Expression and Distribution of the Sweet Taste Receptor Isoforms T1R2, T1R3 in Human and Rat Urinary Bladder.

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Running title: Sweet Taste Receptors T1R2, T1R3 in Urinary Bladder

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Keywords: bladder muscle, overactive bladder, sweet taste receptors, urothelium

ABSTRACT

Purpose: Artificial sweeteners have been shown to augment bladder contraction. We hypothesised that artificial sweeteners activate sweet taste receptors in the bladder; therefore, we investigated the expression of sweet taste receptors in human and rat bladders.

Materials and Methods: Sections of human and rat bladder were cut from paraffin blocks and stained by immunohistochemistry for expression of T1R2 and T1R3 sweet taste receptors. Bladder homogenates were subjected to SDS- polyacrylamide electrophoresis followed by immunoblotting for expression of T1R2 and T1R3 receptors. Strips of rat bladder, with and without the urothelium, were suspended in organ baths and the contractile response to 10Hz electrical field stimulation, in the absence and presence of saccharin 10^{-8} M – 10^{-3} M, was obtained. Responses to KCl in the absence and presence of saccharin and saccharin plus zinc were determined.

Results: T1R2 and T1R3 sweet taste receptors were expressed in the urothelium of human and rat bladder. Immunostaining was evident in the plasma membrane of the three cell types of the urothelium and in particular the umbrella cells. Immunoblotting revealed bands at expected molecular weights in both human and rat bladder homogenates. Saccharin augmented rat bladder smooth muscle contraction to electrical field stimulation only when the urothelium was present in the bladder strip. Zinc blocked the enhancing effect of saccharin on responses to KCl.

Conclusion: T1R2 and T1R3 sweet taste receptors are expressed in the urothelium of human and rat bladder. Activation of these receptors by artificial sweeteners may result in augmentation of bladder contraction.

INTRODUCTION

The International Continence Society definition of overactive bladder (OAB) is urgency with or without urge incontinence, usually with frequency and nocturia[1]. A recent prevalence study, conducted in five countries demonstrated OAB prevalence to be 11.8% (10.9% for men and 12.9% for women) increasing with age[2]. Being so prevalent, OAB has significant socioeconomic burdens and a negative impact on quality of life[3]. The mainstay of therapy is anticholinergic drugs, which are effective but after long term treatment the incidence of adverse effects is high[4]. Newer therapies which enhance the detrusor muscle's relaxation mechanisms[4] are being evaluated, but for now, pharmacotherapy of OAB is limited. **There are, however,** other influences on bladder function such as diet and lifestyle factors.

Studies investigating diet and other lifestyle **factors upon the onset of OAB** have found that risk factors in women include smoking, obesity and daily consumption of carbonated soft drinks[5]. Most surprising is the strong association of the onset of OAB, within a year of follow up, with the daily intake of carbonated soft drinks (OR 1.41 95% CI 1.02-1.95). These beverages contain sugar, high levels of artificial sweeteners, caffeine, preservatives and colorants. It is unlikely that caffeine has a causal role due to the lack of association with coffee found in this study[5]. We have previously investigated the effects of artificial sweeteners and preservatives on isolated detrusor muscle contraction[6, 7]. Acesulfame K and saccharin significantly enhanced the contractile response of rat detrusor muscle to calcium evoked responses[7]. A 1:200 dilution of a well known carbonated soft drink enhanced contractile responses to calcium by over 200%[7], suggesting the combination of chemicals in these drinks has a greater enhancing effect on detrusor muscle contraction than either compound alone. Clinically, consumption of both an artificially

sweetened (“diet”) carbonated soft drink and caffeine free “diet” drink increased urinary frequency, mean urinary urgency and urgency episodes in volunteers[8].

Artificial sweeteners activate the sweet taste receptor dimer T1R2/T1R3, which is mainly located in taste buds on the tongue and exists as a heterodimeric G protein-coupled receptor to α-gustducin. Through this signalling system sweeteners activate phoshpolipase C (PLC) β2-dependent pathways to increase intracellular Ca^{2+} concentration[9]. **The T1R3 monomer also acts as a receptor for “umami” taste, as a dimer with T1R1[10].**

Sweet taste receptors have now been found in different tissues such as the pancreas[11], intestine[12,13] and recently rat and mouse brain[14]. These findings contributed to the formulation of our hypothesis that artificial sweeteners enhance detrusor muscle contraction via activation of the sweet taste receptors T1R2/T1R3 in the urinary bladder.

METHODS AND CHEMICALS

Samples

Bladders were removed from 18 female Wistar rats (150 g -250 g) in accordance with schedule 1 of the Animals (Scientific Procedures) Act 1986. **Human detrusor** muscle biopsies were obtained from five female patients undergoing elective gynaecological surgery by cold cup biopsy. Informed consent was obtained from the patients and the project was approved by the Leicestershire, Northamptonshire and Rutland Research Ethics Committee. Samples were either processed immediately after removal or snap frozen in liquid nitrogen and stored at -80°C for processing at a later date.

