Tumor Necrosis Factor-α Suppresses Sustained Potassium Currents in Rat Small Diameter Sensory Neurons

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Abstract: Tumor necrosis factor-α (TNF-α), a pro-inflammatory cytokine, produces pain and hyperalgesia by activating and/or sensitizing nociceptive sensory neurons. In the present study, using whole-cell patch clamp techniques, the regulation of potassium currents by TNF-α was examined in acutely dissociated small dorsal root ganglion neurons. We found that acute application of TNF-α inhibited, in a dose-dependent manner, the non-inactivating sustained potassium current without changing the rapidly inactivating transient current or the voltage-dependence of steady-state inactivation. The effects of TNF-α on potassium currents were similar to that of prostaglandin E2 as reported previously and also demonstrated in the current study. Furthermore, indomethacin, a potent inhibitor for both cyclo-oxygenase (COX)-1 and COX-2, completely blocked the effect of TNF-α on potassium currents. These results suggest that TNF-α may sensitize or activate sensory neurons by suppressing the sustained potassium current in nociceptive DRG neurons, possibly via stimulating the intracellular production i.e. the synthesis and/or release of endogenous prostaglandins.

Keywords: Tumor necrosis factor-α (TNF-α), dorsal root ganglion (DRG), potassium currents, prostaglandins, indomethacin.

INTRODUCTION

TNF-α, a potent pro-inflammatory cytokine, can be synthesized and released by a variety of cells including neurons, glia, monocytes, fibroblasts, macrophages, endothelial cells, and Schwann cells under normal and pathological conditions [1-3]. TNF-α is also found in normal nucleus pulposus [4] and herniated lumbar disc specimens [5] that are adjacent to the sensory ganglia. The importance of TNF-α in inflammatory and neuropathic pain has been well-documented. TNF-α induces robust mechanical allodynia when applied to the lumbar dorsal root ganglion (DRG) *in vivo* [6]. Neutralizing endogenous TNF-α with antiserums or soluble receptors in neuropathic pain animal models decreases both mechanical and thermal hyperalgesia [7, 8]. Evidence obtained from *in vivo* and *in vitro* electrophysiological studies suggests that TNF-α contributes to pathological pain by enhancing neuronal excitability. Topical application of TNF-α to the DRG *in vitro* increases the excitability [9] and induces or enhances spontaneous activity in DRG neurons of various sizes, effects that are dependent on the protein kinase A (PKA) pathway [10]. Similarly, ectopic discharges can be elicited by applying TNF-α to nerve trunks [11] or injecting into peripheral receptive fields *in vivo* [12]. Aside from the peripheral excitatory effects, TNF-α has also been found to enhance glutamate excitatory synaptic transmission in spinal dorsal horn neurons [13].

TNF-α and other inflammatory cytokines may increase neuronal excitability by directly regulating ion channel activity. For example, acute application of TNF-α rapidly enhances TTX-resistant Na^+ currents in isolated mouse DRG neurons [14]. This effect is thought to be the underlying mechanism for TNF-α-mediated mechanical hypersensitivity. TNF-α also reduces potassium conductance in Aplysia [15], and retinal ganglion neurons in rats [16]. Prostaglandin E2 (PGE2), another potent inflammatory mediator that can be synthesized and released upon exposure to TNF-α through the cyclo-oxygenase (COX) pathway [17-21], also suppresses potassium current in dissociated embryonic rat DRG neurons [22], an effect that is mediated by the PKA pathway [23]. The present whole cell patch clamp study was designed to examine the effects of TNF-α on potassium currents in small adult DRG neurons, and to determine if such effects are related to PGE2 by manipulating the COX pathway.

