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J Dent Res 93(9):911-917, 2014

ABSTRACT

The mechanism of pain in dentine hypersensitivity is poorly understood but proposed to result from the activation of dental sensory neurons in response to dentinal fluid movements. Odontoblasts have been suggested to contribute to thermal and mechanosensation in the tooth via expression of transient receptor potential (TRP) channels. However, a mechanism by which odontoblasts could modulate neuronal activity has not been demonstrated. In this study, we investigated functional TRP channel expression in human odontoblast-like cells and measured ATP release in response to TRP channel activation. Human immortalized dental pulp cells were driven toward an odontoblast phenotype by culture in conditioned media. Functional expression of TRP channels was determined with reverse transcription polymerase chain reaction and ratiometric calcium imaging with Fura-2. ATP release was measured using a luciferin-luciferase assay. Expression of mRNA for TRPA1, TRPV1, and TRPV4 but not TRPM8 was detected in odontoblasts by reverse transcription polymerase chain reaction. Expression of TRPV4 protein was detected by Western blotting and immunocytochemistry. The TRPA1 agonists allyl isothiocyanate and cinnamaldehyde and the TRPV4 agonist GSK1016790A caused a concentration-dependent increase in intracellular Ca²⁺ concentration that was inhibited by the selective antagonists HC030031, AP18, and HC067047, respectively. In contrast, exposure to the TRPV1 agonist capsaicin or the TRPM8 agonist icilin had no effect on intracellular Ca²⁺ concentration. Treatment with allyl isothiocyanate, cinnamaldehyde, or GSK1016790A caused an increase in ATP concentration in culture medium that was abolished by preincubation with TRP channel antagonists. These data demonstrate that activation of TRPA1 and TRPV4 channels in human odontoblast-like cells can stimulate ATP release. We were unable to confirm the presence of thermosensitive TRPV1 and TRPM8 that has previously been reported in odontoblasts.

KEY WORDS: transient receptor potential channel, orofacial pain, signal transduction, dentin, sensation, ion channels.

DOI: 10.1177/0022034514544507

Received April 30, 2014; Last revision June 30, 2014; Accepted July 1, 2014

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

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TRPA1 and TRPV4 Activation in Human Odontoblasts Stimulates ATP Release

INTRODUCTION

Exposed dentine is painful when exposed to changes in temperature or osmolarity. Anatomic and neurophysiologic properties of the tooth suggest that these changes are not detected directly by sensory nerve endings. Instead, dentinal fluid movements are detected by mechanosensitive nociceptive trigeminal nerve endings, the “hydrodynamic theory” of dental pain (Linden and Brannstrom, 1967; Brannstrom and Johnson, 1970). However, direct mechanical activation of the sensory nerves has not been described, and there is doubt whether such small fluid pressure changes could directly activate high-threshold mechanoreceptive nociceptors (Fried *et al.*, 2011). It is thus not clear whether there is direct mechanotransduction by neurons or if other cells (*e.g.*, odontoblasts) may contribute to sensory processing.

The odontoblasts are situated between the dentine and the nerve plexus, containing the majority of dental sensory fiber terminals. They possess cell processes that extend into the dentine tubules (Magloire *et al.*, 2004). Recent studies also suggest that odontoblasts express many ion channels commonly found in sensory neurons, such as mechanosensitive K_{Ca} and TREK-1 potassium channels (Allard *et al.*, 2000; Magloire *et al.*, 2003) and voltage-gated sodium channels (Byers and Westenbroek, 2011).

The transient receptor potential (TRP) family of cation channels play important roles in sensory transduction. The thermosensitive (>42°C) TRPV1, cool-sensitive (<22°C) TRPM8, cold/mechanosensitive TRPA1 and warm/mechanosensitive TRPV4 have received particular attention as a result of *in vivo* experiments, implicating them in nociception (Bevan and Andersson, 2009). Studies of human and rodent odontoblasts have identified expression of TRPV1, TRPM8, TRPA1, and TRPV4, but there is significant disagreement about whether all 4 channels are always expressed. Rat odontoblasts have been suggested to express TRPV1 (Okumura *et al.*, 2005; Tsumura *et al.*, 2012) and TRPA1 (Byers and Westenbroek, 2011), but another study failed to find either channel in reverse transcription polymerase chain reaction (RT-PCR) and Ca²⁺ imaging experiments (Yeon *et al.*, 2009). TRPV1 and TRPV4 were identified in cultured mouse odontoblasts, but no evidence of TRPA1 or TRPM8 expression was identified (Son *et al.*, 2009). TRPV1 and TRPV4 were proposed to mediate Ca²⁺ influx caused by hypotonic membrane stretching in these cells (Sato *et al.*, 2013). Odontoblast-like cells derived from human teeth expressed TRPV1, TRPA1, and TRPM8 (El Karim *et al.*, 2011), and immunostaining of human teeth identified odontoblast expression of TRPV4 (Sole-Magdalena *et al.*, 2011).

