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Src family kinases mediate the inhibition of substance P release in the rat spinal cord by μ-opioid receptors and $GABA_B$ receptors, but not α_2 adrenergic receptors

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Abstract

$GABA_B$, μ-opioid, and adrenergic α_2 receptors inhibit substance P release from primary afferent terminals in the dorsal horn. Studies in cell expression systems suggest that μ-opioid and $GABA_B$ receptors inhibit transmitter release from primary afferents by activating Src family kinases (SFKs), which then phosphorylate and inhibit voltage-gated calcium channels. This study investigated whether SFKs mediate the inhibition of substance P release by these three receptors. Substance P release was measured as neurokinin 1 receptor (NK1R) internalization in spinal cord slices and in vivo. In slices, NK1R internalization induced by high frequency dorsal root stimulation was inhibited by the μ-opioid agonist DAMGO and the $GABA_B$ agonist baclofen. This inhibition was reversed by the SFK inhibitor PP1. NK1R internalization induced by low frequency stimulation was also inhibited by DAMGO, but PP1 did not reverse this effect. In vivo, NK1R internalization induced by noxious mechanical stimulation of the hind paw was inhibited by intrathecal DAMGO and baclofen. This inhibition was reversed by intrathecal PP1, but not by the inactive PP1 analog PP3. PP1 produced no effect by itself. The α_2 adrenergic agonists medetomidine and guanfacine produced a small but statistically significant inhibition of NK1R internalization induced by low frequency dorsal root stimulation. PP1 did not reverse the inhibition by guanfacine. These results show that SFKs mediate the inhibition of substance P release by μ-opioid and $GABA_B$ receptors, but not by α_2 receptors, which is probably mediated by the binding of G protein βγ subunits to calcium channels.

Keywords

Calcium channel; dorsal horn; internalization; neurokinin 1 receptor; primary afferent

Primary afferent activity causes the release of glutamate and substance P into the spinal cord. Most dorsal horn neurons that express the neurokinin 1 receptor (NK1R) for substance P receive synapses from primary afferents and project directly to the brain (Todd et al., 2002; Todd et al., 2005). There is ample evidence that NK1Rs mediate hyperalgesia by increasing the excitability of these neurons (Traub, 1996; Mantyh *et al.*, 1997; De Felipe *et al.*, 1998; Henry *et al.*, 1999; Laird *et al.*, 2001). Substance P release from primary afferents

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is inhibited by several G protein-coupled receptors (GPCRs), including μ-opioid receptors (MORs) (Jessell & Iversen, 1977; Yaksh *et al.*, 1980; Kondo *et al.*, 2005), δ-opioid receptors (Kondo et al., 2005; Overland et al., 2009), $GABA_B$ receptors (Malcangio & Bowery, 1993; Riley *et al.*, 1997; Marvizon *et al.*, 1999a) and adrenergic α_2 receptors (Kuraishi *et al.*, 1985; Pang & Vasko, 1986; Ono *et al.*, 1991a; Bourgoin *et al.*, 1993; Takano *et al.*, 1993). These receptors are present in substance P-containing terminals (Li et al., 1998; Stone et al., 1998; Ataka et al., 2000; Yang et al., 2001).

The conventional idea is that GPCRs inhibit neurotransmitter release through the inactivation of voltage-gated Ca^{2+} [Ca(V)] channels by the binding of G protein β γ subunits (Gβ γ), which is voltage-dependent (Dolphin, 2003; Evans & Zamponi, 2006; Dai *et al.*, 2009). However, recent evidence suggests that some GPCRs may activate other signaling pathways. In primary afferents, Ca(V)2.1 (P/Q-type) and Ca(V)2.2 (N-type) channels control neurotransmitter release and are the ones inhibited by MORs (Rusin & Moises, 1995; Evans & Zamponi, 2006; Dai *et al.*, 2009). Primary afferents contain a unique splice variant of Ca(V)2.2 channels having the 37a exon instead of the 37b exon (Bell et al., 2004; Castiglioni et al., 2006). The 37a exon contains a consensus site for tyrosine phosphorylation by Src family kinases (SFKs) that is absent in the 37b exon, and this makes these channels susceptible to a voltage-independent inhibition by MORs and $GABA_B$ receptors (Diverse-Pierluissi *et al.*, 1997; Strock & Diverse-Pierluissi, 2004; Raingo *et al.*, 2007). A similar voltage-independent inhibition by SFK phosphorylation may affect Ca(V)2.1 (type P/Q) channels (Weiss & Burgoyne, 2001). It has been proposed that, unlike MORs and $GABA_B$ receptors, α_2 receptors do not inhibit Ca(V) channels through SFK phosphorylation, but only through Gβ γ binding (Strock & Diverse-Pierluissi, 2004).

SFKs are a group of ten enzymes that catalyze the phosphorylation of tyrosine residues and that form part of many key signaling pathways in mammalian cells (Thomas & Brugge, 1997). Five SFKs (Src, Fyn, Lck, Lyn and Yes) play important functions in the CNS, particularly in synaptic plasticity, by regulating the activity of Ca(V) channels and NMDA receptors and (Kalia *et al.*, 2004; Xu *et al.*, 2008; Zhang *et al.*, 2008; Chen *et al.*, 2010). Protein tyrosine phosphatases (PTPs) catalyze the dephosphorylation of the tyrosine residues phosphorylated by SFKs (Thomas & Brugge, 1997).

Here we investigate whether SFKs mediate the inhibition of substance P release by MORs, $GABA_B$ receptors and α_2 receptors. We used NK1R internalization to measure substance P release, a method that is more sensitive than radioimmunoassay (Marvizon *et al.*, 2003a), identifies the sites of release (Mantyh et al., 1995; Abbadie et al., 1997; Allen et al., 1997; Honore et al., 1999; Adelson et al., 2009) and reflects NK1R activation (Trafton et al., 1999; Trafton et al., 2001).

Materials and methods

Animals

Animals used in this study were male, Sprague-Dawley rats purchased from Harlan (Indianapolis, IND). A total of 66 rats were used in the study. Spinal cord slices were prepared from 17 juvenile rats (3–5 weeks old). Intrathecal catheters were implanted in 49 adult rats (2–4 months old). The anesthetic used and other procedural details are given below. All animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Efforts were made to minimize the number of animals used and their suffering.

