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GDNF INDUCES MECHANICAL HYPERALGESIA IN MUSCLE BY REDUCING I_{BK} IN ISOLECTIN B4-POSITIVE NOCICEPTORS

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Abstract—We have assessed the mechanism underlying glial cell-derived neurotrophic factor (GDNF)-induced mechanical hyperalgesia in the gastrocnemius muscle, using patch clamp electrophysiology, *in vivo* electrophysiology and behavioral studies. Cultured isolectin B4-positive (IB4+) dorsal root ganglion neurons that innervated this muscle were held under current clamp; the majority developed an increase in action potential duration (a factor of increase of 2.29 ± 0.24, compared to 1.13 ± 0.17 in control, $P < 0.01$) in response to GDNF (200 ng/ml) by 15 min after application. They also demonstrated a depolarization of resting membrane potential, but without significant changes in rheobase, action potential peak, or after-hyperpolarization. Large-conductance voltage- and calcium-activated potassium (BK) channels, which have recently been shown to play a role in the repolarization of IB4+ nociceptors, were inhibited under voltage clamp, as indicated by a significant reduction in the iberiotoxin-sensitive current. *In vivo* single-fiber recording from muscle afferents revealed that injection of iberiotoxin into their peripheral nociceptive field caused an increase in nociceptor firing in response to a 60 s suprathreshold stimulus (an increase from 392.2 ± 119.8 spikes to 596.1 ± 170.8 spikes, $P < 0.05$). This was observed in the absence of changes in the mechanical threshold. Finally, injection of iberiotoxin into the gastrocnemius muscle produced dose-dependent mechanical hyperalgesia. These data support the suggestion that GDNF induces nociceptor sensitization and mechanical hyperalgesia, at least in part, by inhibiting BK current in IB4+ nociceptors.

Key words: pain, potassium channel, growth factor, electrophysiology, behavior.

INTRODUCTION

Two functionally distinct classes of small-diameter nociceptors can be defined by their dependence on different neurotrophic factors, nerve growth factor (NGF) and glial cell-derived neurotrophic factor (GDNF) (Snider and McMahon, 1998; Stucky and Lewin, 1999). GDNF-dependent nociceptors, which can be identified by their binding of isolectin B4 (IB4), terminate in the inner part of lamina II of the spinal dorsal horn. In contrast, IB4-negative (IB4−) neurons terminate in lamina I and the outer part of lamina II (Braz et al., 2005). There has been some controversy over the incidence of this nociceptor subset among muscle afferents, ranging from 15 to 40% (Molander et al., 1987; Pierce et al., 2006), in general a lower proportion than in the skin (Plenderleith and Snow, 1993). However, we have found that IB4+ muscle afferents play a major role in numerous models of inflammatory and ergonomic muscle pain (Alvarez et al., 2012).

In addition to long-term support of target neurons, GDNF can have more short-term effects on ion channels in midbrain (Yang et al., 2001; Wang et al., 2003) and also sensory (Takeda et al., 2010) neurons. The present study was designed to investigate the ionic basis of the mechanical hyperalgesia induced by GDNF application to nociceptors innervating the gastrocnemius muscle in the rat. Toward this end we have characterized action potentials evoked by electrical stimulation of IB4+ muscle afferents *in vitro*, examined BK (also termed K_{Ca}, SLO1, maxi K) current, and evaluated the role of this ion channel in mechanical hyperalgesia in the gastrocnemius muscle in the rat using behavioral and single-fiber *in vivo* electrophysiology studies. BK channels are present in more than 90% of small-diameter IB4+ sensory neurons, but only in a minority of IB4− neurons (Zhang et al., 2010) They play an important role in regulating neuronal excitability (Fakler and Adelman, 2008), and regulate both the frequency and action potential duration in a variety of neurons (Lancaster and Nicoll, 1987; Pineda et al., 1992; Shao et al., 1999; Faber and Sah, 2003; Sausbier et al., 2006; Gu et al., 2007). Although acute modulation of BK channels by glucocorticoids

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Abbreviations: AHP, after-hyperpolarization; ANOVA, analysis of variance; BK channel, large-conductance voltage- and calcium-activated potassium channel; CDK5, cyclin-dependent kinase 5; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tertamethyllindocarbocyanine perchlorate; DRG, dorsal root ganglion; EGTA, ethylene glycol tetraacetic acid; GDNF, glial cell-derived neurotrophic factor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IB4, isolectin B4; IB4+, isolectin B4-positive; IB4−, isolectin B4-negative; *I–V*, current–voltage; NGF, nerve growth factor; NS1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; PI3K, phosphatidylinositol 3-kinase; RET, receptor tyrosine kinase; RMP, resting membrane potential; Src, sarcoma.

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(Lovell et al., 2004) and ethanol (Gruss et al., 2001; Dopico, 2003), both of which affect nociceptive function (Ikeda et al., 2002; Liu et al., 2010b), has been reported, we are not aware of any reports of short-term modulation of BK channels by growth factors.

EXPERIMENTAL PROCEDURES

Ethical approval

Animal care and use conformed to the National Institutes of Health guidelines. The University of California, San Francisco, Institutional Animal Care and Use Committee approved the experimental protocols employed in these experiments.

Animals

Experiments were performed on adult male Sprague–Dawley rats weighing 180–240 g for electrophysiology and 220–240 g for behavioral testing (Charles River, Hollister, CA, USA). Animals were housed in the Laboratory Animal Resource Center of the University of California, San Francisco, under a 12-h light/dark cycle and environmentally controlled conditions (7:00 AM–7:00 PM light cycles; 21–23 °C) with food and water available *ad libitum*.

