IUGA RESEARCH GRANT 2009
FINAL REPORT

PRINCIPAL INVESTIGATOR AND INSTITUTION

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TITLE OF PROJECT

The role and significance of the sweet taste receptor isoforms T1R1, T1R2 and T1R3 in the rodent and human bladder

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ALL COINVESTIGATOR(S) AND INSTITUTIONS

1. Dr Ruth Elliott, Senior Experimental Officer, University of Leicester
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4. Mr Christopher Mayne, Consultant Urogynaecologist, University Hospitals of Leicester

Provide a detailed report of final results obtained and state whether research aims were met

AIM (1)
To confirm the presence of the sweet taste receptor T1R2 protein in rat bladder tissue by immunohistochemistry and Western blot analysis.

Methods

Immunohistochemistry
5 µm bladder and tongue (control) sections were cut from paraffin blocked samples fixed in 4% paraformaldehyde and immunohistochemistry performed using specific polyclonal antibodies to the T1R2, subunits of the receptors. Samples from 10 animals, and the archived material from human samples, were stained using Vector ABC Elite kit containing biotinylated secondary antibodies and 3,3'-diaminobenzidine (DAB) to visualise immunoreactive tissues, with appropriate tissue and assay controls. As a control for antibody specificity, taste receptor antibodies were pre-incubated with the immunizing peptide. Negative controls contained no antibody. Distribution and density of staining will be assessed by image capture.

Western blot
Rat bladder and tongue were removed from rats after schedule 1 culling. Tissues were placed immediately into lysis buffer containing protease and phosphatase inhibitor cocktails and kept on ice. Bladder extracts were prepared by homogenisation followed by differential centrifugation to isolate membrane fractions. Protein estimation of samples was determined by Bio-Rad protein assay. Samples were then subjected to SDS PAGE and

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immunoblotting. The blots were probed with T1R2 polyclonal antibodies and the relative molecular mass identified. As the immunizing peptide was not available for antibodies used for Western blots, rat brain samples were loaded as negative controls.

Results

**Immunohistochemistry**

![Immunohistochemistry of rat bladder sections.](image)

Immunoreactivity for T1R2 taste receptor was observed in the urothelium of all rat bladders examined. As pre-incubation of the T1R2 antibody with the immunizing peptide gave a negative result (no DAB staining), it is possible to conclude that the positive immunoreactivity in the urothelium of rat bladder sections was specific for the T1R2 taste receptor.
Western Blot

Fig 2. Immunoblot of rat bladder probed with T1R2 antibody. Lane 1 marker, 3 rat brain (20 µg), 5 rat tongue (20 µg), 7 rat bladder (10 µg protein), 8 & 9 rat bladder (20 µg protein). As the immunizing peptide was not available from the supplier of these antibodies, rat brain tissue was used as a negative control.

The rat bladder and tongue show specific dark bands at around 100kDa. The predicted molecular weight of T1R2 receptor protein is 95kDa. There is also evidence of some proteolysis in our rat bladder and tongue preparations, in spite of the presence of protease and phosphatase inhibitor cocktails in the lysis buffer, there are also faint bands in both rat bladder and tongue samples at around 55kDa. Both of these bands increased in density with increased protein concentration loaded onto 10% polyacrilamide gels. There are also very dense bands at around 33kDa in both rat bladder and tongue samples. As there is very little in the literature on taste receptor expression in the bladder we have been unable to find published work to compare with our results. However, Mace et al 2007 determined taste receptor expression in the rat small intestine. Our T1R2 protein expression by Western blot was similar to their blots as they also experienced proteolysis of their preparations. They suggested that this proteolysis may be an inherent part of the signaling process or that these taste receptors may be extremely sensitive to proteases during sample preparation. Overall, the reasons for this are unknown.

Conclusion
T1R2 taste receptors, predicted molecular weight 95-100 kDa, are present in the urothelium of rat bladders. The function of these receptors in the bladder requires further investigation.

Problems
As well as problems with sample proteolysis, performing Western blot protocols with taste receptor antibodies has been extremely difficult. The main difficulties have been:

1. Cross reactivity of taste receptor antibodies with BSA

This was a major problem as many commercial antibodies, primary and secondary, contain BSA in the preservative buffer. Cross reactivity was also a problem with 5 % non-fat milk for the blocking step in the immuno-blot protocol. Primary and secondary antibodies that did not contain BSA were identified and only these were subsequently purchased for this study. Non-fat milk for the blocking step was replaced by 2% normal serum made up in TBS buffer containing 0.05% Tween.
2. Inadequate detection by commercial antibodies

Initially, taste receptor antibodies purchased from a commercial supplier did not detect 90-100kDa bands predicted to be the molecular weight of T1R2 taste receptor. Purchasing from a different supplier solved this problem. Overall, all these problems took time to identify.