The expression of sensory ion channels by odontoblasts suggests that they may contribute to sensation in the tooth. However, no evidence has been

provided for a mechanism by which odontoblasts could modulate sensory neuron activity. In many tissues, mechanical stimulation of cells causes ATP release (Praetorius and Leipziger, 2009; Lazarowski, 2012). Sensory fibers in the dental pulp, in the subodontoblastic plexus of Raschkow, and within the odontoblast layer express ionotropic P2X₃ receptors (Alavi *et al.*, 2001; Renton *et al.*, 2003), so a mechanically stimulated release of ATP from odontoblasts could directly activate sensory neurons.

We have previously isolated, immortalized, and characterized odontoblast-like cells from human teeth (Egbuniwe *et al.*, 2011; Egbuniwe *et al.*, 2013). In this study, we investigated functional TRP channel expression in cultured human odontoblasts. We hypothesized that odontoblasts express the mechanosensitive TRP channels TRPA1 and TRPV4 and that activation of these channels would stimulate the release of ATP.

METHODS & MATERIALS

Odontoblast Isolation and Culture

Immortalized human odontoblast-like cells (hOBs) were obtained from extracted human teeth, and an odontoblast phenotype was confirmed by expression and function of specific markers (*e.g.*, collagen 1, alkaline phosphatase), as previously described (Egbuniwe *et al.*, 2011; Egbuniwe *et al.*, 2013). All experiments were approved by the local research ethics committee of King's College London and King's College Hospital Foundation Trust (reference 08/H0808/105).

The hOBs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2,500 U/mL of penicillin, 2.5 mg/mL of streptomycin, 50 µg/mL of ascorbic acid, 10mM β-glycerophosphate, and 100nM dexamethasone (all from Sigma, UK).

Reverse Transcription Polymerase Chain Reaction

hOBs were grown to confluence, and total cellular RNA was isolated with an RNeasy Plus Mini Kit (Qiagen, UK) according to the manufacturer's instructions. Cellular RNA from Chinese hamster ovary (CHO) cells overexpressing rat TRPM8 was kindly supplied by Prof. Stuart Bevan, King's College London, and 1 µg of total RNA was reverse transcribed to cDNA using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, UK). Products were expanded from cDNA by PCR with a Masterplus Sybr Green I dye detection system (Roche, UK) and specific primers (Appendix Table). Products of PCR were visualized with GelRed (Biotium Inc., USA) under ultraviolet light following 1.5% agarose gel electrophoresis.

Western Blotting

hOBs were lysed in phosphate buffered saline (PBS) containing 1% SDS, pH 7.4. Mouse urothelium was surgically separated from TRPV4 wild-type and knockout bladders and homogenized in RIPA buffer (50mM Tris-HCl, 150mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1mM EDTA, pH 7.4) with protease inhibitors (Roche). Lysate (30 µg) was separated by SDS-PAGE (8% acrylamide), transferred to nitrocellulose membranes, and blocked in TBS with 0.1% Tween-20 and 5%

powdered milk for 60 min. Membranes were incubated with rabbit anti-TRPV4 (1:250, AbCam) and mouse anti-GAPDH (anti-glyceraldehyde 3-phosphate dehydrogenase; 1:6,000, Applied Biosystems) overnight at 4°C. Membranes were washed and then incubated with donkey anti-rabbit-IRDye 800 (1:15,000, LI-COR, UK) and goat anti-mouse-Alexa Fluor 680 (Molecular Probes, UK) for 1 hr at room temperature. Immunoreactive proteins were detected with an Odyssey Infrared Imaging System (LI-COR; Grant *et al.*, 2007; Alexander *et al.*, 2013).

Immunocytochemistry

hOBs were fixed in 4% paraformaldehyde for 60 min at 4°C. Coverslips were washed in PBS. Cells were incubated overnight at 4°C with rabbit anti-TRPV4 (1:200, AbCam, UK). Coverslips were washed in PBS containing 0.1% Triton X-100; then, donkey anti-rabbit-Alexa 546 (1:1,000) was added and incubated for 2 hr at room temperature. Coverslips were washed in PBS containing 0.1% Triton X-100 and then mounted on slides with Vectashield with DAPI (Vector Labs, UK), and images were collected with an Axioplan 2 microscope (Zeiss, UK).

Measurement of Intracellular [Ca²⁺]

hOBs were grown to confluence in black-walled 96-well plates. Cells were loaded with Fura-2-AM (2µM) in the presence of probenecid (1mM) for 1 hr at 30°C in Hank's balanced salt solution with 10mM HEPES. Cells were washed, and the mean fluorescence across each well was measured alternately at 340/380-nm excitation and 520-nm emission with a Flexstation (Molecular Devices, Wokingham, UK). Each experimental run lasted 180 s, with agonist treatments at 20 s. In a further series of experiments, cells were pretreated for 15 min with TRP channel antagonists before the start of Ca²⁺ imaging, and the antagonist remained in contact with the cells throughout. Experiments in the absence of external Ca²⁺ were performed with Ca²⁺/Mg²⁺-free Hank's balanced salt solution, supplemented with 10mM HEPES, 1mM Mg²⁺, and 1mM EGTA.

