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ACTIVATION AND RETROGRADE TRANSPORT OF PROTEIN KINASE G IN RAT NOCICEPTIVE NEURONS AFTER NERVE INJURY AND INFLAMMATION

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Abstract—Nerve injury elicits both universal and limited responses. Among the former is regenerative growth, which occurs in most peripheral neurons, and among the latter is the long-term hyperexcitability that appears selectively in nociceptive sensory neurons. Since positive injury signals communicate information from the site of an injury to the cell body, we hypothesize that a nerve injury activates both universal and limited positive injury signals. Studies in *Aplysia* indicate that protein kinase G is a limited signal that is responsible for the induction of long-term hyperexcitability. Given that long-term hyperexcitability contributes to chronic pain after axotomy in rodent neuropathic pain models, we investigated its underlying basis in the rat peripheral nervous system. Using biochemical assays, Western blots, and immunocytochemistry we found that the Type 1α protein kinase G is the predominant isoform in the rat periphery. It is present primarily in axons and cell bodies of nociceptive neurons, including populations that are isolectin B4-positive, isolectin B4-negative, and those that express transient receptor potential vanilloid receptor-1. Surprisingly, protein kinase G is not present in the facial nerve, which overwhelmingly contains axons of motor neurons. Crushing the sciatic nerve or a cutaneous sensory nerve activates protein kinase G in axons and results in its retrograde transport to the neuronal somata in the DRG. Preventing the activation of protein kinase G by injecting Rp-8-pCPT-cGMPS into the crush site abolished the transport. The protein kinase A inhibitor Rp-8-pCPT-cAMPS had no effect. Extracellular signal-related kinases 42/44 are also activated and transported after nerve crush, but in both motor and sensory axons. Chronic pain has been linked to long-term hyperexcitability following a nerve inflammation in several rodent models. We therefore injected complete Freund's adjuvant into the hindpaw to induce an inflammation and found that protein kinase G was activated in the sural nerve and transported to the DRG. In contrast, the extracellular signal-related kinases in the sensory axons were not activated by the complete Freund's adjuvant. These studies support the idea that the extracellular signal-related kinases are universal positive axonal signals and that protein kinase G is a limited positive axonal signal. They also establish the association between protein kinase G, the induction of long-term hyperexcitability, and chronic pain in rodents.

Key words: ERKs, long-term hyperexcitability, nitric oxide synthase, guanylyl cyclase, chronic pain, positive injury signal.

Transecting a peripheral nerve typically axotomizes both motor axons and axons of the primary sensory neurons that mediate nociception, proprioception, thermoreception, and the various kinds of mechanoreception. Interestingly, the changes in gene expression that occur in response to the injury include those that are universal, such as survival and growth, as well as those that are restricted in that they appear only in discrete populations of neurons. Among the latter are alterations in the levels of neurotransmitters (Honore et al., 2000) and ion channels (Ishikawa et al., 1999; Abdulla and Smith, 2002) that occur selectively in nociceptive neurons after peripheral nerve trauma. The change in the composition of ion channels is especially important because it results in a hyperexcitability that enhances inputs to nociceptive circuits in the CNS and, when prolonged, can contribute to chronic pain (Study and Kral, 1996; Ishikawa et al., 1999; Stebbing et al., 1999; Abdulla and Smith, 2002).

The universal and restricted responses both require alterations in gene transcription, but the injuries that induce these responses typically occur in the distal regions of the nerve. How information from the site of a distal lesion is communicated to the transcriptional centers in the cell nucleus is not completely understood, but positive injury signals are thought to have an important role. Positive signals, i.e. axonal proteins that are activated at the site of the injury and retrogradely transported to the cell soma (Johanson et al., 1995; Ambron and Walters, 1996; Schmied and Ambron, 1997; Hanz et al., 2003; Perlson et al., 2004b) monitor the integrity and status of the axon and are known to contribute to nerve regeneration in both vertebrates and invertebrates (Bussmann and Sofroniew, 1999; Perlson et al., 2004a,b). The extracellular signal-related kinases 42/44 (ERKs 1 and 2) are the best characterized positive signals in vertebrates (Perlson et al., 2005) and are likely to be universal signals since they are present in many classes of axons (Johanson et al., 1995) and can activate transcription factors involved in growth and survival. Recent studies in the mollusk *Aplysia californica* suggested that protein kinase G (PKG) is a limited positive signal that is responsible for the induction of a

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Abbreviations: CFA, Complete Freund's Adjuvant; ERKs 1 and 2, extracellular signal-related kinases 42/44; IB4, isolectin B4; LTH, long-term hyperexcitability; LTP, long-term potentiation; NO, nitric oxide; NOS, nitric oxide synthase; PKA, protein kinase A; PKG, protein kinase G; sGC, soluble guanylyl cyclase; TPRV1, transient receptor potential vanilloid receptor-1.

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long-term hyperexcitability (LTH) that appears specifically in nociceptive sensory neurons following a peripheral nerve crush (Walters et al., 1991; Sung and Ambron, 2004; Sung et al., 2004) or inflammation (Clatworthy et al., 1994; Farr et al., 1999). This LTH has well-defined electrophysiological properties (e.g. Walters et al., 1991) and because it appears after both axotomy and inflammation, it differs from the hyperexcitability that can appear in other types of neurons after an axotomy. Significantly, the LTH in the *Aplysia* sensory neurons has the same electrophysiological properties as the persistent hyperexcitability that appears in rat nociceptive neurons in response to nerve injury and inflammation (Sung and Ambron, 2004), both of which are causes of chronic pain.

