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Neuroscience Letters xxx (2014) xxx–xxx

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Prolonged exposure to bradykinin and prostaglandin E2 increases TRPV1 mRNA but does not alter TRPV1 and TRPV1b protein expression in cultured rat primary sensory neurons

Shilpa Mistry[a,1], Cleoper C Paule[a,1], Angelika Varga[a], Andy Photiou[b], Agnes Jenes[a,c], Antonio Avelino[d,e], Laki Buluwela[b], Istvan Nagy[a,*]

[a] Section of Anaesthetics, Pain Medicine and Intensive Care, Department of Surgery and Cancer, Imperial College London, Chelsea and Westminster Hospital, 369 Fulham Road. London, SW10 9NH, United Kingdom
[b] Division of Oncology, Department of Surgery and Cancer Imperial College London, Hammersmith Hospital, Du Cane Road, London, W12 0NN, United Kingdom
[c] Department of Physiology, University of Debrecen, Medical and Health Science Centre, Nagyerdei krt. 98, Debrecen, H-4012, Hungary
[d] Department of Experimental Biology, Faculty of Medicine, Alameda Prof. Hernani Monteiro, 4200-319, Porto, Portugal
[e] IBMC, University of Porto, Rua do Campo Alegre, 823, 4150-180, Porto, Portugal

HIGHLIGHTS

- BK- and PGE_2-evoked changes in TRPV1 and TRPV1b was assessed in cultured DRG neurons.
- Prolonged exposure to BK and PGE_2 increases capsaicin responsiveness of DRG neurons.
- Prolonged exposure to BK and PGE_2 increases TRPV1 but not TRPV1b mRNA expression.
- Prolonged exposure to BK and PGE_2 does not alter TRPV1 or TRPV1b protein expression.
- The role of TRPV1b in regulating channel responsiveness in DRG neurons is challenged.

ARTICLE INFO

Article history:
Received 3 December 2013
Received in revised form 22 January 2014
Accepted 3 February 2014

Keywords:
Pain
Inflammation
Dorsal root ganglion
Capsaicin

ABSTRACT

Sensitisation of the capsaicin receptor, transient receptor potential vanilloid type 1 (TRPV1) ion channel in nociceptive primary sensory neurons (PSN) underlies the development of inflammatory heat hyperalgesia. Removal of the negative-dominant splice variant of the TRPV1 molecule, TRPV1b from TRPV1/TRPV1b heterotetrameric channels, which should be associated with changes in the expression of TRPV1 and TRPV1b transcripts and proteins, has been suggested to contribute to that sensitisation. Respective reverse-transcriptase polymerase chain reaction (RT-PCR) and Western-blotting revealed that both TRPV1 and TRPV1b mRNA, and their encoded proteins are expressed in rat cultured PSN. Sequencing of the RT-PCR products showed that TRPV1b mRNA lacks the entire exon 7. Further, growing PSN for 2 days in the presence of 10 μM bradykinin (BK) and 10 μM prostaglandin E_2 (PGE_2) significantly increases TRPV1 responsiveness and TRPV1 mRNA expression, without producing any changes in TRPV1b mRNA, and TRPV1 and TRPV1b protein expression. These data challenge the hypothesis that alterations in the composition of the TRPV1 ion channel contributes to the sensitisation.

1. Introduction

Transient receptor potential vanilloid type 1 molecules, either on their own, or together with TRPV1 splice variants, or other members of the transient receptor potential vanilloid sub-family, assemble to form the non-selective cationic channel known as the capsaicin receptor (TRPV1 ion channel) [2,6,9,14,25,26]. When one of the splice variants of TRPV1, TRPV1b is co-expressed with TRPV1 in heterologous systems, an inhibitory effect on channel activity, as evoked by various TRPV1 activators, including capsaicin, is seen [14,25,26].

