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Human odontoblasts express functional thermo-sensitive TRP channels: Implications for dentin sensitivity

Ikhlas A. El Karim^a, Gerard J. Linden^a, Timothy M. Curtis^a, Imad About^b, Mary K. McGahon^a, Chris R. Irwin^a, Fionnuala T. Lundy^{a,*}

^aSchool of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, Belfast, UK

^bLaboratoire IMEB, Faculté d'Odontologie, Université de la Méditerranée, Marseille, France

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ABSTRACT

Odontoblasts form the outermost cellular layer of the dental pulp where they have been proposed to act as sensory receptor cells. Despite this suggestion, evidence supporting their direct role in mediating thermo-sensation and nociception is lacking. Transient receptor potential (TRP) ion channels directly mediate nociceptive functions, but their functional expression in human odontoblasts has yet to be elucidated. In the present study, we have examined the molecular and functional expression of thermo-sensitive TRP channels in cultured odontoblast-like cells and in native human odontoblasts obtained from healthy wisdom teeth. PCR and western blotting confirmed gene and protein expression of TRPV1, TRPA1 and TRPM8 channels. Immunohistochemistry revealed that these channels were localised to odontoblast-like cells as determined by double staining with dentin sialoprotein (DSP) antibody. In functional assays, agonists of TRPV1, TRPA1 and TRPM8 channels elicited $[Ca^{2+}]_i$ transients that could be blocked by relevant antagonists. Application of hot and cold stimuli to the cells also evoked rises in $[Ca^{2+}]_i$, which could be blocked by TRP-channel antagonists. Using a gene silencing approach we further confirmed a role for TRPA1 in mediating noxious cold responses in odontoblasts. We conclude that human odontoblasts express functional TRP channels that may play a crucial role in mediating thermal sensation in teeth.

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1. Introduction

Tooth (or dentin) hypersensitivity is an exaggerated response, characterised by short, sharp pain, arising from stimulation of exposed dentin [22]. Dentin hypersensitivity is a common finding in adult populations [20]. The underlying mechanisms of dentin sensitivity, and hypersensitivity, are not clearly understood and of the numerous advocated theories, the most widely accepted is the hydrodynamic theory [10]. This postulates that nociceptive responses may result from the activation of nerve endings resulting from an increase in intradentinal pressure due to fluid movements in dentinal tubules. However, based on this hydrodynamic concept it would be difficult to explain why there is so much variation in dentin sensitivity to different stimuli. It has been proposed that dentinal fluid flow may not be the only mechanism involved in thermal sensation in human teeth [27] and recent studies have demonstrated a lack of correlation between human pain sensation and dentinal fluid movement after cold stimulation [14].

Emerging evidence supports a role for odontoblasts as sensory cells that directly mediate tooth pain sensation [32]. Odontoblasts constitute the outermost cellular layer of the dental pulp and have cytoplasmic extensions which extend throughout the dentin. These odontoblast processes are housed within the dentinal tubules and lie in close proximity to sensory unmyelinated nerve fibres which extend from the dental pulp to the inner half of the dentin [11]. The idea that odontoblasts may sense external stimuli and transduce the signal to nearby nerves cells is supported by the close apposition of the odontoblasts to the dentinal nerve terminals [11,34]. In addition, odontoblasts are known to express several classes of ion channels which have been implicated in sensory reception and signal propagation, including L-type Ca^{2+} channels, mechanosensitive K^+ channels and voltage-gated Na^+ channels [3,33,4]. Although these findings support a role for odontoblasts in sensory transduction in teeth, the precise mechanisms through which these cells detect nociceptive stimuli has yet to be fully elucidated.

Thermal changes in the oral cavity are one of the most common triggers of dental pain [40]. Several members of the transient receptor potential (TRP) superfamily of ion channels are believed to play a critical role in sensory physiology, where they act as transducers for thermal, mechanical and chemical stimuli. Recent studies have shown that some members of the TRPV (vanilloid),

Table 1

Primer sequence and cycling conditions used for gene expression experiments.

Gene	Primer sequence (3' → 5')	Annealing Temp. (°C)	Cycles	Product size (bp)	Accession number
DSPP	Forward CCT AAA GAA AAT GAA GAT AAT T Reverse TAG AAA AAC TCT TCC CTC CTA C	51	36	293	AJ002141
Nestin	Forward GGC AGC GTT GGA ACA GAG GTT GGA Reverse CTC TAA ACT GGA GTG GTC AGG GCT	55	30	718	AJ270321
TRPA1	Forward TGGTGACACAAATAGACCCAGT Reverse TGGGCACCTTTAGAGAGTAGC	60	35	318	AY403101
TRPM8	Forward GATTTTCACCAATGACCGCCG Reverse CCCAGCAGCATTGATGTCG	60	40	503	AY328400
TRPV1	Forward CTCCTACAACAGCCTGTAC Reverse AAGGCCTTCCTCATGCACT	60	35	285	NM_080706

