Bradykinin-Induced NFAT-Dependent Transcription in Rat Dorsal Root Ganglion Neurons

Joshua G. Jackson, Yuriy M. Usachev* and Stanley A. Thayer

Department of Pharmacology, University of Minnesota Medical School
6-120 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455-0217

* Present Address: Department of Pharmacology, The University of Iowa,
2-250 BSB, 51 Newton Road, Iowa City, IA 52242


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Address correspondence to:
S. A. Thayer, Department of Pharmacology, University of Minnesota,
6-120 Jackson Hall, 321 Church Street SE, Minneapolis, MN 55455-0217
Phone: (612) 626-7049
Fax: (612) 625-8408
E-mail: sathayer@umn.edu

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Abbreviations: BK, bradykinin; BDNF, brain-derived neurotrophic factor; [Ca^{2+}]_{i}, intracellular Ca^{2+} concentration; cox-2, cyclooxygenase-2; CPA, cyclopiazonic acid; CSA, cyclosporin A; Ct, crossing threshold; CZP, capsazepine; DRG, dorsal root ganglion; EC_{50}, 50% effective concentration; ER, endoplasmic reticulum; GFP, green fluorescent protein; HHSS, HEPES-Hank’s salt solution; IP_{3}, inositol 1,4,5-triphosphate; NFAT, Nuclear Factor of Activated T-cells; NGF, nerve growth factor; PLA_{2}, phospholipase A_{2}; PLC, phospholipase C; PCR, polymerase chain reaction; NSAIDS, non-steroidal anti-inflammatory drugs; SERCA, sarcoplasmic-endoplasmic reticulum Ca^{2+}-ATPase; TRPV1, transient receptor potential vanilloid receptor 1.
Abstract

Bradykinin produced at sites of tissue injury and inflammation elicits acute pain and alters the sensitivity of nociceptive neurons to subsequent stimuli. We tested the hypothesis that bradykinin could elicit long lasting changes in nociceptor function by activating members of the Nuclear Factor of Activated T-cells (NFAT) family of transcription factors. Bradykinin activation of B2 receptors evoked concentration dependent (EC50 = 6.0 ± 0.3 nM) increases in intracellular Ca2+ concentration ([Ca2+]i) in a proportion of dorsal root ganglion (DRG) neurons in primary culture. These [Ca2+] increases were sensitive to inhibition of phospholipase C (PLC) and depletion of Ca2+ stores. In neurons expressing a green fluorescent protein (GFP)-NFAT4 fusion protein, a two minute exposure to bradykinin induced the translocation of GFP-NFAT4 from the cytoplasm to the nucleus. Translocation was partially inhibited by removal of extracellular Ca2+ and was blocked by inhibition of calcineurin. Furthermore, bradykinin triggered a concentration-dependent increase in NFAT-mediated transcription of a luciferase gene reporter (EC50 = 24.2 ± 0.1 nM). This depended on the B2 receptor, PLC activation and IP3 mediated Ca2+ release. Transcription was not inhibited by capsazepine. Lastly, as indicated by quantitative RT-PCR, bradykinin elicited an increase in cyclooxygenase (cox-2) mRNA. This increase was sensitive to calcineurin and B2 receptor inhibition. These findings suggest a mechanism by which short-lived bradykinin-mediated stimuli can enact lasting changes in nociceptor function and sensitivity.
Tissue damage and inflammation result in the production and release of numerous algesic and pro-inflammatory agents that act to elicit pain or lower the threshold of peripheral nociceptive neurons to painful stimuli. One of these agents, the nonapeptide bradykinin, directly evokes pain, decreases the activation threshold of sensory neurons, and elicits many of the hallmark signs of inflammation, including edema, redness, and local heat (Dray and Perkins, 1993; Marceau and Regoli, 2004).

Bradykinin is produced at the site of tissue injury via cleavage of a kininogen precursor by the protease kallikrein (Dray and Perkins, 1993). Two bradykinin receptors have been identified; a constitutive B₂ and an inducible B₁ receptor. Both receptors are present on the peripheral termini of sensory nerves (Steranka et al., 1988). These receptors couple through heterotrimeric G-proteins to activate phospholipase A₂ (PLA₂) and phospholipase C (PLC). PLC stimulation results in the generation of diacylglycerol and inositol triphosphate (IP₃) and release of Ca²⁺ from the endoplasmic reticulum (ER) (Thayer et al., 1988b). Activation of PLA₂ liberates arachidonic acid leading to the production of prostaglandins by cyclooxygenases and synthesis of 12-hydroperoxy-eicosatetraenoic acid via 12-lipoxygenase (Shin et al., 2002). Downstream targets of these signaling cascades contribute to changes in nociceptor sensitivity.

Bradykinin elicits rapid changes in the response characteristics of sensory neurons. Activation of B₂ receptors decreases the threshold for nociceptor activation by heat and other stimuli by sensitizing transient receptor potential vanilloid receptor (TRPV₁) channels (Chuang et al., 2001; Premkumar and Ahern, 2000). It also inhibits K⁺ conductances in afferent neurons, increasing neuronal excitability (Oh and Weinreich, 2004; Usachev et al., 2002). Bradykinin produces pain hypersensitivity by potentiating
glutamatergic transmission between primary afferents and dorsal horn neurons (Wang et al., 2005).

Tissue damage and inflammation also produce delayed changes in nociceptor function by inducing transcriptional changes. Increased expression of numerous genes including, Substance P, calcitonin gene-related peptide, brain-derived neurotrophic factor (BDNF), growth-associated protein-43, and ion channels Na$_v$1.8, TRPV1 and acid-sensing ion channel (ASIC) are seen in models of inflammation and nerve damage. Further, inflammatory hyperalgesia is mediated in part by phenotypic switches in the sensory modality of DRG neurons that requires changes in gene expression (Woolf and Costigan, 1999).

We were interested in the idea that bradykinin, by regulating [Ca$^{2+}$]$_i$, stimulates changes in gene expression that may underlie long-term changes in the response characteristics of sensory neurons. We hypothesized that bradykinin-induced Ca$^{2+}$ increases would activate members of the NFAT family of transcription factors.