Immunohistochemistry

The distribution of taste receptors, **T1R2 and T1R3,** were analysed in normal human and rat bladder tissue by immunohistochemistry. Tissues were fixed in 4% (w / v) paraformaldehyde, embedded in paraffin blocks, cut into transverse sections (5.0 µM) on a Leica (model RM2035) microtome and left to air dry for 4 – 5 days. After dewaxing and rehydration, endogenous peroxidases were inactivated by application of 6% $H_2 O_2$ (Sigma-Aldrich). The ABC immunoperoxidase system (Vactastain Elite ABC kit, cat. no. PK-6100, Vector **Laboratories Inc)** was used according to the manufacturer's instructions. Briefly, sections were blocked with 10% normal goat serum in TBS-Tween 0.05% for 30 minutes then incubated overnight at 4° C with the following antibodies, all diluted 1:4000 and raised in rabbits: anti – T1R2 receptor (Abcam, cat. no. ab79229 (rat) and cat. no. ab65417 (human)) and anti - T1R3 receptor (Abcam, cat. **n**o. ab79263 (rat) and cat. **n**o. ab65419 (human)) . Sections were incubated in biotinylated goat anti-rabbit immunoglobulin diluted 1:200 in TBS-

Tween 0.05% plus 1% normal goat serum for 30 minutes and then with avidin/biotinylated enzyme macromolecular complex (ABC) for 30 minutes. Immunolabeling was revealed with 3, 3' –daiminobenzidine substrate (DAB, cat. no. SK-4100, Vector Laboratories Inc.), which had been prepared according to the manufacturers instruction. All steps were carried out in TBS – Tween 0.05% with gentle mixing. Sections were dehydrated in IMS, cleared in xylene and cover slip mounted with DPX (Sigma, Dorset, England). Photomicrographs were taken on an Axioplan transmission microscope with a Sony DXC-151P analogue camera connected to a computer running Axiovision image capture and processing software (Axiovision version 4.4, Carl Zeiss Ltd, Welwyn Garden City, Hertfordshire, UK.). As the blocking peptide was unavailable from the supplier, **rat tongue sections were used to demonstrate positive staining and antibody specificity for taste receptors, rat spleen sections, which do not contain taste receptors, were used to control for non specific binding of the rabbit IgG. Bladder sections, in which the primary antibody was omitted, controlled for non-specific binding of the secondary antibody and non specific staining by the reagents used.** Human tongue (Abcam cat. **n**o. ab4375) and human spleen tissue sections (A.H. Taylor, Leicester University) were used as positive and negative controls, respectively.

Western Blotting

Membrane extracts of bladder tissue were prepared by homogenisation in modified RIPA buffer containing 10µl/ml protease inhibitor cocktail (Sigma-Aldrich, product **n**o. 8340) and phosphatase inhibitor cocktail 20 µl/ml (Calbiochem cat. no. 524629) with an Ultra Turrax T8 (IKA, Germany) and differential centrifugation. Protein estimation was obtained using Bio-Rad protein assay (Bio-Rad Laboratories Ltd, Hertfordshire,

UK) and 5, 10 and 20 µg of protein were separated by 10% Tris-HEPES-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Pierce Precise Protein Gels, Thermo Fisher Scientific, Northumberland, UK) and electroblotted onto nitrocellulose membranes. Pierce® chemiliminescent blue prestained peroxidase-labelled protein molecular weight marker (Thermo Fisher Scientific, Northumberland, UK) was used for molecular weight determination and to establish transfer during electroblotting. Blots were blocked with 5% normal goat serum (Vector Laboratories, Inc.) made up in TBS-0.05% Tween 20 (TBS-T) and incubated with, T1R2 and T1R3 (1:1000) sweet taste receptor antibodies overnight at 4°C. After several washes in TBS-T**ween 0.05%**, a peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich, cat. no. A0545) 1:20,000 dilution was added to the blot and left for 1 hour at room temperature. After repeated washing, protein bands were visualised by the enhanced chemiluminescence technique (Immun-Star™ Western C™ Chemiluminescent kit, Bio-Rad, Hertfordshire, UK). As blocking peptides for the taste receptor primary antibodies was not available from the supplier, **HEK293T cell lysate created using plasmid ID RC218543 based on accession number NM_152232 over expressing Homo sapiens taste receptor T1R2 and HEK293T cells using plasmid ID RC221879 based on accession number NM_152228 over expressing Homo sapiens taste receptor T1R3 were used as positive controls. Cells transfected with the empty vector were used as negative controls (Novus Biologicals, Cambridge)**

Functional Studies

Bladders were removed from 11 male Wistar rats (150g-300g), which had been culled in accordance with schedule 1 procedure of the Animal (Scientific Procedures) Act 1986, and were placed into Kreb's solution. Two longitudinal muscle strips per bladder (6 mm x 3 mm x 1 mm) were cut and suspended in a Perspex organ bath chamber of 0.2 ml volume perfused at the rate of 1 ml/min with Kreb's solution aerated with 95 % oxygen and 5 % carbon dioxide at 37°C. The bladder muscle strips were suspended using fine silk sutures. The base of the strip was attached to the bottom of the chamber and the apex to isometric force transducers connected to a four-channel PowerLab system (AD Instruments) running Chart 3.0 (ADInstruments). The strips were allowed to equilibrate for 1 hour under tension of 1 g before experimentation. Bladder strips were stimulated with electrical field stimulation (EFS) using recessed platinum electrodes in the bath chamber connected to a Harvard Dual Impedance Research Stimulator capable of delivering electrical impulses at different frequencies, voltage and pulse width. Frequency responses were obtained by stimulating the muscle strips with 10 Hz, in triplicate, at 50 volts with a pulse width of 0.05 ms.

