The Sensitization of The Transient Receptor Potential Vanilloid 1-Mediated Responses By Prostaglandin and Bradykinin

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Abstract

Transient receptor potential vanilloid type-1 (TRPV1) channels play key roles in chronic pain conditions and are modulated by different inflammatory mediators to elicit heat sensitisation. Bradykinin is a 9-amino acid peptide chain that promotes inflammation. The aim of present study is to investigate how bradykinin and prostaglandin receptors (EP3 and EP4) modulate the sensitisation of TRPV1-mediated responses. Calcium imaging studies of rat dorsal root ganglion (DRG) neurons were employed to investigate the desensitizing responses of TRPV1 ion channels by capsaicin, and the re-sensitization of TRPV1 by bradykinin, then to explore the role EP3 and EP4 receptors in mediating these bradykinin-dependent effects. Immunocytochemistry was used to study the co-expression and distribution of EP4, TRPV1, COX-1 and B2 in rat DRG neurons. Desensitization was seen upon repeated capsaicin application, we show that bradykinin-mediated sensitization of capsaicin-evoked calcium responses in rat DRG neurons occurs is dependent on COX-1 activity, and utilises a pathway that invovles EP4 but not EP3 receptors. The present study provides evidence for a novel signalling pathway through which bradykinin can regulate the TRPV1 ion channel function via the EP4 but not EP3 receptors and provides the anatomical basis for this regulation

1- Introduction

The transient receptor potential vanilloid receptor 1(TRPV1), is a heat-sensitive non-selective cation channel which plays a fundamental role in thermal nociception[1,2]. TRPV1 has essential roles in inflammatory thermal hyperalgesia as illustrated by the observation that TRPV1 knockout mice have reduced thermal inflammation-induced hyperalgesia[3]. Under normal physiological conditions, TRPV1 is widely expressed throughout the central and peripheral nervous systems, including dorsal root ganglion neurons (DRG) [4]. TRPV1 can be activated by noxious heat (>43@C), low pH and by pungent compounds, such as capsaicin[5,6], as well as non-pungent capsaicin-analogs such as arvanil and olvanil[7-10].

Nociceptor sensitization is considered the primary peripheral mechanism underlying primary hyperalgesia[11]. A core feature of nociceptors is that inflammatory mediators, such as prostaglandins and bradykinin, activate their cognate receptors resulting inactivation of signal transduction pathways and enhanced pain sensation[12].

Bradykinin is 9-amino acid peptide chain generated following tissue injury and acts as inflammatory mediator[13]. Bradykinin produces its biological action by activation of two G-protein coupled receptors subtypes, bradykinin receptor type B_1(B_1) and bradykinin receptor type (B_2)[13-15]. Under normal conditions, the B_2 receptors are expressed in most tissues and the inflammatory actions of bradykinin are strongly mediated through the B_2 receptor subtype[16]. B_1receptors are overexpressed in inflammation and also make a contribution to nociception via separate pathways[17-19]. Bradykinin stimulates the formation of prostaglandins in many cell types[20-22] and activation of B_2 (not B_1) receptors can induce the synthesis of prostaglandins in pain sensing adult rat trigeminal ganglia cultures and in isolated rat DRG neurons[21,23]. In

fact, stimulation of B_2 receptors leads to PKC? activation and a phosphorylation dependent increase in the TRPV1 channel current [24]. Bradykinin sensitizes the nociceptor (TRPV1) response to heat and so is able to mediate thermal hyperalgesia and activate these neurons and produce pain[24,25]. Bradykinin sensitizes nociceptors by modulating other ion channels, for example by reducing the activity of potassium channels[26]. Although, several studies demonstrate that bradykinin sensitizes TRPV1 by means of the B2 dependent PLC/PKC??phosphrylation pathway,[21,23,27], we present here evidence that another pathway is involved.

Prostaglandins are generated by either cyclooxygenase 1 or 2 in response to inflammatory insult by damaged cells, and act on a range of prostaglandin receptors on, for example, nociceptor endings. This results in the activation of PLA and PLC-mediated phosphorylation pathways within these endings to elicit changes in nociceptor sensitivity. It is important to note that prostaglandins may also act as intracellular messengers mediating the effects of some ligands, e.g. glutamate acting at metabotropic glutamate receptors, thereby adding to the intricacy of their actions.[21,23,27,28].

In this study we explored the sensitization of TRPV1 receptor by bradykinin, and investigated the role of prostaglandin receptors in mediating the sensitizing effect of bradykinin on TRPV1 receptor, a pathway of potential novel therapeutic importance.

