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The role of TRPM8 in the Guinea-pig bladder-cooling reflex investigated using a novel TRPM8 antagonist

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ABSTRACT

Patients with overactive bladder often exhibit abnormal bladder contractions in response to intravesical cold saline (positive ice-water test). The molecular entity involved in cold sensation within the urinary bladder is unknown, but a potential candidate is the ion channel, transient receptor potential (melastatin)-8 (TRPM8). The objective of the present study was to investigate the role of TRPM8 in a bladder-cooling reflex evoked in anaesthetised guinea-pigs that is comparable to the positive ice-water test seen in patients. Guinea-pig TRPM8 was cloned from L6 dorsal root ganglia (DRG) and expressed in HEK293 cells. Functional agonist- and cold-induced Ca^{2+} influx and electrophysiology assays were performed in these cells, and for comparison in HEK293 cells expressing human TRPM8, using a novel TRPM8 antagonist, the S-enantiomer of 1-phenylethyl 4-(benzyloxy)-3-methoxybenzyl (2-aminoethyl) carbamate hydrochloride (PBMC). Potency data from these assays was used to calculate intravenous infusion protocols for targeted plasma concentrations of PBMC in studies on micturition reflexes evoked by intravesical infusion of menthol or cold saline in anaesthetised guinea-pigs. Tissue expression of TRPM8 in guinea-pig bladder, urethra and in dorsal root ganglia neurones traced from the bladder was also investigated. TRPM8 mRNA and protein were detected in L6 dorsal root ganglia, bladder urothelium and smooth muscle. PBMC antagonised *in vitro* activation of human and guinea-pig TRPM8 and reversed menthol and cold-induced facilitation of the micturition reflex at plasma concentrations consistent with *in vitro* potencies. The present data suggest that the bladder-cooling reflex in the guinea-pig involves TRPM8. The potential significance of TRPM8 in bladder disease states deserves future investigation.

1. Introduction

Transient receptor potential (melastatin)-8/cold and menthol-sensitive receptor-1 (TRPM8/CMR-1) is a non-selective cation channel that is expressed in subsets of rat A-δ and C-fibre sensory neurones (Kobayashi et al., 2005; McKemy et al., 2002) and mouse C-fibre neurones (Peier et al., 2002) and is activated by cold temperatures and agonists such as the cooling agent menthol. TRPM8 mRNA and protein have also been detected in rat and human bladder urothelium (Stein et al., 2004; Mukerji et al., 2006b). Additionally, TRPM8-immunoreactive staining has been observed in sub-urothelial nerve fibres in human bladder and a marked increase in the number of fine-calibre nerve fibres immunoreactive for TRPM8 was found in patients with idiopathic detrusor overactivity and those with painful bladder syndrome compared with control subjects (Mukerji et al., 2006b). Rapid instillation of ice-cold water or saline into the bladder evokes a positive ice-water test (+IWT) in a large proportion of patients with overactive bladder as a consequence of neurological disorders (Geirsson et al., 1993a), in some patients with idiopathic overactive bladder who may have a limited but undetected neuropathy (Geirsson et al., 1993a; 1993b; Mukerji et al., 2006a) and in patients with high-grade bladder outlet obstruction due to benign prostatic hyperplasia (Hirayama et al., 2003). The +IWT

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is distinguished by sustained bladder contractions with fluid expulsion at a threshold volume lower than the normal cystometric capacity for that individual. A bladder-cooling reflex has also been demonstrated in anaesthetised cats (Fall et al., 1990) and anaesthetised guinea-pigs (WO 2005/004883 A2, Gardiner and Westbrook, 2003; Gardiner et al., 2007; Jiang et al., 2008) and in the former is mediated by a spinal pathway triggered by activation of C-fibre afferents in the bladder wall (Jiang et al., 2002; Mazieres et al., 1998). The specific mechanism through which a +IWT is evoked is unknown, however expression of specific ion channels in the bladder which respond to cold stimulation is possible. Potential candidates include TRPM8, transient receptor potential channel ankyrin1 (TRPA1)/ ANKTM1 (Kobayashi et al., 2005; Story et al., 2003) and a background K^+ current that is inhibited by cold (Reid and Flonta, 2001).

In order to investigate the potential role of TRPM8 in the bladder-cooling reflex we presently describe the cloning of guinea-pig TRPM8 from L6 dorsal root ganglia and the potential expression of a truncated form of TRPM8, as proposed to be present in human tissues (Sabnis et al., 2008). In addition, we investigated the tissue expression of TRPM8 in guinea-pig bladder and urethra and in dorsal root ganglia neurones retrogradely traced from the bladder. The effect of a TRPM8/CMR-1 antagonist (WO 2006/040136 A1, WO 2007/017094 A1; Knowlton et al., 2011), namely the S-enantiomer of 1-phenylethyl 4-(benzyloxy)-3-methoxybenzyl(2-aminoethyl) carbamate hydrochloride (PBMC) was tested in functional agonist-induced Ca^{2+} influx and electrophysiology assays in cells expressing recombinant guinea-pig or human TRPM8. Potency data from these assays was used to calculate intravenous infusion protocols for PBMC in studies in anaesthetised guinea-pigs evoked by intravesical infusion of menthol or the rapid instillation of cold saline in a model of the clinical +IWT (Gardiner et al., 2007).