Effects of Saccharin on Bladder Muscle with Urothelium

Bladder muscle strip control contractile responses to 10 Hz EFS was obtained in triplicate. Stimulation was then repeated in the presence of saccharin (Sigma-Aldrich, Dorset, UK) 10^{-8} M, 10^{-7} M, 10^{-6} M, 10^{-5} M, 10^{-4} M and 10^{-3} M. The mean tension in grams was taken from 5 - 6 different bladders and **normalised** to control responses (100%). The results were expressed as % maximum control response.

Effects of Saccharin on Bladder Muscle without Urothelium

The urothelium was carefully removed from rat bladder strips with a scalpel blade. The muscle strips were then treated exactly the same as those with intact urothelium.

Blocking effect of zinc on saccharin

Zinc has been shown to block the action of taste receptors[15-17]. To investigate the action of zinc on the enhancing effect of saccharin on rat bladder contraction, contractile responses to 40 mM KCl in the absence and presence of saccharin 10^{-7} M, Zinc 10^{-6} M and saccharin plus zinc were determined.

Solutions and chemicals

TBS-Tween was made up in distilled water with the following constituents: 20 mM Tris Base, 158 mM NaCl and 0.05% Tween 20 pH 7.5. The composition of Kreb's solution was: NaCl 119 mM, KCl 4.4 mM, $NaHCO_3$ 20 mM, NaH_2PO_4 1.2 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2.5 mM and Glucose 11 mM in distilled water; pH 7.34.

Statistical analysis

Contractile responses to EFS were expressed as mean ± SEM of 6 different experiments. Statistical analysis was carried out using repeated measures A.N.O.V.A followed by Dunnett's multiple comparison (GraphPad Prism 5). A p value <0.05 was considered significant.

RESULTS

Immunohistochemistry

Immunoreactivity to T1R2 and T1R3 was seen throughout the urothelium in human bladder biopsies (Fig 1). The pattern of staining was consistent with a G-protein coupled receptor (GPCR) as no nuclear staining was observed. High power images (x400) demonstrated staining in the cell membrane of the umbrella cells in the urothelium (arrowed). **Positive staining was observed in all cell types of the urothelium. In human bladder sections (Fig 1e and 1h), staining of sub urothelial cells is seen. The smooth muscle in all of the human bladder sections was negative for T1R2/T1R3 sweet taste receptors.** There was no staining in sections where the primary antibody was omitted or in human spleen sections (negative tissue control) **indicating there was no secondary antibody or rabbit IgG non-specific staining, respectively.** Immunostaining in the taste buds of the human tongue section showed a similar pattern of staining to human bladder urothelium, **being** mainly confined to cell membranes (arrowed) with no nuclear staining **confirming specificity of the primary antibodies for sweet taste receptors.**

The rat bladder sections (Fig 2) also demonstrated non-nuclear staining for T1R2 and T1R3 in the cells of the urothelium and sub-urothelium. **Denser staining was noted on the urothelial surface compared to the suburothelium. There was no immunoreactive staining in the smooth muscle of the rat bladder sections.** Rat tongue sections were used as positive control tissue **and demonstrated immunoreactive staining in taste buds. Spleen sections were negative for non-specific staining of the primary antibody.** ~~Brain tissue was not used as a negative tissue control, as with immunoblotting, because of a recent publication[14] suggesting~~

~~taste receptors are present in the brain.~~ The section of rat bladder **omitting** the primary antibody was negative for **non-specific staining by the secondary antibody or reagents used during the procedure.**

Western Blotting

Immunoblotting for T1R2 and T1R3 in human and rat bladder tissue was extremely difficult and some degree of proteolysis **with non-specific bands** was found, which has also been demonstrated by other workers[12]. Like Mace et al[12], we found the addition of phosphatase inhibitor cocktails to the tissue lysis buffer helpful. However, background staining was also problematic when using either bovine serum albumin (BSA) or 5% non-fat milk for blocking. This may be due to some sequence similarities between bovine and T1R proteins. To overcome this problem we found normal serum from the species in which the secondary antibody was raised (in this case normal goat serum) more efficient at reducing background.

