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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

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HIGHLIGHTS
- BK- and PGE2-evoked changes in TRPV1 and TRPV1b was assessed in cultured DRG neurons.
- Prolonged exposure to BK and PGE2 increases capsaicin responsiveness of DRG neurons.
- Prolonged exposure to BK and PGE2 increases TRPV1 but not TRPV1b mRNA expression.
- Prolonged exposure to BK and PGE2 does not alter TRPV1 or TRPV1b protein expression.
- The role of TRPV1b in regulating channel responsiveness in DRG neurons is challenged.

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 E2 (PGE2) 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.

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1. Introduction
The 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 heterological 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 archetypal cell type that expresses TRPV1 [2, 27]. In these neurons, the TRPV1 ion channel, through its increased responsiveness, is essential in

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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 E2 (PGE2; 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' CCTCCA- CAGGCGGATAAG 3' reverse. GAPDH primers were provided by Primerdesign Ltd. Amplification was performed in 3 mM MgCl2, 5 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl2, 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](image-url)

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 E2 (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. p = 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...
prostaglandin E2 (exp) for 2 days. Note that expression of neurons to bradykinin and prostaglandin E2 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.

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).

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

3.2. Exposure of cultured rat PSN to BK and PGE2 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 PGE2-exposed cultures (1.4 ± 0.88%, n = 4, and 1.9 ± 1.12%, n = 4, in naive and BK- and PGE2-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%; n = 4; p = 0.001) and BK- and PGE2-exposed (23.4 ± 5.7%; n = 4; p = 0.01) cultures (Fig. 1B, and Supplementary Figure 2C and D). The relative number of labelled cells in the BK- and PGE2-exposed cultures was significantly higher than that in the naive cultures (p = 0.01).

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

3.3. Exposure of cultured PSN to BK and PGE2 for two days increases TRPV1 mRNA, but does not alter TRPV1b mRNA or TRPV1b protein expression

We assessed TRPV1 and TRPV1b mRNA expression in cultures kept in control medium or in a medium containing BK and PGE2b 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 PGE2-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 TRPV1/1TRPV1b 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 PGE2-exposed cultures.

The anti-TRPV1 antibody recognised two easily distinguishable (at ~110 kDa and ~88 kDa), and faintly visible (~95 kDa), proteins both in the naive and BK- and PGE2-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...
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 kDa 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 PGE2 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 PGE2-exposed = 0.9 ± 0.09, n = 3; TRPV1b ratio in naive/ BK- and PGE2-exposed = 1.12 ± 0.27, n = 3; Fig. 2A and B). Similarly, the inflammatory mediators did not induce significant changes either in TRPV1 (naive/BK- and PGE2-exposed = 0.81 ± 0.02, n = 3), or in TRPV1b (naive/BK- and PGE2-exposed = 1.35 ± 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, TRPV1b that lacks 30 base pairs in exon 7 has also been described [26]. Regardless of the structural differences between TRPV1b and TRPV1b, 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 TRPV1b 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 indicating 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 kDa 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 kDa 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 PGE2 are believed to have the highest importance [15]. Both BK and PGE2 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 capsacin-induced coxal accumulation [3,7,12,13,21,23]. We found that, in addition to increasing capsacin-responsiveness, BK and PGE2, 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 results of the current study is not straightforward. It is possible that some of the effects of BK and PGE2 at the transcription level may be related to their effects on protein expression at the post-transcriptional level. Further studies are required to elucidate the mechanisms underlying the effects of BK on in vivo expression of TRPV1.

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vi tro 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 PGE2-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 PGE2, 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 PGE2. Therefore, the most parsimonious explanation for the development of the sensitised state of TRPV1 is that it is produced by BK- and PGE2-induced post-translational modifications of TRPV1 alone [3,7,12,13,16,22,23]. However, it is also possible that BK- and PGE2 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 TRPV1/1b heterotetramers found in naïve 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-TRPV1 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.

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