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ABSTRACT

Objective and design The aim of this study is to evaluate the expression of the histamine receptors, particularly focusing on the H_4R in human renal tubules.

Material The *ex-vivo* evaluation was carried on specimens from human renal cortex. Primary and immortalized tubular epithelial cells (TECs) and the HK-2 cell line were used as *in vitro* models.

Treatment Cells were pretreated for 10 min with chlorfeniramine maleate 10 µM (H_1R antagonist), ranitidine 10 µM (H_2R antagonist), GSK189254 1 µM (H_3R antagonist) or JNJ7777120 10 µM (H_4R antagonist), and then exposed to histamine (3 pM - 10 nM) for 30 min.

Methods The *ex-vivo* evaluation on specimens from human renal cortex was performed by immunohistochemistry. The expression of histamine receptors on primary and immortalized TECs and the HK-2 cell line was evaluated at both gene (RT-PCR) and protein (immunocytofluorescence) levels. The pharmacological analysis was performed by TR-FRET measurements of second messenger (IP3 and cAMP) production induced by histamine with or without the selective antagonists.

Results Our data revealed the presence of all histamine receptors in human tubules; however, only TECs expressed all the receptors. Indeed, histamine elicited a sigmoid dose-response curve for IP_3 production, shifted to the right by chlorpheniramine maleate, and elicited a double bell-shaped curve for cAMP production, partially suppressed by the selective H_2R, H_3R and H_4R antagonists when each added alone, and completely ablated when combined together.

Conclusions Herein, we report the identification of all four histamine receptors in human renal tubules.

Keywords: Histamine, histamine receptors, human kidney, HK-2 cells, tubular epithelial cells

Introduction

Histamine is a pleiotropic amine ubiquitously distributed, that exerts its effects through four subtypes of G-protein coupled receptors, namely H_1 receptor (H_1R), H_2 receptor (H_2R), H_3 receptor (H_3R) and H_4 receptor (H_4R), differentially expressed in various tissues and cell types. The presence of histamine in the kidney has been detected for decades. It can derive from resident mast cells [1], glomerular cells, which have been reported as a major site of intrarenal histamine synthesis and accumulation [2], and from proximal tubular epithelial cells, where the expression of the enzyme histidine decarboxylase (HDC) has been demonstrated [3].

Indirect evidence sustains the hypothesis that histamine could be involved in renal physiology in humans. In healthy subjects the administration of loading doses of L-histidine led to an increase of histamine concentration in urine, but not in blood [4]. In renal transplant patients the urinary and blood levels of histamine are elevated [5]. Moreover, some evidence suggest that histamine may be involved in diabetes-related kidney disease. Indeed, in the kidney of diabetic rats, histamine was increased compared with controls[6, 7]. Independent studies indicate that histamine regulates the renal microcirculation, by increasing salt and water excretion[8-10], decreasing the ultrafiltration coefficient by reducing the total filtration surface area [10], and increasing renin release [11].

The renal effects of histamine have been first ascribed only to H_1R and H_2R, both identified in the glomeruli [12, 13]. Recently, a possible role for H_4R has been suggested by our research group [14]. While a very low mRNA level of the latest discovered histamine receptor has been reported in the kidney of dog, monkey, rat, mouse, guinea pig and pig [15-20], in 2013 we demonstrated the presence of H_4R in resident renal cells of the loop of Henlé and its profound upregulation in the kidney of diabetic rats [14]. Notably, these data provide the first basis to hypothesize a possible involvement of H_4R in the onset/progression of diabetes-associated renal disease. However, this study, as well as the majority of the previous research, was conducted on rodents and the demonstration of the presence of the histamine receptors in the human kidney

is still lacking. Thus, herein we aimed to extend our previous observation on renal H_4R from rats to humans. The study herein was focused on the renal tubules and the H_4R was evaluated in different cells, by using both the immortalized human proximal tubular epithelial cell line Human Kidney 2 (HK-2), and human tubular epithelial cells (TECs). Moreover, herein we broadened our investigation to all of the four histamine receptors.