In the human bladder homogenates, immunoreactive bands to T1R2 antibody were seen at **96 kDa and 86 kDa**. T1R3 bands were seen at **96 kDa and 89 kDa** (Fig 3a). In the rat bladder **and tongue**, immunoreactive bands to anti- T1R2 antibody was seen at **106 kDa** with very faint bands below this at around 84 kDa (Fig 3b). For the anti -T1R3 antibody, bands were found at **100 kDA and 96 kDa for rat bladder and tongue**. The predicted molecular weight for T1R2 and T1R3 is around 95 kDa. **Positive controls for T1R2 and T1R3 demonstrated specific bands around 100 KDa.**

Functional Studies

Effects of Saccharin on Bladder Muscle with Urothelium

An original Chart recording is shown in Figure 4a, showing the effect of the addition of saccharin (10^{-8} M – 10^{-3} M) on the contractile response of rat bladder strips to EFS 10 Hz (2 stimulations). The basal tension of the strips increased and fell with each addition of saccharin.

Saccharin 10^{-5} M significantly increased the contractile response to EFS 10 Hz by 19.2 ± 2.9 % ($p<0.001$) (Fig 4b)

Effects of Saccharin on Bladder Muscle without Urothelium

An original Chart recording is shown in Figure 5a, showing the effect of saccharin on evoked EFS contractile responses in rat bladder strips with the urothelium removed. Although saccharin failed to have an effect on evoked responses, the basal tension increased slightly after **each** addition of saccharin 10^{-8} M – 10^{-6} M, after which it decreased.

There was no significant difference in the contractile response of rat bladder strips to EFS 10 Hz after the addition of saccharin (10^{-8} M – 10^{-3} M) **compared to control** (Fig 5b). **The amplitude of control responses was increased compared to control responses in bladder strips with the urothelium.**

Blocking effect of zinc on saccharin

Original Chart recordings are shown in figures 6 a-**d**. In figure 6**c** the spontaneous contractions appear increased in amplitude after the addition of saccharin compared

to control (Fig 6a). Saccharin augmented the contractile response of whole rat bladder strips to 40 mM KCl by 27 ± 4.7% ($p<0.05$), this effect was blocked by the addition of zinc sulphate (Fig 6**e**)

DISCUSSION

This study demonstrates for the first time that the sweet taste receptors T1R2 and T1R3 are expressed in the urothelium of human and rat bladders. Rat bladder contractile responses to 10 Hz EFS were significantly augmented by the addition of saccharin. This effect was abolished in bladder strips with the urothelium removed. Zinc sulphate **(blocks taste receptor signalling)** blocked the enhancing effect of saccharin on responses to KCl, suggesting saccharin augments bladder contraction via activation of sweet taste receptors. Thus it appears that sweet taste receptors are functional in human bladders.

Sweet taste in the tongue is mediated by the G-protein-coupled-receptors (GPCR) T1R2 and T1R3 that form a heterodimeric receptor complex[18,19]. T1R2/3 activates the heterotrimeric G-protein gustducin, which uses cyclic nucleotides and inositol-1, 4, 5-trisphosphate (IP_3) as second messengers[20]. Ca^{2+} imaging experiments in isolated taste buds of rat vallate, showed that activation of sweet taste receptors with sucrose increased cyclic adenosine monophosphate (cAMP) and Ca^{2+} uptake but activation with artificial sweeteners resulted in increased IP_3 and Ca^{2+} release[21].

In human and rat bladder homogenised tissue, T1R2 and T1R3 receptors were expressed in both species, **although molecular weights for both taste receptors were higher in rat bladder**, similar to those published for rat small intestine[12] . Immunohistochemistry revealed T1R2 and T1R3 receptors expressed in the urothelium of both human and rat bladder, but not in the detrusor muscle. Receptor expression was seen in the urothelial cell membrane and in particular the umbrella cells in the human bladder sections. To maintain an impermeable barrier during bladder filling, umbrella cells show vesicle trafficking and modulation of the surface area[22]. These cells would be exposed directly to various concentrations of artificial

sweeteners in the urine: urinary saccharin and acesulfame-K levels after intake/excretion studies demonstrate spiking ranges of 0.8 – 80.2 mg/ml and 1.2 – 118.6 mg/ml, respectively[23].

Recently it has become apparent that the urothelial cells have specialised signalling and sensory properties[22]. Urothelial cells release ATP, nitric oxide and acetylcholine and changes in levels of these neurotransmitters resulting from increased levels of $[Ca^{2+}]_i$ may alter afferent nerve activity and bladder function. It is therefore possible that activation of T1R2/3 receptors in the urothelium increases $[Ca^{2+}]_i$ leading to increased neurotransmitter release and excitation of afferent nerves and smooth muscle cells. In our study, saccharin increased the contractile response of rat bladder strips to EFS by 19%. This augmentation of contraction was abolished by the removal of the urothelium, suggesting this effect of saccharin is dependent upon an intact urothelium. **It is also interesting to note that removal of the urothelium increased the control contractile response of rat bladder strips to EFS. Inhibitory factors released by the urothelium, on detrusor muscle contraction, have been previously described[24].** Saccharin also augmented contractile responses to KCl, which depolarises cell membranes; this effect was blocked by the presence of zinc sulphate. Zinc has been shown to inhibit nerve responses to sweet stimuli in mice[15] and sweet taste perception in humans[16] by possibly forming a complex with the extracellular component of the sweet taste receptor[17]. The blockade of saccharin augmentation of contractile response by zinc, suggests the action of saccharin on bladder muscle contraction is via activation of sweet taste receptors.