NFAT activation of gene transcription is regulated by Ca$^{2+}$ (Dolmetsch et al., 1998). In un-stimulated cells, NFAT is phosphorylated and restricted to the cytoplasm. Following an increase in [Ca$^{2+}$]$_i$, cytoplasmic NFAT is dephosphorylated by the Ca$^{2+}$-dependent serine/threonine phosphatase, calcineurin (Beals et al., 1997). This exposes a nuclear localization signal, allowing NFAT to translocate to the nucleus and initiate transcription through interaction with other transcription factors. Four isoforms of NFAT (NFAT1-4) transcription factors have been identified, several of which have been localized to neuronal tissue (Graef et al., 2003; Groth and Mermelstein, 2003), including DRG neurons (Kim et al., 2006). Increases in [Ca$^{2+}$]$_i$ and activation of NFAT-mediated
transcription have been implicated in the maintenance of pain in response to Substance P in spinal neurons (Seybold et al., 2006).

Here we demonstrate that bradykinin-evoked Ca\textsuperscript{2+} release from the ER of rat DRG neurons initiated the translocation of NFAT to the nucleus and subsequent activation of NFAT-dependent transcription. We also demonstrate that bradykinin increases the level of mRNA for the pro-inflammatory enzyme Cox-2 in a calcineurin-dependent manner. These data suggest that in sensory neurons NFAT conveys pro-inflammatory signals to the nucleus to initiate long-term changes in sensory neuron function.
Methods

Cell culture.

Rat DRG neurons were grown in primary culture as described previously (Thayer et al., 1988b). In brief, 1- to 3-day-old Sprague-Dawley rats were killed by decapitation with sharp scissors under a protocol approved by the University of Minnesota Institutional Animal Care and Use Committee. Ganglia were dissected from the thoracic and lumbar regions, incubated at 37°C in collagenase–dispase (V. alginolyticus/B. Polymyxa; 0.8 and 6.4 U/ml, respectively; Roche Diagnostics, Indianapolis, IN) for 45 min, and dissociated by trituration through a flame-constricted Pasteur pipette. Non-neuronal cells attach more readily to substrate than do neuronal cells (Seybold et al., 2006). We plated aliquots of dissociated cells onto HNO3 washed glass coverslips for 1 hr and then replated the unattached neurons onto laminin-coated (50 mg/ml) glass coverslips (25 mm diameter). Cells were grown in Ham’s F12 medium supplemented with 5% heat-inactivated horse serum, 5% fetal bovine serum, 50 ng/ml NGF-7S (mouse submaxillary gland; Sigma), 4.4 mM glucose, 2 mM L-glutamine, modified Eagle’s medium vitamins, and penicillin–streptomycin (100 U/ml and 100 mg/ml, respectively). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO2. As neurotrophins activate NFAT-dependent transcription, serum and NGF were replaced with 1% B27 supplement (Invitrogen) 24 hrs prior to imaging experiments to minimize background NFAT activity (Groth and Mermelstein, 2003). Cells were used on the third and fourth day in vitro.

\[Ca^{2+}\] measurement.
[Ca\(^{2+}\)]\(_i\) was determined with the Ca\(^{2+}\) sensitive fluorescent dye fura-2 (Grynkiewicz et al., 1985). Cells were loaded with indicator by incubation with 5 µM fura-2 acetoxymethyl ester for 45 minutes at 37°C, in HEPES-buffered Hank’s salt solution (HHSS), pH 7.45, containing 0.5% bovine serum albumin. HHSS was composed of the following (in mM): HEPES, 20; NaCl, 137; CaCl\(_2\), 1.3; MgSO\(_4\), 0.4; MgCl\(_2\), 0.5; KCl, 5.4, KH\(_2\)PO\(_4\), 0.4; Na\(_2\)HPO\(_4\), 0.3; NaHCO\(_3\), 3.0; glucose, 5.6.

Coverslips with loaded cells were mounted in a flow-through chamber for viewing (Thayer et al., 1988b) (10 s solution exchange) that was placed on the stage of an inverted Olympus IX70 microscope (Olympus Optical, Tokyo, Japan) equipped with a 40x objective (UApo/340, NA=1.35). Cells were superfused with HHSS at a rate of 1.0-1.5 ml/min for 10 minutes prior to starting an experiment. Fura-2-based digital imaging was performed using an Optoscan monochromater (Cairn Research LTD, Faversham, Kent, UK) rapidly switching excitation between 340 nm (8 nm slit width) and 380 (8) nm.

Emission was detected at 517 (30) nm with a Cascade cooled CCD camera (Roper, Tucson, AZ, USA). Images were acquired and analyzed using Metafluor software (Molecular Devices, Sunnyvale CA, USA).

Fluorescence changes were converted to [Ca\(^{2+}\)]\(_i\) by using the formula [Ca\(^{2+}\)]\(_i\) = \(K_d \beta (R - R_{min})/(R_{max} - R)\), where R is 340/380 nm fluorescence ratio (Grynkiewicz et al., 1985). The dissociation constant (K\(_d\)) for fura-2 was 140 nM and \(\beta\) was the ratio of fluorescence emitted at 380 nm measured in the absence and presence of Ca\(^{2+}\). R\(_{min}\), R\(_{max}\), and \(\beta\) were determined by bathing intact cells in 2 µM ionomycin in Ca\(^{2+}\)-free buffer (1 mM EGTA) and saturating Ca\(^{2+}\) (5 mM Ca\(^{2+}\)). Values for R\(_{min}\), R\(_{max}\), and \(\beta\) were 0.237, 4.10, and 6.52, respectively.
Transfection.

Gene transfer into DRG neurons was performed as described previously (Usachev et al., 2000). Briefly, plasmid DNA was precipitated onto gold particles (1.6 µm) and introduced into DRG neurons using a biolistic particle delivery system (PDS-1000, Biorad, Hercules, CA, USA).

Dual-luciferase-based gene reporter assays.