Chemicals and media

PP1 [1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3, 4-d]pyrimidin-4-amine], PP2 [3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine], PP3 [1-phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine] and Tocrisolve-100 (20% soya oil emulsified in water with Pluronic F68) were from Tocris (Ellisville, MO). Dasatinib was from ChemieTek (Indianapolis, IN). Isoflurane was from Halocarbon Laboratories (River Edge, NJ). Prolong Gold was from Invitrogen (Eugene, OR). Baclofen, DAMGO and other chemicals were from Sigma.

Artificial cerebrospinal fluid (aCSF) contained (in mM) 124 NaCl, 1.9 KCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$ and 10 glucose; K^+-aCSF contained 5 mM of KCl, and sucrose-aCSF contained 5 mM KCl and 215 mM sucrose instead of NaCl (iso-osmotic replacement). All these media were constantly bubbled with 95% O_2/5% CO_2.

Compounds were dissolved in water except for the following. For experiments in slices, PP1, PP2 and PP3 were dissolved at 10 mM in dimethyl-sulfoxide (DMSO) and then diluted to their desired concentrations in aCSF. For intrathecal injection of a 3 nmol dose of PP1, it was dissolved at 10 mM in DMSO and then diluted in saline, resulting in a final concentration of DMSO in the injectate of 3%. For intrathecal injection of a 10 nmol dose of PP1, it was dissolved at 100 mM in Tocrisolve-100 and then diluted in saline, resulting in a final concentration of Tocrisolve-100 in the injectate of 1%.

Spinal cord slices

Spinal cords were obtained from 3–5 weeks old male Sprague-Dawley rats by dorsal laminectomy. The rats were anesthetized with 3% isoflurane in an induction box and kept under isoflurane anesthesia during the extraction of the spinal cord, which took less than 2 min and included euthanasia by bilateral thoracotomy. Coronal slices (400 μm) with one dorsal root were cut with a vibratome (Integraslice 7550PSDS, Campden Instruments USA, Lafayette, IN) from a lumbar spinal cord segment (L2–L4), as described (Marvizon *et al.*, 2003a; Lao & Marvizon, 2005; Adelson *et al.*, 2009). The spinal cord segment was glued vertically to a block of agar on the stage of the vibratome and immersed in ice-cold sucrose-aCSF. Slices were cut using minimum forward speed and maximum vibration while observing them with a stereo microscope mounted over the vibratome. Fiber continuity between the dorsal root and the dorsal horn was assessed by examining the dorsal root and the dorsal surface of the slice with the stereo microscope. Slices were discarded if they did not meet the following criteria: 1) at least 80% of the dorsal funiculus had to be continuous with the dorsal root, and 2) the dorsal root had no cuts or compression damage. Slices were kept for one hour in K^+-aCSF at 35 °C and then in regular aCSF at 35 °C, and were used within 3 hr of preparation.

Dorsal root stimulation of slices

The dorsal root attached to the slice was electrically stimulated using a custom-made chamber, as previously described (Marvizon *et al.*, 2003b; Adelson *et al.*, 2009). Slices were superfused at 3–6 ml/min with aCSF at 35 °C. The root was placed on a bipolar stimulation electrode (platinum wire of 0.5 mm diameter, 1 mm pole separation) in a compartment separated from the superfusion chamber by a grease bridge. The root and the electrodes were covered with mineral oil and any excess aCSF was suctioned away. This ensured that electrical current circulated through the root and that the stimulus was consistent between preparations. Electrical stimulation was generated by a Master-8 stimulator and an Iso-Flex stimulus isolating unit in constant voltage mode (A.M.P. Instruments, Jerusalem, Israel), and consisted of 1000 square pulses of 20 V and 0.4 ms (C-fiber intensity) delivered at 1 Hz or 100 Hz. Slices were superfused at 3–6 ml/min with aCSF at 35 °C. Drugs were present in

the superfusate continuously starting 5 or 10 min before root stimulation. Ten minutes after the stimulus, slices were fixed by immersion in ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in 0.1 M sodium phosphate buffer). A round hole was punched in the ventral horn of the slice ipsilateral to the stimulus in order to identify it in the histological sections after immunohistochemistry.

Intrathecal injections

To deliver drugs to the lumbar spinal cord, rats were surgically implanted with chronic intrathecal catheters inserted between the L5 and L6 lumbar vertebrae (Storkson *et al.*, 1996; Chen & Marvizon, 2009). Rats (2–4 months old rats) were anesthetized with isoflurane (2–4% in oxygen) and kept under anesthesia on a metal platform kept at 35 °C by a feedback device. The skin and muscle were cut to expose vertebrae L5 and L6. A 20G needle was inserted between the L5 and L6 vertebrae to puncture the dura mater, which was inferred from a flick of the tail or paw and the backflow of spinal fluid. The needle was removed and the catheter (20 mm of PE-5 tube heat-fused to 150 mm of PE-10 tube) was inserted into the subdural space and pushed rostrally to terminate over L5–L6. The PE-10 catheter was then tunneled under the skin and externalized over the head. The skin was sutured, and the catheter was flushed with 10 μl saline and sealed. Rats were given an antibiotic (enrofloxacin) and an analgesic (carprofen) for 3 days after surgery. Rats were housed separately and used for the experiment 5–7 days after surgery. A criterion for immediate euthanasia of the rat was the presence of motor weakness or signs of paresis, but this did not occur in any of the rats in this study.

Intrathecal injection volume was 10 μl of injectate plus 10 μl saline flush (Zorman *et al.*, 1982; Jensen & Yaksh, 1984; Aimone *et al.*, 1987; Kondo *et al.*, 2005). This volume leads to the distribution of the injectate over most of the spinal cord, but not into the brain (Yaksh & Rudy, 1976; Chen *et al.*, 2007). Solutions are preloaded, in reverse order of administration, into a tube (PE-10), and delivered with a 50 μl Hamilton syringe within 1 min. The position of the catheter was examined postmortem. We established as exclusion criteria: 1) loss of the catheter, 2) termination of the catheter inside the spinal cord, and 3) occlusion of the catheter tip. Twelve of the 49 rats that were implanted with catheters were excluded from the study according with these criteria.