Nociceptive primary sensory neurons constitute the archetypical cell type that expresses TRPV1 [2,27]. In these neurons, the TRPV1 ion channel, through its increased responsiveness, is essential in

* Corresponding author. Tel.: +44 20 8746 8897; fax: +44 20 3315 5109.
E-mail address: i.nagy@imperial.ac.uk (I. Nagy).
[1] Shilpa Mistry and Cleoper C. Paule contributed equally to this work.

http://dx.doi.org/10.1016/j.neulet.2014.02.006
0304-3940/$ – see front matter © 2014 Published by Elsevier Ireland Ltd.

signalling peripheral inflammatory events to the central nervous system, and subsequently it plays a pivotal role in the development and maintenance of inflammatory heat hyperalgesia and visceral hyper-reflexia [1,4,8]. The co-expression of TRPV1 and TRPV1b molecules in PSN, and the negative dominant effect of TRPV1b on the channel's responsiveness found in heterologous systems [14,25,26] suggest that alterations in the composition of the TRPV1 ion channel might contribute to the inflammation associated increase in the responsiveness of this receptor. Such an alteration, in a long term, should be reflected by altered ratio in the expression of TRPV1 and TRPV1b transcripts and proteins. Hence, we hypothesised that culturing primary sensory neurons in an "inflammation-associated milieu" results in changes in the ratio of TRPV1 and TRPV1b mRNA and protein expressed by those cells.

2. Materials and methods

All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986, the revised National Institutes of Health *Guide for the Care and Use of Laboratory Animals*, the Directive 2010/63/EU of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes and the guidelines of the Committee for Research and Ethical Issues of IASP published in Pain, 16 (1983) 109–110. Every effort was taken to minimize the number of animals used.

2.1. Primary sensory neuron cultures

Cultures were prepared as described previously [17]. Briefly, DRG from the first cervical to the first sacral segment were dissected from 16 terminally anaesthetised Sprague-Dawley rats (80–100 grams) and collected in Ham's nutrient F12 culture medium (Sigma, Poole, UK) that was supplemented with 50 IU/mL penicillin (Invitrogen, Paisley, UK), 50 μg/mL streptomycin (Invitrogen) and 2% Ultroser G (Pall France, St-Germain-en-Laye Cedex France). Ganglia were incubated in 0.125% collagenase (Lorne Diagnostics, reading UK) for 3 h at 37 °C, then triturated and plated on poly-DL-ornithine (Sigma)-coated glass coverslips in the supplemented culture medium. Cells were cultured either in the absence (naive) or presence (exposed) of the inflammatory mediators, prostaglandin E_2 (PGE_2; Sigma; 10 μM) and bradykinin (BK; Sigma; 10 μM) for 2 days at 37 °C in the presence of 5% carbon dioxide.

2.2. Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA content was extracted both from naive and exposed cultures using the RNeasy Protect Mini Kit (Qiagen, Crawley, UK). The extracted RNA was reverse-transcribed using SuperScript II cDNA synthesis reagents (Invitrogen). The primer sequences were as follows: 5′-TGGAGGTGGCAGATAACACA-3′ forward and 5′ CCTTCCA-CAGGCCGATAGTA -3′ reverse. GAPDH primers were provided by Primerdesign Ltd. Amplification was performed in 3 mM $MgCl_2$, 5 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide mix, pH 8.3 and 1.25 U of Go-Taq Flexi DNA polymerase (Promega, UK). The amplification reaction consisted of 30 cycles with 30 s of denaturation at 96 °C, 1 min annealing at 59 °C, and 3 min extension at 72 °C in a thermal cycler (Eppendorf-Mastercycler Personal, UK). PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide. Expression was quantified by intensity measurements with the ImageJ software package. Intensity values were normalised to that of GAPDH.

2.3. Sequencing of rat TRPV1b

The RT-PCR products were purified from the agarose gels following electrophoresis using the QIAquick Gel extraction kit