AIM (2)
To examine rat bladder tissue for the expression of the taste receptor proteins T1R1 and T1R3 by immunohistochemistry and Western blot analysis.

Methods

Immunohistochemistry, SDS PAGE and immunoblot protocols as described for T1R2

Messenger RNA expression
Tissue samples will be snap-frozen in liquid nitrogen and stored at -80°C. Tissue extracts will be subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) with gene specific primer sets for both human and rodent T1R2 and T1R3 with appropriate controls. Reaction products will be resolved on agarose gels and comparison to size-specific markers. Eluted products will be sequenced to ensure specificity of amplification.

Results

Immunohistochemistry

Fig 3. Immunohistochemistry of five rat bladder sections probed with T1R1 antibody. Immunoreactivity for T1R1 taste receptor was negative in four out of five rat bladder sections investigated. Section C shows some patchy immunoreactivity in the urothelium of the rat bladder. In section F the T1R1 antibody has been pre incubated with the immunizing peptide.
Fig 4. Immunohistochemistry of four rat bladder sections probed with T1R3 antibody. Immunoreactivity to T1R3 sweet taste receptor was seen mainly in the urothelium of rat bladders. If the T1R3 antibody was pre-incubated with the immunizing peptide.

**Western Blot**

Fig 5. Immunoblot of rat bladder probed with T1R1 antibody. Lane 1 is the marker, 3 rat tongue (20 µg), 5 rat brain (20 µg), 7 rat bladder (5 µg), 8 rat bladder (10 µg), 9 rat bladder (20 µg).

Rat tongue and bladder samples show very faint bands at around 90kDa. The T1R1 antibody detects bands of approximately 93kDa. As with the immunoblot for T1R2 taste receptor, there is evidence of some proteolysis in our samples. The rat tongue preparation also shows a dense band of around 40kDa which is very faint in the rat bladder preparations. The negative control (rat brain) has a band at around 30kDa, which suggests the bands at this molecular weight in our samples are not specific for T1R1.
It was extremely difficult to obtain a reasonable blot with the T1R3 antibody. There was a significant amount of background in spite of using 2% normal goat serum for blocking. However, the blot shows faint bands at around 104 kDa in both the rat tongue and bladder. In the rat bladder samples this band increases in density with increasing amount of protein loaded onto the gel. A darker band is seen at around 90kDa in both the tongue and bladder samples, which increases in intensity in rat bladder samples. This antibody detects bands of approximately 95kDa. At around 76kDa there it a dark band in the bladder samples which appears as a very faint band in the tongue. Other bands at 45, 40 and 30 kDa were found in both rat bladder and tongue samples. The brain sample was negative.

mRNA expression
RT-PCR with primer sets for rodent T1R3 have so far been negative for mRNA expression. It is highly likely that that this has been due to technical issues rather than the absence of T1R3 mRNA. Quantification of RNA has showed adequate volumes, however, other possible problems are:

1. Primer annealing temperature is too low
2. Quality of cDNA made from mRNA (action of AMV-RT)
3. Taq polymerase out of date or contaminated

These possible problems are currently being investigated.

Conclusions
The rat bladder expresses T1R3 taste receptor but it does not consistently express T1R1 taste receptors.

AIM (3)
To examine human bladder tissue samples for the presence of T1R1, T1R2 and T1R3.

Methods
Human bladder biopsies were removed from female patients undergoing routine gynecological surgery. The study was granted ethics committee approval and bladder biopsies were removed after informed consent was obtained from the patients.
Immunohistochemistry as previously described.

**Results**

![Image of immunohistochemistry results](image)

Fig 7. Immunohistochemistry of human bladder biopsy section probed with taste receptor antibodies T1R1, T1R2 and T1R3. A  immunoreactivity to T1R1 antibody, B after pre-incubation of T1R1 antibody with immunizing peptide. C-G 5 different human bladder biopsy sections showing immunoreactivity to T1R2 antibody. Section H is the same bladder biopsy as D but the T1R2 antibody was pre-incubated with the immunizing peptide. I & J are the same human bladder biopsy section showing immunoreactivity to T1R3 antibody. K after pre-incubation of the T1R3 antibody with the immunizing peptide.

Immunoreactivity for T1R1, T1R2, and T1R3 taste receptors is present in the urothelium of human bladder biopsies examined. Further investigations are required to confirm these results.