METHODS

Animals

Young female Sprague-Dawley rats (body weight 120-180 g) were housed one or two per cage under a controlled diurnal cycle of 12 h light and 12 h dark with free access to water and food. The ambient environment was maintained at constant temperature (22 ± 0.5°C) and relative humidity (60-70%). All the surgical procedures and the experimental protocols were approved by the institutional animal care and use committees of the University of Arkansas for Medical Sciences (Little Rock, Arkansas, USA) and University of Cincinnati (Cincinnati, OH, USA).

Cell Culture

Rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). The bilateral L4 and L5

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DRGs were isolated and the sheath was carefully removed in ice-cold normal Ringer solution. The connective tissue was digested by exposure to Ca^{2+}-free solution containing 1.0% collagenase II for 30 min at 37°C followed by washout in normal Ringer solution for another 10 min. DRGs were then dissociated by trituration with fire-polished pasteur pipettes. DRG cells were plated onto poly-D-lysine coated glass coverslips in Medium199 (Sigma, St. Louis, MO, USA) containing 10% heat-inactivated FBS and 1000 U/ml each of penicillin and streptomycin. DRG cells were incubated at 37°C for 12-24 hours before recording.

Electrophysiology

Coverslips with cells were transferred to a recording chamber, which is mounted on the stage of an inverted microscope (IX71, Olympus America Inc., Center Valley, PA), and observed under differential interference contrast. The recording chamber was continuously perfused at room temperature with oxygenated bath solution at a flow rate of 2 ml/min.

Whole cell voltage-clamp recordings of small DRG neurons (diameter less than 30 μm) were conducted at room temperature with an AxoPatch-200B amplifier (Molecular Devices Corp., Sunnyvale, CA). Patch pipettes (2-4.0 MΩ) were fabricated from borosilicate glass (Sutter Instruments, Novato, CA). The cell capacitance artifact was canceled by the nulling circuit of the recording amplifier. Ohmic leakage currents were subtracted using the P4/±4 subtraction protocol. Voltage errors were minimized by using ≥80% series resistance compensation. The current was filtered at 5 kHz and sampled at 50 kHz. Data were acquired on a Pentium IV computer with the Clampex 8.0 program.

Potassium currents were evoked by depolarizing voltage steps from -60 mV to +50 mV following a 1-s prepulse to -120 or -30 mV for total and non-inactivating currents, respectively. The rapidly inactivating transient current was obtained by subtracting the non-inactivating current from the total. The steady-state inactivation (measuring the total current) protocol consisted of a 1-s conditioning prepulse to potentials ranging between -100 mV and -10 mV followed by depolarizing voltage steps to +50 mV.

Solutions and Chemical Application

The normal bath solution contained (in mM) 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with NaOH, and the osmolarity adjusted to ~300 - 310 mOsm with sucrose. The bath solution for recording K^+ currents contained (in mM) 130 Choline Cl, 5 KCl, 1 $MgCl_2$, 2 $CoCl_2$, 10 HEPES and 10 glucose. The pH was adjusted to 7.4 with Tris-base, and the osmolarity was adjusted to ~300 - 310 mOsm with sucrose. The pipette solution contained (in mM) 140 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 11 EGTA, 10 HEPES, 2 Mg ATP and 1 Li GTP. The pH was adjusted to 7.2 with Tris-base, and osmolarity to ~290 - 300 mOsm with sucrose. Voltages were not corrected for liquid junction potentials, which were estimated to be <10 mV in all cases.

Recombinant human TNF-α (R and D Systems, Minneapolis, MN) was dissolved in 0.1% bovine serum albumin (BSA) in buffered saline to a concentration of 100 ng/ml and stored at -80°C in 10 μl aliquots for later use. PGE2 and indomethacin were both dissolved in ethanol and diluted to the final concentration of 1 μM for PGE2 and 10 μM for indomethacin prior to use. Control solutions for TNF-α, PGE2 and indomethacin contained the same amount of BSA or ethanol.