Measurement of ATP Release

hOBs were grown to confluence in 4-well plates. Cell culture medium was replaced 2 hr before experiments with fresh medium alone or medium containing TRP channel antagonists. In one set of experiments, 100-µL samples of medium were collected 1, 5, or 15 min after the medium was replaced with warmed fresh medium. In another, cells were treated for 15 min with TRP channel agonists, and then 100-µL samples of medium were collected. ATP content in culture medium was assayed with the CellTiter-Glo kit (Promega, UK) according to the manufacturer's protocol, with an ATP standard curve (1nM-1µM). Duplicate measurements were made from each well and an average taken of the measurements from 2 wells to give $n = 1$.

Materials

Allyl isothiocyanate (AITC), capsaicin, cinnamaldehyde (CA), GSK1016790A, HC030031, HC067047, icilin, and

probenecid were all obtained from Sigma. AP18 was obtained from Tocris, UK. Fura-2-AM was obtained from Molecular Probes (Invitrogen, UK). All compounds were dissolved in DMSO and diluted in culture medium / reaction buffer as appropriate.

Analysis of Data

Graphical data are presented as mean \pm SEM. TRP channel agonist responses in the Ca^{2+} imaging experiments were calculated as maximum Δ ratio in the presence of the agonist minus the mean baseline ratio. Sigmoidal curves were fitted to the concentration-response data via GraphPad Prism software. ATP release following medium change was compared with a non-parametric Kruskal-Wallis test, followed by Dunn's posttest. ATP release in response to TRP channel agonists was compared with unpaired *t* tests.

RESULTS

TRP Channel Expression in Cultured Human Odontoblasts

Expression of TRP channels in cultured hOBs was initially examined by RT-PCR. Specific primers amplified products for GAPDH and the TRP channels TRPA1 and TRPV1 from total cellular cDNA (Figure 1a). A doublet band was observed with primers specific to TRPV4 (Grant *et al.*, 2007), whereas no band was seen for TRPM8 (Figure 1a). Expansion of products with primers for 2 phenotypic markers of odontoblasts—alkaline phosphatase and collagen 1—was also observed (Figure 1b). To confirm the ability of the TRPM8 primers to amplify a product, a further RT-PCR reaction was run with cDNA from hOBs and CHO-TRPM8 cells. A band for TRPM8 was detected only with CHO-TRPM8 cDNA.

Expression of TRPV4 protein was studied in hOB lysates by Western blotting. To confirm antibody specificity, urothelial (a tissue strongly expressing TRPV4; Mochizuki *et al.*, 2009) lysates from wild-type and TRPV4 knockout mice were also assayed. A band at 37 kDa, corresponding to GAPDH, was seen in all lysates (Figure 1c). A relatively weak double band (98 and 104 kDa) corresponding to TRPV4 was seen in the hOB lysate, and stronger bands of the same size were observed in wild-type urothelial lysate. No bands at these sizes were seen in urothelial lysates from TRPV4 knockout mice (Figure 1c). Immunostaining of hOBs with anti-TRPV4 also demonstrated expression of protein, and specificity of antibody binding was further supported by the absence of fluorescence in cells exposed to secondary antibody alone (Figure 1d).

We attempted to extend these experiments to investigate expression of TRPA1 and TRPV1 protein in hOBs. Various commercially available antibodies were tested by Western blotting on lysates of dorsal root ganglia from wild-type, TRPA1 knockout, and TRPV1 knockout mice. We were unable to identify any antibodies that specifically recognized bands corresponding to the molecular weights of these proteins.