Drugs

Unless specifically stated, all chemicals were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Retrograde dye injection

Dorsal root ganglion (DRG) neurons innervating the gastrocnemius muscle were identified by their uptake of the retrograde tracer 1,1′-dioctadecyl-3,3,3′,3′-tertamethyllindocarbocyanine perchlorate (DiI). Rats were subjected to gaseous anesthesia (isofluorane, 3%), and an incision made to expose a section of biceps femoris muscle. A 30-g needle was passed through the biceps femoris muscle and into the belly of the gastrocnemius muscle; then 2 μl dimethyl sulfoxide containing 2% DiI was slowly injected into each of three sites within the muscle. Each injection was performed over 2 min, followed by observation for 1 min to observe for any leakage. In a separate experiment, the overlap in fluorescence between DiI (injected into the muscle) and water containing 2% Fast Blue dye (applied to the surface of the muscle) was examined. No overlap in fluorescence was observed.

DRG cell-culture

DRGs were surgically removed from rats 5–8 days after DiI injection. Rats were decapitated and the vertebral column excised. The column was then opened up on the ventral side, and L4-L5 DRGs were removed and de-sheathed. Ganglia were treated with collagenase (0.125% in Neurobasal-A medium; Invitrogen, Carslbad, CA) for 90 min at 37 °C, and then treated with 0.25% trypsin in Ca^{2}- and Mg^{2+}-free phosphate-buffered saline (PBS; Invitrogen) for 10 min, followed by trituration (in Neurobasal-A) to produce a single-cell suspension. The suspension was centrifuged at 1000 RPM and re-suspended in Neurobasal-A medium supplemented with 50 ng/ml NGF, 100 U/ml penicillin/streptomycin and B-27 (all Invitrogen). Cells were then plated on coverslips coated with poly-DL-ornithine (0.1 mg/ml) and laminin (5 μg/ml; Invitrogen), and incubated at 37 °C in 5% CO_2.

Imaging and cell counting

Cells were stained with fluorescein isothiocyanate-conjugated IB4 (Invitrogen), at a concentration 10 μg/ml (in water), 10 min before use. DiI-labeled cells were identified under epifluorescence illumination. The proportion of IB4 + cells was established from 20 coverslips, across three separate cultures. Cell counting occurred within 30 h of cell dissociation to minimize the impact of culture conditions on the survival of different subpopulations of neurons. Neurons were identified by morphology and phase-brightness.

In vitro electrophysiology

Electrophysiology was performed using an Axopatch 200A amplifier and pCLamp 8.2 software (Molecular Devices, Sunnyvale, CA, USA). DRG neurons were subjected to current- or voltage-clamp after 2–36 h in culture. For current-clamp, the external solution contained (in mM): NaCl (140), KCl (3), $MgCl_2$ (1), $CaCl_2$ (1), glucose (10), HEPES (10), adjusted to pH 7.3 and 320 mOsm. The pipette solution contained: KCl (140), EGTA (1); NaCl (10); MgATP (2); HEPES (10), adjusted to pH 7.3 and 310 mOsm.

Cells were held in the whole-cell configuration at −60 mV, following seal formation (seal resistance > 1 GΩ). Whole-cell capacitance and series resistance were compensated (80%) using the amplifier circuitry. Cells with a series resistance > 10 MΩ were not used for experimentation. Following the switch to current clamp, current steps of 50 pA were applied to find the minimum stimulus intensity required to elicit an action potential stimulation (rheobase). Eight hundred ms steps were then applied every 2 min for the duration of the experiment. After the determination of rheobase, GDNF (200 ng/ml) or vehicle (external solution) was applied directly to the bath.

BK current–voltage (*I–V*) relationships were obtained by applying test pulses (from −90 to +60 mV in 10 mV increments) for 500 ms, from the holding potential of −60 mV. This was repeated until the currents had stabilized. At this point, the BK channel blocker iberiotoxin (100 nM) was added to the bath. A test pulse to +60 mV was elicited at 5 s intervals, and a corresponding reduction in the current response occurred with the onset of channel block. Once channel block had reached its maximal level, the *I–V* protocol was run again. For voltage-clamp experiments the external solution contained (in mM): choline-Cl (130); KCl (5); $CaCl_2$ (2.5); $MgCl_2$ (0.6); HEPES (5); glucose (10), adjusted to pH 7.4 and 320 mOsm. The pipette solution contained: KCl (30); K-methanesulfonate (110); $MgCl_2$ (1); HEPES (10); EGTA (0.1), adjusted to pH 7.2 and 310 mOsm.

Data analysis

Various characteristics of the rheobase action potentials were analyzed. Action potential duration was defined as the width at the membrane potential halfway between resting membrane potential (RMP) and action potential peak. Action potential peak refers to the maximal depolarization that occurred during the action potential. The after-hyperpolarization (AHP) refers to the maximal repolarization following the action potential. Offline analysis of I-V curves involved subtraction of the iberiotoxin-resistant current from the total current; this was defined as the BK channel component of the current. Currents were normalized according to cell capacitance, as determined by the amplifier compensation circuitry. Data were analyzed and plotted using Origin 6.1 software (OriginLab, Northampton, MA, USA).

Mechanical nociceptive threshold in gastrocnemius muscle

Mechanical nociceptive threshold in the gastrocnemius muscle was measured as previously described (Alvarez et al., 2011).