After measuring potassium currents in control solution, TNF-α at different doses (0.001, 0.01, 0.1, 1 or 5 ng/ml) was bath applied to DRG neurons for 15 min before repeating the same protocols for potassium current recording. The duration of TNF-α application was based on our previously reported finding that it takes more than 15 min for TNF-α to elicit C-fiber firing in an *in vitro* nerve-DRG preparation [10]. PGE2 was bath applied to dissociated neurons for 5 min prior to the measurement of its effect on potassium currents. To determine if the regulation of potassium currents by TNF-α is mediated by the COX pathway, indomethacin (Sigma, St. Louis, MO), a potent blocker for both COX-1 and COX-2, was applied to neurons for 15 min followed by mixture of indomethacin and TNF-α for additional 15 min. To avoid repetitive drug application, only one cell was studied per concentration per dish.

Data Analysis

All data are expressed as means ± S.E.M. The voltage dependence of activation of the potassium currents was fitted with the Boltzmann function: $G/G_{max} = \delta/[1+\exp(V_{0.5}-V_m)/\kappa]$. G is the conductance which is calculated by $G=I/(Vm-E_k)$ where I is the potassium current, V_m is the voltage step and E_k is the potassium equilibrium potential which is calculated to be -84 mV for our intra-and extra cellular potassium concentrations. G_{max} is the maximal conductance obtained at the membrane potential of + 50 mV prior to drug application. δ is a factor to account for inhibition by TNF-α or PGE2. $V_{0.5}$ is the membrane potential for half-activation or inactivation. κ is the slope factor. An inhibitory effect was defined as reducing the G/G_{max} by greater than 5% at +50 mV. For inactivation the relation $G/G_{max} = c + \{(1-c)/[1+\exp(V_m -V_{0.5})/\kappa]$ was used where c is the fraction of non-inactivating current. Currents were normalized with respect to G_{max}. Normalized currents (G/G_{max}) were plotted as a function of membrane potential. Two-way repeated measures ANOVA (RM ANOVA) with pairwise multiple comparison (Holm-Sidak method) was used to determine at which voltage levels the differences between control and drug treatment were significant if an overall effect of drug treatment was observed. $P<0.05$ is considered statistically significant.

RESULTS

Acute Application of TNF-α Suppresses Sustained Potassium Current Without Affecting the Rapidly Inactivating Component

Topical application of TNF-α (1 ng/ml) for 15 min suppressed the total potassium current in 8 of the 11 neurons tested (P=0.006) (Fig. **1A** and **D**). The 3 cells that did not show response to TNF- α had both sustained and transient components. Three of the 8 cells responded to TNF- α only had sustained component. Potassium current reduction by 1 ng/ml TNF began at 4-5 min after TNF-α application. The inhibitory effects increased over time but the current