The *Aplysia* studies showed that nerve crush initiates a cascade at the injury site that begins with the activation of nitric oxide synthase (NOS) and the subsequent formation of nitric oxide (NO). The NO activates soluble guanylyl cyclase (sGC), which produces cGMP, thereby activating a Type 1α PKG. The activated PKG is then transported retrogradely to the cell bodies of the injured nociceptive sensory neurons where it activates a transcriptional program resulting in LTH. Blocking any step in the NOS–sGC–PKG pathway prevents the appearance of LTH (Sung et al., 2004). These studies suggested that PKG is a limited positive signal because the retrogradely transported active PKG did not appear in the cell bodies of non-sensory neurons even though their axons were injured (Sung et al., 2004).

The nociceptive systems in *Aplysia* and the rat have many properties in common (Sung and Ambron, 2004) and we have now used two well-characterized models of chronic pain, axotomy (Decosterd et al., 2002) and CFA-induced inflammation (Gould, 2000), to investigate the response of the NOS–sGC–PKG cascade in the rat. We found that PKG is activated and transported after both types of trauma and that this occurred primarily in the axons of nociceptive neurons. In contrast, the ERKs were activated and transported in the axons after the axotomy, but did not respond to the inflammation. Thus, following a nerve injury, PKG behaves as a limited positive axonal signal and the ERKs as a universal axonal signal. Our findings also extend recent studies showing that the activation of PKG is linked to both LTH and nociception in rodents (Tegeder et al., 2004; Song et al., 2006).

EXPERIMENTAL PROCEDURES

All studies were performed in accordance with the proposals of the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee at the Columbia University in accordance with the guidelines provided by the National Institutes of Health. Efforts were made to minimize the number of animals and yet still yield statistically significant results.

In vivo nerve crush

Adult male Sprague–Dawley rats (200–250 g) were anesthetized with a cocktail containing ketamine and xylazine intraperitoneally before all the surgical procedures. For studies of sciatic nerve or sural nerve injury, either the right sciatic nerve proximal to the bifurcation of tibial and common peroneal nerves, or the right sural nerve after it separates from the tibial nerve, was crushed by a fine hemostat forceps for 30 s (Decosterd et al., 2002). In both cases, muscle and skin were closed in two layers. For cutaneous nerve injury, the lateral cutaneous branches of the 9th to 12th intercostal nerves were exposed and crushed as described above. The crush/ligation protocol was carried out as described (Ambron et al., 1995).

Inflammation in rats

A unilateral, acute inflammatory lesion was produced by injecting 100 μl of Complete Freund's Adjuvant (CFA) (Sigma, St. Louis, MO, USA) into the plantar surface of the hindpaw under isoflurane anesthesia as described (Ma and Woolf, 1996).

Kinase assays

PKG activity was assayed as described (Sung et al., 2004).

Western blotting

Protein samples were resolved on 10% SDS polyacrylamide gels and subsequently transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA); the blots were probed with various gene-specific primary antibodies and appropriate horseradish peroxidase-conjugated or Alexa fluor-680 or infrared fluorescent-labeled IRDye 800 secondary antibodies. Immunoreactivity was detected using the Pico-tag chemiluminescence system (Pierce, Rockford, IL, USA) or with an Odyssey infrared imaging system (LI-COR Inc., Lincoln, NE, USA).

Immunohistochemistry

After anesthesia, rats were transcardiac-perfused with saline followed by 4% paraformaldehyde. Following perfusion, the DRG and/or nerve segments were removed, post-fixed in 4% paraformaldehyde for 2 h, cryo-protected in 15% sucrose and then frozen for cryostat sectioning. Following sectioning, primary antibodies were diluted in TBS supplemented with 0.1–0.5% Triton X-100 in TBS and 5% goat serum, and incubated overnight at 4 °C. After several washes, an AlexaFluor-594 or -488 conjugated secondary antibody (Molecular Probes, Eugene, OR, USA) was applied for an hour at room temperature. Subsequently, the cells were visualized by confocal fluorescence microscopy (LSM510 confocal microscope, Carl Zeiss Inc., Thornwood, NY, USA), and images captured.

Other materials

Bovine PKG 1α protein, Rp-8-pCPT-cGMPS, and Rp-8-pCPT-cAMPS were purchased from Calbiochem (La Jolla, CA, USA). Biotin-labeled isolectin B4 (IB4) lectin and CFA were purchased from Sigma. The following antibodies were obtained and used according to the manufacturer's instructions: TPRV1 antibody from Chemicon (Temecula, CA, USA); non-phosphorylated MAPK (ERK1and2) antibody from Cell Signaling Technology; anti-PKG 1α and anti-PKG1β from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phosphoERK1 and 2, βIII-tubulin, NF-200, and anti-α-actin, from Sigma-Aldrich; antibody to type-I PKG c-terminal (657–671) was obtained from Calbiochem; S100-1β antibody was purchased from East Acres Biological (Southbridge, MA, USA).

RESULTS

Type I alpha PKG is present in axons of sensory, but not motor neurons

There are three isoforms of PKG in vertebrates, two of which are soluble (Type 1α and β), and one that is asso-