**CONCLUSIONS**

All research aims have been met. We have demonstrated for the first time that sweet taste receptors T1R2 and T1R3 are expressed in human and rat bladders and are located specifically in the urothelium and not in the detrusor muscle. The taste receptor T1R1, although expressed in the human bladder is not consistently expressed in the rat bladder. Previous work from this group has demonstrated increased isolated rat bladder muscle contraction in the presence of individual artificial sweeteners (Dasgupta et al 2006). Our hypothesis is that artificial sweeteners activate taste receptors in the urothelium raising intracellular [Ca^{2+}] resulting in augmented detrusor muscle contraction. Further investigation is required to confirm our preliminary findings and to investigate secondary signaling pathways following activation of sweet taste receptors in the bladder.
FUTURE WORK

Over the next six months, we plan to conduct further experiments to confirm the initial findings of differential T1R1 expression in rodent and human bladder. The presence of both T1R2 and T1R3 is consistent with our hypothesis that a functional sweet taste receptor exists in the bladder (T1R2/T1R3 dimer forms the sweet taste receptor) so we will also begin to examine second messenger pathways which may be involved using urothelial cell cultures to examine calcium flux and cGMP, diacyl glycerol and phospholipase A2. The work we have completed, and the early work planned with the remainder of the grant should allow us to be in a position to submit grant application for significant funding in the New Year.

We are therefore requesting a six month “time only” extension for the grant to allow us to complete this work.

References


I. Was an abstract submitted to the IUGA annual meeting? Yes

Presentation Title: THE EXPRESSION OF T1R2 TASTE RECEPTOR IN HUMAN AND RAT URINARY BLADDER.

Presentation Start/End Time: Friday, Jun 19, 2009, 11:48 AM -12:00 PM

Objectives: To investigate sweet taste receptor T1R2 expression in human and rat bladder.

Background:
Consumption of carbonated soft drinks is associated with onset of OAB symptoms within the next 12 months (OR 1.62, 95% CI 1.18, 2.22)\(^1\). These drinks contain artificial sweeteners saccharin, acesulfame K, aspartame. We have shown that artificial sweeteners increased rat bladder contraction via increased Ca\(^{2+}\) influx through L-type calcium channels\(^2\). Sweeteners activate T1R2 and T1R3 taste receptors which are coexpressed as a heterodimeric G-protein-coupled receptor complex. Downstream signalling is via increased IP\(_3\) and raised intracellular Ca\(^{2+}\) concentration. Our hypothesis is that artificial sweeteners increase bladder contraction via sweet taste receptors in bladder smooth muscle. This study investigated the expression of T1R2 taste receptors in human and rat bladder.

Methods:

Immunocytochemistry

Immunocytochemistry was performed on 5\(\mu\)m formalin fixed human and rat bladder sections using specific polyclonal antibodies to T1R2 receptors. Sections were stained using 3,3\(^'\) - dianinobenzidine to visualise immunoreactive tissues. Rat tongue was used as positive control and the specificity of the T1R2 antibody was determined by incubation with the blocking peptide.

Western blot

Rat bladder and tongue samples were homogenised and differentially centrifuged then subjected to SDS-PAGE. Each lane was loaded with 20\(\mu\)g of protein. Proteins were transferred onto PVDF membrane for immunoblotting and probed with T1R2 antibodies and the relative molecular mass identified.
Functional studies.
Rat bladder strips were prepared with and without urothelium and placed in a perfusion organ bath. Responses were obtained to 10Hz electrical field stimulation after the strips were incubated with saccharin $10^{-8}$M - $10^{-3}$M. The effect of zinc (which blocks T1R2) on whole rat bladder strips in the presence of saccharin was also determined.

![Fig1. T1R2 taste receptor immunohistochemistry of human (A) and rat bladder (B).](image)

Results:
Specific immunostaining for T1R2 receptors was seen in human and rat bladder urothelium (Fig 1). No staining was observed when sections were co-incubated with the blocking peptide. Immunoblot of rat bladder probed with T1R2 antibody showed evidence of proteolysis, as previously observed in intestinal smooth muscle\(^3\). Specific dark bands were seen at 55 kDa and 30 kDa in bladder, and at 110 kDa and 40 kDa in the rat tongue.

Functional studies showed that saccharin increased contractile response of rat bladder strips by 18 ± 2%. The effect was abolished when the urothelium was removed. Saccharin increased the response to 40mM KCl by 27 ±4.7% and this effect was blocked by zinc.

Conclusions:
Taste receptor T1R2 is expressed in the urothelium of human and rat bladder. These receptors are functional and mediate artificial sweeteners augmentation of bladder contraction.

References:

Presenter:  
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II. Was a manuscript submitted to IUJ? No, PENDING.