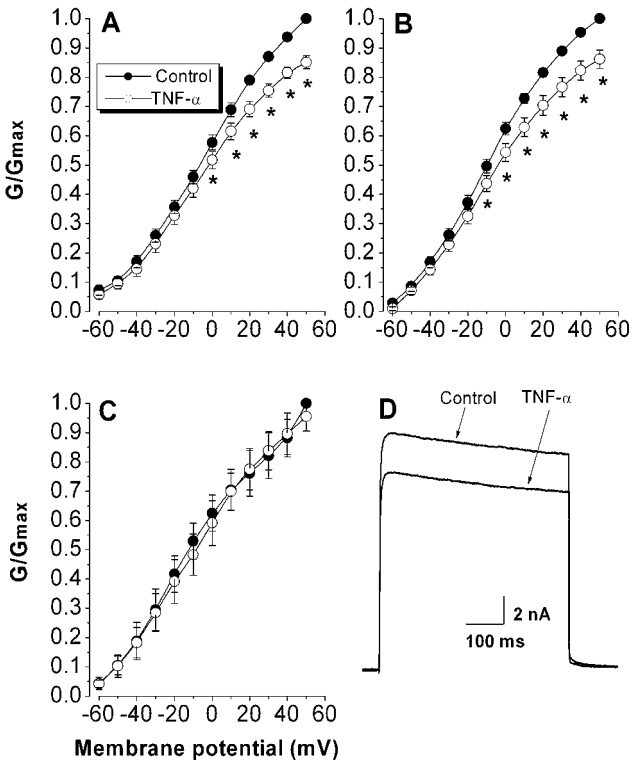

Fig. (1). Effects of TNF-α on potassium currents. TNF-α (1 ng/ml) suppressed the activation of total (**A**) and sustained potassium currents (**B**), but did not change the rapidly inactivating transient current (**C**). The potassium current was normalized by the maximum conductance obtained from control at +50 mV, and fitted by the Boltzmann equation, and plotted as a function of membrane potential. The activation protocol consisted of 500-ms depolarizing voltage steps to potentials between –60 and 50 mV after a 1-s voltage step to either –120 (**A**) or –30 mV (**B**).

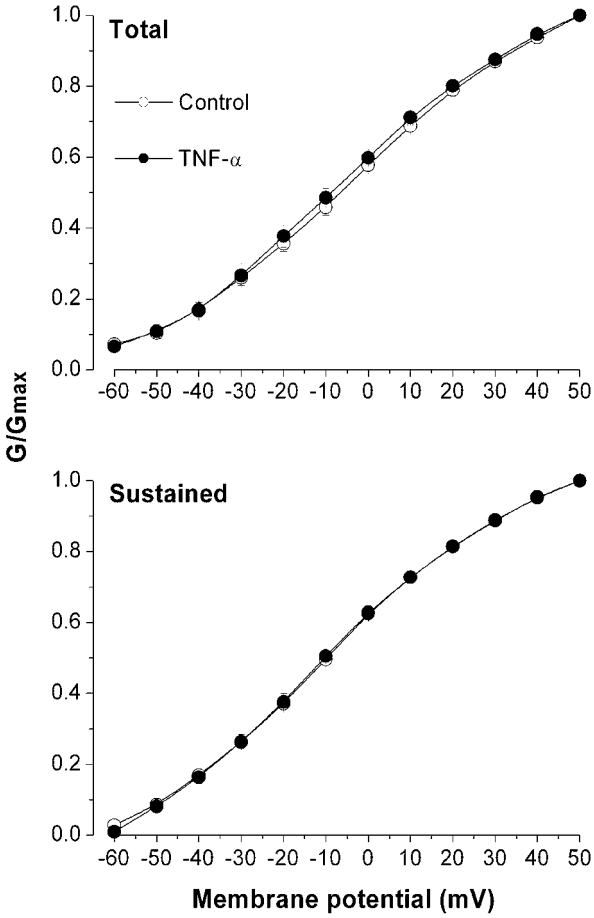

Fig. (2). The potassium current suppression by TNF-α is not associated with a shift in the voltage dependence for activation. Currents from control and TNF-α treatment were independently normalized by the respective G_{max} obtained at +50 mV and plotted as a function of membrane potentials.

reduction by TNF-α seemed to peak after 15-16 min of the drug application (data not shown).

The average reduction in G/G_{max} measured at +50 mV was 15% ranging from 7% to 23%. Further analyses revealed that TNF-α suppressed the sustained non-inactivating potassium currents (P=0.003) (Fig. **1B**) but did not affect the rapidly inactivating potassium currents (P=0.504, two-way) (Fig. **1C**). The G/G_{max} measured at +50 mV was reduced by an average of 13% (n=8, Fig. **1B**) for sustained non-inactivating K^+ currents but less than 5% (n=8, Fig. **1C**) for rapidly inactivating currents. Two-way RM ANOVA indicated that the effect of TNF-α was significant at individual voltages above 0 mV on the total potassium current (P=0.009 at 0 mV) (Fig. **1A**) and above -10 mV on the sustained potassium current (P=0.02 at -10 mV) (Fig. **1B**).