In conclusion, the sweet taste receptors, T1R2 and T1R3, have been shown to be expressed in the urothelium of human and rat bladder for the first time. Saccharin

enhanced rat bladder contraction only when the urothelium was intact and zinc blocked the effect of saccharin. These results suggest that artificial sweeteners enhance bladder contraction via activation of functional T1R2/T1R3 taste receptors in the urothelium.

ACKNOWLEDGEMENTS

Funding for this project was provided by a grant from the International Urogynecological Association.

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LEGENDS

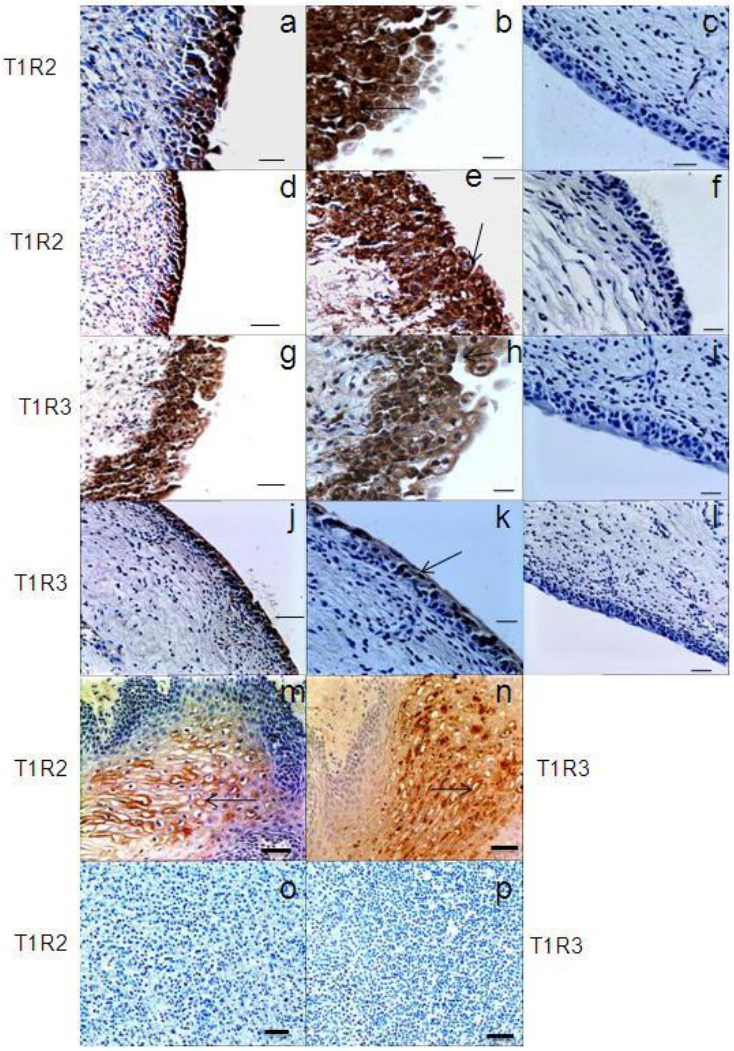

Fig 1. Photomicrographs of human bladder biopsy sections showing immunoreactivity staining of sweet taste receptor T1R2/T1R3 in the urothelium. Immunoreactivity staining (brown) of T1R2/T1R3 receptors is shown in two human bladder sections at different magnifications (a – k), staining in human tongue sections as positive **tissue** control (m, n), human spleen sections showing no staining as negative **tissue** control (o,p) and human bladder sections with no primary antibody added (c,f,**i,**l). Arrows indicate staining in cell plasma membranes**. Scale bar 20µm except for (a,d,g,j) scale bar 50µm.**