Noxious mechanical stimulation

A noxious mechanical stimulus was used to induce NK1R internalization in vivo, and was given 5–7 days after implanting the intrathecal catheters. Rats were anesthetized with isoflurane (2–3%) in an induction box and kept under isoflurane anesthesia until they were euthanized. After the intrathecal injections, one hind paw was clamped with a hemostat (closed to the first notch) for 30 sec (Le Bars et al., 1987). Ten minutes later, rats were euthanized with pentobarbital (100 mg/Kg). Rats were fixed immediately by aortic perfusion of 100 ml phosphate buffer (0.1 M sodium phosphate, pH 7.4) containing 0.01% heparin, followed by 400 ml of ice-cold fixative (4% paraformaldehyde, 0.18% picric acid in phosphate buffer).

Antibody characterization

The NK1R antibody was rabbit antiserum # 94168, made at CURE: Digestive Diseases Research Center, UCLA, under the sponsorship of Dr. Nigel Bunnett, UCSF. It was generated in rabbits using a peptide corresponding to the C-terminus of the rat NK1R (amino acids 393–407) coupled to KLH (Grady *et al.*, 1996). It labeled by immunofluorescence cells transfected with rat NK1R, and it did not label nontransfected cells. Staining of the transfected cells was eliminated by preadsorption with its immunizing

peptide. In Western blots from cells transfected with the NK1R, it produced a single band corresponding to a molecular weight of 100 kDa (Grady *et al.*, 1996).

Immunohistochemistry

Spinal cord slices were fixed, cryoprotected, frozen and re-sectioned at 25 μm in a cryostat as described (Marvizon *et al.*, 2003a; Adelson *et al.*, 2009). Rats were fixed by aortic perfusion as described above, and the L4–L5 lumbar spinal cord segments were similarly processed and sectioned at 25 μm in the coronal plane (Chen *et al.*, 2007; Lao *et al.*, 2008). Sections were washed four times and then incubated overnight with the NK1R antiserum diluted 1:3000 in phosphate-buffered saline containing 0.3 % Triton X-100, 0.001 % thimerosal and 10 % normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA). After three washes, the secondary antibody (1:2000, goat anti-rabbit IgG coupled to Alexa Fluor 488, Molecular Probes-Invitrogen, Eugene, OR) was applied at for 2 hours. Sections were washed four more times, mounted on glass slides, and coverslipped with Prolong Gold (Molecular Probes-Invitrogen). All incubations were done at room temperature.

Quantification of NK1R internalization

The amount of NK1R internalization was quantified using a standard method (Mantyh *et al.*, 1995; Marvizon *et al.*, 2003a; Adelson *et al.*, 2009). NK1R neurons were visually counted while classifying them as with or without internalization, using a Zeiss Axio-Imager A1 microscope with a 63x oil immersion (numerical aperture [NA] 1.40) objective. The criterion for having internalization was the presence in the neuronal soma of ten or more NK1R endosomes, defined as a small region of bright staining separated from the cell surface (Fig. 1). The person counting the neurons was blinded to the treatment. All NK1R neurons in lamina I were counted in each histological section. In experiments in slices, at least three sections per slice were counted. In experiments in vivo, at least four sections were counted in the L4–L5 spinal segment for each rat.

Confocal microscopy

Images were acquired using a Zeiss LSM 710 confocal microscope (Carl Zeiss, Inc., Thornwood, NY), with 10x (NA 0.3) and 63x oil immersion (NA 1.4) objectives. Excitation light for the Alexa Fluor 488 fluorophore (emission peak 519 nm) was provided by the 488 nm line of an argon laser and the emission window was 500–580 nm. The pinhole was 1.0 Airy unit: 38.2 μm for the 10x objective and 51.5 μm for the 63x objective, as determined by the confocal microscope software. Images were acquired in grayscale as confocal stacks of sections of 1024×1024 pixels. Photomultiplier gain and offset was individually adjusted for each image to avoid pixel saturation and loss of background detail. Each section was averaged 2 or 4 times to reduce noise. The separation between confocal sections, optimized by the confocal microscope software using the Nyquist equation, was 5.98 μm for the 10x objective and 0.38 μm for the 63x objective.

Image processing

Images of the dorsal horn obtained with the 10x objective were used to show the location of the neurons imaged with the 63x objective (Fig. 1). The program Imaris 6.1.5 (x64, Bitplane AG, Zurich, Switzerland) was used to crop the images in three dimensions. Images at 10x were cropped in x-y to 1024 × 768 pixels, and in the z dimension to the two brightest optical sections. Images at 63x were cropped in x-y to show the soma and proximal dendrites of the target neurons, and in the z dimension into three optical sections through the middle of the soma. Occasionally, several neurons were cropped from the same confocal stack. Image resolution was preserved in the cropping, so that pixels in Fig. 1 correspond to the pixels

acquired by the confocal microscope. Voxel dimensions were 830 × 830 × 5983 nm with the 20x objective and 132 × 132 × 383 nm with the 100x objective. After cropping, a two-dimension projection picture was generated in Imaris and imported into Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA), which was used to make slight adjustments in the gamma of the images so that important details are clearly visible in Fig. 1. Adobe Photoshop was also used to compose the multi-panel figures and to add text and arrows.

Data analysis

Data were analyzed using Prism 5 (GraphPad Software, San Diego, CA). Statistical analyses consisted of two-way ANOVA and Bonferroni's post-hoc test. Statistical significance was set at 0.05. The two variables were "drugs" (drug combinations) and "stimulus" (comparing the ipsilateral and contralateral sides). The Bonferroni's post-hoc test was applied to the variable "drugs" to compare effects on the ipsilateral side.