TRPM (melastatin) and the TRPA (ankyrin) subfamilies encode “thermo-TRPs” that respond to moderate or noxious changes in the external temperature. Examples include TRPV1 which responds to noxious heat, and TRPM8 and TRPA1 which mediate cool and noxious cold sensations, respectively [12,41,47,7]. Heat-sensitive TRP channels have been demonstrated in rat trigeminal ganglion neurons [39] and TRPV1 is known to be expressed in rat odontoblasts [37,45]. No studies to date have addressed the expression and functional significance of thermo-sensitive TRP channels in human odontoblasts. In the present study, we show that human odontoblasts express TRPA1, TRPV1 and TRPM8 at

both gene and protein level and confirm their functionality in response to chemical agonists and thermal stimuli.

2. Materials and methods

2.1. Cell culture

Immature permanent third molar teeth were obtained in accordance with French ethics legislation (informed patient consent and Institutional Review Board approval of the protocol used). Human odontoblast-like cells were derived from dental pulp as previously

Fig. 1. Molecular expression of thermo-sensitive TRP channels in human odontoblast-like cells using PCR and western blotting. (A) PCR product for odontogenic markers dentin sialophosphoprotein (DSPP) (left lane) and nestin (right lane) in human odontoblast-like cells. (B–D) 2% Agarose gel electrophoresis showing PCR amplification product for TRPA1, TRPV1 and TRPM8 respectively, all PCR products were detected at the predicted sizes (Table 1). (E–G) Western blots for TRPA1, TRPV1 and TRPM8, respectively, in odontoblast-like cells detected at the predicted sizes. Representative results from at least three independent experiments are shown.

described [1]. In brief, dental pulp cells derived from dental pulp explants were grown in minimal essential medium with L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin and 100 µg/ml streptomycin. To differentiate pulp cells to odontoblasts the media were supplemented with 2 mM β-glycerophosphate. The odontoblastic phenotype of the cells was confirmed by the expression of the odontoblast markers dentin sialophosphoprotein (DSPP) and nestin. Cells in passage 4–6 were used in all subsequent experiments.

2.2. Dental pulp slice preparation

Dental pulp slices were prepared from healthy molar teeth obtained with the approval of the Office for Research Ethics Committees (Northern Ireland). Immediately after extraction, teeth were placed on ice cold Hanks solution and all gingival and periodontal tissues were removed with scalpel blades. Teeth were then clamped to the specimen chuck of an ISOMET Low Speed Saw (Buehler, Lake Bluff, IL), and sectioned transversely through the pulp, at a speed of 100 RPM, into 500 µm thick slices. During sectioning, the tooth/blade interface was continually bathed in cold Hanks solution. Slices were collected immediately after sectioning

and washed with Hanks solution prior to incubation in a solution containing 2 mg/ml collagenase Type 1 A (Sigma, UK) for 30 min at 37 °C. Following incubation, slices containing both dentin and pulp tissue were washed in Hanks solution and placed in glass dishes pre-coated with poly D-lysine and maintained in minimal essential medium with L-glutamine, supplemented with 10% FBS, 100 UI/ml penicillin and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C.

2.3. RNA extraction, cDNA synthesis, and reverse transcriptase-PCR

For RNA extraction cells were grown to 70% confluence in T75 flasks in minimal essential medium with L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin, 100 µg/ml streptomycin and 2 mM β-glycerophosphate. Total RNA from cells grown in culture was prepared using the RNeasy MiniKit (Qiagen) according to the protocol provided by the manufacturer. cDNA was synthesized from 1 µg of extracted RNA with the Superscript™ First Strand Synthesis System (Invitrogen, Paisley, UK) and an oligo dT primer mix, according to the manufacturer's guidelines. For conventional RT-PCR sense and antisense PCR primers specific to human TRPA1, TRPV1 and TRPM8 and the

Fig. 2. Confocal microscopy images of double staining immunocytochemistry demonstrating localisation of TRP channels to cultured human odontoblast-like cells. Odontoblast-like cells identified by positive staining with dentin sialoprotein (DSP) (green) were also positive for TRPA1 (red) and co-localisation was evident when images were merged (yellow). Similarly expression of TRPV1 and TRPM8 co-localised with DSP is shown. A representative image of TRPM8 antibody pre-incubated with blocking peptide showed no TRPM8. Cells were counterstained with DAPI for nuclear enhancement (blue).

odontogenic markers nestin and DSPP were used (Table 1). PCR reactions were carried out using Platinum *Taq* DNA polymerase (Invitrogen) and reaction conditions as listed in Table 1. PCR products were analyzed by electrophoresis in 2% agarose gel and visualized by Ethidium Bromide staining. Parallel reactions were run for each RNA sample in the absence of reverse transcriptase to confirm that there was no genomic DNA contamination.