2-Materials and Methods

The study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies[29].

2.1 Calcium imaging experiments

2.1.1 Cell culture:

60 male Wistar rats (200–300g) were killed by stunning and cervical dislocation under Schedule 1 according to the United Kingdom Animals (Scientific Procedures) Act 1986. DRGs were removed from male Wistar rats and collected in cold (4°C) (HBSS; Gibco BRL). Using sterile scissors, the DRG were chopped into small fragments to increase surface area for enzyme digestion. Ganglia were incubated in (papain (1mg/ml) and L-cysteine (2mg/ml) in HBSS (Gibco BRL) for 15 min at 37°C. Ganglia were then replaced in (dispase (0.8%) and collagenase type F (1mg/ml,)) in HBSS for 22-30 min at 37°C, during the incubation a mechanical trituration was performed by using a sterile, fire-polished glass Pasteur pipette to enhance the disaggregation of neurons in the clamp. the cell suspension was centrifuged for 5 min at 1000g at 4°C this was then followed by the resuspension of the cell pellets with sterile HBSS to remove the enzyme. Cell pellets were resuspended in 1ml of feeding media, and the DRG neurons were plated onto the 16mm coverslips (Scientific Laboratory Supplies) coated with poly-D-lysine (0.1mg/ml, Sigma Aldrich) and laminin (20μg/ml, Sigma Aldrich). Cultures were maintained for 2d in feeding medium containing Ham's F-12 supplemented with 10% horse serum (Sigma Aldrich), 2mM glutamine (Sigma Aldrich), 10ng/ml nerve growth factor (NGF) (Sigma Aldrich),and 1% penicillin/streptomycin (enzyme activity = 5,000units/ml penicillin and5,000μg/ml streptomycin, Gibco at 37°C in humidified air with 5% CO_2.

2.1.2 Calcium imaging:

Cells were pre-incubated with a normal Ringer's solution (in mM: NaCl 14, HEPES 10, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$1.8 and glucose 5), including Fluo-4 (2.5μM; 45min) as the fluorescent Ca^{2+} indicator. Following the loading process, the cells were washed with a normal Ringer's solution to ensure that the excess Fluo-4 AM was removed. The coverslips were transferred to the perfusion chamber constantly supplied with Ringer's solution through the perfusion system. The whole experiment was performed in a darkened room to keep the background illumination at the lowest level and prevent bleaching of the fluorophore.

The increase in fluorescence associated with Ca^{2+}binding can be monitored using an excitation wavelength of 488nm with detection at $>$ 500 nm. Images were taken using an Olympus inverted microscope with a 10x lens and an Olympus fluoview 300 (version 4.2) software. At the end of each experiment, the cells were challenged with a 60 mM KCl Ringer's solution to confirm the viability of the cells because a High-K^+

Ringer's solution induces depolarization only in living cells. The change in fluorescence was normalized as F510 self-ratio (F510 S-R) by this equation

$$F510\ S-R = \frac{fluorence\ intensity - background\ intensity}{baseline\ intensity - background\ intensity}$$

For Ca^{2+} response experiments, all raw data were transferred from the image J into Excel sheets for primary calculation, followed by transfer them to GraphPad and analyzing them using GraphPad Prism version 6.00 (GraphPad Software, LaJolla, California, USA).

2.1.3. Drug administration

A u-tube system (Fig 1) was used for the rapid application of capsaicin. The u-tube was placed close to a group of cells of interest, and this was attached to a gravity-feed perfusion system through microbore Teflon PTFE tubing (O.D. 0.042", I.D. 0.022" (Cole Palmer). The tubing had previously been coated with a siliconising agent (sigmacote, Sigma-Aldrich), and the application of capsaicin was controlled by a 3-way solenoid valve (The Lee Company, England) connected to pulse generator unit (High Med, England). The drug application lasted 10s for all experiments involving capsaicin.

capsaicin was applied twice with a 10 min rest period, at the second application the magnitude of the calcium responses was diminished compared to the response obtained following the first capsaicin application (mean ± sem) response ratio which is described as the (F510 self-ratio) of the second response as a percentage of the first response (F510 self-ratio).

The different chemical reagents used to inhibit the sensitisation effect of bradykinin on capsaicin cells were started 10–30 min before the challenge with the first capsaicin and continued until the experiment done. Each chemical was dissolved in the appropriate solvent at the desired concentration, according to the manufacturer's recommendations. All reagents were diluted to the desired concentration in a normal Ringer's solution (pH 7.4) before the experiments (table1).