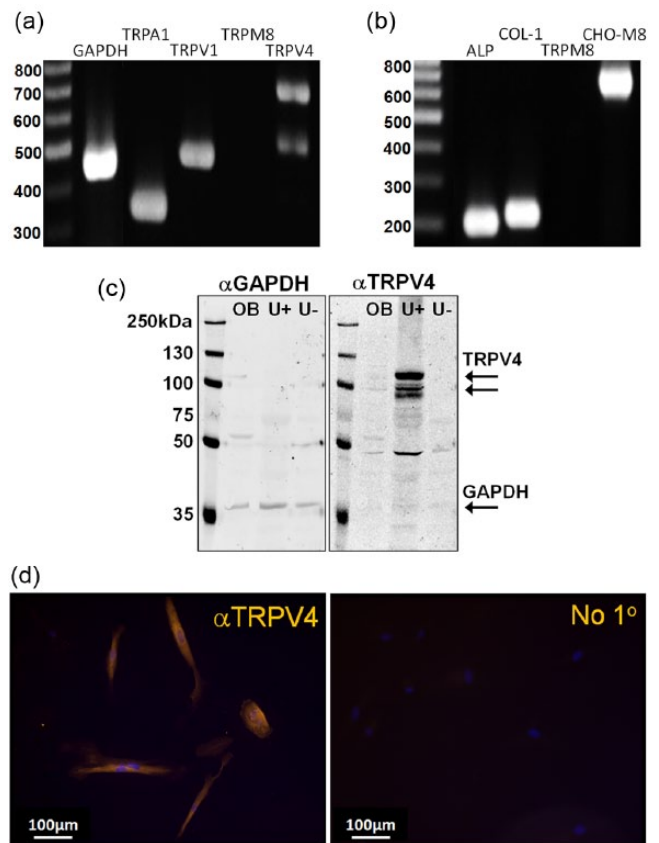


Figure 1. Expression of transient receptor potential channels in cultured human odontoblast-like cells (hOBs). (a) Reverse transcription polymerase chain reaction with primers selective for GAPDH (product size = 425 bp), TRPA1 (381 bp), TRPV1 (452 bp), and TRPV4 (512/692 bp) produced bands, but no product of TRPM8 cDNA was expanded. (b) Primers selective for alkaline phosphatase (ALP; 196 bp) and collagen 1 (COL-1; 213 bp) produced bands from hOB cDNA, whereas primers selective for TRPM8 produced a product only from CHO-TRPM8 cDNA (681 bp) but not from hOBs. (c) Lysates (30 μ g of total protein) from hOBs (OB), wild-type mouse urothelium (U+), and TRPV4 $^{-/-}$ mouse urothelium (U-) showed expression of GAPDH (37 kDa, left blot). Lysates from hOBs and wild-type mouse urothelium showed the double band corresponding to TRPV4 (98 and 104 kDa, right blot), but it was absent in TRPV4 $^{-/-}$ mouse urothelium. (d) Immunostaining of hOBs with rabbit anti-TRPV4 (1:200) and donkey anti-rabbit-Alexa 546 (1:1,000) revealed positive TRPV4 immunoreactivity that was not absent in the “no primary” cells. Images are representative of data obtained from *n* = 3 independent experiments.

Identification of Functional TRP Channel Expression in Odontoblasts by Ca^{2+} Fluorimetry

Exposure of cultured hOBs to the TRPV4 agonist GSK1016790A caused a concentration-dependent increase in intracellular Ca^{2+} concentration, with EC_{50} = 30nM (Figure 2a). The TRPA1 agonists AITC and CA also caused concentration-dependent increases in $[Ca^{2+}]_i$, with EC_{50} = 6 μ M and 36 μ M, respectively (Figure 2b and c). In contrast, the TRPV1 agonist capsaicin and

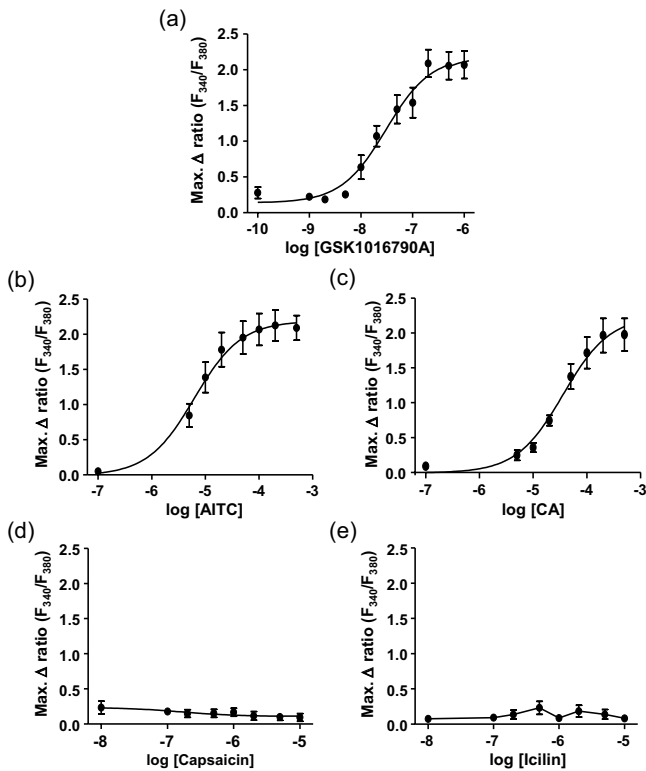


Figure 2. The effect of agonists of TRPV4, TRPA1, TRPV1, and TRPM8 on $[Ca^{2+}]_i$ in cultured human odontoblast-like cells. (a) The TRPV4 agonist GSK1016790a (0.1 nM–1 μM) and the TRPA1 agonists (b) allyl isothiocyanate (AITC; 0.1–500 μM) and (c) cinnamaldehyde (CA; 0.1–500 μM) caused a concentration-dependent increase in intracellular Ca^{2+} concentration. (d) The TRPV1 agonist capsaicin (10 nM–10 μM) and (e) the TRPM8 agonist icilin (10 nM–10 μM) had no effect on intracellular Ca^{2+} concentration. $n = 3$ –7 triplicate measurements.