When the potassium currents recorded under control conditions and following 15 minutes of treatment with TNF-α were normalized by the respective G_{max} obtained at +50 mV, it revealed that TNF-α-induced suppression of potassium current was not associated with a shift in the voltage dependence for activation (Fig. **2**).

In addition to 1 ng/ml TNF-α, four other doses (0.001, 0.01, 0.1, or 5 ng/ml) were tested in different neurons. Because the rapidly inactivating transient current was not affected by 1 ng/ml TNF-α, in the following studies only the non-inactivating sustained potassium current was examined. As shown in Fig. (**3**), the inhibitory effect of TNF-α on sustained potassium current was dose-dependent. TNF-α at 0.001 ng/ml did not have a statistically significant effect on the potassium currents (P=0.788, compared with control). Significant reduction of potassium currents was observed at 0.01 ng/ml (P=0.04, compared to 0.001 ng/ml). The effect approached apparent maximum within the 1 to 5 ng/ml range of concentrations of TNF-α (P>0.05, compared between 1 and 5 ng/ml of TNF-α).

The effect of TNF-α on the voltage dependence of steady-state inactivation was also determined for the total voltage-activated potassium currents. The protocol consisted of a 1-s conditioning prepulse to potentials ranging between -100 and -10 mV followed by a depolarizing voltage step to a +20 mV test pulse for 1 second. Two-way RM ANOVA used to compare before and after TNF-α treatment failed to detect significant changes at any voltage levels (P=0.947, n=7) (Fig. **4**).

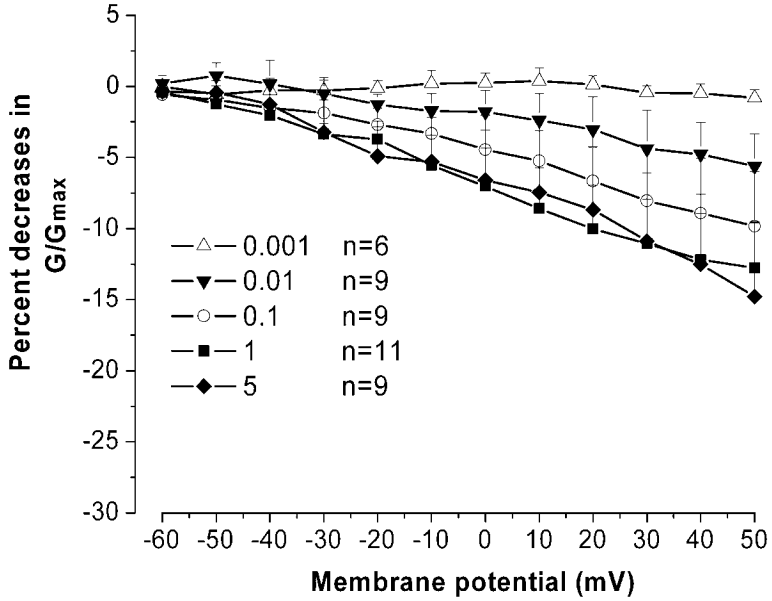

Fig. (3). The dose-dependence of the inhibitory effect of TNF-α on non-inactivating sustained potassium current. TNF-α-induced potassium current suppression was tested at different concentrations (0.001-5 ng/ml). Each data point represents the percent decrease of the normalized current (G/G_{max}) over control after TNF-α application.