Table 1: The concentrations and solvents applied to investigate their role in capsaicin sensitization by bradykinin.

Reagent	Solvent	Concentration
Thapsigargin	DMSO	2μM
GW627368	DMSO	1μM
RHC-80267	DMSO	20μM
NS398	DMSO	10μM
SC560	DMSO	100nM

2.1.3 Data analysis:

Treatment effects were statistically analyzed by one-way analysis of variance ANOVA, followed by post-hoc analysis using the Bonferroni correction for multiple comparisons in Graphpad Prism Software (GraphPad Software, La Jolla,California USA). Student's t-test was used when comparisons were restricted to two experimental groups. A P value of < 0.05 were considered statistically significant.

2.2 Immunohistochemistry experiments

2.2.1 Triple-label immunocytochemistry:

25 male Wistar rats were killed by stunning and cervical dislocation under Schedule 1 of the United Kingdom Animals (Scientific Procedures) Act 1986. Then lumbar (L4-L6) DRG were excised under aseptic conditions and axons were trimmed away using micro-scissors. DRG were immersed in Tissue-Tek (Thermo Scientific,

Raymond Lamb) and rapidly frozen in a hexane bath cooled with dry ice (-73°C) for 20 min in a Dewar flask and then transferred for storage at -20°C until used. Tissue blocks were trimmed and 10μm sections of DRG were cut using an ultracryomicrotome (model OTF, Hacker-Bright OTF, Fairfield, NJ), and thawed onto poly-L-lysine coated glass slides (VWR International, Lutterworth, UK) and allowed to air dry.

Sections were fixed with 2% paraformaldehyde in 0.1 M phosphate buffer for 10min at ambient temperature, followed by a wash in PBS for 15 min. For the dilution of both primary and secondary antibodies, PBS with 10% of host- directed serum was used.

The sections were incubated in a blocking buffer for 30 min at room temperature in an incubation box. Triton X-100 is a detergent and facilitates the penetration of the antibody into the tissue sections and the cells. Sections were washed in PBS (2 x 15 min) and were incubated in the appropriate primary antibody overnight in an incubation box at 4°C (the primary antibodies for EP_4 (goat anti-rabbit, 1:200), TRPV1 (goat anti-mouse; 1:1000) and B_2 (goat anti-donkey; 1:200))

Control experiments were performed to determine the level of nonspecific binding and to achieve this primary antibody was omitted from the incubation medium. The sections were then washed in PBS (6 x 10 min) after which they were placed in a solution containing the appropriate secondary antibody donkey anti-rabbit (for EP_4) DyLight 405 conjugated secondary antibody (1:1000; Jackson ImmunoResearch Labs and a donkey anti-goat (for B_2) TR- conjugated secondary antibody (1:400; Jackson ImmunoResearch Labs) and a donkey anti-mouse (for TRPV1) AF-conjugated secondary antibody (1:500; Jackson ImmunoResearch Labs). and incubated for 2 hours at room temperature. Sections were then washed in PBS (2x15 min) and tissue sections were mounted using a glycerol-based, aqueous, antifade mountant, Citiflour (UKC Chem. Laboratory, Kent, UK).

The mounted sections were photographed using a Nikon Labphot 2A epifluorescence microscope using the appropriate filter set (FITC-absorbance peak = 492nm, emission peak = 520nm; TR-absorbance = 596nm, emission peak= 620nm) for each fluorophore with a x20 objective lens. Publication-quality micrographs were captured using laser-scanning microscope confocal microscope (Olympus FluoView® 300), employing an Argon ion (488nm) and green HeNe (543nm) lasers.

2.2.2 Co-localization of EP_4 and COX-1

Dual-label immunocytochemistry was conducted according to the same procedure as mentioned previously in Section 2.4 and the DRG sections were incubated overnight at 4°C with the primary antibody for EP_4 (1:200; Cayman Chemical) and a COX-1 antibody (1:200; Cayman Chemical) raised in mice. The DRG sections were then washed with PBS six times for 10 min each followed by incubation with the host-directed secondary antibody (EP_4 primary, Texas Red-conjugated goat anti-rabbit, 1:500 dilution ; COX-1 antibody, Alexa-Fluor 488-conjugated goat anti-mouse, 1:200). The DRG sections were then washed again twice with PBS, mounted and photographed.