DRG neurons were transferred to serum-free Ham’s F12 medium supplemented with 1% B27 (Invitrogen) 24 hrs post plating. Five hours following serum removal, DRG neurons were co-transfected with plasmids encoding a luciferase (firefly) based reporter of NFAT activity (pNFAT-luciferase; Graef et al., 1999) and a constitutively active Renilla luciferase under the control of a TK promoter (pRL-TK; Promega, Madison, WI) at a ratio of 5:1. The neurons were stimulated 2 hrs post-transfection and returned to culture medium for an additional 16-18 hours. Neurons from a single coverslip were lysed and the activity of each reporter measured on a TD 20/20 luminometer (Turner Biosystems, Sunnyvale, CA) using the Dual-Luciferase® Reporter Assay (Promega). The expression of firefly luciferase was normalized to constitutively expressed Renilla luciferase activity to correct for differences in transfection efficiency. Within each experiment, treatments were conducted in duplicate to triplicate. Each experiment was conducted at least three times on cultures prepared from separate litters.
Simultaneous confocal imaging of $[Ca^{2+}]$, and GFP-NFAT4.

DRG cultures were transfected with a plasmid encoding GFP-NFAT4 fusion protein (Tomida et al., 2003). Neurons were transferred to serum-free Ham’s F12 medium supplemented with 1% B27 (Invitrogen) 24 hrs post plating. 48 hours post transfection cultures were loaded with X-Rhod-1 AM (2 µM; room temperature; 45 min). Cells were rinsed in HHSS and the indicator allowed to de-esterify for 15 min prior to the start of the experiment. GFP and $[Ca^{2+}]$ imaging were performed on a Fluoview 300 laser scanning confocal microscope attached to an inverted microscope (Olympus IX70) equipped with a PlanApo 60x objective (NA 1.40) (Olympus Optical, Tokyo, Japan). GFP-NFAT4 and X-rhod-1 were excited with the 488 nm (Argon) and 540 nm (He-Ne) laser lines and the fluorescence imaged at 510-540 and $>605$ nm, respectively (Jackson and Thayer, 2006).

Quantitative Real-time PCR.

DRG neurons were prepared using the pre-plating protocol. Medium was replaced with serum-free Ham’s F12 medium supplemented with 1% B27 two hours after plating. On day 2 in vitro, growth medium was removed and neurons stimulated by application of bradykinin (1 µM; 2 min). Stimulation was terminated by removal of bradykinin and return of growth medium. Total RNA was isolated from DRG neurons using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA) seven hours after bradykinin stimulation and further purified by DNase digestion. RNA was reverse transcribed into cDNA using a QuantiTect RT-PCR kit (Qiagen). Real-Time PCR was performed using Sybr Green Master Mix (Applied Biosystems, Foster City, CA, USA) on an Applied Biosystems 7300 Real-Time PCR System. Samples were run through 40 cycles (95°C for 10 sec, 58°C for
33 sec, 72°C for 33 sec). Each cDNA sample was run in duplicate for the target (e.g. Cox-2) and the normalizing gene (S15). Experiments were repeated at least three times using RNA collected from at least three separate neuronal platings. Amplicon specificity was determined by melting curve analysis and gel electrophoresis. Primer pair sequences were validated previously (Groth et al., In Press) and are as follows: BDNF (GenBank Accession number NM_007540) Forward primer 5’-CCA TAA AGG ACG CGG ACT TGT ACA-3’ and reverse primer 3’-AGA CAT GTT TGC GGC ATC CAG-3’; COX-2 (S67722) forward primer 5’-GCT GCT GCC GGA CAC CTT CA-3’ and reverse primer 5’-AGC AAC CCG GCC AGC AAT CT-3’; and S15 (BC094409) forward primer 5’-CCG AAG TGG AGC AGA AGA AG-3’ and reverse primer 5’-CTC CAC CTG GTT GAA GGT C-3’. All primers were synthesized by Integrated DNA Technologies (Coralville IA, USA). Cycle thresholds (Ct) were calculated automatically using the Applied Biosystems software to minimize user bias. Fold increases in mRNA expression for each sample were calculated using the Pfaffl correction of the Livak method in which Ct values of target genes are normalized to that of S15 (Pfaffl, 2001) using the following equation: 

\[
\text{Fold increase} = \left(\frac{E_{\text{target}}}{E_{S15}}\right)^{\Delta \text{Ct S15(control-treated)}}
\]

where E is the efficiency of the primer. To determine individual primer efficiencies we serially diluted cDNA samples and calculated Ct values for each dilution. Ct values were plotted against the log [cDNA] and the efficiency calculated from the formula \(E = 10^{(-1/\text{slope})}\).
Results

**Bradykinin activates B$_2$ receptors to mobilize IP$_3$-sensitive Ca$^{2+}$ stores in DRG neurons**

We monitored changes in [Ca$^{2+}$]$_i$ in a field of DRG neurons grown in primary culture using fura-2 based digital [Ca$^{2+}$]$_i$ imaging. The resting [Ca$^{2+}$]$_i$ prior to bradykinin exposure was 71 ± 1 nM (n = 1176 neurons). Application of bradykinin (2 min) by superfusion elicited concentration-dependent increases in [Ca$^{2+}$]$_i$ (Fig 1A). As only a subset of DRG neurons express receptors for bradykinin (Thayer et al., 1988a), we separated non-responders from weak responders by maximally stimulating the cells with 1 µM bradykinin 20 minutes after the application of the test concentration. Neurons that failed to display a net increase in [Ca$^{2+}$]$_i$ of at least 25 nM in response to this second application of bradykinin were considered non-responders and not included in further analysis. Fitting of the bradykinin concentration response data with a logistic equation determined a 50% effective concentration (EC$_{50}$) of 6.0 ± 0.3 nM. This concentration response profile is similar to those seen in DRG neurons and other cell types (Lo and Thayer, 1993).

Bradykinin (10 nM; 2 min) evoked reproducible increases in [Ca$^{2+}$]$_i$ from the same DRG neuron (Fig. 1B). The amplitude of the first [Ca$^{2+}$]$_i$ response to 10 nM bradykinin was 84 ± 5 nM. The amplitude of the second bradykinin-evoked response (Peak 2) was 86 ± 6 % (n = 169 neurons) of the first [Ca$^{2+}$]$_i$ transient (Peak 1), indicating that a 2 min exposure to 10 nM bradykinin did not appreciably desensitize its receptor.
and that sufficient time elapsed to allow refilling of the Ca$^{2+}$ stores. As individual DRG neurons vary greatly in the amplitude of their bradykinin-induced Ca$^{2+}$ responses, we normalized the amplitude of the second response to that of the first response (Peak 2/Peak 1). We used this as an assay to test the regulation of bradykinin-induced Ca$^{2+}$ signals.