2.4. Western blotting

Cultured cells were lysed for 30 min on ice with 10 mM Tris-HCL buffer (pH 7.4) containing 1% SDS and 'Complete' protease inhibitor cocktail (Roche, UK). Total protein in the cell lysate was determined using the bicinchoninic acid (BCA) protein assay and equal amounts of protein (27 µg) were fractioned by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose. The blots were blocked in 5% non fat milk for 1 h prior to probing with rabbit polyclonal primary antibody to TRPA1 (ab31486; Abcam, UK), or TRPM8 (ab3243; Abcam, UK), or mouse monoclonal antibody to TRPV1 (H00007442-M01; Abnova, USA) at 1:500 dilution overnight. Detection of the bound primary antibody was achieved using appropriate anti-species antibody conjugates (Dako, Denmark) and chemiluminescent substrates. All western blotting and PCR images were obtained using a Syngene G box imaging system.

2.5. Immunohistochemistry

For double staining immunohistochemistry, cells were grown on cover slips, acetone-fixed and processed for indirect immuno-

histochemistry using primary rabbit polyclonal antibodies against human TRPA1(ab68846; Abcam, UK), TRPV1 (ab63083; Abcam, UK) and TRPM8 (ACC-049; Alomone Labs, Jerusalem) and mouse monoclonal anti-human dentin sialoprotein (DSP) antibody (Sc-73632; Santa Cruz, USA). Appropriate anti-species Alex Fluor 488 secondary antibody conjugates and Alexa Fluor 594 (Invitrogen, UK) were used for secondary detection. Briefly, specimens were incubated with primary rabbit antisera diluted 1:200 overnight at room temperature and then washed extensively with PBS before they were incubated overnight with DSP antibody (1:200). Following washing with PBS a 1:500 dilution of secondary antibody was then applied for 45 min at 37 °C. Tissue sections were washed again before mounting in VectaShield with DAPI (4',6 diamidino-2-phenylindole) (Vector Lab., Burlingame, CA) to counterstain the nuclei. Control sections were incubated with non-immune serum in place of primary antibody. As additional controls, antibodies were pre-incubated with blocking peptides for 30 min prior to staining as described above.

For immunohistochemical localisation of TRPA1, TRPV1 and TRPM8 in human pulp tissue, healthy third molar teeth were fixed in a 4% formol solution and demineralised in a solution of 3.4% sodium formate in 17% formic acid until fully decalcified for histological treatment. Samples were routinely processed through a series of graded alcohols and xylene, and finally embedded in paraffin wax. Rehydrated paraffin embedded sections (7 µm) from human third molars were incubated with rabbit and mouse monoclonal antibody against TRPV1 (H00007442-M01; Abnova, USA) at 1:200 dilution and anti-human TRPA1 antibody (ab31486; Abcam, UK) or TRPM8 antibody (ACC-049; Alomone

Fig. 3. Immunohistochemical localisation of (A) TRPA1, (B) TRPV1 and (C) TRPM8 in human odontoblasts *ex-vivo*. Streptavidin-biotin staining of decalcified sections of healthy molar teeth. The odontoblast layer (OD) is the outermost cell layer of the dental pulp (P) and is in close contact with dentin (D). TRPA1 is abundantly expressed in odontoblasts and sparsely in the cell rich zone layer underneath the odontoblasts (A). Both TRPV1 and TRPM8 are also detected mainly on odontoblasts (B and C), and there was also evidence of staining in dentinal tubules (B, arrows). Sections incubated in the absence of antibodies showed no staining; a representative image is shown in (D). All sections were counterstained with H&E. Scale bar = 50 µm.

Labs, Jerusalem) at 1:100 dilution overnight at 4 °C. Staining was revealed using the labelled streptavidin–biotin method (LSAB) (DakoCytomation, USA) according to the manufacturer's instructions.

Fluorescence immunohistochemistry images were obtained using a Leica TCS SP5 confocal microscope and light microscopy images were visualised using a Leica DMLB microscope.

2.6. $[Ca^{2+}]_i$ measurements

$[Ca^{2+}]_i$ was estimated fluorometrically in both cultured and native odontoblasts, still embedded within dental pulp slice preparations, loaded with the intracellular Ca^{2+} probe fura-2AM. Cultured cells were grown on glass coverslips overnight in minimal essential medium with L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 UI/ml penicillin and 100 µg/ml streptomycin prior to incubation in the dark with fura-2AM (5 µM) for 1 h at

37 °C. Similarly dental pulp slice preparations were loaded with fura-2AM (20 µM) for 1 h at 37 °C.

