (c) were taken at the times marked by the sauce letters in the trace immediately above. Broken lines indicate the original holding current.

hyperpolarizing command potential (at -60 mV) which is a voltage-independent 'leak' current (Adams et al. 1982*a,b*). When NKA was continuously applied to the external solution for more than 1 min , the I_{NKA} returned to the original holding current (Fig. 4B). The inhibition of the I_M also declined with time during prolonged application of NKA. A rebound enhancement of the I_M was seen in 8 out of 9 cells (Fig. 4Bc).

Effect of NKA on the activation curve of the I_M

The ganglion cells were continuously voltage-clamped at the bottom of the activation curve of I_M , -70 mV , and subjected to depolarizing voltage-steps between

Fig. 5. Effect of NKA on the steady-state activation curve of the I_M . A: sample records of the I_M tail current obtained before (left panel) and during (right panel) application of NKA (100 nM). The DRG neuron was initially held at -70 mV and then subjected to depolarizing voltage command pulses with duration of 300 ms . The amplitude of I_M tail currents was measured 10 min after termination of the depolarizing voltage-commands. B: graph shows the relationship between the amplitude of the tail current (ordinate) and holding voltage (abscissa). The data were obtained from the records shown in (A). The maximum amplitude of the tail current obtained at $+20 \text{ mV}$ represents 1.0 . Open and filled circles were obtained in the absence and presence of NKA (100 nM), respectively. Curves were drawn according to the Boltzmann equation (see Text).

-60 and $+20 \text{ mV}$, of 300 ms duration (Tokimasa and Akasu, 1990*a,b*). The outward tail current was measured 10 ms after termination of the depolarizing step command (Fig. 5A). NKA (100 nM) depressed

the I_M ; the tail current of the I_M was depressed by $56 \pm 8\%$ ($n=43$) at all holding potentials tested. The amplitude of the tail current relative to its maximum value ($I/I_{\max}$) was plotted as a function of membrane potential (V) to obtain the steady-state activation curve of the I_M (Fig. 5B). The tail current of the I_M appeared at a potential of -60 mV and increased in amplitudes as the membrane of DRG cells was depolarized. The maximum amplitude ($I_{\max}$) of the tail current was obtained between $+10$ and $+20$ mV, indicating the top of the activation curve ($n=8$). The curve could be described by Boltzmann equation, $I/I_{\max} = [1 + \exp((A-V)/k)]^{-1}$, where A and k denote the half-activation voltage and the slope factor, respectively (Tokimasa and Akasu, 1990a). The half-activation voltage was -32.4 ± 1.3 mV ($n=4$) and -30.8 ± 0.9 mV ($n=4$), respectively in the absence and presence of NKA (100 nM). The slope factors were 21.1 ± 0.7 mV ($n=4$) in control and 19.5 ± 1.1 mV ($n=4$) in the presence of 100 nM NKA.

Effect of NKA on background K^+ current, $I_{K(B)}$

The contribution of non-M type K^+ current to the I_{NKA} was examined at the resting membrane potential where the I_M is strongly deactivated. DRG neurons were voltage-clamp at -60 mV and were superfused with an M-solution containing nominally zero Ca^{2+} , Co^{2+} ($300 \mu M$) and Cs^+ (1 mM) to eliminate the I_M , Ca^{2+} -induced K^+ current and H-current (Tokimasa and Akasu, 1990a,b). Hyperpolarizing voltage-commands (400 ms in duration) from -60 to -120 mV produced a square (ohmic) inward current associated with no obvious current relaxation (Fig. 6A). Bath-appli-

cation of NKA (100 nM) produced a 0.5–1 nA (0.23 ± 0.05 nA, $n=10$) inward shift of the holding current at -70 mV and depressed the amplitude of ohmic currents produced by hyperpolarizing voltage-steps (Fig. 6A). NKA (100 nM) decreased the slope conductance by 5–20 nS ($n=4$) at potentials between -60 and -130 mV. In this particular neuron, the linear I - V

Fig. 6. Effect of NKA on background K^+ current, $I_{K(B)}$. DRG neuron superfused with the M-solution containing nominally 0 mM Ca^{2+} , Co^{2+} ($300 \mu M$) and Cs^+ (1 mM). A: the neuron was initially held at -60 mV. Ohmic (square) current, $I_{K(B)}$, was produced by hyperpolarizing step-pulse from -60 to -110 mV. Left and middle records and are the I_M taken before and after application of NKA ($1 \mu M$). Right record was taken 25 min after withdrawal of NKA. B: current-voltage relationships obtained from a DRG cell superfused with an external solution as described in (A). Open and filled circles were taken before and during the application of NKA ($1 \mu M$). The neuron was initially held at -60 mV.

curve intersected the control curve at -96 mV. From 4 cells, the reversal potential of the I_{NKA} was -96.2 ± 4.1 mV (Fig. 6B).