2.2.3 Co-localization of B_2 and COX-1

Dual-label immunocytochemistry was conducted according to the same procedure as mentioned previously in Section 2.4 The DRG sections were incubated with the primary antibody for B_2 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA raised in goat) and the COX-1 antibody (1:200; Cayman Chemical) overnight at 4°C. The DRG sections were then washed with PBS six times for 10 min each. This was followed by incubation with a host-directed AlexaFluor conjugated anti-mouse secondary antibody (1:200 dilutions, Jackson ImmunoResearch Labs) and a Texas Red conjugated anti-goat secondary antibody (1:200 dilution, Jackson ImmunoResearch Labs). The DRG sections were then washed again twice with PBS, mounted and photographed.

2.2.4 Morphometric data analysis

Six sections from six different DRGs from at least different 3 rats were used for morphometric analysis of each experiment. By using a computerized image analysis system (image J), the cell area and the number

of labelled and unlabeled cells was determined. The size (in μm2) of DRG neurons was classified based on their cross-sectional area being small ($< 1000\mu m^2$) or intermediate to large ($> 1000\mu m^2$) (Chopra et al. 2000, Hammond 2006) using images taken with a Nikon (Labophot 2A) microscope (x 20 objective). A chi-square test was performed to compare the distribution of different proteins. For EP_4, COX-1, and B_2 localization experiments, data were pooled forms multiple experiments.

2.3 Chemicals:

All chemical reagents and solvents were obtained from Sigma, unless otherwise stated. The EP_4 and COX-1 antibodies were purchased from Cayman. The bradykinin antibody and RHC-80267 was from Santa Cruz Biotechnology, Inc. H89 and GF109203X were from Enzo Life Science (UK) LTD. SC560 was from Cambridge Biosciences LTD. Capsaicin, bradykinin, GW627368 and L-798106 were purchased from Tocris Bioscience (UK).

3- Results

3.1 Calcium imaging results

3.1.1 Enhancing effect of BK and PGE_2 on capsaicin evoked responses in isolated DRG neurons

Desensitization was observed upon repeated capsaicin application with a response ratio of 46.9 ± 3.6% (Fig 2A). (PGE (500nM, 3 min) perfused into the bath before the second capsaicin application enhance the capsaicin evoked responses with a response ratio = of 108.7 ± 12.5% (Fig 2B and D). In addition, bradykinin (100nM) via the bath perfusion system has ability to reverse desensitization of capsaicin responses (Fig 2C) with a response ratio of 115±11.8% (Fig 2E). In bradykinin experiments, coverslips were incubated with thapsigargin (2μM) before applying bradykinin in order to block the release of calcium from intracellular stores thus enabling the investigation of the signaling modulation by alternate pathways.

3.1.2 COX-1 but not COX-2 signaling mediates the bradykinin-enhancing capsaicin responses

The figure 3A and B shows representative traces of the effect of either SC560 (cox-1 inhibitor) or NS398 (COX-2 inhibitor) on calcium responses upon repeated capsaicin applications in the presence of bradykinin (100nM; 3 min). The SC560; 100nM), but not the COX-2 inhibitor (NS398; 10μM), had a significant inhibitory effect on the bradykinin-induced sensitization of capsaicin-induced calcium responses in DRG neurons (Fig 3 A and B). The response ratios of first and second capsaicin responses in the presence of bradykinin + SC560 and for bradykinin + NS398 were 56.9±8.1% (*$P < 0.05$ by one-way ANOVA followed by a Bonferroni's multiple comparison test) and 84.7 ± 10.6% ($P > 0.05$), respectively (Fig 3C).

3.1.3 EP_4 but not EP_3 signaling mediates bradykinin-enhancing capsaicin responses

The observation that bradykinin-mediated sensitization of capsaicin-induced calcium responses in cultured DRG neurons is inhibited by COX enzyme inhibitors and thus involves prostaglandins, led to speculation that prostaglandins, of unknown type, could be released from DRG neurons to act in an autocrine manner. Since it is already known that the EP_4 receptors for PGE_2 are important in the sensitisation of capsaicin responses by PKA modulation of TRPV1 ion channels (Lin et al. 2006), we examined the effect of EP receptor antagonists on the bradykinin-mediated sensitisation of capsaicin responses in cultured DRG neurons.

Figure 4A shows traces of calcium responses that resulted from the repeated capsaicin application (80nM; for 10 sec via U tube) in the presence of bradykinin (100nM; 3 min), after treatment with an antagonist cocktail of an EP_4 inhibitor (GW627368, 1μM) and an EP_3 inhibitor (L-798106; 1μM) for 10 min. In the presence of an antagonist cocktail, bradykinin failed to sensitise the second capsaicin-induced calcium response. The histogram in figure 4C shows that the mean response ratio for bradykinin + AC was 58.7 ±7 % which was significantly reduced (* $P<0.05$, one-way ANOVA and Bonferroni's multiple comparison test) from the mean response ratio observed with bradykinin alone.