TRPM8 agonist icilin, both at concentrations up to 10 μM, had no effect on intracellular Ca^{2+} concentration (Figure 2d and e).

Preincubation of hOBs for 15 min with the TRPV4 antagonist HC067047 caused a concentration-dependent reduction in the increase in $[Ca^{2+}]_i$ caused by 200 nM GSK1016790A, with $IC_{50} = 100$ nM (Figure 3a). The TRPA1 antagonist HC030031 caused concentration-dependent inhibition of the increase in $[Ca^{2+}]_i$ to 200 μM AITC or CA with $IC_{50} = 67$ μM and 30 μM, respectively (Figure 3b and c). Similarly, the TRPA1 antagonist AP18 caused concentration-dependent inhibition of the increase in $[Ca^{2+}]_i$ to 200 μM AITC or CA with $IC_{50} = 6$ μM and 3 μM, respectively (Figure 3d and e).

In the absence of external Ca^{2+} , no increase in intracellular Ca^{2+} concentration occurred following exposure to GSK1016790A, AITC, or CA. In the same experiment, cells exposed to 1 μM GSK1016790A, 500 μM AITC, or 500 μM CA in the presence of external Ca^{2+} showed a robust increase in $[Ca^{2+}]_i$ (Figure 4).

Activation of TRPA1 and TRPV4 in Odontoblasts Stimulates ATP Release

Changing the hOB cell culture medium caused a release of ATP, with the concentrations remaining elevated in cell culture

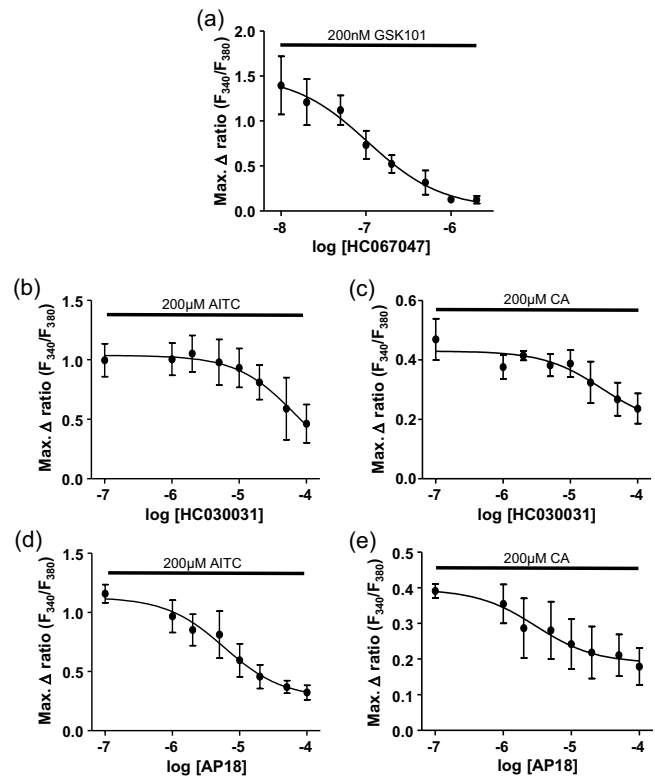


Figure 3. The effect of pretreatment with transient receptor potential channel antagonists on changes in $[Ca^{2+}]_i$ in cultured human odontoblast-like cells induced by TRPV4 and TRPA1 agonists. (a) The TRPV4 antagonist HC067047 (10 nM–2 μM) caused a concentration-dependent reduction in the increase in intracellular Ca^{2+} concentration caused by 200 nM GSK1016790A. The TRPA1 antagonist HC030031 (0.1–100 μM) caused a concentration-dependent reduction in the increase in intracellular Ca^{2+} concentration caused by (b) 200 μM AITC and (c) 200 μM CA. The TRPA1 antagonist AP18 (0.1–100 μM) caused a concentration-dependent reduction in the increase in intracellular Ca^{2+} concentration caused by (d) 200 μM allyl isothiocyanate (AITC) and (e) 200 μM cinnamaldehyde (CA). $n = 4$ triplicate measurements.

medium 1 and 5 min after medium change. The concentration had returned to its basal value by 15 min (Figure 5a). Thus, to avoid any artifact from mechanical stimulation of cells during pipetting, the effects of TRP channel agonists on ATP concentration were assessed 15 min after application.

Application of 100 nM GSK1016790A to hOBs caused a significant increase in ATP concentration in the cell culture medium (Figure 5b). This was prevented by preincubation with 10 μM HC067047 (Figure 5c). Application of 500 μM AITC or 500 μM CA to hOBs also caused an increase in ATP concentration in the cell culture medium (Figure 5d and f). Preincubation with 100 μM AP18 prevented this increase in both cases (Figure 5e and g). Application of 10 μM capsaicin (vehicle: 2.91 ± 1.41 nM vs. capsaicin: 2.92 ± 0.72 nM) or 10 μM icilin (vehicle: 2.61 ± 0.87 nM vs. icilin: 3.26 ± 1.50 nM) had no effect on the concentration of ATP in the cell culture medium.

