Acute Topical Application of Tumor Necrosis Factor α Evokes Protein Kinase A-Dependent Responses in Rat Sensory Neurons

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Acute topical application of tumor necrosis factor α evokes protein kinase A-dependent responses in rat sensory neurons. J Neurophysiol 88: 1387–1392, 2002; 10.1152/jn.00020.2002. Local perfusion of the dorsal root ganglion (DRG) with tumor necrosis factor (TNF) 88: 1387–1392, 2002; 10.1152/jn.00020.2002. Local perfusion of the dorsal root ganglion (DRG) with tumor necrosis factor α (TNF-α) in rats induces cutaneous hypersensitivity to mechanical stimuli. Thus we investigated the cellular mechanisms of TNF-α-induced mechanical hyperalgesia. The L₄ and L₅ DRGs with the sciatic nerves attached were excised from rats for in vitro dorsal root microfilament recording. After baseline recording for 15 min, TNF-α (0.001, 0.01, 0.1, or 1 ng/ml) was applied to the DRG for 15 min, followed by washout for at least 30 min. Alternatively, H-89 or Rp-cAMPS, two specific cAMP-dependent protein kinase (PKA) inhibitors, was added to the perfusion solution for 15 min prior to TNF-α application. TNF-α (1 ng/ml) induced neuronal discharges in 67% (14/21) of C fibers and 27% (4/15) of Aβ fibers when applied topically to the DRG. Acute TNF-α application not only evoked discharges in silent fibers, but also enhanced ongoing activity of spontaneously active fibers and increased neuronal sensitivity to electrical stimulation of the peripheral nerves. H-89 (10 μM) and Rp-cAMPS (100 μM) each completely blocked the TNF-α-evoked response in most C and Aβ fibers tested but did not affect fiber conductivity. Our results demonstrate that exogenous inflammatory cytokines such as TNF-α can elicit a PKA-dependent response in sensory neurons and thus strongly suggest that endogenous TNF-α may contribute to the development of certain pathological pain states.

INTRODUCTION

Proinflammatory cytokines such as tumor necrosis factor α (TNF-α) are involved in the development of inflammatory and neuropathic pain behaviors. The level of TNF-α in inflamed tissue was significantly increased after intradermal injection of endotoxin (Kanaan et al. 1998). Conversely, the hyperalgesic effect induced by carrageenan was limited by an antiserum to endotoxin (Kanaan et al. 1998). In neuropathic animal models, it has been shown that TNF-α mediates thermal as well as mechanical hyperalgesia after nerve injury (George et al. 2000; Ignatowski et al. 1999; Sommer et al. 1998a,b; Nicol et al. 1997). Nicol et al. (1997) found that chronic treatment of the DRG cells with TNF-α enhanced capsaicin sensitivity of isolated sensory neurons. The enhanced sensitivity is likely to be mediated by the neuronal production of prostaglandins, as treating the cells with cyclo-oxygenase-2 (COX) inhibitors blocked the TNF-α-induced sensitivity enhancement. Some of these data can be used to explain, at least partially, the hyperalgesic effects of TNF-α when administered acutely to the nerve trunk (Sorkin and Doom 2000; Wagner and Myers 1996) or when injected subcutaneously into the rat hindpaw (Perkins and Kelly 1994; Woolf et al. 1997).

The role of cyclic AMP-dependent protein kinase (protein kinase A, PKA) pathway in the maintenance of inflammatory pain was demonstrated recently by the finding that PKA inhibitors reduced hyperalgesia induced by hyperalgesic agents (e.g., prostaglandin E₂, and purine) (Aley and Levine 1999). Furthermore, a role for PKA in mediating nerve injury-induced neuropathic pain was indicated in a study demonstrating that intrathecal spinal delivery of H-89 (a specific PKA inhibitor) reduced the hyperalgesia resulting from a unilateral tight ligation of L₄ and L₅ spinal nerves (Hua et al. 1999).

In the present study, using extracellular electrophysiological techniques, we examined the effects of acute application of TNF-α on the excitability of DRG neurons. The intracellular transduction cascade, in particular the role of PKA pathway in mediating the interaction between TNF-α and neuronal excitability, was also investigated.