Figure 4B shows representative traces of the effect of the EP_4 antagonist or an EP3 antagonist on capsaicin-induced calcium responses in the presence of bradykinin (100nM for 3 min). The EP_4 inhibitor, GW627368

(1μM), but not the EP_3 antagonist, L-798106 (1μM), significantly inhibited bradykinin-induced sensitisation of capsaicin sensitive neurons. The response ratios of first and second capsaicin responses in the presence of bradykinin + GW627368 and for bradykinin + L-798106 were 48.4 ± 7.6 % (*P < 0.05 one-way ANOVA followed by Bonferroni's multiple comparison test) and 90.7 ± 10.6 % (P >0.05), respectively.

3.1.4. DAG lipase is required for bradykinin-induced enhancing of capsaicin cells

The results above have shown that bradykinin-induced sensitisation of capsaicin-sensitive neurons is mediated by EP4 and cyclooxygenase. To investigate this further we looked at the DAG lipase branch of the PLC pathway since PLC activation results in the production of DAG which can then metabolised to arachidonic acid by cyclooxygenase enzymes, particularly the constitutively expressed isoform, COX-1. To confirm this, experiments were conducted to examine the effect of DAG lipase inhibitor, RHC-80267, on the bradykinin-induced enhancement of capsaicin-induced calcium responses in cultured DRG neurons.

Figure 5A representative traces showing the effect of the DAG lipase inhibitor RHC-80267 on capsaicin responses and its effect on bradykinin mediated sensitisation following bradykinin application. Capsaicin (80nm; 10seconds) was applied twice in absence and presence of BK (100nM; 3-minutes, bar) with second application in presence of BK and the DAG lipase inhibitor, RHC-80267 (20μM; 20minutes).

RHC-80267 (20μM; 20 min) attenuated the bradykinin-induced sensitisation of capsaicin-induced calcium responses (Fig 5B). The response ratio of the first and second capsaicin responses was 61.9± 4.9 % in the presence of (RHC-80267 +bradykinin) which was significantly reduced compared to the effect of bradykinin alone (115±11.8 %).(** $P< 0.01$, one-way ANOVA by Bonferroni's multiple comparison tests).

3.2 Immunohistochemistry experiments

3.2.1 Triple-label immunocytochemistry

To confirm the presence of three proteins, TRPV1, $EP_4$and B_2, in same cell, triple-label immunostaining with EP_4 (blue, 1:200), TRPV1 (green, 1:1000) and B_2 (red, 1:200) were conducted. Confocal images shown in figure 6A illustrate immunolabelling for the three proteins of interest.

Morphometric analyses of six ganglia have shown that all the three proteins were present 21.7% of DRG neurons. The size-frequency histogram presented in figure 6B shows that triple-labelling was limited to small neurons (96.4%, <1,000 μm^2), with only a small number of medium sized neurones labelled (3.6%, 1,000 -2,000μm^2), and no large neurons (>2,000 μm^2) labelled. The mean area of labelled neurons was 651.9 ± 15.2 μm^2.

3.2.2 Co-localization of EP_4 and COX-1 in dorsal root ganglia (DRG)

When the DRG cells were stained with two antibodies for both of EP_4 and COX-1, there was a co-expression of the two proteins. An example of EP_4 and COX-1 dual labelling is shown in figure 7A using confocal imaging of DRG neurons EP_4 specific antibody (red) and the COX-1 specific antibody (green). The frequency histogram of cell size derived for six ganglia in figure (7B) shows that 36% of DRG neurons express both EP_4 receptor and the COX-1 proteins, with a mean cell area of 849.1± 22.4μm^2.The dual labelling occurred mainly in small neurons (77.8%, <1000 μm^2), with fewer medium (20.1%, 1,000-2,000 μm^2) and large neurons (2.1%, >2,000 μm^2) labelled.

3.2.3 Co-localization of B_2 and COX-1 in dorsal root ganglia (DRG)

When the DRG cells were stained with antibodies for both B_2 and COX-1, there was a clear co-expression of the two proteins. An example of B_2 and COX-1 dual labelling is shown in figure 8A where confocal images have been taken using a B_2 spedific antibody (red) and a COX-1 specific antibody (green). The histogram of cell size frequency derived for six ganglia shown in figure 8B shows that 39.2% of DRG neurons express both B_2 receptor and COX-1 proteins, with a mean cell area of 955.7 ± 28.1 μm^2.