DISCUSSION

In this study, we have identified functional expression of the ion channels TRPA1 and TRPV4 in cultured hOBs. We were unable to find evidence for expression of TRPV1 or TRPM8. We have shown for the first time that hOBs can release ATP following activation of TRPA1 and TRPV4, suggesting a possible contribution to sensation in the tooth.

Identification of alkaline phosphatase and collagen 1 expression supports our previous finding that these cells maintain an odontoblast phenotype in culture (Egbuniwe *et al.*, 2011; Egbuniwe *et al.*, 2013). Expression of TRPV4 (mRNA and protein) and TRPA1 (mRNA) was also identified in these cells. Expression of TRPV4 protein was identified by Western blotting and immunocytochemistry. The TRPV4 agonist GSK1016790A caused a concentration-dependent increase in $[Ca^{2+}]_i$ with $EC_{50} = 32nM$, a similar order of magnitude to the 43nM that we previously observed in a human keratinocyte cell line (Alexander *et al.*, 2013). The increase in $[Ca^{2+}]_i$ was inhibited in a concentration-dependent manner by the TRPV4 antagonist HC067047. Removal of Ca^{2+} from the extracellular media prevented any increase in $[Ca^{2+}]_i$ following exposure to GSK1016790A, suggesting that it occurs via entry of Ca^{2+} through TRPV4. Together, the presence of TRPV4 mRNA and protein, as well as calcium increases following exposure to a TRPV4 agonist, suggests that cultured hOBs express functional TRPV4. Immunohistochemical identification of TRPV4 in odontoblasts in human teeth (Sole-Magdalena *et al.*, 2011) suggests that this expression also occurs *in situ*.

The TRPA1 antagonists AITC and CA both caused concentration-dependent increases in $[Ca^{2+}]_i$. Two different TRPA1 antagonists inhibited the increase in $[Ca^{2+}]_i$ caused by either AITC or CA. Removing Ca^{2+} from the extracellular media prevented any increase in $[Ca^{2+}]_i$ following exposure to AITC or CA, suggesting that each was triggering an influx of extracellular Ca^{2+} through TRPA1. Together, the presence of TRPA1 mRNA and calcium increases following exposure to TRPA1 agonists suggests that cultured hOBs express functional TRPA1. Odontoblasts in human teeth were positive for TRPA1 expression following immunohistochemical staining, suggesting that this expression is also present *in situ* (Kim *et al.*, 2012).

Although TRPV1 mRNA was identified by RT-PCR, no increase in $[Ca^{2+}]_i$ followed exposure to the TRPV1 agonist capsaicin up to a concentration of 10 μ M, well above the EC_{50} value of 300nM at hTRPV1 (McNamara *et al.*, 2005). Furthermore, exposure of hOBs to 10 μ M capsaicin had no effect on ATP release. These findings suggest that TRPV1 mRNA is not translated into mature protein or that the mature protein does not reach the cell surface. Another explanation could be that the TRPV1 mRNA is translated into a capsaicin-insensitive splice variant of the channel, several of which have been described.

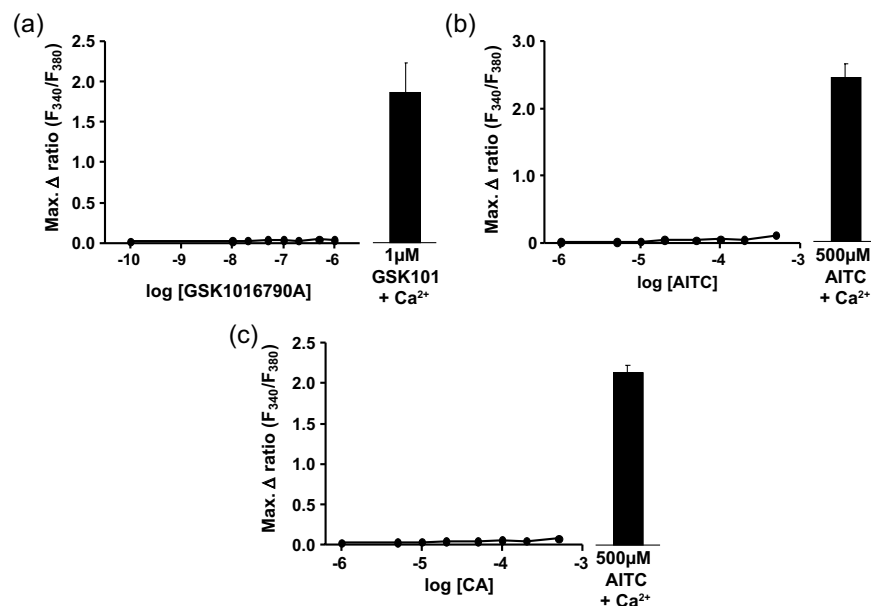


Figure 4. Removal of extracellular Ca^{2+} abolished the increase in $[Ca^{2+}]_i$ induced by (a) GSK1016790A (0.1 nM–1 μ M), (b) allyl isothiocyanate (AITC; 1–500 μ M), and (c) cinnamaldehyde (CA; 1–500 μ M) in cultured human odontoblast-like cells. The highest doses of each agonist induced a robust increase in $[Ca^{2+}]_i$ in the same cells when extracellular Ca^{2+} was present. $n = 3$ triplicate measurements.