2. Materials and methods

2.1. Cloning and expression of guinea-pig TRPM8 (gpTRPM8)

Dorsal root ganglia (L6) were collected from 3 male guinea-pigs. Division of urothelium from underlying detrusor layer was performed by microdissection and tissues preserved in RNALater. TRPM8 was cloned from guinea-pig dorsal root ganglion cDNA using the primers (5′)CCACCATGTCCTTCGAGGGAGC(3′) and (5′) TTATTTGATTTTAGCAGCAATCTCCTTCAGG(3′) and the pCDNA3.1™ Directional TOPO® Expression kit (Invitrogen Ltd.), according to the manufacturer's instructions.

2.2. Cell line generation and recombinant pharmacology

2.2.1. Cell lines

An expression construct for human TRPM8 (hTRPM8) in pCDNA3.1 (Invitrogen Ltd.) was provided by Pfizer Ltd. Japan. Human embryonic kidney 293 (HEK293) cells were transfected with hTRPM8.pcDNA3.1 using Lipofectamine™ 2000 according to the manufacturer's guidelines. A stably transfected clone was isolated by selection in media containing Geneticin® (0.05%). Stably expressing cells were maintained in Dulbecco Modified Eagle Medium supplemented with 10% foetal bovine serum, sodium pyruvate, non-essential amino acids and Geneticin® (0.05%), in a humidified incubator (37 °C in 5% CO_2). Transient expression of gpTRPM8 was achieved using the Freestyle™ 293 Expression system (Invitrogen Ltd), according to the manufacturer's instructions.

2.2.2. Agonist inhibition assays

Cells (1×10^4 or 1×10^5 cells/well) were seeded into 384 or 96-well black-walled clear-bottom poly-D-lysine-coated assay plates (Greiner Bio-One, Frickenhausen, Germany/Corning Ltd, Sunderland, UK) and incubated for 24 h at 37 °C under a humidified 5% CO_2 atmosphere. Assays were performed using a functional drug screening system (FDSS6000; Hamamatsu, Shizuoka, Japan) or fluorometric imaging plate reader (FLIPR 96; Molecular Devices, Wokingham, UK) and Calcium 4 assay kit (Molecular Devices, Sunnyvale, CA). Dye loading in Hanks balanced salt solution (HBSS) containing 20 mM HEPES buffer and 2.5 mM probenecid was performed for 1 h at 37 °C. Changes in fluorescence were measured every 1 s for 60 s. Initially cells were treated with a concentration response curve of menthol, WS-12 (a menthol derived selective TRPM8 agonist, Sherkheli et al., 2008) or PBMC. After 15 min a fixed concentration of menthol (15 μM) or WS-12 (300 nM) was added (to approximate an EC_{70}–EC_{80} concentration based on previous data; pEC_{50} values from current studies are presented in the data analysis section) to every well and the plate read as before for 60 s. Data were obtained as fluorescence ratio of the baseline corrected maximal signal responses. All solutions were pre-warmed to 37 °C prior to addition.

2.2.3. Cooling-induced activation assay

Human embryonic kidney 293 cells transiently expressing hTRPM8 were maintained at 37 °C throughout experiments until activation was performed by controlled decrease in temperature. Cells were incubated with Fluo4 dye, washed and resuspended at 10^6 cells/ml for plating at 100 μl per well in the presence of a range of concentrations of PBMC (0.325–333 nM). Assays were performed using an RT-PCR ABI7700 (Applied Biosystems) to enable controlled sample temperature decrease to an activation temperature of 10 °C. Calcium flux was measured in each well and IC_{50} curves were plotted from calculated fluorescence ratio values at 37 °C and 10 °C.

2.2.4. Electrophysiology

Cultured hTRPM8 HEK293 cells were seeded (4000 cells/ml) onto 12 mm poly-D-lysine/laminin coated coverslips (100 μl per coverslip) and maintained in an incubator for at least 24 h prior to experimentation. Transiently transfected gpTRPM8 HEK293 cells were similarly plated (5000 cells/ml) and allowed to settle for more than 1 h. Recordings were carried out up to 48 h after plating.

For recordings, a coverslip was transferred to a recording chamber (Warner RC-26; Harvard Apparatus, Kent, UK) and superfused with external solution (composition in mM: 139 NaCl, 5.4 KCl, 2.4 $CaCl_2$, 1.7 $MgCl_2$, 10 HEPES and 10 glucose, pH 7.4 adjusted with NaOH) at a rate of 2 ml/min. Perforated-patch recording methods were used for hTRPM8 cells, patch pipettes were dipped in pipette solution (composition in mM: 110 K aspartate, 30 KCl, 10 NaCl, 1 $MgCl_2$, 10 HEPES and 0.05 EGTA, pH 7.4 adjusted with NaOH) for a period of 15–25 s and then back-filled with amphotericin B-containing pipette solution (300 μg/ml). After obtaining a giga-seal, a period of 5–15 min was allowed for membrane perforation. Whole-cell patch recordings were performed on gpTRPM8 transients using pipette solution of composition (in mM) 140 CsCl, 10 EGTA, 2 MgATP, 10 HEPES and 1 $CaCl_2$.

Currents were amplified by an Axopatch 200B or multiclamp 700A (Axon Instruments, Foster City, CA, USA), filtered at 5 kHz, digitised at 10 kHz and relayed, *via* a Digidata 1320 interface, directly onto the hard drive of a PC running pClamp data acquisition software (version 9.2). Recordings were neither capacity- nor series resistance-compensated. All experiments were conducted at