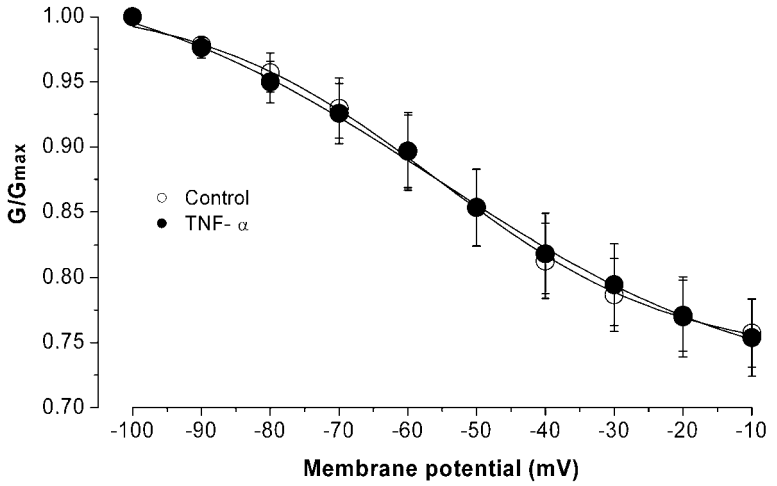

Fig. (4). Acute bath application of TNF-α did not change the steady-state inactivation of the total potassium current. Current was normalized by the maximum conductance and fitted by the Boltzmann equation, and plotted as a function of membrane potential. The inactivation protocol consisted of a 1-s voltage step to potentials from -100 to -10 mV followed by depolarizing voltage step to +50 mV.

The Inhibitory Effects of TNF-α on Potassium Current are Similar to that of PGE2 and are Blocked by Indomethacin

Because PGE2 has also been found, in a previous study, to reduce potassium currents in DRG neurons [22], and TNF-α may stimulate synthesis/release of endogenous PGE2, we tested the possibility that potassium inhibition by TNF-α may have been caused by synthesis/release of PGE2. We first tested the effect of PGE2 on the voltage-dependent activation of the sustained potassium currents and the voltage-dependent inactivation of the total currents. As shown in Fig. (**5**), similar to TNF-α, 5 min after application, PGE2 (1 μM) inhibited the sustained non-inactivating potassium currents (Fig. **5A**, p=0.009) without altering the voltage-dependence of steady-state potassium inactivation (Fig. **5B**, P=0.176). After PGE2 application, the G/G_{max} measured at + 50 mV decreased by 14% (n=9).

We then attempted to further examine the inhibitory effect of TNF-α on potassium current by blocking PGE2 synthesis/release using indomethacin, one of the potent cyclooxygenase inhibitors that blocks both COX1 and COX2 pathways. Indomethacin (10 μM) alone was applied to the dissociated cells for 15 min followed by indomethacin plus TNF-α for another 15 min. Pretreatment of the sensory neurons with indomethacin completely blocked the inhibitory effects of TNF-α on sustained potassium currents (P=0.557, compared between INDO and TNF-α + INDO, n=10) (Fig. **6**). Compared to the control value (G/G_{max}=1),