One variant was identified in murine sensory neurons, where it negatively regulated the function of the capsaicin-sensitive isoform (Wang *et al.*, 2004). An N-terminal variant TRPV1 was also proposed to mediate depolarization of magnocellular neurosecretory cells by thermal and hyperosmotic stimuli in mice (Sudbury *et al.*, 2010). Further investigation of TRPV1 expression and cellular localization is necessary to understand this discrepancy between expression and function data.

In contrast to our findings, several previous studies have identified functional capsaicin-sensitive TRPV1 in odontoblasts (e.g., Okumura *et al.*, 2005; El Karim *et al.*, 2011; Sato *et al.*, 2013), although another study failed to do so (Yeon *et al.*, 2009). Species differences in the odontoblast expression of TRP channels may explain some of this variability. Additionally, some data supporting an expression of TRPV1 are based on immunohistochemical staining, which must be interpreted with caution as TRPV1 antibodies bind nonspecifically to tissues lacking TRPV1 (Everaerts *et al.*, 2009). However, El Karim *et al.* (2011) studied human odontoblasts isolated and cultured with a methodology similar to that used in this study and identified increased $[Ca^{2+}]_i$ following exposure to capsaicin or noxious heat. It is notable that the mean fluorescence changes to capsaicin and heat in their study are very small with a large variability and the number of experimental repeats is not described, so it is possible that these were just nonspecific fluctuations in $[Ca^{2+}]_i$ in a few cells that coincided with capsaicin or heat application. Cells isolated from dental pulps also exhibit neuronal phenotypes (D'Anto *et al.*, 2006), so there is the possibility that the studies identifying odontoblast TRPV1 have actually isolated precursors displaying a mix of neuronal and odontoblast characteristics. A recent study using a TRPV1 reporter mouse to overcome antibody specificity problems identified only TRPV1 expression in neurons and a small subset of vascular smooth muscle

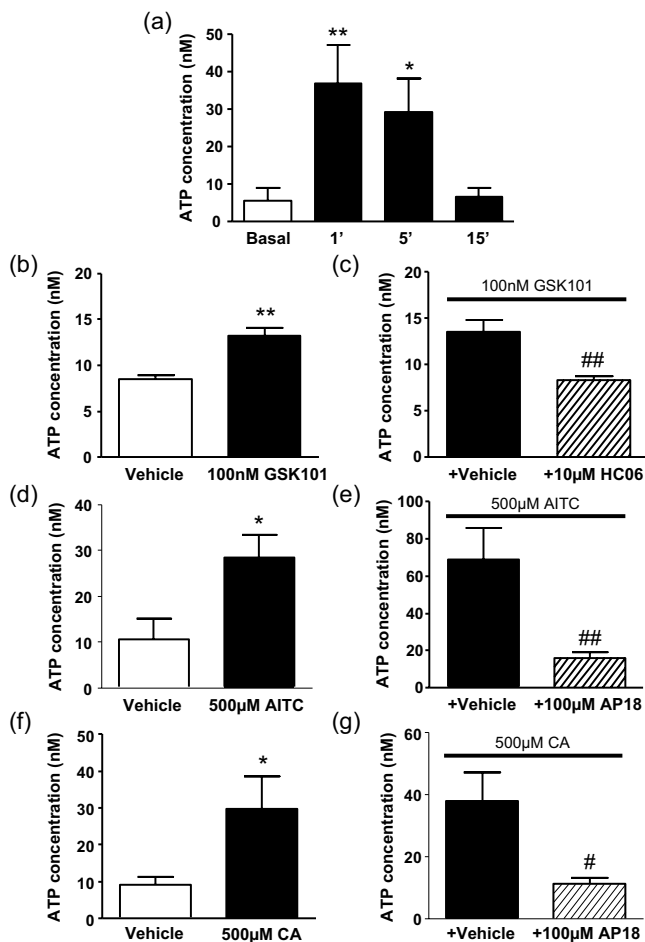


Figure 5. Release of ATP from cultured human odontoblast-like cells. (a) Changing the cell culture medium caused an increase in extracellular ATP concentration 1 and 5 min afterward, which returned to basal levels by 15 min. (b) 100nM GSK1016790A, (d) 500µM allyl isothiocyanate (AITC), and (f) 500µM CA caused a significant increase in extracellular ATP concentration, measured 15 min after agonist administration. These increases were significantly inhibited by pretreatment with (c) 10µM HC067047 or (e, g) 100µM AP18. * $p < .05$, ** $p < .01$ vs. vehicle/basal groups. # $p < .05$, ## $p < .01$ vs. agonist + antagonist vehicle groups. $n = 8$ -10 groups of paired duplicate measurements.