Bradykinin acts through either of two G-protein coupled receptors (B$_1$ or B$_2$) present in sensory neurons. The response to bradykinin was abolished by application of the B$_2$ receptor antagonist HOE140 (1 µM; n = 9 neurons), indicating that B$_1$ receptor activation is unlikely to contribute to the responses we observed (Fig. 1C). B$_2$ receptors couple to several signaling cascades. To assess the contribution of extracellular Ca$^{2+}$ to the bradykinin response, we applied Ca$^{2+}$-free media (20 µM EGTA) to the cells one minute prior to and throughout application of bradykinin. Removal of extracellular Ca$^{2+}$ decreased the amplitude of the [Ca$^{2+}$]$_i$ response by 35% relative to control (n = 46 neurons; p < 0.01), suggesting that intracellular Ca$^{2+}$ release was sufficient to account for much of the bradykinin-induced Ca$^{2+}$ signal (Fig. 1D). Because removal of extracellular [Ca$^{2+}$] only partially inhibited the Ca$^{2+}$ signal, we next tested the contribution of the intracellular Ca$^{2+}$ stores to the bradykinin-induced [Ca$^{2+}$]$_i$ increase using the sarcoplasmic-endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) inhibitor cyclopiazonic acid (CPA; 5 µM). Inhibition of ER Ca$^{2+}$ pumps depleted the ER Ca$^{2+}$ stores, and decreased the amplitude of [Ca$^{2+}$]$_i$ transients evoked by bradykinin by 56% (n = 66 neurons; p < 0.01).

Bradykinin is known to evoke IP$_3$-mediated Ca$^{2+}$ release in neurons. To verify that this signaling pathway was required for BK-induced Ca$^{2+}$ responses, we treated DRG
neurons with the phospholipase C inhibitor U-73122. U-73122 decreased the amplitude of the second Ca\(^{2+}\) transient by 86% (n = 19; p < 0.01). However, this response was not inhibited by its inactive analog U-73343 (1 µM; Peak 2 / Peak 1 = 0.78 ± 0.13; n = 15), demonstrating the specificity of the U-73122-induced block. DRG neurons also possess ryanodine-sensitive Ca\(^{2+}\) stores (Shmigol et al., 1995). We tested the contribution of these stores to the bradykinin-induced \([Ca^{2+}]_i\) transients by application of ryanodine. Ryanodine (1 µM) failed to inhibit the response (Peak 2 / Peak 1 = 0.91 ± 0.08; n = 36).

The immunosuppressant cyclosporine A interacts with multiple proteins involved in Ca\(^{2+}\) signaling including calcineurin, immunophilins, and the mitochondrial permeability transition pore (Snyder et al., 1998). These interactions can exert complex effects on Ca\(^{2+}\) signals. However, bradykinin-evoked \([Ca^{2+}]_i\) transients were not affected by application of cyclosporin A (10 µM; Peak 2 / Peak 1 = 0.89 ± 0.17; n = 14).

Bradykinin decreases the temperature threshold for vanilloid receptor activation and increases the membrane current activated by heat (Cesare et al., 1999). Activation of protein kinase C by bradykinin or phorbol esters, although controversial (Bhave et al., 2003), has been proposed to directly evoke TRPV1 dependent currents and Ca\(^{2+}\) increases (Premkumar and Ahern, 2000), an effect suppressed by the competitive TRPV1 antagonist capsazepine. We tested whether \([Ca^{2+}]_i\) transients evoked by bradykinin were due to influx through TRPV1 channels by applying the TRPV1 competitive antagonist capsazepine. Capsazepine (1 µM) did not affect the \([Ca^{2+}]_i\) response (Peak 2 / Peak 1 = 0.79 ± 0.1; n = 30), suggesting that TRPV1-mediated Ca\(^{2+}\) influx did not contribute to the bradykinin-evoked \([Ca^{2+}]_i\) increase.
Bradykinin triggers translocation of NFAT

In addition to generating acute pain, bradykinin elicits long lasting changes in the sensitivity of nociceptors to subsequent painful stimuli (Woolf and Costigan, 1999). Here we tested the hypothesis that bradykinin-evoked \([\text{Ca}^{2+}]_i\) increases triggered the activation and translocation of the transcription factor NFAT. DRG neurons were transfected with a plasmid encoding enhanced green fluorescent protein fused to the N terminus of NFAT4 (GFP-NFAT4) using a gene gun (Tomida et al., 2003). Previous work demonstrated that this method preferentially transfects sensory neurons without impairing physiological function (Usachev et al., 2000).