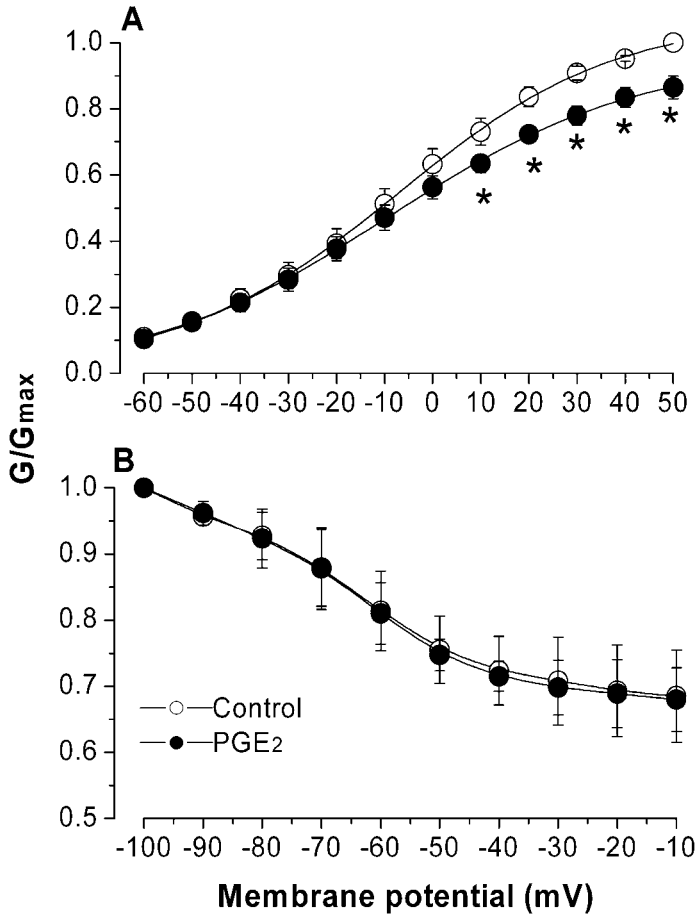

Fig. (5). Acute application of PGE2 for 5 minute inhibited the non-inactivating potassium current without affecting the voltage-dependence of steady-state inactivation.

the G/G_{max} measured at +50 mV was 0.87 ± 0.04 in TNF-α treated cells (n=8) and 0.96 ± 0.01 in cells pretreated with TNF-α plus indomethacin. This result indicates that indomethacin may block the inhibition of TNF-α on sustained potassium current by decreasing the synthesis/release of PGE2. In cells treated with indomethacin alone, no change in the activation of potassium currents was observed.

DISCUSSION

In the present study, we found that TNF-α, a potent pro-inflammatory cytokine, inhibits the non-inactivating sustained potassium current in small DRG neurons. This inhibition was mimicked by PGE2 application and blocked by pretreatment of the cells with indomethacin, a potent blocker of the COX activity. The rapidly inactivating potassium current and the steady-state inactivation were not affected by acute TNF-α application, a result which is similar to that with PGE2 obtained from our current study and reported earlier by [22].

Potassium currents have an important role in modulating neuronal excitability, and downregulation of potassium channels is thought to contribute to the increased excitability and generation/patterning of spontaneous activity in sensory neurons following peripheral nerve injury [24-26]. Potassium currents are not always reduced under pathological conditions; they may be upregulated to compensate increased sodium currents as recently described by our group in an inflammatory pain model [27].

There are three major components of voltage-gated potassium current in mammalian sensory neurons: non-inactivating current, fast and slow transient currents [28]. [29] further classified voltage-gated potassium currents into six subtypes in adult DRG neurons based on distinct biophysical and pharmacological properties. In general, both sustained and transient components are capable of regulating action potentials of neurons. Non-inactivating current is important in limiting repetitive firing by holding the membrane potential close to the potassium equilibrium potential. The transient potassium current modulates the repolarization of single action potential, the time required to reach the threshold to fire an action potential, and repetitive firing [30-32]. Our finding that TNF-α only inhibits sustained non-inactivating current suggests that TNF-α-evoked firing or enhancement of spontaneous activity, as reported in our previous studies [9, 10], may be caused by a decrease of potassium currents and associated oscillations of the resting membrane potential of the neuron.

The effects of TNF-α reported here and in our previous publications are most likely due to the endogenous synthesis/release of PGE2. The reasons are: 1) the reduction