METHODS

Extracellular electrophysiological recording

Male Sprague-Dawley rats (150–200 g, n = 54) were anesthetized with pentobarbital sodium (40 mg/kg ip). The L₄ and L₅ ganglia with attached dorsal roots (length: about 2 cm) and sciatic nerve (length: about 3 cm) were dissected and placed in a recording chamber (Zhang et al. 1997). The DRG was perfused at a rate of 5 ml/min with oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH₂PO₄, 24 NaHCO₃, 10 dextrose, 1.2 MgCl₂, and 1.2 CaCl₂ (pH = 7.3). The perfusion solution was heated to maintain the bath temperature at 37°C. The dorsal root was led out of this chamber into an adjacent mineral oil-filled chamber where...
Microfilament recordings were performed. The spinal/sciatric nerve (length: about 3 cm) was contained in an adjacent chamber with mineral oil and placed in contact with a bipolar stimulating electrode. Each chamber was separated by petroleum jelly (Vaseline) to prevent the solutions from different chambers from intermixing.

Dorsal root microfilaments were teased apart under a dissecting microscope. The proximal end of a dissected microfilament was placed on a fine silver electrode for single fiber recording. The discharges of single fibers were displayed on a digital oscilloscope and collected via Spike 2 data-acquisition system (Cambridge Electronic Design, Cambridge, UK) on a Pentium III PC. The conduction velocity of each fiber was obtained via electrical stimulation delivered to the sciatic nerve.

**Drug preparation and application**

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. The H-89 (Sigma Chemicals, St. Louis, MO) was dissolved in methanol at 0.5 mM and diluted to final concentration of 10 μM (containing methanol 0.0025%, vol/vol) prior to recordings. Rp-cAMPS (Sigma Chemicals) was dissolved in distilled water and diluted to 100 μM immediately prior to drug application. The pH for all drugs used in the present study was 7.3.

To determine whether the acute application of exogenous proinflammatory cytokines directly evokes action potentials in the somata of lumbar ganglia, TNF-α (0.001, 0.01, 0.1, or 1 ng/ml) was applied to the DRG for 15 min after a 15-min baseline recording. Alternatively, either Rp-cAMPS or H-89 was applied to the DRG before the TNF-α application to test the involvement of the PKA pathway. Briefly, H-89 or Rp-cAMPS was applied to the DRG for 15 min, followed by Rp-cAMPS or H-89 plus TNF-α for another 15 min. In some experiments, after 30–40 min washout with ACSF, the same dose of TNF-α (containing the same vehicle as the first application) was once again applied to the DRG for 15 min.

**Data acquisition and analyses**

With the employment of Spike 2 data-acquisition system, we could examine up to three C or Aβ fibers with different amplitudes during each trial. A mean basal ("control") discharge rate was computed as the mean number of spikes/s (±SE) for 15 min before delivery of TNF-α. Spontaneously active was defined as fibers with any number of spikes in 5-min interval. For each fiber, the maximal or peak effect of TNF-α on the fiber’s activity was defined as the discharge rate in the 5-min interval (spikes·s⁻¹·15 min⁻¹) following drug administration that exhibited the greatest increase from the peak basal rate. Student’s two-tailed t-test was used to compare the response latencies between silent and spontaneously active fibers. The criterion for significance was P < 0.05.

**RESULTS**

**Silent vs. spontaneously active C and Aβ fibers**

Myelinated Aβ and unmyelinated C fibers were activated using an in vitro nerve-DRG-dorsal root preparation by electrical stimulation applied to the sciatic nerve in 54 rats. A total of 148 fibers were activated and identified as C fibers with conduction velocity ranging between 0.48 and 1.79 m/s (mean conduction velocity: 0.98 ± 0.05 m/s). Eight-five of the 148 C fibers (74 silent and 11 spontaneously active) could be isolated using the Spike 2 acquisition program and thus were used for the present electrophysiological study. The discharge rate was low and extremely variable, ranging between 1 and 150 spikes in a 5-min interval. Eight of 11 fibers had less than 12 spikes in a 5-min interval. The mean discharge rate over 15 min for the 11 spontaneously active C fibers was 0.11 ± 0.04 spikes·s⁻¹·15 min⁻¹. The discharge pattern was irregular for all but one fiber, which exhibited a short burst-type discharge. Of 34 Aβ fibers used in this study, 30 were silent and 4 were spontaneously active with conduction velocity >15 m/s (Harper and Lawson 1985; Villiere and McLachlan 1996) and a low discharge rate of 0.03 ± 0.02 spikes·s⁻¹·15 min⁻¹ (n = 4).