The coverslips bearing the adherent cells or dental pulp slice preparations were then placed in a recording chamber mounted on the stage of an inverted microscope (Nikon Eclipse TE2000) and superfused with Hank's solution at 37 °C. Only cells on the rim of the dental pulp corresponding to the anatomical location of native odontoblasts were used for recording from pulp slice preparations. The temperature of the chamber was controlled with an inbuilt Peltier device (PTC-10; ALA Scientific). Alternating excitation wavelengths of 340 and 380 nm light were delivered from a dual monochromator (5 nm bandwidth) using a light chopper (Cairn Research Ltd., Faversham, UK). Emitted fluorescence was measured from the side port of the microscope via an adjustable rectangular window, a filter (510 nm) and a photon counting photomultiplier tube (PMT) in the light path. Fluorescence

Fig. 4. Pharmacological activation of thermo-sensitive TRP channels in odontoblast-like cells. Each panel consists of a representative trace of $[Ca^{2+}]_i$ in response to both agonist and antagonist and a bar chart of summary of average responses. (A) Significant increase in $[Ca^{2+}]_i$ in response to 100 µM Icilin which was blocked in the presence of 10 µM RuR. (B) The response of odontoblast-like cells to 50 µM cinnamaldehyde was attenuated by 20 µM HC-030031. (C) The odontoblasts response to 10 µM capsaicin induced significant increase in $[Ca^{2+}]_i$, which was blocked by 10 µM capsazepine. (D) and (E) the significant increase in $[Ca^{2+}]_i$ in response to 1 mM menthol was only blocked in the presence of 10 µM capsazepine (capz) but retained in the presence of RuR, indicating a selective TRPM8 response. Bar charts represent mean effect of individual agonist compared to baseline and antagonists, error bars indicate SEM, ANOVA, ** $p \leq 0.01$; * $p \leq 0.05$.

Fig. 4 (continued)

equipment was controlled by Acquisition Engine (Cairn) software (V1.1.5) which was also used for analysis of the fluorescence data. At the end of each experiment, background fluorescence was quantified by incubation of the cells with 5 mM Mn^{2+} in Ca^{2+} -free solution. Changes in the ratio of the background-corrected fluorescence emitted at each excitation wavelength ($R = F_{340}/F_{380}$) was used as a measure of changes in the cytoplasmic Ca^{2+} concentration [21]. A positive Ca^{2+} response was defined as a change in the F_{340}/F_{380} ratio >4 standard deviations above the background noise.

Cells were stimulated with the agonists capsaicin, menthol, cinnamaldehyde and icilin. Thermal stimuli were induced by increasing or decreasing temperature in the recording chamber at a rate of 0.13 $^{\circ}C/s$ using an inbuilt Peltier device (PTC-10; ALA Scientific). The TRP antagonist ruthenium red (RuR), the selective TRPV1 antagonist capsazepine (capz) and TRPA1 selective antagonist (HC-030031) were used to block the corresponding agonist and thermal responses.

2.7. Small interference RNA (siRNA)

Gene knockdown of human TRPA1 was performed by using HP Genome Wide siRNA (Qiagen UK). For siRNA transfection cells were grown in six-well plates to 30–50% confluence in minimal essential medium with L-glutamine supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 $\mu g/ml$ streptomycin. 6 μl TRPA1 siRNA was incubated with 14 μl transfection reagent (InterferinTM, Autogen Bioclear, UK) in 180 μl serum free media for 15 min at room temperature to allow for formation of transfection complexes. The mixture was then added to each of the culture wells containing 2 ml of minimal essential medium with L-glutamine supplemented with 10% fetal bovine serum and the cells incubated for 72 h (final siRNA concentration, 50 nM).

Western blot analysis was performed to confirm knockdown of TRPA1 protein expression. All experimental procedures were performed 24 h following the 72 h transfection period.

2.8. Drugs and solutions

Capsaicin, menthol, icilin, cinnamaldehyde, capsazepine, ruthenium red and β -glycerophosphate were obtained from Sigma UK, TRPA1 selective antagonist (HC-030031) from Tocris, UK, and fura-2AM from Enzo Life Sciences, UK. The physiological solution used contained 140 mM NaCl, 5 mM KCl, 5 mM D-glucose, 1.3 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (free acid), pH 7.4.

Agonists and antagonists were used at concentrations previously described as follows; 100 μM icilin [47,18], 50 μM cinnamaldehyde [7,19] 20 μM HC-030031 [19], 10 μM capsaicin [45], 10 μM capsazepine [12], 10 μM RuR [12] and 1 mM menthol [39].

2.9. Data analysis

Data are presented as means $\pm$ SEM. For statistical analysis paired and unpaired Student *t*-tests or one way analysis of variance (ANOVA) were used with $p \leq 0.05$ considered statistically significant.