NFAT localization and activity is controlled by its phosphorylation state (Crabtree and Olson, 2002; Groth and Mermelstein, 2003). NFAT is dephosphorylated by the phosphatase calcineurin and subsequently translocates to the nucleus where it interacts with other transcription factors to initiate transcription. We quantified NFAT translocation by measuring the mean GFP fluorescence from regions in the cytoplasm and nucleus and calculating the nuclear-to-cytosolic ratio (GFP-NFAT4\(_{\text{n/c}}\)). \([\text{Ca}^{2+}]_i\) was monitored simultaneously using the fluorescent \(\text{Ca}^{2+}\) indicator x-rhod-1 AM (Kd = 700 nM). Resting GFP-NFAT4\(_{\text{n/c}}\) was 0.29 ± 0.01 (n=116 neurons). Application of bradykinin (1 \(\mu\)M) elicited an increase in \([\text{Ca}^{2+}]_i\) (\(\Delta F/F_0 = 0.8 \pm 0.1; \ n = 17\)) and a corresponding translocation of GFP-NFAT4 into the nucleus (GFP-NFAT4\(_{\text{n/c}}\) = 0.9 ± 0.2; (Fig. 2A & B). The \([\text{Ca}^{2+}]_i\) elevation was rapid and transient, comparable in waveform to that recorded with fura-2 (Fig 1B), whereas the translocation developed slowly, reaching a maxima over the course of 20-30 minutes. GFP-NFAT4 continued to accumulate in the nucleus even after \([\text{Ca}^{2+}]_i\) had recovered to basal levels. Thus a
transient bradykinin-elicited [Ca\(^{2+}\)]\(_{i}\) increase can trigger long-lasting changes in cell signaling. Depolarization-induced activation of voltage-gated Ca\(^{2+}\) channels is a powerful activator of NFAT in hippocampal neurons (Graef et al., 1999). Depolarization of DRG neurons with 90 mM K\(^+\), evoked a large increase in [Ca\(^{2+}\)]\(_{i}\) (\(\Delta F/F_0 = 1.58 \pm 0.21; n = 21\)) that elicited robust translocation of GFP-NFAT4 (GFP-NFAT4\(_{i/c}\) = 1.4 \pm 0.2; Fig 2C). Pretreatment with the calcineurin inhibitor CSA (10 \(\mu\)M; 10 min) blocked NFAT translocation (GFP-NFAT4\(_{i/c}\) = 0.22 \pm 0.06; n = 4), but did not appear to inhibit the bradykinin-induced [Ca\(^{2+}\)]\(_{i}\) increase (\(\Delta F/F_0 = 0.85 \pm 0.15;\) Fig. 3A and Fig 1D), consistent with translocation requiring NFAT dephosphorylation. GFP-NFAT4 translocation was triggered by Ca\(^{2+}\) release from the ER evoked by 5 \(\mu\)M CPA (GFP-NFAT4\(_{i/c}\) = 2.4 \pm 0.8; \(\Delta F/F_0 = 0.9 \pm 0.35; n = 4;\) Fig 3B). To verify that the bradykinin-mediated NFAT translocation was dependent upon Ca\(^{2+}\) release, we applied bradykinin (1 \(\mu\)M) in Ca\(^{2+}\)-free solution (+20 \(\mu\)M EGTA). In the absence of extracellular Ca\(^{2+}\), bradykinin still elicited a [Ca\(^{2+}\)]\(_{i}\) increase (\(\Delta F/F_0 = 0.73 \pm 0.22; n = 4\)) and a corresponding translocation of NFAT to the nucleus (GFP-NFAT4\(_{i/c}\) = 0.4 \pm 0.1; Fig. 3C), although to a lesser degree than in normal Ca\(^{2+}\) buffer (Fig. 2B). These data suggest that Ca\(^{2+}\) mobilization from the ER and Ca\(^{2+}\) influx contribute to the bradykinin-induced translocation of NFAT.

**Bradykinin evokes NFAT-dependent transcription**

Bradykinin-induced translocation of GFP-NFAT4 indicates that bradykinin activates NFAT-dependent transcription. To determine whether bradykinin activates endogenous NFAT in DRG neurons, we transfected DRG cultures with a NFAT-
luciferase (firefly) expression reporter (Graef et al., 1999). Firefly luciferase activity (relative light units; RLU\textsubscript{Firefly}) was normalized to co-transfected, constitutively expressed renilla luciferase activity (RLU\textsubscript{Renilla}). In un-stimulated neurons, the resting ratio was 0.22 ± 0.02 (n = 15). Stimulation with bradykinin caused a concentration-dependent increase in NFAT-dependent transcription. Fitting of the concentration response data with a logistic equation yielded an EC\textsubscript{50} of 24.2 ± 0.1 nM (Fig. 4A). A 1 µM concentration of bradykinin maximally stimulated NFAT-dependent transcription (RLU\textsubscript{Firefly} / RLU\textsubscript{Renilla} = 0.40 ± 0.04; n = 15; p < 0.05; Fig 4B). Values were comparable to those evoked by a 2 min depolarization in 90 mM K\textsuperscript{+} (RLU\textsubscript{Firefly} / RLU\textsubscript{Renilla} = 0.38 ± 0.03; n = 15; p < 0.05). Mobilization of Ca\textsuperscript{2+} stores with CPA produced a smaller increase in NFAT-mediated transcription (RLU\textsubscript{Firefly} / RLU\textsubscript{Renilla} = 0.29 ± 0.08; n = 3). As shown in Figure 4C, pretreatment with CSA (10 µM; 15 min) abolished bradykinin-induced NFAT-dependent transcription (0.20 ± 0.02; p < 0.05; n = 6), consistent with the inhibition of NFAT translocation by this drug (Fig. 3A). This effect was not due to changes in IP\textsubscript{3}-mediated [Ca\textsuperscript{2+}]\textsubscript{i} increases as CSA failed to alter the amplitude of bradykinin-induced [Ca\textsuperscript{2+}]\textsubscript{i} transients (Fig 1D and Fig 3A).

Bradykinin may act through several different pathways. To further elucidate the mechanism of bradykinin-induced NFAT-dependent transcription, we tested several of these possibilities. Stimulation of NFAT-dependent transcription by bradykinin was inhibited by the selective B\textsubscript{2} receptor antagonist HOE 140 (1µM; RLU\textsubscript{Firefly} / RLU\textsubscript{Renilla} = 0.21 ± 0.02; p < 0.05; n = 3; Fig 4C), consistent with the complete block of the bradykinin-induced [Ca\textsuperscript{2+}]\textsubscript{i} increase produced by this drug (Fig. 1C & D). Bradykinin-elicted transcription was blocked by the PLC antagonist U-73122 (1 µM; RLU\textsubscript{Firefly} /
RLU_{Renilla} = 0.31 \pm 0.03; n = 9; p < 0.05). The specificity of this inhibition was demonstrated by the failure of its inactive analog U-73343 (1 \mu M; RLU_{Firefly} / RLU_{Renilla} = 0.43 \pm 0.05; n = 9) to affect luciferase expression. While these data imply that IP3-mediated [Ca^{2+}]_{i} release was necessary, we tested this explicitly using the IP3 receptor antagonist xestospongin C. Pretreatment (15 min) with xestospongin C (500 nM) completely inhibited the bradykinin-mediated increase in luciferase activity (500 nM; RLU_{Firefly} / RLU_{Renilla} = 0.21 \pm 0.03; n = 5; p < 0.05). This inhibition was not mimicked by blockade of caffeine-sensitive Ca^{2+} stores by application of ryanodine (1 \mu M; RLU_{Firefly} / RLU_{Renilla} = 0.45 \pm 0.1; n = 5). Bradykinin sensitizes the TRPV1 receptor to heat (Chuang et al., 2001). However, bradykinin-mediated increases in NFAT-dependent transcription were not antagonized by pretreatment (1 \mu M; 15 min) with capsazepine (RLU_{Firefly} / RLU_{Renilla} = 0.43 \pm 0.1; n = 3).