The dual labelling occurred mainly in small neurons (69.9%, <1,000 μm^2), some medium sized neurons (23.1%, 1,000-2,000 μm^2) and only very few large neurons (7%, >2,000 μm^2) dual labelled.

4- Discussion

The current study illustrates a novel signaling pathway in DRG neurons through which bradykinin can regulate the magnitude of TRPV1 channel responses. It shows that the bradykinin sensitization of capsaicin-evoked calcium responses in rat DRG neurons occurs as a result of formation of PGE2 by COX-1 which mediates sensitization of TRPV1 via the EP_4 but not EP_3 receptors. Inhibition of DAG-lipase significantly attenuated the bradykinin mediated sensitisation effect on capsaicin responses which confirms the DAG lipase branch of the PLC pathway.

The findings that bradykinin-induced sensitizating effect of capsaicin-evoked calcium responses in the DRG neurons is mediated by the formation of the prostaglandin E2 is consistent with other many *in vivo* studies demonstrating that bradykinin-evoked hyperalgesia was mediated by prostaglandins[28,30-32].

However, this study shows the co-localization of COX-1 with other proteins including EP_4, B_2 and TRPV1 involved in the proposed signaling pathway providing an anatomical for the proposed signaling pathway. It is particularly noteworthy that the immunocytochemical and morphometric data shows that B_2, EP_4, TRPV1 and COX-1 are expressed mainly in a subpopulation of putatively defined nociceptors in the rat lumbar DRG, i.e. mall-medium diameter neurons. COX-1 expression in rat DRG neurons was reported some time ago using different techniques such as western blotting[33,34],immunocytochemistry[35,36],and RT-PCR[33,36]although it was not demonstrated that it played a role in nociceptor signaling per se. . Likewise, , it has been known for several years that bradykinin, B_2 receptor mRNA is present in small DRG neurons as well[37,38], and Wang and colleagues showed that B_2 expression in occurs mainly in small peripherin positive DRG neurons which are a sub-type of nociceptors[39]. Several studies have demonstrated EP_4 expression and localization in DRG neurons in rats [40-42], and Ma and colleagues reported that the EP_4, TRPV1 and the inducible COX-2 protein is expressed in L4-6 DRG and plantar skin in rats. Following the repeated restraint stress, the level of expression of the EP4 and TRPV1 were significantly elevated in both L4-6 DRG and plantar skin, while the levels of COX-2 remained unchanged[43].

The finding in this study that COX-1 but not COX-2 is involved in bradykinin-mediated sensitization of capsaicin-evoked calcium responses is consistent with an earlier report studying the potentiating effect of bradykinin on capsaicin elicited calcium uptake in rat DRG[22] although the role of specific prostaglandin receptors was not demonstrated in that study Interestingly, several studies showed that the effect of bradykinin on sensory neurons can be abolished by NSAIDs which inhibit COX enzymes (COX-1 and COX-2). For example, bradykinin has a sensitizing effect to heat in C-polymodal nociceptors on rat skin nerve preparations through the cyclooxygenase (COX) pathway[44].

In the current study, the bradykinin-mediated sensitization of capsaicin-evoked calcium responses was significantly diminished in presence of EP_4 antagonist (GW627368) but not the selective EP_3 antagonist (L-798106). Figure 9 shows a summary of the possible signaling pathways that mediate bradykinin-sensitizing effect on TRPV1. These findings confirm the role EP_4 receptor in mediating bradykinin effects on TRPV1. In agreement with this finding, deleting EP_4 receptors but not the EP_1, EP_2 or EP_3 receptors from mice, reduced inflammation in vivo[45]. A role of EP_4 receptors in nociception has previously demonstrated since the oral administration of the EP_4 receptor antagonist, CJ-023,423 significantly diminished thermal hyperalgesia and mechanical allodynia in the carrageenan model of inflammation in rodents[46]. In addition to the novel pathway suggested here, a previous study has shown that pre-treatment of DRG neurons with PGE2, EP_4 and EP_2 agonists but not EP_3 agonists augments tetrodotoxin-resistant (TTX-R) Na^+ currents suggesting that specific prostaglandin moieties can mediate sensitization via different ion channel species..