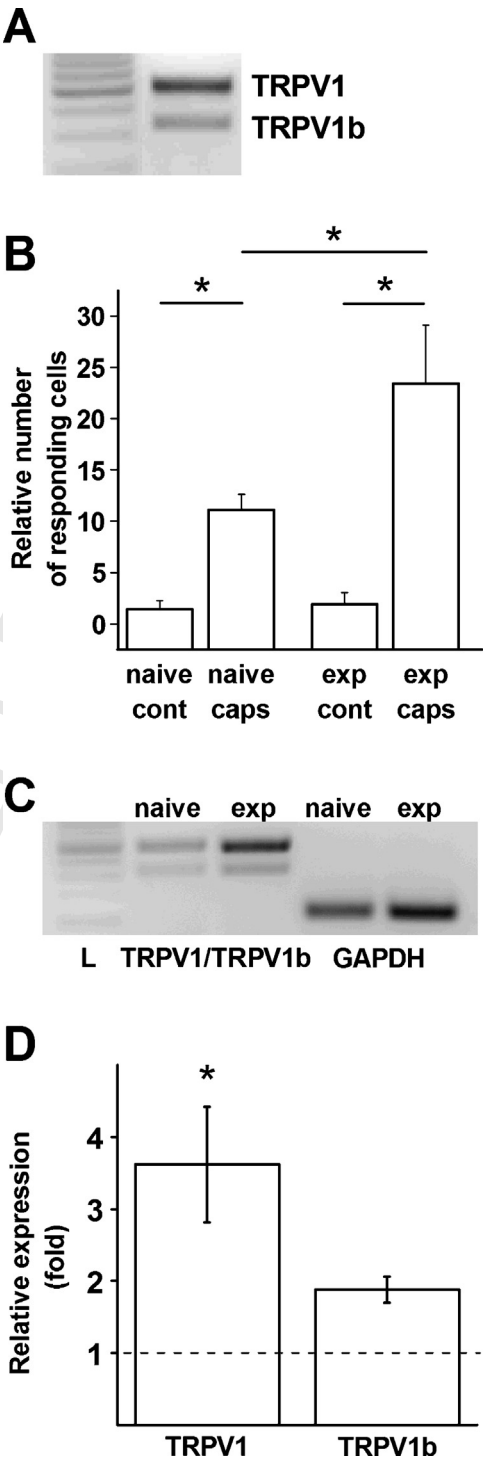

Fig. 1. (A) Agarose gel electrophoresis of RT-PCR products for TRPV1 and TRPV1b from cDNA made to RNA isolated from cultured primary sensory neurons. Note that with the TRPV1 primers we used, two products were detected: while one of the products was ~500 bp, the other was ~325 bp. (B) Relative number (number of labeled cell/number of total cells) of primary sensory neurons showing capsaicin-independent or 30 nM capsaicin-evoked cobalt accumulation following growing in control medium (naive) or in the presence of 10 μM bradykinin and 10 μM prostaglandin E_2 (exp) for two days. Note that addition of capsaicin to the cobalt buffer (see Methods) produced a significant increase in the number of cells accumulating cobalt in both conditions. Note also that the capsaicin-induced increase in the relative number of neurons showing cobalt accumulation is higher in the inflamed than in the naive condition. $n = 4$ at each data point. (C) Agarose gel electrophoresis of RT-PCR products for GAPDH, TRPV1 and TRPV1b from cDNA made to RNA isolated from cultured primary sensory neurons, which were grown either in control medium (naive) or in the presence of 10 μM bradykinin and 10 μM

(Qiagen, UK) and the microcentrifuge protocol. Sequencing was carried out using the ABI Prism automated sequencing system (Applied Biosystems 3100 16-capillary Genetic analyzer, Japan) using the primers used previously for RT-PCR (Fig. 1).

2.4. Western blotting

For preparation of whole cell extracts, or membrane proteins, of neurons, NP40 cell lysis buffer (Invitrogen, Carlsbad, CA) or ProteoJET™ Membrane Protein Extraction Kit (Fermentas, Burlington, Canada) were applied, respectively. Proteins were then denatured in NuPAGE LDS sample buffer (Invitrogen) at 70 °C for 10 min, fractionated by NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen) and blotted onto PVDF membrane with the use of iBlot® Dry Blotting System (Invitrogen). After blocking with 5% non-fat dry milk, the membrane was incubated with goat anti-TRPV1 polyclonal antibody (Santa Cruz, San Diego, CA) in a dilution of 1:200 at room temperature for 2 h. Horseradish peroxidase-conjugated donkey anti-goat IgG (1:10000, Santa Cruz) was applied to the membrane for 1 h, followed by detection using Western blotting luminal reagent (Santa Cruz). Membrane was stripped with 0.2 M glycine stripping buffer supplemented with 0.5% Tween-20 (pH 3.0) at room temperature for 30 min and re-probed with rabbit anti-β-actin as a loading control (1:1000, Cell Signaling Technology, Danvers, MA). Expression was quantified by intensity measurements with the ImageJ software package. Intensity values were normalised to that of β-actin.