Discussion

Present study showed that NKA (3 nM- 1 μ M) caused the depolarization associated with spontaneous firing of action potentials in neurons of bullfrog DRG. Under voltage-clamp, NKA caused the inward current, I_{NKA} , associated with a decreased membrane conductance. The I_{NKA} reversed polarity at the equilibrium potentials of K^+ . The substitution of Cs^+ for K^+ in the pipette-solution, which generally blocks K^+ channels in neuronal cells (Rudy, 1988; Hille, 1992), strongly reduced the I_{NKA} . Ba^{2+} , a channel blocker (Adams et al. 1982*a,b*; Tokimasa and Akasu, 1990*b*), also reduced the I_{NKA} at -60 mV. These results suggest that the I_{NKA} is produced mainly by depression of a K^+ conductance in DRG neurons. The I_{NKA} was not blocked by TEA (40 mM), Cs^+ (2 mM), 4-AP (1 mM) and apamin (50 nM), suggesting that the delayed rectifier current, the inward rectifier current and A-current are not involved in the I_{NKA} in neurons of bullfrog DRG (Tokimasa and Akasu, 1990*a,b*). SP has been reported to suppress the $I_{K(Ca)}$ in neurons of guinea-pig submucous and myenteric ganglia (Akasu and Tokimasa, 1989; Morita and Katayama, 1992). However, the $I_{K(Ca)}$ may not contribute to the I_{NKA} in bullfrog DRG neurons, because 1) the I_{NKA} can be recorded from DRG neurons that had been dialyzed with a pipette solution containing nominally 0 mM Ca^{2+} and BAPTA (1 mM) and 2) I_{NKA}

was not blocked in a nominally Ca^{2+} -free solution containing Co^{2+} (300 μ M) and Mg^{2+} (11 mM) and 3) apamin (30 nM) did not block the I_{NKA} .

It has been reported that the I_M occurs at relatively depolarized membrane potentials in bullfrog DRG neurons (Tokimasa and Akasu, 1990*b*). In the present study, the I_{NKA} was associated with suppression of the I_M relaxation produced by hyperpolarizing voltage-command from -30 to -60 mV. NKA did not alter the activation and deactivation time courses, or the reversal potential, of the I_M . The activation curve for the M-conductance (G_M) showed that NKA did not change the half activation voltage. These results indicate that NKA reduces the maximum G_M but does not change the opening and closing kinetics of M-channels. Thus, the suppression of I_M may contribute to the mechanism underlying the I_{NKA} at relatively depolarized membrane potentials in neurons of bullfrog DRG. The present study clearly showed that NKA produced the current response in at the resting membrane potential of -72.2 ± 1.6 mV, where the M-channels are deactivated (Adams et al. 1982*a,b*; Tokimasa and Akasu, 1990*a,b*). This implies that a potassium current other than the I_M is involved in the I_{NKA} at the resting membrane potential. An ohmic inward current produced by a voltage-command from the resting membrane potential to hyperpolarizing potentials was depressed during the I_{NKA} . I - V curves showed that the I_{NKA} reversed polarity at the equilibrium potential of K^+ . Such a NKA-sensitive voltage-independent K^+ current occurring at the resting membrane potential has been termed a background potassium current ($I_{K(B)}$) in bullfrog DRG neurons (Ishimatsu,

1994). The NKA-induced inward current and is due primarily to the reduction of $I_{K(B)}$ at the resting membrane potential.

Tachykinins are putative transmitter peptides of primary sensory neurons mediating the transmission of sensory information (see Review Otsuka and Yoshioka, 1993). Tachykinins cause enhancement or depression of nociceptive responses in substantia gelatinosa cells of the cat dorsal horn, by modifying both the release of a sensory transmitter and the membrane excitability of the postsynaptic membrane (Henry, 1980; Otsuka and Yanagisawa, 1987; Otsuka and Yoshioka, 1993). NKA may depolarize the central terminals of primary afferent neurons, resulting in the facilitation of nociceptive transmission in spinal cord.

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