Importantly the experiments described in this study were conducted in the presence of thapsigargin to inhibit the release of the calcium from intracellular stores in response to the activation by bradykinin. This strategy enabled us to investigate the pathway of prostaglandin formation upon bradykinin application excluding the effects of PLC mediated of calcium release from internal stores via $InsP_3$ receptors in the endoplasmic reticulum which would have interfered with our intracellular measurements of intracellular calcium changes. The finding in this study that blocking DAG-lipase significantly attenuated the bradykinin

mediated sensitisation effect on capsaicin responses is consistent with earlier report studying bradykinin activation of PLC in DRG neurons[47]. The generation of IP3, increase of intracellular calcium, and liberation of DAG activates the PKC pathway. It is suggested, therefore, that bradykinin can modulate TRPV1 through PKC mediated phosphorylation of the channel[47,48]. In our proposed model, Prostaglandins released in response to BK receptor activation may act on the same cells or it can diffuse freely to other cells or the same cell and bind to prostaglandin receptors on the cell surface. The resulting activation of EP4 receptors will result in TRPV1 phosphorylation via PKA leading to nociceptor sensitization.

The antagonists for EP_4 receptors used in previous studies for pharmacological inactivation of EP_4 subtype are not selective, and many of these compounds act at multiple prostanoid receptor subtypes. However, the present data indicate that GW627368, a highly potent and selective antagonist of EP_4 receptors[49], produces antihyperalgesic effects in animal models of inflammatory pain. Thus, specific blockade of the EP_4 receptor signaling may represent a novel therapeutic approach for the treatment of inflammatory pain, which retains the therapeutic benefits of NSAIDs and COX inhibitors without the gastrointestinal, cardiovascular, and renal side effects.

5- Conclusion

In conclusion, the present study indicated for the first time that prostaglandins, acting through the EP_4 receptor for PGE2 mediate the bradykinin-induced sensitization of capsaicin-evoked calcium responses in rat DRG neurons. This study also illustrates that modulation of TRPV1 might occur through one or more signalling pathways including the PLC-DAG-PKC pathway and a pathway that involves arachidonic acid -DAG lipase – COX –PGE2. These results shed light on the molecular mechanism underlying the signaling pathway for bradykinin in cases of thermal allodynia and hyperalgesia and help to describe the nociceptive hypersensitivity observed in primary afferent neurons. These findings could lead to the discovery of new and more effective targets for treating pain. Therefore, blocking EP_4 receptors could be a novel therapeutic avenue to prevent the development of chronic pain with fewer side effects.

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Figure legends

Fig 1: Schematic diagram represent the drug application via U tube to DRG neurons.

Fig 2. A) Representative traces of capsaicin (Cap) induced calcium responses to 80nM capsaicin applied twice for 10 seconds with a 10 minute interval between each application. KCl (60mM) was applied at the end of the experiment to confirm cell viability. **B)**Representative traces showing the sensitisation effect of 500nm PGE2 on capsaicin elicited calcium responses. Capsaicin (80nM; 10seconds) was applied twice with a 10 minute interval between each application. The second capsaicin application was performed in presence of PGE2 (500nM; 3-minutes). **C)** Representative traces showing the sensitisation effect of bradykinin (100nM; 3minutes) on capsaicin-evoked calcium response in cultured DRG neurons. Capsaicin (80nM; 10seconds) was applied twice with 10 minutes interval between each application. Bradykinin (100nM; 3-minutes) was also present during the second capsaicin application. **D)** Histogram showing the mean ± SEM response ratio (where the second response expressed as a percentage of the first one) for 98 cells (6 coverslips) in control and 115 cells (8 coverslips) with PGE2 exposed conditions. These were significantly different (two-tailed t-test, *P$<$ 0.05). **E)** Histogram showing the mean ± SEM response ratio (where the second response expressed as a percentage of the first one) for 98 cells (6 coverslips) in control and (101 cells; 6 coverslips) for the BK condition (*P$<$ 0.05).

Fig 3. COX-1 but not COX-2 is required for bradykinin mediated modulation of capsaicin-sensitive cells. **A)** Representative traces show the effect of the COX-2 inhibitor (NS398; 10μM) on capsaicin responses and its effect on sensitisation following bradykinin application. Capsaicin (80nm; 10seconds) was applied twice in the absence and presence of BK (100nM; 3-minutes, bar) with second application and in presence of NS398. **B)** Representative traces show the effect of the COX-1 inhibitor (SC560; 100nM) on capsaicin responses and its effect on sensitisation following bradykinin application. Capsaicin (80nm; 10seconds) was applied twice in the absence and presence of BK (100nM; 3-minutes, bar) with second application and in presence of SC560. **C)** Histogram showing mean ±SEM response ratio for control (98 cells; 6 coverslips) and in presence of the BK (101 cells; 6 coverslips), then in the presence of the BK along with each inhibitor. The number in parentheses indicates sample size the dashed line represents the bradykinin response ratio, (*P $<$ 0.05, one-way ANOVA followed by Bonferroni's multiple comparison test).