2.5. Cobalt uptake

Cobalt uptake was used to ascertain BK and PGE_2 induced increase in TRPV1 ion channel responsiveness [19,21]. Briefly, cells attached to the coverslips were washed twice for 2 min at 37 °C with buffer A (in mM: NaCl 57.5, KCl 5, $MgCl_2$ 2, HEPES 10, glucose 12, sucrose 139; at pH 7.4). Cells were then incubated for 5 min at 37 °C in cobalt-uptake buffer (buffer A and 5 mM $CoCl_2$), which contained capsaicin (30 nM; Tocris, UK). In control experiments, cells were incubated for 5 min in capsaicin-free cobalt-uptake buffer. Cobalt accumulated in responding cells was visualized by 2% beta-mercaptoethanol dissolved in buffer A. Cells were put into 70% ethanol for 30 min, and the coverslips were mounted on glass slides with glycerol. Analysis of the cobalt uptake was carried out as described previously [19,21] using a light microscope attached to a CCD camera (Hamamatsu, Japan) and a PC running the Qwin software package (Leica, UK). Images for the analysis were selected systematically randomly. The staining intensity of at least 100 neurons was measured in each coverslip. Following establishing the threshold intensity for labelling, capsaicin responsiveness was expressed as the number of neurons labelled by cobalt accumulation above the threshold in the total number of cells.

2.6. Statistical analysis

Normalised changes in mRNA and protein expressions were analysed by the unpaired Student's *t*-tests. In the cobalt uptake experiments, the relative number of labelled cells found in each condition was averaged and analysed by the unpaired Student's *t*-tests or multiple analysis of variances (ANOVA) as appropriate. After ANOVA, the significance of the differences was assessed by Fischer's post-hoc least significant difference test. Differences were regarded as significant at $p < 0.05$. Data are expressed as mean ± standard error means, n refers to the number of cultures on which the given experiment was carried out. Each culture was prepared from a single experimental animal.

3. Results

3.1. Both TRPV1 and TRPV1b are expressed in cultured PSN

Rat TRPV1b lacks exon 7 either completely [20,29] or in part [14]. Hence, as expected, our primers flanking exon 7 amplified two products (Fig. 1A). The larger product had a size close to that predicted for the full length rat TRPV1 sequence, whereas the smaller product size was ~325 base pairs (Fig. 1A). DNA sequencing of the RT-PCR products confirmed that the respective sequence of the larger and smaller products corresponded to the relevant section of rat TRPV1 transcript (NM_031982, NCBI) with differences reflecting alternative splicing of 180 bases of sequence, encompassing exon 7 (Supplementary Fig. 1).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.02.006http://dx.doi.org/10.1016/j.neulet.2014.02.006.

3.2. Exposure of cultured rat PSN to BK and PGE_2 for two days increases the responsiveness of the cells to capsaicin

In cultures, incubated in capsaicin-free cobalt uptake buffer, only few neurons exhibited labelling above the detection threshold both in the naive and BK- and PGE_2-exposed cultures (1.4 ± 0.88%, $n = 4$, and 1.9 ± 1.12%, $n = 4$, in naive and BK- and PGE_2-exposed cultures, respectively; Fig. 1B, and Supplementary Figure 2A and B). The proportions of labelled cells in the control experiment between the two conditions were not significantly different from each other.

Addition of 30 nM capsaicin to the cobalt uptake buffer resulted in a significant increase in the number of labelled cells both in the naive (11.1 ± 1.5%; $n = 4$; $p = 0.001$) and BK- and PGE_2-exposed (23.4 ± 5.7%; $n = 4$; $p = 0.01$) cultures (Fig. 1B, and Supplementary Fig. 2C and D). The relative number of labelled cells in the BK- and PGE_2-exposed cultures was significantly higher than that in the naive cultures ($p = 0.01$).

Supplementry material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.neulet.2014.02.006http://dx.doi.org/10.1016/j.neulet.2014.02.006.