**Bradykinin increases transcription of Cox-2 mRNA**

Bradykinin is produced at sites of tissue injury (Barlas et al., 1985) and increases in bradykinin concentration have been noted at sites of inflammation (Hargreaves et al., 1988). We tested whether bradykinin activation of NFAT increased the expression of BDNF and Cox-2 using quantitative real-time PCR. Both genes have NFAT-binding sites in their promoter regions (Groth and Mermelstein, 2003; Iniguez et al., 2000) and both proteins are implicated in inflammatory pain (Svensson and Yaksh, 2002; Woolf and Costigan, 1999). Bradykinin (1 \mu M; 2 min) was applied to cultured rat DRG neurons and RNA harvested 7 hrs post-stimulation. Bradykinin produced a 2.0 \pm 0.43 (n = 5) fold increase in Cox-2 mRNA relative to sham treated controls. To determine whether this
response depended on calcineurin activation, we pretreated neurons with CSA (10 µM) 15 min prior to and during bradykinin exposure. RNA was harvested 7 hours after stimulation with bradykinin. Pretreatment with CSA not only blocked the bradykinin-mediated increase in Cox-2 mRNA, but reduced Cox-2 mRNA to below control levels (0.51 ± 0.23, n = 3), suggesting tonic NFAT-mediated transcription of Cox-2. Bradykinin-induced Cox-2 mRNA expression was attenuated by pretreatment with HOE140 (1 µM; 1.18 ± 0.19; n = 3), suggesting that it depends on the selective activation of the bradykinin B2 receptor. Activation of NFAT increases the transcription of BDNF in hippocampal neurons (Groth and Mermelstein, 2003). However, we did not detect any significant changes in BDNF mRNA (0.7 ± 0.3 fold change; n=3) in DRG neurons when challenged with bradykinin.

Discussion

Application or injection of bradykinin directly elicits pain and sensitizes DRG neurons to painful stimuli (Dray and Perkins, 1993). We describe for the first time, a pathway linking bradykinin-induced mobilization of Ca²⁺ stores to translocation of NFAT, initiation of transcription, and subsequent increases in mRNA for the pro-inflammatory enzyme cyclooxygenase-2. This mechanism may provide a pathway by which bradykinin may contribute to long lasting changes in the sensitivity of sensory neurons to painful stimuli.

Bradykinin-induced regulation of Ca²⁺ release and NFAT activation
A subset of DRG neurons expresses receptors for bradykinin (Cesare et al., 1999; Thayer et al., 1988a). These receptors are preferentially found on small-sized DRG neurons involved in nociception (Dray and Perkins, 1993), suggesting that the gene expression studies described in Figures 4 and 5 likely underestimate the magnitude of the bradykinin-evoked responses because only approximately 30% of the DRG neurons express the appropriate receptor. Bradykinin stimulation increases [Ca\textsuperscript{2+}]\textsubscript{i} via Ca\textsuperscript{2+} release from the ER and influx across the plasma membrane. Our results build upon the previously established model where bradykinin activation of B\textsubscript{2} receptors triggers the cleavage of phosphatidyl 4,5 bisphosphate by PLC to diacylglycerol and IP\textsubscript{3} with the subsequent release of Ca\textsuperscript{2+} from intracellular stores (Dray and Perkins, 1993; Thayer et al., 1988a). In our neuronal cultures, we showed that bradykinin was capable of eliciting Ca\textsuperscript{2+} release from the ER in a manner that was attenuated by PLC inhibition with U-73122 and by depletion of the ER with CPA, suggesting that bradykinin triggered Ca\textsuperscript{2+} release from intracellular stores. That neither of these treatments completely blocked bradykinin-induced [Ca\textsuperscript{2+}]\textsubscript{i} transients suggests that influx also contributed to these increases. Further, while our results demonstrate that IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release is sufficient to trigger translocation of NFAT to the nucleus, the extent of translocation in Ca\textsuperscript{2+}-free solution was decreased relative to control (Fig 3B). In contrast, bradykinin-induced NFAT-mediated transcription was completely blocked by xestospongin C, suggesting that Ca\textsuperscript{2+} store mobilization is necessary for bradykinin-triggered NFAT-dependent transcription. Perhaps, xestospongin inhibits capacitative calcium entry secondary to blocking IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release. There is a precedent in neurons for activation of NFAT by other mechanisms. Ca\textsuperscript{2+} entry via L-type Ca\textsuperscript{2+} channels (Graef et
al., 2003) and IP3-mediated Ca$^{2+}$ release (Groth and Mermelstein, 2003) both activate NFAT3 in hippocampal neurons, suggesting that NFAT signaling can be initiated through multiple mechanisms linked to increases in [Ca$^{2+}$].

Addition of growth factors stimulates the activation of NFAT in hippocampal neurons (Groth and Mermelstein, 2003). DRG cultures were maintained in serum-free medium without growth factors to avoid masking changes in NFAT activation subsequent to bradykinin challenge. These conditions likely dampened the responsiveness of DRG neurons to bradykinin as expression of B$_2$ receptors may be regulated by NGF (Lee et al., 2002b).

**NFAT integrates pro-inflammatory signals**

While acute changes in neuronal activity may be accomplished through modulation of pre-existing proteins (e.g. phosphorylation, glycosylation), long-term changes require transcription and additional protein synthesis. NFAT(1-4) proteins are poised to transduce electrical, growth factor, and inflammatory signals to regulate synaptogenesis (Yoshida and Mishina, 2005), axonal outgrowth (Graef et al., 2003) and survival (Benedito et al., 2005). Here we demonstrate that bradykinin drives translocation of NFAT4, a major NFAT isoform in DRG neurons (Kim et al., 2006). We further demonstrate that bradykinin is capable of stimulating NFAT-dependent transcription. In hippocampal and spinal neurons, the growth factors NGF and BDNF initiate NFAT-dependent transcription (Groth and Mermelstein, 2003; Seybold et al., 2006). In spinal neurons, NFAT-dependent transcription is triggered by the algesic agent Substance P. These results suggest that a number of algesic and pro-inflammatory agents
initiate signaling cascades that converge on the NFAT transcription factors. This in turn suggests a role for NFAT in integrating responses to pain and inflammation in sensory neurons as shown here as well as in spinal neurons (Seybold et al., 2006).