Fig 4. A) Representative traces showing the effect of prostaglandin receptor antagonists applied as a cocktail on the capsaicin-induced calcium responses in cultured DRG neurons response, and their effect on bradykinin mediated sensitisation of this response. Capsaicin (80nm for 10 seconds) was applied twice with a 10 minutes interval between each application in absence and presence of BK (bar, 100nM for 3 minutes) with second application in presence of antagonist cocktail for 10minutes. **B)** Representative traces showing the effect of prostaglandin receptor antagonists (EP4 antagonist, GW627368 or the EP_3 antagonist, L-798106) on the capsaicin-induced calcium responses in cultured DRG neurons response, and their effect on bradykinin mediated sensitisation of this response. Capsaicin (80nm for 10 seconds) was applied twice with a 10 minutes interval between each application in absence and presence of BK (bar, 100nM for 3 minutes) with second application in presence of antagonist (500nM for; 10 minutes).Histogram showing the mean peak heights (F510 S-R) ± SEM for repeated capsaicin application in presence of BK (100nM, 3-minutes) and (EP4 antagonist, GW627368X) before second capsaicin application, **C)** Histogram showing mean ±SEM response ratio for 98 cells (6 coverslips) in control and 101 cells (6 coverslips) with BK. then in the presence of the BK along with each antagonist. The number in parentheses indicate number of cells, the dashed line represents the bradykinin response ratio for comparison, (*P $<$ 0.05, one-way ANOVA followed by Bonferroni's multiple comparison test).

Fig 5. Bradykinin enhances capsaicin responses and requires DAG lipase activity. **A)** Representative traces

showing the effect of the DAG lipase inhibitor RHC-80267 on capsaicin responses and its effect on bradykinin mediated sensitisation following bradykinin application. Capsaicin (80nm; 10seconds) was applied twice in absence and presence of BK (100nM; 3-minutes, bar) with second application in presence of BK and the DAG lipase inhibitor, RHC-80267 (20μM; 20minutes). **B)**Histogram showing mean±SEM response ratio for control (98 cells; 6 coverslips), in the presence of bradykinin (101 cells; 6 coverslips), and in the presence of bradykinin + RHC-80267 (158 cells; 4 coverslips). The dashed line represents the bradykinin response ratio for comparison (**P < 0.01, ****P < 0.0001, one-way ANOVA followed by Bonferroni's multiple comparison test).

Fig 6. Triple label of EP_4, TRPV1 and B_2 in (L4-L6) rat DRG neurons. **A)** Individual DRG neurons labelled with EP_4 (green), B_2 (red), and TRPV1 (green) antibodies. The panel on the right shows a composite image of triple labels clearly demonstrating neurons that are labelled with three antibodies. **B)** Cell size frequency distribution derived from 6 ganglia triple labelled with EP_4, TRPV1 and B_2. Triple labelling was observed mainly in small cells (<1,000 μm^2) with a mean cell area of 651.9± 15.2 μm2.

Fig 7. Co-localisation of EP_4 and COX-1 in lumbar (L4-L6) rat DRG neurons. **A)** Individual DRG neurons labelled with COX-1 (green) and EP_4 (red) antibodies. The panel on the right shows a composite image of two labels clearly demonstrating neurons that are labelled with both antibodies.**B)** Cell size-frequency histogram derived from 6 ganglia shows that 36% of DRG neurons expressed both EP_4 and COX-1 proteins and 77% of dual labelled cells have cell area less than 1,000μm2 with a mean cell area of 849.1± 22.4μm2.

Fig 8. Co-localisation of COX-1 and B_2 in lumbar (L4-L6) rat DRG neurons. **A)** Individual DRG neurons labelled with COX-1 (green) and B_2 (red) antibodies. The panel on the right shows a composite image of two labels clearly demonstrating neurons that are labelled with both antibodies.**B)** Cell size- frequency histogram derived from 6 ganglia shows that 39% of DRG neurons expressed both B_2 and COX-1 proteins. The frequency histogram shows that 69% of dual labelled cells have cell area less than 1,000μm2 with a mean cell area of 955.7± 28.1μm2.

Fig 9 : A summary of the signalling pathways on the DRG neurones by bradykinin and PGE_2.