3.3. Exposure of cultured PSN to BK and PGE_2 for two days increases TRPV1 mRNA, but does not alter TRPV1b mRNA or TRPV1 and TRPV1b protein expression

We assessed TRPV1 and TRPV1b mRNA expression in cultures kept in control medium or in a medium containing BK and PGE_2 by semi-quantitative RT-PCR (Fig. 1C and D). Both the size of the RT-PCR products (Fig. 1C) and the sequences (data not shown) of both products were identical to the respective products and sequences in naive and BK- and PGE_2-exposed cultures. By measuring intensity of the products we found that while the expression of TRPV1 mRNA was significantly increased (362 ± 80%, $n = 4$; $p = 0.016$) that of TRPV1b mRNA was not (188 ± 18%, $n = 4$; $p = 0.07$; Fig. 1C and D). These changes meant that the TRPV1/TRPV1b mRNA expression ratio was significantly ($p = 0.028$) increased from 1.07 ± 0.2 ($n = 4$) in the naive cultures to 1.86 ± 0.18 ($n = 4$) in the BK- and PGE_2-exposed cultures.

The anti-TRPV1 antibody recognised two easily distinguishable (at ~110 kDa and ~88 kDa), and one faintly visible (~95 kDa), proteins both in the naive and BK- and PGE_2-exposed cultures in the whole cell lysates (Fig. 2A). In the membrane fractions, a fourth easily distinguishable protein (~75 kDa) has also been identified in

prostaglandin E_2 (exp) for 2 days. Note that exposure of neurons to bradykinin and prostaglandin E_2 induced changes in TRPV1 and TRPV1b mRNA expression. (D) Relative changes in the expression of TRPV1 and TRPV1b mRNA in primary sensory neuron cultures produced by the inflammatory mediators. Note that the relative expression of TRPV1 mRNA increased significantly, whereas the expression of TRPV1b mRNA was not changed. $n = 4$ at each data point.

Please cite this article in press as: S. Mistry, et al., Prolonged exposure to bradykinin and prostaglandin E2 increases TRPV1 mRNA but does not alter TRPV1 and TRPV1b protein expression in cultured rat primary sensory neurons, Neurosci. Lett. (2014), http://dx.doi.org/10.1016/j.neulet.2014.02.006