Results

Effect of SFK inhibitors on MOR and $GABA_B$ receptor inhibition of substance P release in spinal cord slices

We hypothesized that the inhibition of substance P release produced by MORs and $GABA_B$ receptors is mediated by SKFs. If so, then SFK inhibitors should reverse the inhibition produced by MOR and $GABA_B$ receptor agonists. In a first experiment, substance P release was induced in rat spinal cord slices by electrical stimulation of the dorsal root at high frequency (100 Hz), using stimulation parameters previously found to be optimal to induce substance P release (Adelson *et al.*, 2009). Substance P release was measured as NK1R internalization in lamina I neurons. The stimulus induced NK1R internalization in the ipsilateral dorsal horn, but not contralaterally. Superfusing the slices with the MOR agonist DAMGO (1 μM, Fig. 2A) or the $GABA_B$ receptor agonist baclofen (30 μM, Fig. 2B) significantly inhibited the evoked NK1R internalization. The concentration of DAMGO was chosen based on its concentration-response curve to induce MOR internalization in rat spinal cord slices (IC_{50} = 30 nM, 95% CI = 20–45 nM) (Marvizon *et al.*, 1999b). Moreover, 1 μM DAMGO inhibited NK1R internalization induced by dorsal root stimulation in slices, and this inhibition was reversed by 10 μM naloxone (Kondo *et al.*, 2005). The concentration of baclofen was chosen based on its concentration-response to inhibit NK1R internalization induced by dorsal root stimulation in slices (IC_{50} = 1.5 μM, 95% CI = 0.4–5.5 μM) (Lao & Marvizon, 2005). Therefore, the chosen concentrations of DAMGO and baclofen are near-saturation for their target receptors.

To determine whether the inhibition produced by MORs and $GABA_B$ receptors was mediated by SFKs, we used PP1 and PP2, which are selective inhibitor of SFKs, particularly of Lck and Fyn (Hanke *et al.*, 1996; Liu *et al.*, 1999). PP1 and PP2 inhibited human T-cell proliferation with IC_{50}s ranging from 0.5 μM to 26 μM, depending of the stimulus used to induce proliferation (Hanke *et al.*, 1996). Therefore, we chose a concentration of 10 μM for PP1 and PP2. As shown in Fig. 2, PP1 reversed the inhibition of the evoked NK1R internalization by DAMGO or baclofen, but produced no effect by itself. Inhibition by baclofen was also reversed by PP2. PP1 and PP2 were applied to the slices for 1 hr before the stimulus and then together with the agonist. This length of time appears to be necessary for inhibition of SFKs and/or de-phosphorylation of their substrates by PTPs in primary afferent neurons (McRoberts *et al.*, 2007; Chen *et al.*, 2010). Two-way ANOVA of the data with DAMGO (Fig. 2A) revealed significant effects of the variables 'stimulus' (F_1=1346, p<0.0001), 'drugs' (F_3=81, p<0.0001) and their interaction (F_3=70, p<0.0001). Similarly, two-way ANOVA of the data with baclofen (Fig. 2B) revealed significant effects of

'stimulus' (F_1=761, p<0.0001), 'drugs' (F_3=16, p<0.0001) and their interaction (F_3=14, p<0.0001).

Next, we investigated whether the inhibition of substance P release by DAMGO and its reversal by the SFK inhibitor PP1 also occurred when substance P release was evoked by low frequency (1 Hz) stimulation of the dorsal root (Fig. 3). Because this stimulus induces a relatively small amount of NK1R internalization (Marvizon *et al.*, 1997;Marvizon *et al.*, 2003b;Adelson *et al.*, 2009), we increased it by using peptidase inhibitors (captopril and thiorphan, 10 μM) to reduce substance P degradation (Marvizon *et al.*, 2003b;Adelson *et al.*, 2009). Stimulation at 1 Hz with peptidase inhibitors induced a somewhat higher amount of NK1R internalization than 100 Hz stimulation without peptidase inhibitors (Fig. 2). DAMGO (10 μM) substantially inhibited the evoked NK1R internalization, but this time the inhibition was not reversed by 10 μM PP1. As before, PP1 was applied to the slices for 1 hr before the stimulus and then together with DAMGO. Two-way ANOVA of data in Fig. 3 revealed significant effects of the variables 'stimulus' (F_1=98, p<0.0001), 'drugs' (F_2=34, p<0.0001) and their interaction (F_2=29, p<0.0001).

SFK inhibitors reversed MOR and $GABA_B$ receptor inhibition of substance P release in vivo

This experiment was performed in vivo by inducing substance P release with a noxious stimulus: clamping one hind paw with a hemostat for 30 sec, under anesthesia (Mantyh *et al.*, 1995; Lao *et al.*, 2008; Chen & Marvizon, 2009). This stimulus induced abundant NK1R internalization in lamina I of the ipsilateral L4–L5 segments (Fig. 1B), but none contralaterally (Fig. 1A). Quantitative results are shown in Fig. 4. The MOR agonist DAMGO (2 nmol, Fig. 1C) or the $GABA_B$ agonist baclofen (50 nmol) injected intrathecally 10 min before paw clamp significantly reduced the evoked NK1R internalization. This intrathecal dose of DAMGO was chosen because 1 nmol DAMGO strongly inhibited NK1R internalization and produced near-maximal analgesia in previous studies (Trafton *et al.*, 1999; Kondo *et al.*, 2005). Both effects were reversed by naloxone (Kondo *et al.*, 2005). Likewise, the dose of baclofen was chosen based on its dose-responses to inhibit NK1R internalization induced by noxious stimulation (Riley *et al.*, 2001) and to produce thermal antinociception (Malan *et al.*, 2002). In both cases the effect of baclofen was near-maximal at 40 nmol.

To study the involvement of SFKs, the SKF inhibitor PP1 was injected intrathecally 1 hr before the injection of DAMGO or baclofen and then coinjected with these compounds. At a dose of 3 nmol, PP1 produced a near complete reversal of the inhibition by baclofen and a partial reversal of the inhibition by DAMGO (Fig. 4). Increasing the dose of PP1 to 10 nmol (PP1* in Fig. 4, see 'Chemicals and media' in Material and Methods) resulted in a near complete reversal of the inhibition by DAMGO, as can be observed in the example image in Fig. 1D. PP1 by itself (3 nmol intrathecally) did not affect the evoked NK1R internalization ipsilaterally or induced NK1R internalization contralaterally, showing that SFKs do not affect the internalization mechanism of NK1Rs. The inactive PP1 analog PP3 (3 nmol) or vehicle (1 % Tocrisolve-100) did not affect the inhibition produced by DAMGO or baclofen, indicating that the effects of PP1 were mediated by SFKs. Two-way ANOVA of the data in Fig. 4 revealed significant effects of the variables 'stimulus' (F_1=1435, p<0.0001) and 'drugs' (F_8=37, p<0.0001) and their interaction (F_8=37, p<0.0001). These results indicate that inhibition of substance P release by MORs and $GABA_B$ receptors is mediated by SFKs.