3. Results

3.1. Molecular identification of thermo-sensitive TRP channels in human odontoblasts

The odontoblastic phenotype in cultured cells was confirmed by expression of the odontoblast markers DSPP and nestin (Fig. 1A). Gene and protein expression of TRPA1, TRPV1 and TRPM8 in

Fig. 5. Recording of $[Ca^{2+}]_i$ (top trace) and bath temperature (bottom trace) during application of thermal stimuli to odontoblast-like cells. (A) Capsaicin responsive cell showed response to heat (threshold 43 ± 2 °C) and (B) summary of cells response ($n = 10$) to heat (threshold 43 ± 2 °C). (C) Response of capsaicin responsive cell to heat threshold (43 ± 2 °C) was abolished in the presence of TRPV1 antagonist capsazepine. (D) Summary of cell responses to heat with and without capsazepine, error bars indicate SEM, paired Student t test $**p \leq 0.01$, unpaired Student t test $*p \leq 0.05$.

human odontoblast-like cells was demonstrated with PCR and western blotting. Electrophoresis of PCR products revealed strong intensity bands corresponding to predicted sizes of TRPA1, (Fig. 1B), TRPV1 (Fig. 1C) and TRPM8 (Fig. 1D). TRPA1, TRPV1 and TRPM8 proteins were detected at their expected molecular masses of 140 kDa, 95 kDa, and 128 kDa, respectively (Fig. 1E–G).

Double staining immunohistochemistry revealed localisation of TRPA1, TRPV1, and TRPM8 to cultured odontoblast-like cells (Fig. 2). Identification of an odontoblast phenotype was determined by positive staining with DSP antibody. Co-localisation of TRP channels to these cells was evident when the confocal images were merged.

Furthermore TRPA1, TRPV1 and TRPM8 were also detected in native odontoblasts from healthy wisdom teeth. TRPA1 was abundantly expressed in odontoblasts and more sparsely expressed in some of the cells in the cell rich zone (Fig. 3A). Similarly TRPV1 and TRPM8 were expressed mostly by odontoblasts (Fig. 3B and C), with staining also evident in the dentinal tubules (Fig. 3B).

3.2. Functional expression of thermo-sensitive TRP channels in human odontoblasts

3.2.1. TRP ligands induce changes in $[Ca^{2+}]_i$ in odontoblast-like cells

The functional expression of TRPA1, TRPM8 and TRPV1 was initially investigated using Ca^{2+} microfluorimetry in cultured cells by

measuring global changes in $[Ca^{2+}]_i$. In a control experiment to study the temporal profile of odontoblast responses to TRP ligands we found that odontoblasts respond to TRP ligands, icilin, capsaicin and menthol and that some of the cells respond to multiple agonists (Supplementary Fig. 1).

TRPA1 is a polymodal channel that is activated by a number of chemical agonists including icilin and cinnamaldehyde [47,7]. In this study 100 μ M icilin and 50 μ M cinnamaldehyde produced significant increase in $[Ca^{2+}]_i$, which was attenuated in the presence of RuR ($p < 0.01$, $n = 22$) and the TRPA1 antagonist HC-030031 ($p = 0.03$, $n = 14$, Fig. 4A and B).

Capsaicin is a specific TRPV1 agonist [12] and at 10 μ M concentration it produced a significant change in $[Ca^{2+}]_i$ that was blocked by the selective TRPV1 antagonist capsazepine ($p \leq 0.001$, $n = 27$, Fig. 4C). Menthol is classical agonist for TRPM8 but can also activate TRPA1 [41,25]. At 1 mM concentration menthol produced a significant increase in $[Ca^{2+}]_i$ and to differentiate TRPM8 from TRPA1 responding cells we studied the effects of antagonists on the menthol responses. We found that in some of the cells the menthol response was blocked by the TRPM8 antagonist capsazepine [28,51] and retained in the presence of RuR ($p = 0.001$, $n = 8$, Fig. 4D and E) indicating functional expression of TRPM8 in these cells, while in other cells the menthol responses were not blocked in the presence of capsazepine (data not shown).

Fig. 6. Recording of $[Ca^{2+}]_i$ (top trace) and bath temperature (bottom trace) during application of cold stimuli to odontoblast-like cells. (A) Menthol responsive cell responded to cold (threshold $22 \pm 3^\circ C$) and (B) summary of cells response ($n = 8$) to cold stimuli (threshold $22 \pm 3^\circ C$). (C) Responses to moderate cold (threshold $22 \pm 3^\circ C$) are blocked in the presence of the TRPM8 antagonist capsazepine. (D) Summary of responses to moderate cold in the presence or absence of capsazepine, error bars indicate SEM, paired Student t test ** $p \leq 0.01$, unpaired Student t test ** $p \leq 0.01$.

