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CHEMICAL MEDIATORS ENHANCE THE EXCITABILITY OF UNMYELINATED SENSORY AXONS IN NORMAL AND INJURED PERIPHERAL NERVE OF THE RAT

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Abstract—Ectopic excitation of nociceptive axons by chemical mediators may contribute to symptoms in neuropathic pain. In this study, we have measured the excitability of unmyelinated rat C-fiber axons in isolated segments of sural nerves under different experimental conditions. (1) We demonstrate in normal rats that several mediators including ATP, serotonin (5-HT), 1-(3-chlorophenyl)biguanide (5-HT3 receptor agonist), norepinephrine, acetylcholine and capsaicin alter electrophysiological parameters of C-fibers which indicate an increase of axonal excitability. Other mediators such as histamine, glutamate, prostaglandin E_2 and the cytokines tumor necrosis factor α, interleukin-1β and interleukin-6 did not produce such effects. (2) The effects of several mediators were tested after peripheral nerve injury (partial ligation or spared nerve injury). Sural nerves from such animals did not show significant changes when compared with controls. (3) We tested whether the effects of chemical mediators on axonal excitability are due to actions on the sensory C-fiber afferents or the postganglionic sympathetic efferents. In order to distinguish these effects, we performed surgical sympathectomy of the lumbar sympathetic chain, including the L3, L4 and L5 ganglia. Sympathectomy did not markedly influence the effects of mediators on axonal excitability (except that the norepinephrine effect was significantly diminished). In conclusion, our data suggest a constitutive rather than inducible expression of axonal receptors for some chemical mediators on the axonal membrane of unmyelinated fibers. Most of the changes in axonal excitability take place in sensory C-fiber afferents rather than in postganglionic sympathetic efferents. Thus, it is possible that certain immune and glial cell mediators released in or around the nerve following injury or inflammation influence the excitability of intact nociceptive fibers. This mechanism could contribute to ectopic excitation of axons in neuropathic pain.

Key words: axon, threshold tracking, excitability, nerve injury, sympathetic, pain.

Unmyelinated C-fiber and thinly myelinated Aδ-fiber nociceptors transduce external noxious stimuli into electrical activity. This activity is conducted to the spinal cord, and after transmission in central pathways, to higher centers, where pain sensation is experienced. Nociceptor function is considerably modified in response to tissue damage, inflammation, or injury of the nervous system, which can result in chronic neuropathic pain. Changes in threshold and excitability of nociceptors can be localized to the peripheral terminals, the site of axonal injury, or the central synapses, all of which can contribute to pain hypersensitivity (Scholz and Woolf, 2002). Early work concentrated on the idea that the cut ends of nociceptors become hyperexcitable and generate ectopic action potentials, resulting in increased activity. This leads to sensitization of central neurons and neuropathic pain (Devor, 2001). However, recent work has emphasized the role of intact nociceptive axons in the generation and maintenance of neuropathic pain (Campbell, 2001). For example, blocking peripheral input from intact spinal nerves (L3 and L4) by transection of their dorsal roots or application of a local anesthetic to their roots, after neuropathic injury (L5 and L6 spinal nerve ligation), reduces some signs of pain behavior (Yoon et al., 1996). Mechanical hyperalgesia after L5 spinal nerve lesion is reversed following L4 dorsal rhizotomy, and thus develops and persists independent of input from injured afferents but due to interactions between the degenerating fibers of the injured spinal nerve and the intact fibers of adjacent spinal nerves (Li et al., 2000). Likewise, mild irritation of the L4 spinal nerve and application of mechanical stimuli to the ipsilateral paw significantly augment the development of mechanical allodynia after L5 and L6 spinal nerve ligation, suggesting that afferent activity of the intact L4 spinal nerve aids in the development of neuropathic pain (Lee et al., 2003). Dorsal root ganglion (DRG) neurons with intact axons show changes in molecular phenotype that contribute to pain behavior following partial nerve injury (Obata et al., 2003). In fact, it is not necessary to lesion any nociceptive fibers in order to elicit neuropathic pain. Unilateral L5 ventral root transection produces rapid, robust and prolonged bilateral mechanical allodynia, cold allodynia and short-term thermal hyperalgesia and leads to significant inflammation in the DRG, sciatic nerve and muscle fibers (Li et al., 2002). Degeneration of myelinated efferent fibers following L5 ventral rhizotomy induces spontaneous activity in uninjured C-fiber afferents (Wu et al., 2002) and both L5 ventral rhizotomy and L5 ganglionectomy produce mechanical hyperalgesia (Sheth et al., 2002). These findings suggest that neuropathic pain can

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Abbreviations: ACh, acetylcholine; ACSF, artificial cerebrospinal fluid; BSA, bovine serum albumin; CAP, compound action potential; DRG, dorsal root ganglion; IL, interleukin; m-CPBG, 1-(3-chlorophenyl) biguanide; NE, norepinephrine; PBS, phosphate buffered saline; PGE_2, prostaglandin E_2; TH, tyrosine hydroxylase; TNF, tumor necrosis factor; TRPV1, vanilloid receptor; 5-HT, 5-hydroxytryptamine.

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be induced without damage to sensory neurons, but through exposure of intact sensory axons to the milieu of Wallerian degeneration and inflammation after nerve injury. Moreover, a focal inflammation of peripheral nerve, in the absence of axonal damage, produces neuropathic pain; exposure of a healthy sciatic nerve to an inflammatory stimulus such as yeast cell walls (zymosan) (Chacur et al., 2001), carrageenan or complete Freund's adjuvant (Eliav et al., 1999) initiates immune activation followed by rapid and significant allodynia and hyperalgesia. Thus, intact axons can be sensitized to elicit neuropathic pain.