A SFK inhibitor did not reverse the α_2 adrenergic inhibition of substance P release

Substance P release from primary afferent terminals is also inhibited by α_2 adrenergic receptors (Kuraishi *et al.*, 1985; Pang & Vasko, 1986; Ono *et al.*, 1991b; Bourgoin *et al.*, 1993; Takano *et al.*, 1993; Overland *et al.*, 2009). Accordingly, we investigated whether α_2 receptor agonists inhibit NK1R internalization evoked by stimulating spinal cord slices at the dorsal root at high and low frequencies, and whether this inhibition is mediated by SFKs.

When NK1R internalization was evoked with high frequency (100 Hz) stimulation, the α_2 agonist guanfacine (10 nM or 100 nM, Fig. 5A) produced a trend towards inhibition that was not significant in a two-way ANOVA (F_1=358, p<0.0001 for 'stimulus'; F_3=1.46, p=0.25 for 'guanfacine', and F_3=0.53, p=0.66 for their interaction). The SFK inhibitor PP1 (10 μM) produced no significant effects, either.

We then evoked NK1R internalization with low frequency (1 Hz) stimulation of the dorsal root. As in the experiment in Fig. 2, peptidase inhibitors (captopril and thiorphan, 10 μM) were used to reduce substance P degradation and hence boost NK1R internalization. In these conditions, the α_2 agonists guanfacine and medetomidine at a concentration of 10 nM produced a small but statistically significant inhibition of the evoked NK1R internalization (Fig. 5B). Guanfacine produced no inhibition at a higher concentration (1 μM). Inhibition of substance P release by 10 nM guanfacine was not reversed by PP1 (10 μM, Fig. 5B). If anything, PP1 showed a trend (not significant in the Bonferroni's post-hoc test) to increase the inhibition by guanfacine. Here again, PP1 was applied to the slices for 1 hr before the stimulus and then together with guanfacine. Two-way ANOVA of the data in Fig. 5B revealed significant effects of the variables 'stimulus' (F_1=998, p<0.0001), 'drugs' (F_4=7.2, p=0.0002) and their interaction (F_4=6.18, p=0.0002). These results indicate that inhibition of substance P release by α_2 receptors is not mediated by SFKs.

Discussion

Our results show that blockade of SFKs reverses the inhibition of substance P release produced by MORs and $GABA_B$ receptors, but not by α_2 receptors.

SFKs mediate the inhibition of substance P release by MORs and $GABA_B$ receptors

Our results, together with previous studies in cell expression systems (Raingo *et al.*, 2007) and in cultures of embryonic chicken sensory neurons (Diverse-Pierluissi *et al.*, 1997; Strock & Diverse-Pierluissi, 2004), support the two mechanisms for the inhibition of substance P release by GPCRs illustrated in Fig. 6. One of these mechanisms is the voltage-dependent blockade of Ca(V) channels by Gβ γ binding (Dolphin, 2003; Evans & Zamponi, 2006; Dai *et al.*, 2009). The second mechanism involves the activation of SFKs, which then inhibit Ca(V) channels by tyrosine phosphorylation. Since the entry of Ca^{2+} in the terminal through Ca(V) channels triggers glutamate and substance P release, blockade of the channels results in inhibition of transmitter release.

Ca(V) channels present in primary afferent terminals are mainly Ca(V)2.2 (N-type), although Ca(V)2.1 (P/Q-type) channels are also present (Rusin & Moises, 1995; 1998). In nodose and dorsal root ganglia, $GABA_B$ receptors inhibit exclusively Ca(V)2.2 channels, whereas MORs inhibit both Ca(V)2.2 and Ca(V)2.1 channels (Rusin & Moises, 1995; 1998). Ca(V)2.2 channels in primary afferent neurons have a unique splice variant of their α1 subunit containing the 37a exon instead of the 37b exon (Raingo *et al.*, 2007), which is largely absent in other CNS neurons (Bell *et al.*, 2004; Castiglioni *et al.*, 2006). Importantly, the 37a exon, but not the 37b exon, contains a tyrosine residue susceptible to SFK phosphorylation (Raingo *et al.*, 2007). In HEK293 cells expressing Ca(V)2.2e[37a] channels, MORs and $GABA_B$ receptors activate SFKs, which phosphorylate Ca(V)2.2

channels, inhibiting them. Similar findings were obtained in cultures of embryonic chicken sensory neurons (Diverse-Pierluissi *et al.*, 1997; Strock & Diverse-Pierluissi, 2004), where Ca(V)2.2 channels were found to form a complex with Src kinase (Richman *et al.*, 2004) and Src activation by $GABA_B$ receptors inhibited Ca(V)2.2 channels (Schiff *et al.*, 2000). Although SFK inhibition of Ca(V)2.1 channels has not been studied in primary afferents, SFKs were found to phosphorylate and inhibit Ca(V)2.1 channels in adrenal chromaffin cells (Weiss & Burgoyne, 2001). Hence, this may contribute to the MOR inhibition of substance P release.

Our data show that SFK-mediated inhibition of Ca(V) channels does occur in mammalian primary afferents and plays a key role in controlling substance P release. When substance P release was evoked by high frequency stimulation, SFK inhibitors reversed most of the inhibition by $GABA_B$ receptors and MORs, indicating that SFKs is the primary inhibitory mechanism in these conditions. In contrast, when substance P release was evoked by low frequency stimulation, the SFK inhibitor PP1 did not reverse the inhibition by MORs. A possible explanation for this frequency dependence is that high frequency firing of primary afferents produces a large depolarization of their terminals, which hinders the inhibition of Ca(V) channels by Gβ γ binding (Raingo *et al.*, 2007). In addition, our results suggest that the SFK mechanism is turned off during low frequency firing of primary afferents, and that Gβ γ binding mediates MOR inhibition in these conditions.