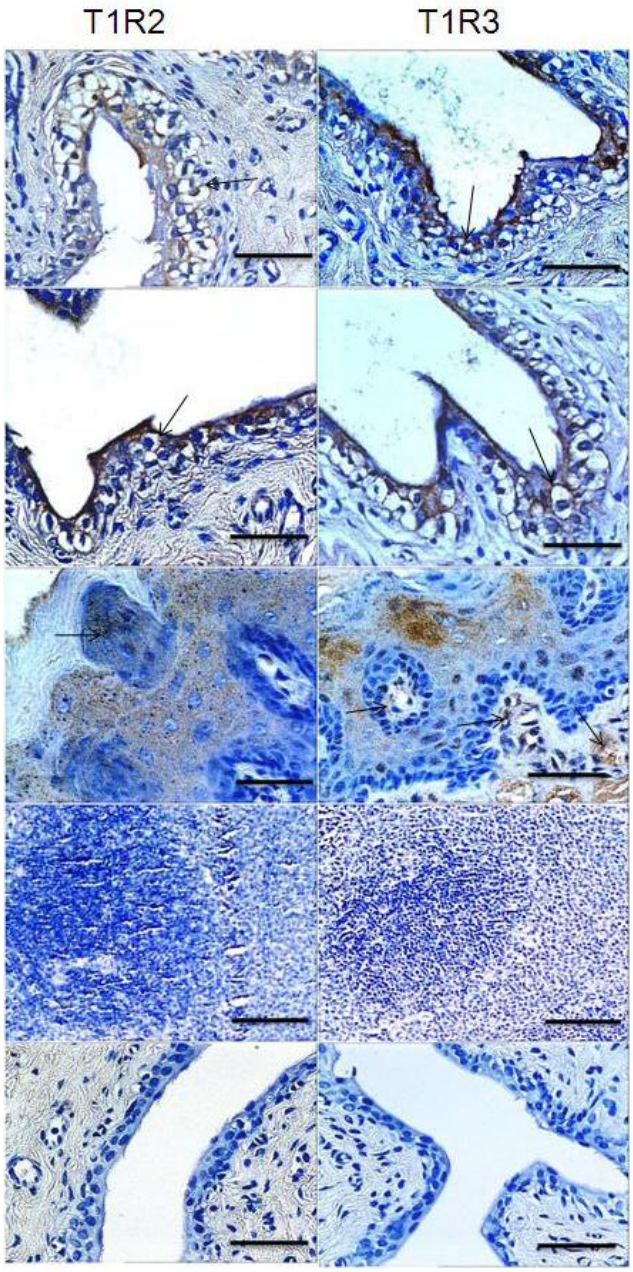

Fig 2. Photomicrographs of whole rat bladder sections showing immunoreactivity staining of sweet taste receptor T1R2/T1R3 in the urothelium. Staining (brown) of sweet taste receptors in the urothelium of two different bladder sections (a - d), rat tongue section showing staining of sweet taste receptors used as a positive **tissue** control (e,f), rat spleen sections showing no staining used as a negative **tissue** control (g,h) and rat bladder sections with no primary antibody added (i,j). Arrows indicate immunoreactivity staining in cell plasma membranes. **Scale bar 50µm.**

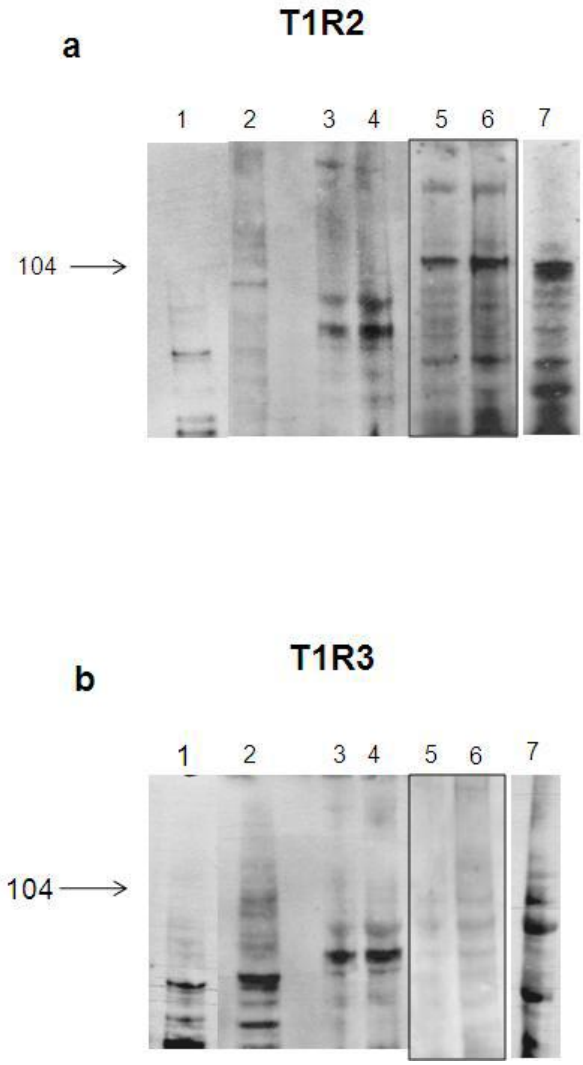

Fig 3. Immunoblot results for human and rat bladder homogenates probed with T1R2 and T1R3 primary antibodies.

(3a) negative control 20 µg protein (1), positive control 20 µg protein (2), human bladder 10 µg protein (3), human bladder 20 µg protein (4), rat bladder 10 µg protein (5), rat bladder 20 µg protein (6), rat tongue 20 µg protein (7)

(3b) negative control 20 µg protein (1), positive control 20 µg protein (2), human bladder 10 µg protein (3), human bladder 20 µg protein (4), rat bladder 10 µg protein (5), rat bladder 20 µg protein (6), rat tongue 20 µg protein (7)