3.2.2. Thermal stimuli induce changes in $[Ca^{2+}]_i$

To examine the effect of thermal stimuli on odontoblasts, the temperature in the recording chamber was altered. Increasing the temperature from 36 to 46 °C evoked a substantial increase in $[Ca^{2+}]_i$ in capsaicin responsive cells which was evident at $43 \pm 2^\circ C$ ($p = 0.002$, $n = 10$, Fig. 5A and B). The response to heat in capsaicin sensitive cells was consistently blocked in the presence of the TRPV1 antagonist capsazepine ($p = 0.03$, Fig. 5C and D).

Reducing the bath temperature also resulted in an increase in $[Ca^{2+}]_i$, and to investigate odontoblast responses to cold stimuli we exposed the cells to two different cold thresholds. In a set of experiments cells were exposed to moderately cold stimuli by lowering the bath temperature from 30 to 15 °C. In these experiments cells responded by increase in $[Ca^{2+}]_i$, when the temperature in the recording chamber was in the range of $22 \pm 3^\circ C$. To test whether these cold responses were mediated via TRPM8, cells were first stimulated with menthol and the menthol responsive cells were exposed to moderate cold $22 \pm 3^\circ C$. As shown in Fig. 6A and B these cells responded by significant increase in $[Ca^{2+}]_i$, ($p = 0.002$, $n = 8$). In addition these responses to moderate cold were abolished when the cells were incubated with capsazepine prior to cold stimulation (Fig. 6C and D, $p = 0.01$).

In another set of experiments odontoblasts were exposed to noxious cold by lowering the bath temperature from 20 to 10 °C and at this temperature the cells responded by significant

increase in $[Ca^{2+}]_i$. As detailed above for TRPM8, the response of cells to the TRPA1 agonist, cinnamaldehyde, was first tested prior to exposure to noxious cold. The majority of cinnamaldehyde and icilin responsive cells responded to cold stimulation when the temperature in the recording chamber was in the range of $12 \pm 2^\circ C$ ($p = 0.001$, $n = 23$, Fig. 7A and B, and data not shown). Furthermore the response of icilin and cinnamaldehyde sensitive cells to cold ($12 \pm 2^\circ C$) was attenuated in the presence of the selective TRPA1 antagonist HC-030031 ($p = 0.03$, Fig. 7C and D), and RuR (data not shown).

3.3. TRPA1 siRNA

To further study the role of TRPA1 in mediating cold responses in odontoblasts we performed a gene silencing approach using TRPA1 siRNA. Treatment of odontoblasts with 50nM TRPA1 siRNA resulted in a significant reduction in TRPA1 protein expression as shown by western blotting ($p = 0.001$, Fig. 7E). When transfected cells were assayed for functionality there was a reduction in the magnitude of the responses to cinnamaldehyde and cold threshold ($12 \pm 2^\circ C$) compared to control cells transfected with scrambled siRNA ($p = 0.04$, $n = 12$, Fig. 7F and G). The viability of the transfected cells after cold stimulus application was evaluated by application of 1 μM ionomycin and transfected cells showed a robust increase in $[Ca^{2+}]_i$, (mean 0.5 ΔR).

Fig. 7. Responses of odontoblast-like cells to noxious cold stimulation. (A) Cinnamaldehyde responsive cell showed response to cold stimulus, (threshold $12 \pm 2^\circ\text{C}$) and (B), bar chart summary of cell responses to noxious cold stimuli ($n = 23$). Cell responses to cold were attenuated pharmacologically with TRPA1 antagonist M HC-030031 (C and D) and with gene suppression of TRPA1. (E) Western blot analysis of TRPA1 protein following 72 h cells transfection with TRPA1 siRNA and control scrambled siRNA. Protein expression decreases in a concentration dependent manner with significant knockdown at 50 nM compared to control. (F) TRPA1 siRNA transfected cell (ii) showing no response to cinnamaldehyde or cold (threshold $12 \pm 2^\circ\text{C}$), while a control cell (i) showed response to both. (G) Bar chart of average responses of control and TRPA1 siRNA transfected cells to cold ($n = 12$) (threshold $12 \pm 2^\circ\text{C}$), error bars indicate SEM, paired Student *t* test ** $p \leq 0.01$, * $p \leq 0.05$.

3.3.1. TRP ligands induce changes in $[\text{Ca}^{2+}]_i$ in native odontoblasts *ex vivo*

In addition to cultured odontoblasts we also examined the *ex-vivo* functional expression of thermo-sensitive TRP channels in native odontoblasts using dental pulp slices preparations obtained from freshly extracted healthy molar teeth. Using fura2-AM we found that the TRP agonists cinnamaldehyde, menthol and capsaicin induced increases in $[\text{Ca}^{2+}]_i$ similar to those observed in cultured odontoblasts (Figs. 8A and B).