Given that all of these growth and inflammatory signals converge on NFAT activation, it remains to be seen to what extent these signals summate. Our data demonstrate that increasing bradykinin concentration increases NFAT-dependent transcription (Fig 4A) suggesting that NFAT acts as an integrator capable of producing graded changes in gene expression in response to these convergent signals, rather than acting as an all-or-none switch. This idea fits with previous studies that showed NFAT-dependent transcription was regulated in part by the presence of transcriptional partners, such as AP-1 (Groth and Mermelstein, 2003). It is also consistent with the analgesic properties of cyclosporine (Lee et al., 2002a). Further, we noted a 4-fold rightward shift in the concentration response profile of bradykinin-induced NFAT-dependent transcription ($EC_{50} = 24.2 \pm 0.1$) relative to the that of bradykinin-evoked $[Ca^{2+}]_i$ increases ($EC_{50} = 6.0 \pm 0.3$). The response profile for bradykinin-evoked $[Ca^{2+}]_i$ increases is in close agreement with previously observed bradykinin-evoked IP$_3$ production (Thayer et al., 1988a). Bradykinin B$_2$ receptor activation of PLC increases both IP$_3$ and diacylglycerol. Increased concentrations of bradykinin may be required to activate transcription factors downstream of diacylglycerol production and PKC activation that partner with NFAT to initiate transcription.

**Bradykinin and Prostaglandins in Peripheral Sensitization**
The concentration of bradykinin increases during acute (surgery) and chronic (rheumatoid arthritis) inflammation (Hargreaves et al., 1988). Many of the pro-inflammatory effects of bradykinin in peripheral neurons have been linked to the synthesis and release of prostanoids including PGE$_2$ and PGI$_2$ that are known to sensitize nociceptors (Dray and Perkins, 1993). The rate-limiting step in prostaglandin synthesis is the conversion of arachidonic acid to prostaglandin H$_2$, catalyzed by cycloxygenase enzymes (cox-1 and cox-2). While cox-1 is constitutively expressed in many tissues, cox-2 expression is increased in response to tissue injury and inflammation. The role for cyclooxygenase (and prostanoid) involvement in pain and inflammation is supported by the analgesic properties of non-steroidal anti-inflammatory drugs (NSAIDS). Activation of nociceptors by bradykinin is attenuated by pretreatment with NSAIDS (Dray et al., 1992), further supporting a link between cyclooxygenase activity and bradykinin.

The mechanisms coupling bradykinin exposure to long-term elevation of prostanoids have received little attention. Cox-2 expression in other tissues is regulated by several Ca$^{2+}$-activated transcription factors, including CREB and NF-κB (Svensson and Yaksh, 2002). Cox-2 expression, driven by calcineurin and NFAT signaling, has been demonstrated in vascular smooth muscle (Robida et al., 2000), spinal neurons (Groth et al., In Press) and T lymphocytes (Iniguez et al., 2000). Our results demonstrate that bradykinin can elicit B$_2$ receptor and calcineurin-dependent increases in cox-2 mRNA in sensory neurons. While these results do not explicitly address whether bradykinin induced cox-2 expression is involved in chronic pain syndromes in vivo, they do suggest a potential mechanism whereby short-lived stimuli may contribute to long-term sensitization produced by inflammation.
Conclusions

Bradykinin activated the transcription factor NFAT in DRG neurons. This activation depended on B2 receptor activation of phospholipase C and subsequent Ca\textsuperscript{2+} release to trigger calcineurin-dependent NFAT translocation to the nucleus and subsequent NFAT-dependent transcription. Further, our data demonstrate that activation of this cascade leads to increases in mRNA for the pro-inflammatory enzyme cyclooxygenase-2. This suggests a potential mechanism by which bradykinin may elicit long lasting changes in the sensitivity of sensory neurons to painful stimuli.
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Footnotes

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Correspondence: S. A. Thayer, Dept. of Pharmacology, University of Minnesota Medical School, 6-120 Jackson Hall, 321 Church St., S. E., Minneapolis, MN 55455

voice: 612-626-7049; fax: 612-625-8408; e-mail: sathayer@umn.edu
Figure Legends

Figure 1. Bradykinin elicited [Ca\(^{2+}\)]\(_i\) increases in rat DRG neurons in culture.

[Ca\(^{2+}\)]\(_i\) was measured in a field of DRG neurons in culture using fura-2-based digital [Ca\(^{2+}\)] imaging as described in Methods. Drugs were applied by superfusion at the times indicated by the horizontal bars.

A. Bradykinin elicited a concentration dependent increase in [Ca\(^{2+}\)]\(_i\) in rat DRG neurons. Data points represent at least three experiments and are expressed as mean ± S.E.M. Curves were fit by a logistic equation of the form \(\Delta [Ca^{2+}] = Ca^{2+}_{max} / (1 + 10^{(logEC_{50} - X)})\), where X is the logarithm of the bradykinin concentration, \(Ca^{2+}_{max}\) is the baseline-corrected peak [Ca\(^{2+}\)], and the Hill coefficient = 1.

B. Bradykinin (BK; 10 nM; 2 min application) elicited [Ca\(^{2+}\)]\(_i\) increases of reproducible amplitude. Bradykinin was applied at 20 minute intervals as indicated.

C. The bradykinin-induced [Ca\(^{2+}\)]\(_i\) increase was blocked by the B\(_2\)-selective antagonist HOE140 (1 \(\mu\)M).

D. Bar chart depicts the effect of inhibitors using the testing paradigm depicted in C. Concentrations of inhibitors are as follows: HOE140, 1 \(\mu\)M (n = 9); Cyclosporin A, 1 \(\mu\)M (n = 14); U-73122, 1 \(\mu\)M (n = 19); U-73343, 1 \(\mu\)M (n = 15); 0 Ca\(^{2+}\) (Ca\(^{2+}\)-Free medium + 20 \(\mu\)M EGTA; n = 46 neurons); Capsazepine, 1 \(\mu\)M (n = 30); Ryanodine, 1 \(\mu\)M (n = 19); Cyclopiazonic acid, 5 \(\mu\)M (n = 66). Each experiment was performed on at least three fields of cells; n values represent the number of cells. Data are presented as mean ± S.E.M. and significance was determined by analysis of variance using Dunnett’s post hoc test. **p < 0.01 relative to control.
Figure 2. Bradykinin triggers translocation of GFP-NFAT4 coincident with [Ca\textsuperscript{2+}]\textsubscript{i} increases.