SFKs do not mediate the inhibition of substance P release by α_2 adrenergic receptors

We show that the α_2 agonists guanfacine and medetomidine inhibit NK1R internalization evoked by dorsal root stimulation in spinal cord slices, although their effect was small and detected only at low concentrations. Similar low concentrations (1–10 nM) of the α_2 receptor agonist clonidine were found to inhibit the release of calcitonin gene-related peptide (CGRP) from rat spinal cord slices (Overland *et al.*, 2009). Notably, inhibition of substance P release by guanfacine was only statistically significant when using low frequency stimulation. This is consistent with the idea that α_2 receptors do not inhibit Ca(V) channels through SFK phosphorylation (Strock & Diverse-Pierluissi, 2004), but only through the voltage-dependent binding of Gβ γ (Fig. 6) (Dolphin, 2003; Evans & Zamponi, 2006; Dai *et al.*, 2009). Thus, terminal depolarization produced by high frequency stimulation would prevent Gβ γ binding and therefore inhibition by the α_2 receptors. Further support for this idea comes from the fact that the SFK inhibitor PP1 did not reverse the inhibition of substance P release by guanfacine.

A previous study in vivo by Nazarian et al. (2008) failed to detect inhibition of the evoked NK1R internalization by the α_2 agonists ST-91 and dexmedetomidine (an enantiomer of medetomidine). This study used two different noxious stimuli to induce NK1R internalization: formalin injection and paw compression, and several doses of dexmedetomidine (0.3–10 μg or 1.3–43 nmol). We attribute the differences from our study to two possible causes. First, Nazarian et al. anesthetized the rats with pentobarbital, which may have blocked the effect of the α_2 receptors. Second, these intense noxious stimuli probably induce high frequency firing of primary afferents, which we found hinders the inhibition by α_2 receptors. Nazarian et al. pointed out that α_2 receptor inhibition of substance P release was detected in studies using spinal cord slices (Pang & Vasko, 1986; Ono *et al.*, 1991b; Takano *et al.*, 1993) but not in vivo. However, the two studies in vivo (Lang *et al.*, 1994; Zhao *et al.*, 2004) that they cite were conducted in cats, suggesting that there may be species differences. Another study done in vivo in rabbits (Kuraishi *et al.*, 1985) did report a noradrenergic inhibition of substance P release. In any case, the results by Nazarian et al. agree with our observation that the inhibition of substance P release by α_2 receptors is much less robust than the inhibition produced by MORs and $GABA_B$ receptors. It is possible that α_2 receptors in primary afferents work primarily in synergy with opioid receptors. Overland

et al. (2009) recently reported a strong synergy between α_2 and δ-opioid receptors to inhibit CGRP release from primary afferents and to produce analgesia. Another study (Tan *et al.*, 2009) found that α_{2A} receptors associate with MORs in primary afferents: they co-internalize and cross-desensitize.

Physiological relevance

An intriguing possibility is that the SFKs activated by MORs and $GABA_B$ receptors phosphorylate proteins other than the Ca(V) channels. Thus, we recently reported that NMDA receptors in primary afferent terminals require SFK phosphorylation to be activated (Chen *et al.*, 2010). Although it is not clear whether these SFKs are the same that are activated by the MORs, this possibility is supported by another recent report (Zhou *et al.*, 2010) showing that MORs present presynaptically in primary afferent terminals induce long-term potentiation of these synapses, and that this requires activation of NMDA receptors.

Pathological states like inflammation and neuropathic pain probably induce changes in MORs, $GABA_B$ receptors and α_2 receptors, or in their signaling mechanisms, which may be result in hyperalgesia and altered responses to analgesics. The fact that NK1R antagonists reverse the hyperalgesia and allodynia produced by nerve injury (Cumberbatch *et al.*, 1998; Cahill & Coderre, 2002) indicates that substance P does play a role in neuropathic pain. However, after nerve injury substance P synthesis decreases in C-fibers (Hokfelt *et al.*, 1994) and occurs de novo in A-fibers (Marchand *et al.*, 1994; Noguchi *et al.*, 1994; Noguchi *et al.*, 1995; Fukuoka *et al.*, 1998). Therefore, it has been proposed that A-fibers are the source of substance P in neuropathic pain. Indeed, Malcangio et al. (2000) found that Aβ-fiber stimulation induced substance P release after spinal nerve lesion. However, other investigators studying nerve injury models could not detect substance P immunoreactivity in A-fiber terminals (Noguchi *et al.*, 1995; Hughes *et al.*, 2007) or substance P release induced by A-fiber stimulation (Allen *et al.*, 1999; Hughes *et al.*, 2007). Instead, Allen et al. (1999) found that nerve transection resulted in an increase in substance P release induced by C-fiber stimulation.

Morphine loses its effectiveness in neuropathic pain in humans (Arner & Meyerson, 1988; Portenoy *et al.*, 1990) and when injected intrathecally in rodent nerve injury models (Mao *et al.*, 1995; Ossipov *et al.*, 1995b; a; Wegert *et al.*, 1997). This seems to be largely caused by the downregulation of MORs in primary afferents (Zhang *et al.*, 1998; Kohno *et al.*, 2005). Ca(V)2.2 channels possessing the 37a exon and susceptible to SFK inhibition are also downregulated in neuropathic pain (Altier *et al.*, 2007). However, the effect of this loss of the 37a exon is unclear, in view that $GABA_B$ receptors in primary afferents, whose effect is also mediated by SFKs, do not lose effectiveness in neuropathic pain. Unlike MORs, $GABA_B$ receptors in primary afferents are not downregulated by nerve injury (Engle *et al.*, 2006). $GABA_B$ receptor agonists decrease neuropathic pain in rodents (Chen & Pan, 2003; Franek *et al.*, 2004; Urban *et al.*, 2005), and in humans are particularly effective to treat trigeminal neuralgia (Idanpaan-Heikkila & Guilbaud, 1999; Deseure *et al.*, 2003) and in conjunction with spinal cord electrical stimulation (Lind *et al.*, 2004; Lind *et al.*, 2008).