(Cavanaugh *et al.*, 2011), so expression of TRPV1 in odontoblasts would be surprising.

We were also unable to find evidence for expression of TRPM8 mRNA or protein in the hOBs. Confirmation that our TRPM8 primers were working correctly was provided by their amplification of the correct product from cDNA from CHO-TRPM8 cells. As with TRPV1, this was in contrast to some studies (*e.g.*, El Karim *et al.*, 2011) but supporting the observation of others (*e.g.*, Son *et al.*, 2009). Similar to the putative TRPV1 responses, the putative TRPM8 responses to cold and icilin described by El Karim *et al.* (2011) are small in magnitude with a high degree of variability, so they may have been nonspecific fluctuations in cytoplasmic calcium. Most pertinently, none of the studies proposing expression of TRPV1 and TRPM8 as odontoblast thermosensors have explained how this would fit

with the hydrodynamic theory. It is widely accepted that thermal changes are transduced indirectly via changes in dentine fluid volume that activate mechanosensory proteins on sensory cells (neurons, odontoblasts, or both). TRPV4 and TRPA1 activation by mechanical stimuli has been described (Suzuki *et al.*, 2003; Kwan *et al.*, 2006; Brierley *et al.*, 2011), indicating that odontoblasts with these channels have the potential to detect pressure changes caused by changes in dentine fluid volume.

Although expression of TRPV4 and TRPA1 suggests that odontoblasts are mechanosensitive, they could not contribute to dental pain without some means to modulate the activity of sensory neurons. In many tissues, mechanical stimulation of cells induces ATP release, so we investigated whether a similar release could be triggered by stimulation of hOBs. Aspirating and replacing the cell culture medium to mechanically stimulate the cells caused an increase in ATP concentration 1 and 5 min later, but this returned to basal levels by 15 min. To ensure that observed changes in ATP concentration in TRP agonist experiments were due to chemical activation of TRP channels rather than inadvertent mechanical stimulation of cells, we measured the ATP concentration in the culture medium 15 min after agonist administration.

Application of GSK1016970A to hOBs caused an increase in ATP concentration in the culture medium, which was inhibited by the TRPV4 antagonist HC067047. Similarly, exposure to either AITC or CA caused an increase in extracellular ATP concentration that was inhibited by the TRPA1 antagonist AP18. This suggests that activation of TRPV4 or TRPA1 triggers a release of ATP from hOBs. Basal and evoked concentrations of ATP in the nanomolar range are similar in magnitude to those described in bulk phase in previous studies (Praetorius and Leipziger, 2009). TRPV4-induced release of ATP has been described in urothelial cells (Mochizuki *et al.*, 2009) and keratinocytes (Mihara *et al.*, 2011). Mechanical activation of TRPV4 by urothelial stretch and consequent release of ATP to activate P2X₃ receptors on sensory neurons plays a key role in detecting bladder filling (Gevaert *et al.*, 2007). Dental sensory neurons adjacent to the odontoblasts express P2X₃ receptors (Alavi *et al.*, 2001; Renton *et al.*, 2003). The cell surface enzyme nucleoside triphosphate diphosphohydrolase 2, which hydrolyzes extracellular ATP, is expressed by sensory neurons and odontoblasts (Liu *et al.*, 2012). The ability of hOBs to release ATP, the presence of ATP-sensitive receptors on dental sensory neurons, and the presence of enzymes necessary to terminate ATP signaling all suggest that odontoblasts could play a physiologic role in modulating sensory neuron activity in the tooth.

In conclusion, we have shown that hOBs express functional TRPV4 and TRPA1 and that activation of these channels triggers ATP release. This mechanism may contribute to dentine hypersensitivity by allowing odontoblasts to sense changes in dentine fluid pressure and release ATP to activate adjacent sensory neurons. However, direct mechanical activation of TRPV4 and TRPA1 in human odontoblasts, with subsequent ATP release, still remains to be identified. While this article was under revision, Shibukawa *et al.* (2014) published a report describing TRP channel-dependent release of ATP from cultured rat odontoblasts in response to mechanical stimulation, suggesting that this mechanism may be common to dental nociception in humans and rodents.

ACKNOWLEDGMENTS

These studies were supported by a Biomedical Research Centre award to the Guy's & St Thomas' NHS Foundation Trust in partnership with King's College London, a training grant from the Royal College of Surgeons (England), and a Capacity Building Award in Integrative Mammalian Biology funded by the Biotechnology and Biological Sciences Research Council, British Pharmacological Society, Higher Education Funding Council for England, Knowledge Transfer Network, Medical Research Council. The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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