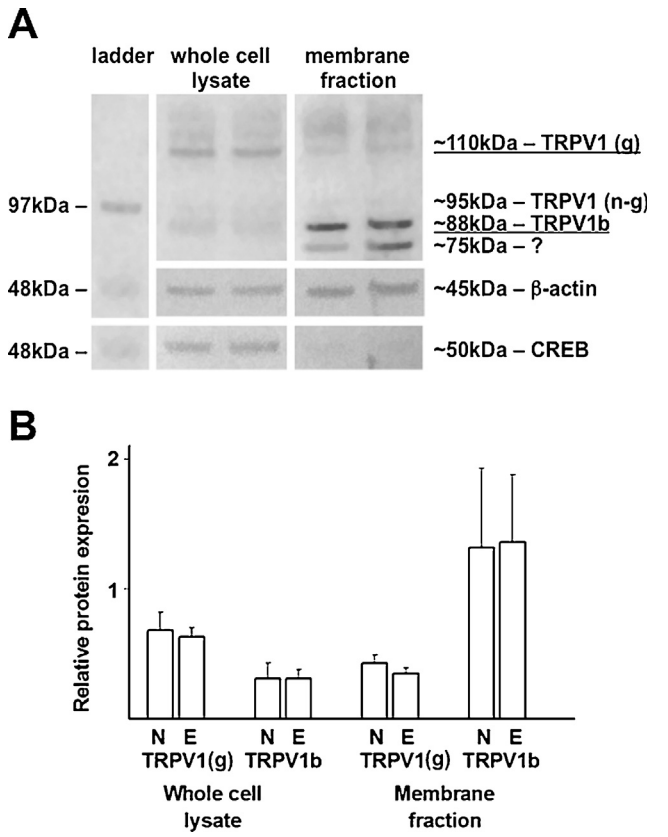

Fig. 2. (A) Representative immunoblotting for TRPV1 and TRPV1b in whole cell lysate and in membrane fraction from cultured primary sensory neurons grown in control medium (naive) or in the presence of 10 μM bradykinin and 10 μM prostaglandin E_2 (exp) for two days. Approximate molecular weights are indicated at the right side of each blot. Note that TRPV1 is expressed predominantly in glycosylated form (TRPV1 (g)) both in the whole cell lysate and the membrane fraction, whereas TRPV1b (~88 kDa) is expressed predominantly in the membrane fraction. The non-glycosylated form of TRPV1 (TRPV1 (n-g)) is only faintly visible in the membrane fraction. (B) Quantitative data of TRPV1 and TRPV1b expression in whole cell lysate and membrane fraction of primary sensory neurons grown in control medium (*N*) or in the presence of 10 μM bradykinin and 10 μM prostaglandin E_2 (E) for two days. Expression of TRPV1 and TRPV1b was normalised to the expression of β-actin. Note that growing of the neurons in the presence of bradykinin and prostaglandin E_2 did not induce significant change in the expression of TRPV1 or TRPV1b either in the whole-cell lysates of membrane fraction ($n = 3$).

both conditions (Fig. 2A). The ~110 kDa and ~95 kDa proteins correspond to the predicted molecular weight of the fully glycosylated full length TRPV1 and the non-glycosylated full length TRPV1, respectively [10,18]. Given that the TRPV1b mRNA contains 180 less bases than the TRPV1 mRNA (current study), and that Vos and colleagues [25] identified rat TRPV1b as an ~88 kD protein in rat DRG, it is feasible to assume that the ~88 kDa protein revealed in our Western blots by the TRPV1 antibody is the TRPV1b protein. The ~75 kDa protein found in the membrane fraction may be a product formed during the preparation of the fractions, as it could not be seen in whole cell lysates (Fig. 2A). Due to its faint appearance, the expression of the ~95 kDa protein was not quantified. Nevertheless, the non-glycosylated form of TRPV1, due to its low level of expression, might contribute little to TRPV1-mediated responses in cultured primary sensory neurons.

We found a marked difference between TRPV1 and TRPV1b expression in the whole cell lysates and membrane fractions (Fig. 2A and B). We also found that BK and PGE_2 did not induce significant change in the expression of either the TRPV1 or TRPV1b protein in the whole cell lysates (TRPV1 ratio in naive/ BK- and PGE_2-exposed $= 0.9 \pm 0.09$ ($n = 3$); TRPV1b ratio in naive/ BK- and PGE_2-exposed $= 1.12 \pm 0.27$ ($n = 3$); Fig. 2A and B). Similarly, the inflammatory mediators did not induce significant changes either in TRPV1 (naive/BK- and PGE_2-exposed $= 0.81 \pm 0.02\%$, $n = 3$), or in TRPV1b (naive/BK- and PGE_2-exposed $= 1.35 \pm 0.42$, $n = 3$) protein expression in the membrane fractions (Fig. 2A and B).

4. Discussion

4.1. The rat TRPV1b splice variant

Sequencing the two RT-PCR products generated by primers flanking exon 7 revealed that while the larger product corresponds perfectly to the appropriate section of rat TRPV1 mRNA [2], the smaller transcript lacks 180 bases of this mRNA. Those 180 bases represent the entire exon seven [9]. While this splicing is different from that described previously as rat TRPV1b, it is similar to that seen for human TRPV1b [14].

Previously, a splice variant, TRPV1β that lacks 30 base pairs in exon 7 has also been described [26]. Regardless of the structural differences between TRPV1b and TRPV1β, both splice variants fail to respond to vanilloids and protons, and have attenuated responses to noxious heat [14,25,26]. When co-expressed with the full length TRPV1, both TRPV1b and TRPV1β reduce the responsiveness of the ion channel in a ratio-dependent manner [26]. Hence, we regard the smaller transcript amplified from rat DRG in our samples the rat homologue of TRPV1b.

Semi-quantitative RT-PCR analysis, in this study, revealed a TRPV1/TRPV1b mRNA expression ratio of ~1 in rat cultured PSN. This ratio is significantly smaller than that found by Vos and colleagues (~14) [25] in intact human DRG indicting that rat cultured PSN express more copies of TRPV1b mRNA or/and less copies of TRPV1 mRNA than human PSN *in situ*.

Our findings indicate that both the TRPV1 and TRPV1b transcripts are translated into proteins in cultured PSN. We did not study the amino acid sequence of the ~88 kD protein identified by the TRPV1 antibody we used. Further, direct identification of TRPV1b is not feasible at present as no antibody recognising this splice variant specifically and selectively is available. Nevertheless, based on the size of the TRPV1b mRNA and on previous findings [25], it is feasible to assume that the ~88 kD protein recognised by the TRPV1 antibody is the TRPV1b protein. Quantification of the TRPV1 and TRPV1b immunoblots showed that, in the whole cell lysate, the relative expression level of TRPV1 and TRPV1b proteins corresponds well to the relative expression level of TRPV1 and TRPV1b mRNA. Intriguingly however, the relative expression level of the TRPV1b protein in the membrane fraction was almost 3 times higher than that of the TRPV1 protein.