After nerve injury, spinal application of the α_2 adrenergic agonist clonidine produced antinociception in both rats (Luo *et al.*, 1994; Duflo *et al.*, 2002) and humans (Eisenach *et al.*, 1995). However, it is not clear whether this effect is mediated by α_2 receptors in primary afferents or in dorsal horn neurons. Primary afferents express α_{2A} receptors, whereas dorsal horn neurons express α_{2C} receptors (Stone *et al.*, 1998). Since expression of α_{2A} receptors decreases after nerve injury (Stone *et al.*, 1999; Leiphart *et al.*, 2003), it is possible that adrenergic effects on primary afferents decrease in neuropathic pain. Alternatively, α_2 receptor analgesia may switch from α_{2A} to α_{2B} receptors after nerve injury (Duflo *et al.*, 2002; Leiphart *et al.*, 2004). In addition, changes in the signaling system used by the α_2

receptors after nerve injury (Bantel *et al.*, 2005) may be important in determining their analgesic effect.

In conclusion, knowledge of the signaling systems used by MORs, $GABA_B$ receptors and α_2 adrenergic receptors in primary afferent terminals is important to understand the mechanisms of action of analgesic drugs and how these mechanisms change during chronic pain.

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Abbreviations

aCSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
Ca(V)	voltage-gated calcium channel
CGRP	calcitonin gene-related peptide
CI	confidence interval
DMSO	dimethyl-sulfoxide
DAMGO	[D-Ala^2, NMe-Phe^4, Gly-ol^5]-enkephalin
DRG	dorsal root ganglia
Gβ γ	G protein β γ subunits
GPCR	G protein-coupled receptor
MOR	μ-opioid receptor
NA	numerical aperture
NK1R	neurokinin 1 receptor
PKC	protein kinase C
PP1	1-(1,1-Dimethylethyl)-1-(4-methylphenyl)-1H-pyrazolo[3, 4-d]pyrimidin-4-amine
PP2	3-(4-chlorophenyl)-1-(1,1-dimethylethyl)-1H-pyrazolo[3,4-d]pyrimidin-4-amine
PP3	1-Phenyl-1H-pyrazolo[3,4-d]pyrimidin-4-amine
PTP	protein tyrosine phosphatase
SFK	Src family kinase

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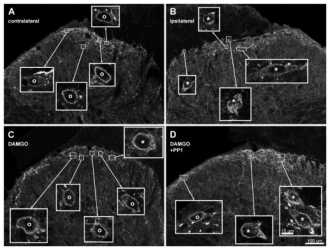

Fig. 1. Confocal images of NK1R neurons in lamina I after noxious stimulation of the hind paw in vivo

Rats received a noxious stimulus (clamp for 30 sec) in the hind paw to induce substance P release and were fixed 10 min later for NK1R immunohistochemistry. Images were taken from sections of the L4–L5 spinal segments. **A, B:** Rats received an intrathecal injection of saline 10 min before the stimulus; A and B correspond to the contralateral and ipsilateral dorsal horns, respectively, of the same histological section. **B:** Rats received an intrathecal injection of DAMGO (2 nmol) 10 min before the stimulus; ipsilateral dorsal horn. **C:** Rats received intrathecal injections of DAMGO (2 nmol) and PP1 (10 nmol) 10 min before the stimulus and of PP1 (10 nmol) 70 min before the stimulus; ipsilateral dorsal horn. Main panels: images taken with a 10x objective, with a voxel size of 830 × 830 × 5983 nm and 2 confocal planes. Insets: images of lamina I neurons taken with a 63x objective, with a voxel size of 132 × 132 × 383 nm and 3 confocal planes. Neurons with NK1R internalization are indicated with "*" and neurons without internalization by "o". Scale bars (in panel D) are 100 μm for the main panels and 10 μm for the insets.

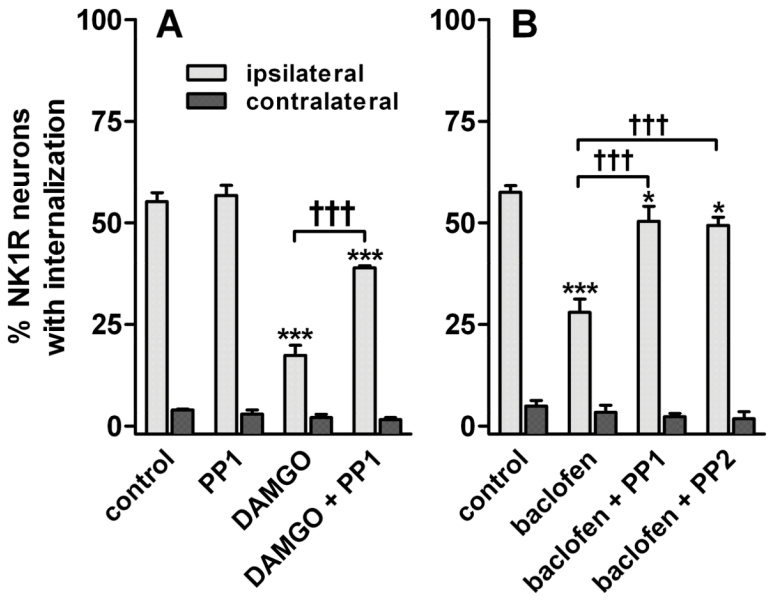

Fig. 2. NK1R internalization induced by 100 Hz dorsal root stimulation of spinal cord slices: SFK inhibitors reversed the inhibition by DAMGO and baclofen

Spinal cord slices (n=3–4) were stimulated at the dorsal root at 100 Hz to induce substance P release. **A.** Slices were superfused with aCSF (control), 10 μM PP1, 1 μM DAMGO or DAMGO + PP1. **B.** Slices were superfused with aCSF, 30 μM baclofen, baclofen + 10 μM PP1 or baclofen + 10 μM PP2. Treatments with PP1 and PP2 were preceded by 1 hr preincubation with these drugs at 10 μM. Two-way ANOVA: $p<0.0001$ for stimulus and drugs. Bonferroni's post-hoc test: *, $p<0.05$, ***, $p<0.001$ compared to control; †††, $p<0.001$ as indicated.