**Acute application of TNF-α evoked responses in DRG neurons with slow-conducting C fibers and fast-conducting myelinated Aβ fibers**

A total of 20 C fibers were tested with 1 ng/ml of TNF-α. 7 fibers were spontaneously active and 13 fibers were initially quiescent. TNF-α elicited discharges in 7 of 13 silent fibers, enhanced the firing rate by at least 30% in 6 of the 7 spontaneously active fibers (Fig. 1) and suppressed the firing rate in 1 spontaneously active fiber. The average latency for TNF-α to evoke discharges in initially quiescent fibers was 21 ± 7 min (range, 5–43 min). In four fibers, TNF-α evoked discharges...
within 15 min of drug application. The remaining fibers started responding after the beginning of the washout. The latency between TNF-α application and enhancement of the spontaneously active fibers (17 ± 5 min) was shorter but not significantly different from that of silent fibers (P > 0.05, Student’s t-test). The evoked activity usually lasted for more than 30 min in response to a 15-min TNF-α application. After a 30-min washout, 50% of the tested fibers returned to basal level, but the remaining fibers did not recover for at least 1 h after TNF-α application. However, in two initially quiescent fibers, the evoked response lasted for only 10–20 min.

TNF-α was tested twice in one spontaneously active fiber. The first TNF-α application increased peak discharge rate from 0.02 to 0.27 spikes/s, with a latency of 2.6 min. After 30-min washout, a subsequent application of TNF-α at the same dose evoked a weaker response (peak discharge rate of 0.07 spikes/s) and a longer latency (30 min).

Three lower doses of TNF-α (0.001, 0.01, and 0.1 ng/ml) were also tested. None of six fibers that were tested with 0.001 ng/ml of TNF-α responded. Of the seven fibers (including 3 fibers that did not respond to 0.001 ng/ml) that were tested with 0.01 ng/ml, only two responded (including 1 that did not respond to 0.001 ng/ml) and had a latency of 30 and 44 min, respectively. Of 19 fibers (18 silent, 1 spontaneously active) treated with 0.1 ng/ml, 9 fibers exhibited enhanced firing with an average response latency of 23 ± 4 min. Only two of these fibers recovered after 30-min washout with ACSF alone. Of the 19 fibers tested with 0.1 ng/ml of TNF-α, 8 were also tested once with TNF-α at 0.01 ng/ml. No higher doses than 0.1 ng/ml of TNF-α were tested prior to the application of 0.1 ng/ml in any of the 19 fibers. Although variable doses of TNF-α elicited different response latencies, the peak discharge rates were not significantly different (P > 0.05, ANOVA; Fig. 2).

TNF-α not only evoked discharges in silent C fibers but also enhanced neuronal sensitivity to electrical stimulation of the peripheral nerves. In 32 C fibers, a single stimulation of the nerve only evoked a single action potential prior to TNF-α application. However, 15 min after TNF-α application (1 ng/ml), the same current pulse evoked a long-lasting burst in 7 of 32 fibers (22%; Fig. 3). The duration of electrically evoked firing varied among different fibers and ranged from 5 to 30 s,
Excitatory effect of TNF-α was blocked by H-89 or Rp-cAMPS

A total of eight C fibers (2 spontaneously active) were treated with H-89 (10 μM) for 15 min, followed by application of TNF-α (1 ng/ml) plus H-89 for another 15 min. None of the eight fibers responded to TNF-α. However, after the washout with ACSF for 30–60 min, TNF-α (1 ng/ml) induced responses in six of the eight fibers that had not responded to TNF-α plus H-89 (Fig. 5).

In five silent Aβ fibers, TNF-α failed to evoke responses when applied to the DRG together with H-89. After washout with ACSF alone for 30 min, a second application of TNF-α without H-89 evoked responses in four of five fibers tested. The average response latency was 10 ± 3 min, and mean peak discharge rate was 0.34 ± 0.25 spikes s⁻¹. 5 min⁻¹. None of the four fibers recovered from TNF-α-induced response after the 30-min washout period.

In a separate experiment, six C fibers were treated with Rp-cAMPS (100 μM) for 15 min, followed by Rp-cAMPS and TNF-α (1 ng/ml) for another 15 min. No neuronal discharge was elicited in five of six fibers tested while a weak and transient response was induced in only one fiber. After 30-min washout with ACSF, TNF-α alone was added to the perfusion solution, and a response was elicited in four of six fibers tested, including the fiber that had responded previously. For this fiber, TNF-α alone evoked a much stronger response than did TNF-α plus Rp-cAMPS in the previous test. Alternatively, in three C fibers, TNF-α (1 ng/ml), instead of Rp-cAMPS, was first applied to the DRG for 15 min prior to the treatment with Rp-cAMPS. TNF-α alone evoked a robust discharge in all three fibers. However, the second application of TNF-α plus Rp-cAMPS failed to evoke any action potentials in one of three fibers and evoked a transient weak response in the remaining two C fibers tested (Fig. 6).

Although PKA inhibitors (H-89 or Rp-cAMPS) blocked the acute TNF-α-elicited responses, the fiber conductivity, as examined by electrical stimuli of the peripheral nerve, was not interrupted in any fibers during or after the administration of the PKA inhibitors.