4.2. Inflammatory mediator-induced changes

Although, inflammation is associated with the production, release and accumulation of a myriad of agents, among them, regarding the development and maintenance of pain, BK and PGE_2 are believed to have the highest importance [15]. Both BK and PGE_2 induce rapid TRPV1 phosphorylation which results in a significant reduction in the heat threshold of the ion channel [3,7,12,13,23]. Due to the integration of effects on the channel's gating apparatus produced by various TRPV1 activators [11,24], that reduction results in increased TRPV1-mediated responses including capsaicin-induced cobalt accumulation [3,7,12,13,21,23]. We found that, in addition to increasing capsaicin-responsiveness, BK and PGE_2, during prolonged application, also up-regulates *trpv1* transcription, which results in uneven increase in TRPV1 and TRPV1b mRNA expression. In contrast to this finding, no evidence for increased *trpv1* transcription was found in our recent *in vivo* experiment [5]. The discrepancy in gene transcription between the *in vivo* and *in*

vitro studies could be due to the differences in the mediators found in inflamed tissues [15,28] and used to create the inflammation-associated milieu in the present study, as well as in the proportion of the sampled PSN which were exposed to the mediators *in vitro* and *in vivo*.

In spite of the BK- and PGE_2-induced up-regulation in TRPV1 mRNA expression, TRPV1 protein expression was not changed either in whole cell lysates or membrane fractions. This mismatch between changes in TRPV1 mRNA and protein expression could be due to yet unidentified regulatory mechanisms. Alternatively, it could be due to activity-dependent increase in the turnover of the TRPV1 protein. Such an increase is highly likely to occur in the presence of BK and PGE_2, because the ambient temperature in the incubator should keep the post-translationally-modified channel activated to a certain level [3,7,12,13,23].

While TRPV1 and TRPV1b protein expression was not altered, the capsaicin-induced, hence TRPV1 ion channel-mediated cationic influx was significantly enhanced by exposing neurons to BK and PGE_2. Therefore, the most parsimonious explanation for the development of the sensitised state of TRPV1 is that it is produced by BK- and PGE_2-induced post-translational modifications of TRPV1 alone [3,7,12,13,16,22,23]. However, it is also possible that BK- and PGE_2 exposure induces, without changing TRPV1 and TRPV1b protein expression, re-arrangement of TRPV1 and TRPV1b subunits. Such a re-arrangement might result in the formation of TRPV1 and TRPV1b homotetramers in inflamed conditions from TRPV1/TRPV1b heterotetramers found in naive conditions. Hence, the higher number of fully responsive TRPV1 homotetramers, without increased TRPV1 protein expression, could mediate the increased capsaicin-evoked responses. Clearly, providing evidence for such a re-arrangement requires further studies, which, at present, are hindered, by the lack of a selective and specific anti-TRPV1b antibody. Nevertheless, the high level of expression of the negative dominant TRPV1b splice variants in the membrane fraction suggests that TRPV1b could indeed have an important role in regulating the responsiveness of the channel, and supports the view that changes in the composition of the ion channel could contribute to increased responsiveness of the capsaicin receptor in inflammatory conditions.

Acknowledgements

This work was supported by a Biotechnology and Biological sciences Research Council – GlaxoSmithKline CASE award (Cleoper C Paule); a National Institute of Academic Anaesthesia project grant (Agnes Jenes), European Commission Marie Curie Intra-European Fellowship (254661; Angelika Varga) and Wellcome Trust project grant (061637/Z06/Z; Andy Photiou).

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Please cite this article in press as: S. Mistry, et al., Prolonged exposure to bradykinin and prostaglandin E2 increases TRPV1 mRNA but does not alter TRPV1 and TRPV1b protein expression in cultured rat primary sensory neurons, Neurosci. Lett. (2014), http://dx.doi.org/10.1016/j.neulet.2014.02.006