4. Discussion

The present report demonstrates for the first time that human odontoblasts express functional thermo-sensitive TRP channels, TRPA1, TRPV1 and TRPM8. Using PCR and western blotting we demonstrated gene and protein expression of these channels and our immunohistochemical results confirmed their cellular localisation as has previously been described for non neuronal TRP channels [28,9]. The cultured odontoblast-like cells used in this study were derived from pulp tissue explants using a previously validated method [1,4]. The odontoblastic phenotype of the cells was confirmed using gene expression

of the odontogenic markers DSPP and nestin [2]. To further confirm localisation of TRP channels to human odontoblasts we performed double staining immunohistochemistry with antibody to the odontoblast marker dentin sialoprotein (DSP) and showed co-localisation of TRPA1, TRPV1 and TRPM8 with DSP positive cells. In addition to well-defined expression by odontoblasts, DSP has also been shown to be very minimally expressed by osteoblasts [42]. However, osteoblasts are not part of the cellular population in the dental pulp and therefore do not confound interpretation of our double staining with DSP as a demonstration of TRP channels co-localisation with cells of an odontoblastic phenotype.

We have also demonstrated by immunohistochemistry *ex-vivo* expression of TRPA1, TRPV1 and TRPM8 in native odontoblasts in dental pulp of healthy third molars. These results provide conclusive evidence for gene and protein expression of thermo-sensitive TRP channels in human odontoblasts.

In addition to odontoblasts, our immunohistochemical studies revealed expression of TRP channels in some of the cells of the dental pulp *ex-vivo*. The dental pulp is populated by different cell types including fibroblasts, undifferentiated mesenchymal cells and defence cells. The undifferentiated mesenchymal cells can differenti-

Fig. 7 (continued)

ate into odontoblasts following injury and damage to odontoblasts. It is tempting to speculate that expression of TRP channels in pulp cells is likely to be conserved in the undifferentiated mesenchymal cells. Previous work on ion channels in undifferentiated dental pulp cells showed expression of sodium and potassium channels [15,16], which was later confirmed in differentiated odontoblasts [3,4].

TRP channels respond to stimuli by activation of calcium fluxes. We therefore used ratiometric calcium imaging to test the functionality of these channels. Ratiometric calcium imaging was performed to record changes in $[Ca^{2+}]_i$ in response to TRP chemical agonists and thermal stimuli. We have demonstrated that odontoblasts, whether they are cultured or native cells within the dental pulp, can be stimulated by TRP ligands.

Odontoblasts showed responses to capsaicin which was abolished in the presence of capsazepine indicating functional expression of TRPV1. TRPV1 is a capsaicin gated ion channel that responds to heat ($>43^\circ\text{C}$) [12] and has been shown to play an essential role in inflammatory thermal hyperalgesia [17]. Previous work reported the expression of TRPV1 in undifferentiated dental pulp cells, and although its functionality was not investigated via change in $[Ca^{2+}]_i$, it was shown that capsaicin induced IL-6 production in dental pulp cells via activation of TRPV1 [35]. In the present report we demonstrated for the first time that in addition to activation by capsaicin, TRPV1 channels in human odontoblasts can also be activated by noxious heat, indicating a potential role in thermal transduction in human teeth and mediation of thermal hyperalgesia associated with pulpal inflammation. It is well known that TRPV1 is sensitised by several inflammatory mediators [53] and since dentin sensitivity has been reported to increase during pulp inflammation [36] it is tempting to speculate that thermal hyperalgesia associated with pulp inflammation could be mediated via TRPV1.

As well as TRPV1, odontoblasts also express functional TRPA1 and TRPM8, as indicated by their response to icilin, menthol and cinnamaldehyde. Icilin and menthol induced different responses in odontoblastic cells. Icilin responses were blocked by RuR in most but not all of the cells. Menthol produced a highly efficacious response that was blocked in the presence of capsazepine but persisted in the presence of RuR indicating expression of TRPM8 as previously described [41]. However, in some other cells the response was not blocked by capsazepine and since menthol is also known to activate TRPA1 [25,50], it is possible that these menthol responses were mediated via TRPA1. Activation of many of the cells by the TRPA1 and TRPM8 agonists cinnamaldehyde, menthol and icilin and the demonstration of their expression and cellular localisation in human odontoblasts by western blotting and immunohistochemistry provides strong evidence that human odontoblasts express functional TRPA1 and TRPM8. In recent reports expression of TRPM8 and TRPA1 was not demonstrated in mouse and rat odontoblasts [45,52]. However, since species differences in TRP channel expression and function have previously been observed [29,13], this difference in TRPA1 and TRPM8 expression between mouse/rat and human odontoblasts may simply reflect different physiological relevance in different species.