DISCUSSION

The present study demonstrated the ability of TNF-α to evoke discharges in DRG neurons with myelinated or unmyelinated axons. This effect is likely mediated by a PKA pathway as pretreatment of the ganglia with specific PKA inhibitors blocked TNF-α-induced responses.

A suppressive effect was only observed in a minority of spontaneously active C fibers even when higher dose of TNF-α (1 ng/ml) was employed. In previous studies, it was found that the ongoing activity was decreased in the majority of spontaneously active C fibers in response to higher doses of TNF-α (>0.05 ng/ml) applied topically to the nerve trunk (Sorkin et al. 1997). It is possible that the sensitivities to exogenous TNF-α are different between nerve fibers and cell bodies. The use of human, instead of rat, TNF-α in the present study might be another contributory factor to the discrepancy in TNF-α sensitivity between our and other studies. Although the amino acid sequence homology of human and rat TNF-α (Kwon 1993), it is expected that human TNF-α might have lower affinity to rat receptors.

Most fibers responded to TNF-α after exposure of at least 10-min TNF-α duration. The relatively long response latency suggests that an intracellular signal transduction pathway may have been involved in TNF-α-evoked responses. A similar response pattern has been observed recently. In a study exploring the effect of PKA on the modulation of spontaneous...
activity, most fibers responded to PKA activators after 15 min of topical application (Hu et al. 2001). The variability in response latency and recovering time might be due to the time for the drug to reach the somata that are located at different levels beneath the surface of the ganglion. The discrepancy in response latency between current results and results reported previously (Sorkin et al. 1997) may lie in the use of TNF-α at different pH. The use of different types of TNF-α (rat vs. human) in two studies might be another cause of the discrepancy in response latency.

The firing frequency elicited by TNF-α is greater in quiescent fibers than in spontaneously active fibers. The differences might be explained by partial phosphorylation of certain ion channels contributing to the generation of spontaneous activity. Earlier studies have shown that spontaneous activity of DRG neurons can be increased by protein phosphatase inhibitors such as okadaic acid (Hu et al. 2001), suggesting that ion channel phosphorylation may be involved in the signal transduction pathways that modulate spontaneous activity. These channels are likely potassium channels as demonstrated in earlier studies on TNF-α (Diem et al. 2001). A partial phosphorylation might have caused inactivation of certain numbers of potassium channels and decreased potassium conductance, which resulted in reduced magnitude of responses of spontaneously active fibers to any further drug applications.

Subsequent experiments with specific PKA inhibitors, H-89 and Rp-cAMPS, demonstrated that TNF-α-induced responses are PKA dependent. These results agree with previous reports that G-protein-mediated activation of PKA is one of the several signal transduction pathways that can be activated by TNF-α (Pan et al. 1997). It has been assumed that sensitization of nociceptors is due to increased concentrations of cAMP/Ca2+ in the sensory neurons (Cui and Nicol 1995; Ferreira 1993). This hypothesis is supported by results from a recent study in which blocking the PKA pathway with H-89 or Rp-cAMPS suppressed spontaneous activity of Aβ and Aδ fibers (C fibers were not tested) originating in the ganglia subjected to a previous chronic compression injury. Increasing intracellular cAMP level, on the other hand, enhanced ongoing spontaneous activity of DRG neurons (Hu et al. 2001). Thus it is likely that the excitatory effects of acute application of TNF-α on the DRG somata may have resulted from elevated intracellular cAMP level (Ebadi et al. 1997). The latencies for TNF-α to evoke neuronal responses were shorter in both A and C fibers tested previously with H-89 plus TNF-α. This suggests that earlier application of TNF-α may have partially sensitized the tested fibers, although no discharges were evoked in the presence of H-89.

The present study has demonstrated a PKA-mediated TNF-α response, a mechanism that is similar to prostaglandins E2 (PGE2)-induced sensitization of DRG neurons (Cui and Nicol 1995; Evans et al. 1999; Lopshire and Nicol 1998). It is possible that topical application of TNF-α may have caused PGE2 release through activation of COX-2 pathway as suggested previously (Nicol et al. 1997). However, a more extensive study is needed to determine if acute TNF-α application-evoked responses can be blocked by specific COX-2 inhibitors.

Clinically, lumbar ganglia and the adjacent dorsal roots are exposed to inflammatory cytokines (e.g., TNF-α) released from a ruptured lumbar disk (Kang et al. 1996). Our findings that TNF-α may evoke discharges in DRG neurons with myelinated as well as unmyelinated axons strongly suggest that, in addition to cutaneous hyperalgesia in inflammatory and neuropathic animal models, inflammatory cytokines also contribute to the initiation and maintenance of low back pain in patients with the preceding pathological conditions.

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