Immune cells are activated both in the periphery and the CNS in response to tissue damage, inflammation or mechanical nerve lesion and may increase nociception through the release of inflammatory mediators and cytokines (Tracey and Walker, 1995; Watkins et al., 2001). Recent studies have demonstrated the role of inflammatory cells including neutrophils (Perkins and Tracey, 2000), macrophages (Liu et al., 2000), mast cells (Zuo et al., 2003) and T lymphocytes (Moalem et al., 2004), as well as their mediators in the peripheral mechanisms of neuropathic pain. Probable peripheral mediators are nerve growth factor, serotonin (5-hydroxytryptamine, 5-HT), prostaglandin E_2 (PGE_2), bradykinin, noradrenaline, adenosine, ATP, histamine (Tracey and Walker, 1995; Wood and Docherty, 1997) and the proinflammatory cytokines tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and IL-6 (DeLeo and Yezierski, 2001; Sommer and Kress, 2004). Inflammatory mediators in the periphery are normally thought to sensitize nociceptors by acting on the peripheral nerve terminals (Woolf and Salter, 2000), or near a lesion site following axotomy (Michaelis et al., 1998). However, the immune cells which release inflammatory mediators may be concentrated at some distance from the nociceptive terminals, and may induce pain due to nerve inflammation in the absence of trauma. An alternative is that some mediators act on receptors located on the intact axon.

Evidence has accumulated that receptors for neurotransmitters alter electrophysiological properties of myelinated and unmyelinated axons in the trunk of peripheral somatosensory nerves. For example, axonal GABA receptors have been demonstrated in rat dorsal roots (Bhisitkul et al., 1987; Liske and Morris, 1994). Neuronal nicotinic acetylcholine receptors (nAChRs) enhance the excitability of unmyelinated axons in peripheral nerves of cat and man (Douglas and Ritchie, 1960; Lang et al., 2003) and induce a rise of the intra-axonal Ca^{2+} concentration in axons of rat optic nerve (Zhang et al., 2004). The effect of capsaicin, a potent agonist at the vanilloid receptor (TRPV1) on compound C- fiber action potentials (Hayes et al., 1984; Baranowski et al., 1986) is accompanied by a rise in intracellular Ca^{2+} concentration (Mayer et al., 1999) and axonal release of CGRP (Bernardini et al., 2004). Also, expression of TRPV1 in the axonal membrane has been demonstrated by immunocytochemistry (Tominaga et al., 1998). Receptors for ATP and adenosine in peripheral nerves of rat, mouse, and man enhance axonal excitability by activation of purinergic P2X and A2 receptors (Irnich et al., 2002; Lang et al., 2002; Labrakakis et al., 2003). The presence of receptors for 5-HT has also been demonstrated on primary afferent fibers in functional (5-HT3; Zeitz et al., 2002) and immunohistochemical studies (5-HT2A; Carlton and Coggeshall, 1997).

We have recently demonstrated that adenosine sensitizes C-fibers in fascicles of human nerve, probably by activation of adenosine A2 receptors, and that ATP sensitizes C-fibers in rat sural nerve by activating P2X receptors (Irnich et al., 2002; Lang et al., 2002). In addition, acetylcholine (ACh) increases axonal excitability in human C-fiber axons (Lang et al., 2003). However, whether such changes in axonal excitability are modulated by nerve injury, and what subtype of unmyelinated fibers is responsible have not been addressed. In the present study, we repeated some of the above findings and have tested the effect of several other chemical mediators on axonal excitability in rat sural nerve, using electrophysiological recording by the *in vitro* threshold tracking technique. We examined whether peripheral nerve injury, which is associated with neuropathic pain, alters the drug-induced changes in axonal excitability. Finally, we identified whether effects on axonal excitability result from actions on the sensory C-fiber afferents or the postganglionic sympathetic efferents, by comparing axonal excitability in sural nerves derived from normal and sympathectomized rats.

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats (Biological Resources Center, University of New South Wales, or Animal Resources Center, Perth, Australia, $n=49$) at 8–12 weeks of age were used. All procedures were approved by the Animal Care and Ethics Committee of the University of New South Wales and adhered to the guidelines of the Committee for Research and Ethical issues of the International Association for the Study of Pain. All efforts were made to minimize the number of animals used and their suffering.

Preparation of nerves and experimental setup

Rat sural nerves were removed from Wistar rats that were deeply anesthetized and subsequently killed with an overdose of sodium pentobarbitone (120 mg/kg i.p.). Isolated nerves were immediately put into fresh artificial cerebrospinal fluid (ACSF) solution containing (in mM) NaCl 117, KCl 3.6, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25, and D-glucose 11; pH 7.4. The nerves were desheathed under a microscope and then held at each end by suction electrodes in an organ bath. One suction electrode was used to elicit action potentials, while the other was used as a recording electrode (Grafe et al., 1997). The distance between stimulating and recording electrodes was approximately 4 mm. The organ bath (volume: 2 ml) was continuously perfused with ACSF solution at a flow rate of 8 ml/min and a temperature of 30 °C. The perfusion solution was bubbled with 95% O_2–5% CO_2. Drug application lasted 3 min, and the interval between drug applications was normally about 10 min.

Threshold tracking

Axonal excitability was measured using the threshold tracking technique, making use of the QTRAC program (Institute of Neurology, London, UK). QTRAC is a flexible, stimulus-response data-acquisition program, originally written for studies of human nerves *in vivo* (Bostock et al., 1998) but also suitable for electro-