Odontoblasts also produced variable responses to cold stimulation. In line with previous evidence for cold induced responses in neuronal and non neuronal TRPM8 expressing cells [41,28], we showed that some of the menthol sensitive odontoblasts responded to reducing temperature ($22 \pm 3^\circ\text{C}$) by an increase in $[Ca^{2+}]_i$ indicating that odontoblasts may sense cold via TRPM8. This TRPM8 mediated cold response was further confirmed by attenuation of the response by capsazepine in agreement with previous reports [41,28]. Odontoblasts also responded to noxious cold at a threshold of $12 \pm 2^\circ\text{C}$. This response was attenuated in the presence of the TRPA1 selective antagonist HC-030031. TRPA1 has been

Fig. 8. Native odontoblast response to TRP ligands. Odontoblasts on the rim of the dental pulp slice were used for fluorometric calcium measurements. (A) Low magnification image of tooth slice showing dentin (D), pulp (P) and odontoblasts layer between (arrows). On the right phase contrast image of pulp slice odontoblasts (box area) where fluorometric calcium recording was carried out. (B) Representative traces showing that TRP channel agonists cinnamaldehyde, menthol and capsaicin elicited rises in $[Ca^{2+}]_i$ in native odontoblasts.

proposed to be activated by temperatures that are perceived as noxiously cold [47,7], however this mode of activation remains controversial [23,8,19,24]. To further explore the role of TRPA1 in cold responses in odontoblasts we performed siRNA knockdown of TRPA1. We demonstrated a reduction in responses to the specific TRPA1 agonist cinnamaldehyde and cold responses at a threshold of 12 ± 2 °C, suggesting a role for TRPA1 in mediating noxious cold sensation in line with previous reports [47,7,19,24].

Our findings clearly demonstrate a potential role for TRPM8 and TRPA1 in transduction of cold responses in human odontoblasts; however, the contributions of other cold transduction molecules cannot be ruled out. In addition to TRPM8 and TRPA1 several other potassium and sodium channels have been implicated in cold transduction [43,49]. Whether these ion channels play a role in cold transduction in odontoblasts has yet to be elucidated and thus the present report is the first to describe a molecular mechanism for cold transduction in human odontoblasts.

Previous reports have shown that odontoblasts are excitable cells that can generate action potentials [4] and our current findings demonstrate that odontoblasts can sense environmental stimuli through expression of TRP Channels. However, it is not yet clear how odontoblasts signal to and communicate with nociceptive afferent terminals in the dental pulp, and both electrical and paracrine paths of communication have been suggested. Odontoblasts lie in close proximity to afferent terminals, but in the absence of gap junctions between odontoblasts and nerves it has been suggested that communication between odontoblasts and nerves could be an ephaptic transmission, in which electric fields generated by odontoblasts may alter the excitability of neighbouring neurons [4]. Alternatively the release of intercellular signalling molecules such as ATP from odontoblasts is a poten-

tial mechanism of paracrine communication that merits further investigation.

Functional expression of thermo-sensitive TRP channels in human odontoblasts may indicate a crucial role for these cells not only in transduction of thermal stimuli in human teeth but also in mediating pain and inflammation. Tooth pain is generally induced by thermal changes in the oral cavity and the peripheral location of odontoblasts as the outermost cell layer of the dental pulp make them suitable candidates to sense external stimuli in a manner similar to that reported in skin keratinocytes. The expression of thermo-sensitive TRP channels has been reported in keratinocytes to mediate not only thermo-sensation [30], but also other functions that might be important for neuronal responses. It has been suggested that TRP channels in non-neural tissues could act in concert with neurons to mediate pain and inflammation. Indeed activation of thermo-sensitive TRP channels was shown to induce release of inflammatory cytokines [35,44,6] that may promote inflammation directly or sensitise the sensory neurons to augment neurogenic inflammation and nociceptive signalling [46].

TRPV1 and TRPA1 co-localise with peptidergic sensory neurons expressing substance P and calcitonin gene related peptide. Pungent irritants such as capsaicin and mustard oil induce neurogenic inflammation through their respective receptors TRPV1 and TRPA1 [48,5]. The role of neuropeptides in pulpal pain and inflammation has previously been described [31] and recently pulpal fibroblasts and odontoblasts have been shown to express neuropeptides [26,38]. The possibility that TRPA1 and TRPV1 channels in dental pulp cells could play a role in the release of neuropeptides and thus contribute to the development of neurogenic inflammation merits further investigation.

In summary, we provide the first evidence that human odontoblasts express functional thermo-sensitive TRP channels suggesting that these cells might act in concert with sensory neurons to perceive thermal stimuli and to activate adaptive responses to environmental stressors.

Conflict of interest statement

The authors declare no conflicts of interest.

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