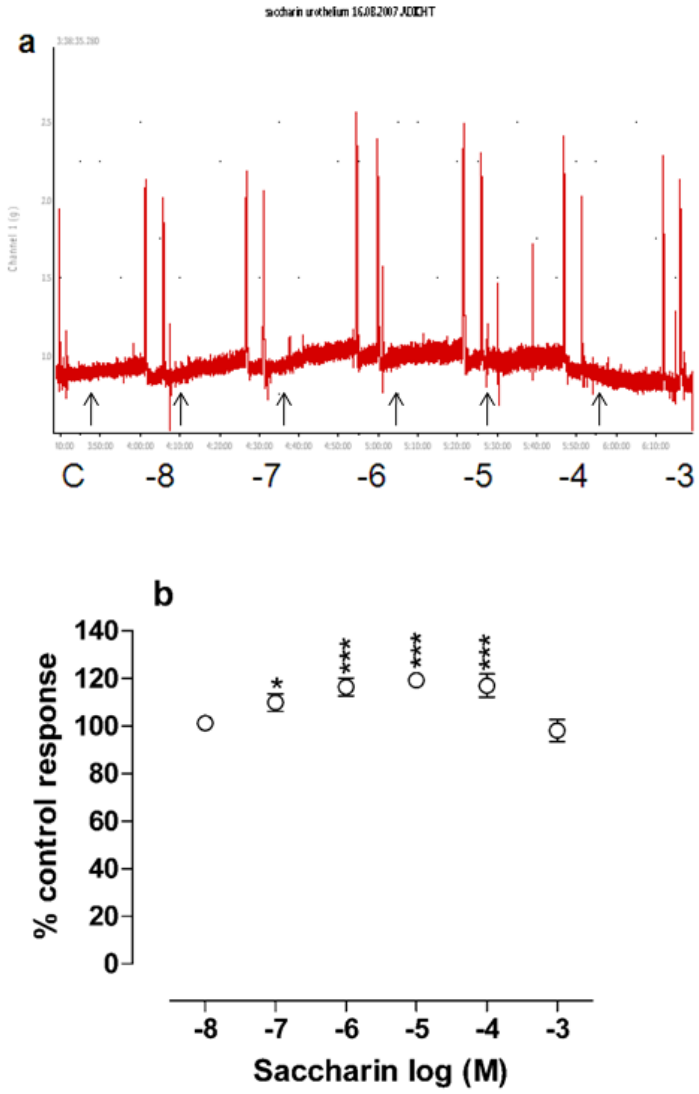

Fig 4. Effect of different concentrations of saccharin (10^{-8} M – 10^{-3} M) on the contractile response of whole rat bladder strips to electrical field stimulation 10 Hz.

(A) An original Chart recording. (B) Normalised contractile responses to EFS 10 Hz in the presence of saccharin. Each data point represents the mean of 6 experiments plus SEM. *p <0.05, ***p <0.001 (Repeated measures ANOVA followed by Dunnett's multiple comparison) **Arrows in panel a) indicate the addition of saccharin**