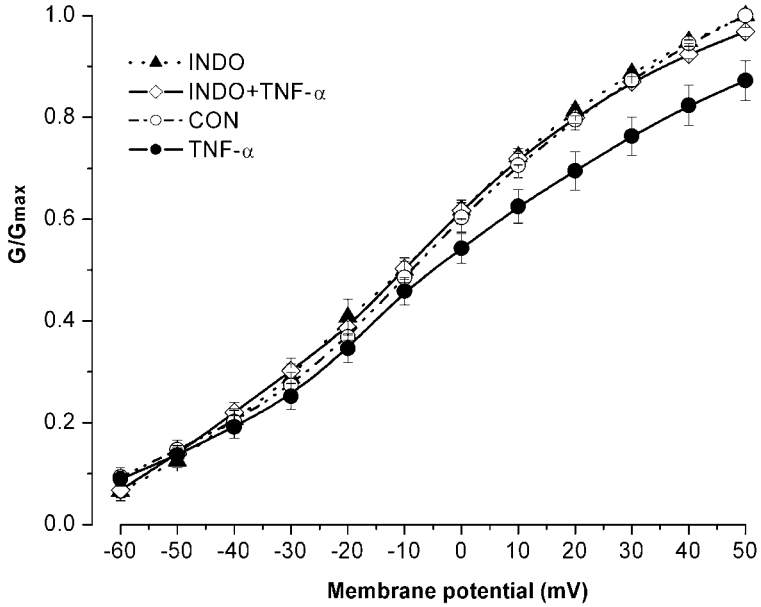

Fig. (6). Pretreatment of the DRG cells with indomethacin completely blocked TNF-α-induced potassium current inhibition. Indomethacin alone had no effect on the potassium currents.

of sustained potassium current by TNF-α is blocked by the COX inhibitor indomethacin; 2) effects of TNF-α on the activation of potassium current are similar to those of PGE2: inhibition of sustained current without altering the activation of transient potassium current or the steady-state inactivation; 3) the relatively longer time (e.g., 15 min) required for TNF-α to reduce potassium current or to evoke firing; and 4) the involvement of PKA pathway in both TNF-α evoked firing and in PGE2-induced suppression of potassium currents in sensory neurons [10, 23]. In addition, [33] reported in an earlier study that enhancement of the capsaicin-evoked current in nociceptive DRG neurons by TNF-α also was blocked by a specific COX-2 inhibitor, which provides further evidence that TNF-α can enhance the sensitivity of sensory neurons by the neuronal production of prostaglandins. Finally, TNF-α is known to activate p38 MAPK, which may increase PGE2 production via PLA2 to produce arachidonic acid, the PGE2 precusor [34].

Although not tested, it is possible that effects of TNF-α on potassium current may involve prostaglandins other than PGE2 because prostaglandins such as PGF2 and PGI2 are also regulated by COX pathway and their expression may be reduced by COX inhibitors [19, 22]. Finally, we do not believe the effects reported here are due to an indirect effect on satellite glia or other non-neuronal cells because results were performed in low density, acute cultures, in which non-neuronal cells were very sparse and glia-derived intercellular mediators would be diluted by the culture medium.

In addition to indirectly regulating potassium currents in sensory neurons, TNF-α may directly act on some ion channels such as sodium channels. [14] recently tested the effects of TNF-α on TTX-resistant sodium channels in isolated mouse DRG neurons and found that acute application of TNF-α rapidly enhanced TTX-resistant Na^+ currents. This potentiation of TTX-resistant currents by TNF-α is mediated by the p38 mitogen-activated protein kinase pathway through TNFR1 receptor. Further study is needed for us to determine whether direct effect of TNF-α on potassium channel may exist and which TNF receptor mediates the effects of TNF- α on potassium currents.

The inhibitory effect of TNF-α on potassium current in sensory neurons may play an important role in abnormal neuronal activity under various pathological conditions. Sensitization of sensory neurons by exposure to TNF-α released from the ruptured nucleus pulposus is thought to contribute to pathogenesis of low back pain or radicular pain. In nerve-injury neuropathic pain models, immunohistochemical staining demonstrated increased expression of TNF-α and TNFR1 receptor in DRG neurons, satellite glia, and macrophages ipsilateral and contralateral to the nerve injury [3]. Although our recent studies using the multiplex technique failed to detect changes in TNF-α protein levels in the inflamed or spinal nerve ligated DRG [35, 36], the receptor upregulation may account for the important contribution of TNF-α to abnormal neuronal firing and development of pain.

In summary, the pro-inflammatory cytokine TNF-α may sensitize sensory neurons by suppressing the non-inactivating sustained potassium current, which may be secondary to the neuronal production of endogenous prostaglandins.

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