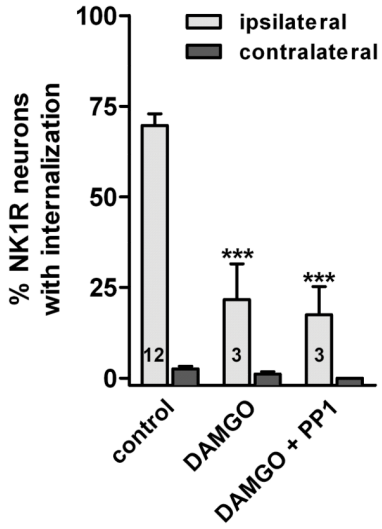

Fig. 3. NK1R internalization induced by 1 Hz dorsal root stimulation of spinal cord slices: a SFK inhibitor did not reverse the inhibition by DAMGO

Spinal cord slices were stimulated at the dorsal root at 1 Hz to induce substance P release while being superfused with the peptidase inhibitors captopril (10 μM) and thiorphan (10 μM) alone (control), with 1 μM DAMGO or with 1 μM DAMGO + 10 μM PP1. Treatment with PP1 was preceded by 1 hr preincubation with 10 μM PP1. Two-way ANOVA: $p<0.0001$ for stimulus, drugs and interaction. Bonferroni's post-hoc test: ***, $p<0.001$ compared to control. Number of slices (n) for each data set is given inside the columns.

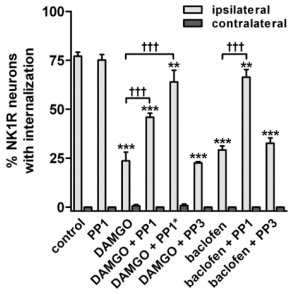

Fig. 4. SFK inhibitors reverse the inhibition by DAMGO and baclofen of NK1R internalization induced by noxious stimulation in vivo

Rats (*n*=3) received an i.t. injection of 3 nmol PP1 (PP1*: 10 nmol), 3 nmol PP3, or no injection. One hour later they received an i.t. injection of DAMGO (2 nmol) or baclofen (50 nmol) with or without PP1 or PP3, as indicated. Noxious stimulation (hindpaw clamp for 30 sec) was delivered 10 min after the second injection, and rats were fixed 10 min later. NK1R internalization was measured in the L4–L5 spinal segments. Two-way ANOVA: $p<0.0001$ for 'drugs' and 'stimulus'. Bonferroni's post-hoc test: ***, $p<0.001$, **, $p<0.01$ compared to control; †††, $p<0.001$ as indicated.

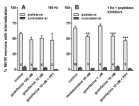

Fig. 5. Inhibition of NK1R internalization by α_2 adrenergic agonists in spinal cord slices

Spinal cord slices were stimulated at the dorsal root to evoke substance P release. The α_2 agonists medetomidine and guanfacine were superfused to the slices at the concentrations indicated. PP1 (10 μM) was applied in a 1 hr preincubation and then added to guanfacine in the superfusate. Number of slices (*n*) for each data set is indicated by numbers inside the columns. **A.** 100 Hz stimulation; two-way ANOVA: $p<0.0001$ for 'stimulus', $p=0.25$ for 'drugs', $p=0.66$ for their interaction. **B.** 1 Hz stimulation in the presence of peptidase inhibitors (captopril and thiorphan, 10 μM); two-way ANOVA: $p<0.0001$ for 'stimulus', $p=0.0002$ for 'drugs', $p=0.0002$ for their interaction. Bonferroni's post-hoc tests: **, p<0.01, ***, p<0.001 compared to control.

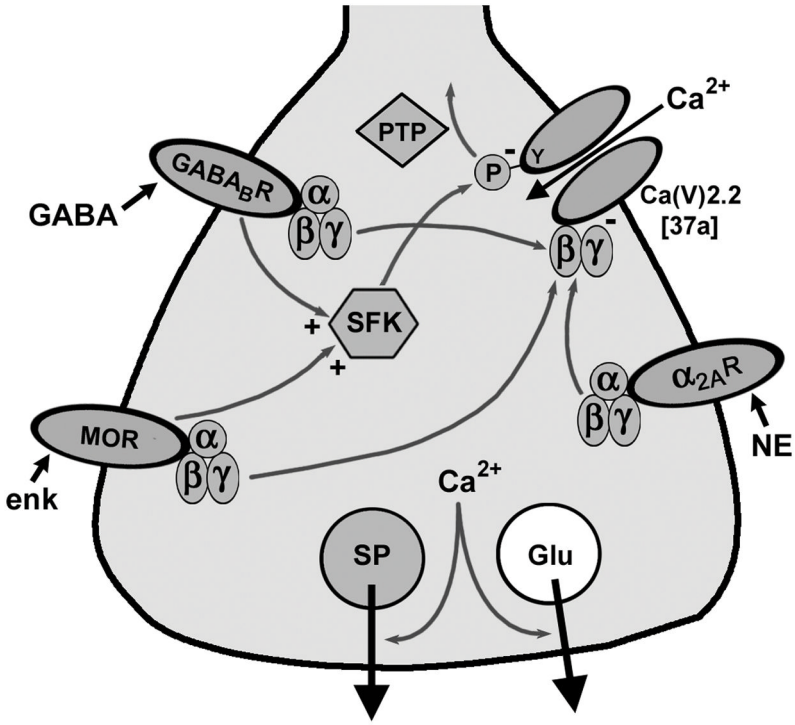

Fig. 6. Mechanisms of inhibition of substance P release from primary afferent terminals

Substance P (SP) and glutamate (Glu) are released when Ca^{2+} enters the terminal through Ca(V) channels. MORs, $GABA_B$ receptors ($GABA_B$Rs) and α_{2A} receptors inactivate (-) Ca(V)2.2 through Gβ γ. MORs and $GABA_B$Rs also inactivate Ca(V)2.2 by Tyr (Y)-phosphorylation by SFKs. NE: norepinephrine; enk: enkephalin.