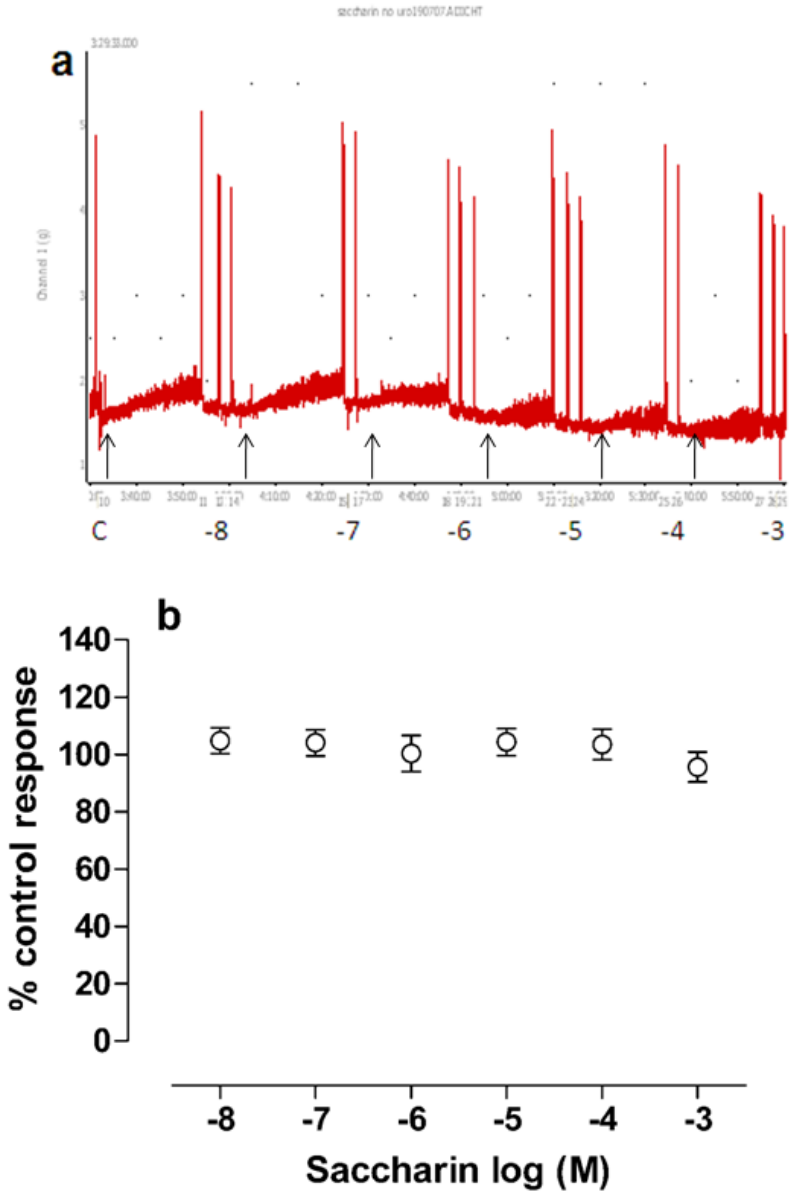

Fig 5. Effect of different concentrations of saccharin (10^{-8} M – 10^{-3} M) on the contractile response of rat bladder strips, with the urothelium removed, to electrical field stimulation 10 Hz. (A) An original Chart recording. (B) Normalised contractile responses to EFS 10 Hz in the presence of saccharin. Each data point represents the mean of 6 experiments plus SEM. **Arrows in panel a) indicate the addition of saccharin**

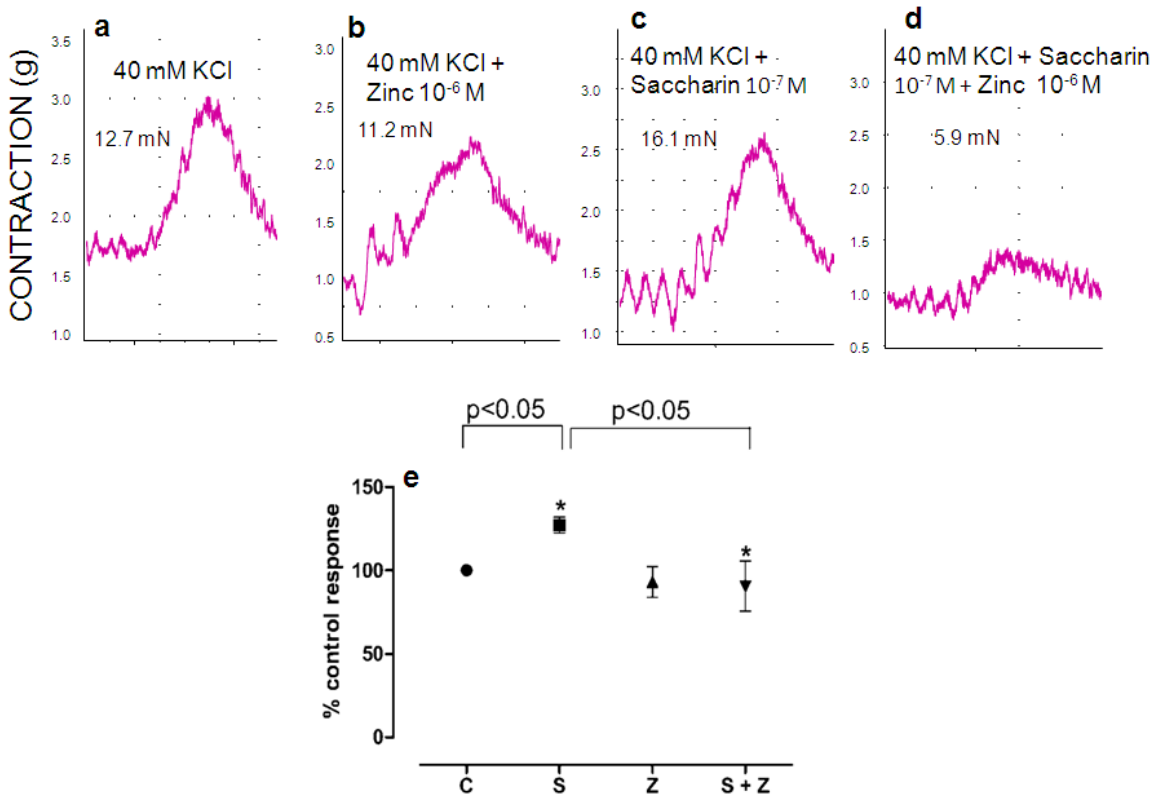

Fig 6. The effect of saccharin (10^{-7} M) and saccharin plus zinc sulphate (10^{-6} M) on the contractile response of rat bladder strips to 40 mM KCl. (a-**d**) Original Chart recordings showing the effect of **zinc** (b), **saccharin (c)** and saccharin plus zinc sulphate (**d**) on the contractile response to KCl. The inhibitory effect of zinc sulphate on contractile response is particularly pronounced in this example. (**e**) normalised data showing (C) control response, (S) effect of saccharin, (Z) effect of zinc sulphate and (S+Z) the effect of saccharin plus zinc sulphate on the response to KCl. Each data point represents the mean of 4 experiments plus SEM. * $p<0.05$ (ANOVA followed by Bonferroni's multiple comparison)