[Ca\textsuperscript{2+}]\textsubscript{i} and GFP-NFAT4 were simultaneously imaged in single, transfected DRG neurons in culture using the argon and HeNe laser lines of a confocal microscope to excite the GFP-NFAT4 and the Ca\textsuperscript{2+} indicator X-Rhod-1. Drugs were applied by superfusion at the times indicated by the horizontal bars.

A. Representative images depict translocation of GFP-NFAT4 from the cytosol to the nucleus. GFP-NFAT4 was localized primarily to the cytosol in un-stimulated neurons. Images were acquired at times indicated in B.

B. Application of 1 \textmu M bradykinin (BK; 2 min) elicited a transient increase in [Ca\textsuperscript{2+}]\textsubscript{i} (red) and increased the proportion of GFP-NFAT4 residing in the nucleus (NFAT (nuclear/cytoplasmic); black). The proportion of GFP-NFAT4 residing in the nucleus was quantified as a ratio of the mean GFP fluorescence from the nucleus relative to the cytoplasm. Changes in X-Rhod-1 (red) fluorescence (\Delta F) were normalized to its initial fluorescence intensity (F\textsubscript{0}) from a region encompassing the DRG cell body.

C. Depolarization (90 mM K\textsuperscript{+}; 2 min) elicited an increase in [Ca\textsuperscript{2+}]\textsubscript{i} (red) and increased the proportion of GFP-NFAT4 residing in the nucleus (NFAT (nuclear/cytoplasmic); black).

Figure 3. Bradykinin-induced GFP-NFAT4 translocation depends on Ca\textsuperscript{2+} release and calcineurin activation.

[Ca\textsuperscript{2+}]\textsubscript{i} and GFP-NFAT4 were simultaneously imaged in single, transfected DRG neurons in culture using the argon and HeNe laser lines of a confocal microscope to excite the
GFP-NFAT4 and the Ca^{2+} indicator X-Rhod-1. Drugs were applied by superfusion at the times indicated by the horizontal bars.

A. Representative trace depicts the effects of calcineurin inhibition (1 µM CSA) on GFP-NFAT4 translocation. CSA inhibited GFP-NFAT4 translocation without altering the amplitude of the [Ca^{2+}]_{i} transient (n = 4). One of four bradykinin-induced [Ca^{2+}] responses in CSA exhibited oscillations, a response also seen in untreated cells (n = 2) that showed robust GFP-NFAT4 translocation (GFP-NFAT4(n/c) = 1.1).

B. Representative trace depicts the effect of store mobilization on GFP-NFAT4 localization. Blocking SERCA with CPA (5 µM; 30 min) elicited a [Ca^{2+}]_{i} increase that caused GFP-NFAT4 translocation (n = 4).

C. Ca^{2+}-mediated GFP-NFAT4 translocation occurs in the absence of Ca^{2+} influx. Representative trace shows bradykinin-evoked (1 µM) [Ca^{2+}]_{i} increases and GFP-NFAT4 translocation in Ca^{2+}-free medium (20 µM EGTA; n = 4).

Figure 4. Bradykinin stimulates NFAT-dependent transcription.

Luciferase activity, expressed as the ratio of firefly to Renilla luminescence was measured in a population of transfected DRG neurons as described in the Methods. Data are presented as mean ± S.E.M. Significance was determined by Student’s t-test.

A. Bradykinin elicited concentration-dependent increases in NFAT-dependent transcription. Data points represent four separate experiments from different neuronal platings for which full concentration responses were run in parallel. Curves were fit by a logistic equation of the form RLU_{Firefly} / RLU_{Renilla} = R_{min} + [(R_{max} - R_{min}) / (1 + 10^{(LogEC_{50} - X)})], where X is the logarithm of the bradykinin concentration, R_{min} is the...
RLU\textsubscript{Firefly} / RLU\textsubscript{Renilla} in unstimulated cells, R\textsubscript{max} is RLU\textsubscript{Firefly} / RLU\textsubscript{Renilla} following 1 µM bradykinin, and the Hill coefficient = 1. *p < 0.05 vs control.

B. Activation of NFAT-dependent transcription is not Ca\textsuperscript{2+}-source specific. Bradykinin (1 µM BK; 2 min n = 15), depolarization (90 mM K\textsuperscript{+}; 2 min; n = 15), and CPA (5 µM; 2 min; n = 3) all elicit NFAT-dependent transcription. *p < 0.05 vs control.

C. Bradykinin-induced increases in NFAT-dependent transcription require activation of B\textsubscript{2} receptors, calcineurin, and IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release. Coverslips were pre-incubated (15 min) with either HOE140 (1 µM; n = 3), U-73122 (1 µM; n = 9), U-73343 (1 µM; n = 9), CSA (1 µM; n = 6), capsazepine (CZP; 1 µM; n = 3), ryanodine (1 µM; n = 5), or xestospongin C (xest c; 500 nM; n = 5) before being challenged with bradykinin 100 nM. * p < 0.05 vs control, # p < 0.05 vs bradykinin.

Figure 5. Bradykinin stimulates increased Cox-2 expression. Quantitative, real-time PCR experiments were performed as described in Methods. Bradykinin (1 µM; 2 min) stimulation of cultured DRG neurons increased Cox-2 mRNA (n = 5). This effect was attenuated by pretreatment with CSA (10 µM; 15 min; n = 3) and HOE140 (1 µM; 15 min; n = 3). Changes in gene expression are reported as a ratio of Fold Change relative to untreated control, both normalized to an internal standard (S15). Data are presented as mean ± S.E.M. and significance was determined by Student's t-test. *p < 0.05 vs control; # p < 0.05 vs bradykinin.
Figure 2

A

B

C
Figure 3

(A) NFAT (nuclear/cytosolic) vs. Time (s) with BK stimulation.

(B) NFAT (nuclear/cytosolic) vs. Time (s) with CPA stimulation.

(C) NFAT (nuclear/cytosolic) vs. Time (s) with 0 Ca²⁺ stimulation.