Materials and Methods

Reagents

All reagents and chemicals used were from Sigma–Aldrich (St. Louis, MO) unless otherwise noted. GSK189254 (PubMed CID 24768876) was kindly supplied by Prof. E. Masini, University of Florence, Italy. Cell media and cell reagents were from Lonza group Ltd. (Allendale, NJ, USA). Hanks Balanced Salt Solution was from GIBCO (Grand Island, NY). RevertAid™ First Strand cDNA Synthesis Kit as well as SuperSignal West Pico Chemiluminescent Substrate were from Thermo Fisher Scientific Inc. (Rockford, IL, USA), EuroTaq DNA polymerase was from Euro-clone (Milan, Italy). Sequence-specific oligonucleotide primers were purchase from Sigma-Genosys (Milan Italy). The antibodies for histamine H_1R (H300, sc-20633) and H_2R (S20, sc-33973) as well as UltraCruz™ Autoradiography Film were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA), while anti-rabbit and anti-mouse IgG HRP-linked antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA) and the swine anti-Goat IgG antibody from Cedarlane Labs (Ontario, Canada). BCA protein assay was from Pierce Biotechnology Inc. (Rockford, IL, USA) and PVDF membrane from Millipore (Bradford, MA, USA). The LANCE® Ultra cAMP Detection Kit and the IP-One HTRF® assay kit were from PerkinElmer Inc. (Waltham, MA, USA) and Cisbio Bioassays (France), respectively.

Histamine dihydrochloride (PubMed CID 5818), chlorpheniramine maleate (PubMed CID 5281068), Ranitidine hydrochloride (PubMed CID 3033332), GSK189254 and JNJ7777120 (PubMed CID 4908365) were dissolved in dimethyl sulfoxide, and the final drug concentrations were obtained by dilution of stock

solutions in the experimental buffers. The final concentration of the organic solvent was less than 0.1%, which had no effect on cell viability.

Antibodies

H_3R and H_4R were detected by using the anti-hH_3R (349–358) and the anti-hH_4R (374–390) antibodies produced and validated for detecting both human and rodent H_3R and H_4R in the School of Biological and Biomedical Sciences, Durham University [21-27]. Briefly, anti-hH_3R antibody was generated coupling the peptide corresponding to the amino acids RLSRDRKVAK Cys of the human and rat H_3R to thyroglobulin by m-maleimidobenzoic acid N-hydroxysuccinimide ester coupling method as described previously [6] and antibodies were raised in rabbits to the resultant conjugate. Anti-peptide antibody production was monitored by enzyme-linked immunosorbent assay with the peptide H_3 (349–358) as the antigen. The anti-hH_4R antibody was generated against the last 17 amino acids of the C-terminal tail of the H_4R conjugating the peptide corresponding to the amino acids CIKKQPLPSQHSRSVSS to thyroglobulin by the cysteine-coupling method [28]. The resultant conjugate was used to generate polyclonal antibodies in rabbits. The selectivity of the anti-hH_4R antibody was confirmed by blockade with the C-terminal peptide of the H_4R and a lack of cross-reactivity with the human H_3R, the most closely related G-coupled receptor [21]. The antibodies for histamine H_1R (H300, sc-20633) and H_2R (A20, sc-19773), were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA).

Immunohistochemistry

Conventional immunohistochemical procedures were employed as described previously [14, 29, 30]. Briefly, immunoperoxidase staining for H_1R, H_2R, H_3R and H_4R was performed on 5 μm sections for formalin-fixed tissue from 12 patients who underwent elective nephrectomy as first line therapy for renal carcinoma at the Urology Surgery of the A.O.U. Città della salute e della Scienza of Turin, Italy, after approval of the Ethical Committee for the use of human tissue of the Department of Medical Sciences of

the University of Turin, Italy. Incubation with sodium citrate 50 mM pH 8.4 for 30 min at room temperature followed by sodium citrate 50 mM pH 8.4 for 30 min at 80°C was performed for antigen retrival. Sections were incubated overnight with anti-H_1R (1.3 µg/ml), anti-H_2R (1.3 µg/ml), anti-H_3R (1 µg/ml) or anti-H_4R (2 µg/ml) receptor subunit at 4°C, followed by a three-layer streptavidin–biotin–peroxidase complex staining method (Vectastain ABC Elite kit and 3',3-diaminobenzadine tetrahydrochloride, DAB; Vector Laboratories, Inc.). Tissue was also screened in the absence of primary antibody, where in all cases, a major part of the staining was abolished (data not shown). All sections were stained or immunostained in a single session to minimize artifactual differences in the staining. Photomicrographs of the histological slides were acquired randomly with a digital camera connected to a light microscope equipped with a ×20 objective (Olympus BH2).

Cell cultures

In this study, the following cell types primary (p) and immortalized (i) TECs from human renal cortex and HK-2 cell line were used. Primary cultures were obtained from the normal portion of cortex surgically removed kidneys (n=5) as described previously [31]. The healthy tissue was derived from kidney samples of patients who underwent unilateral nephrectomy due to renal carcinomas as first line treatment. To our knowledge, no other relevant pathology was diagnosed in the medical history of each patient enrolled and no drugs able to induce histamine release were used in treatment. Briefly, after dissection of the cortex and passage through a graded series of meshes, a mixed population of pTECs was plated in DMEM containing 1 mg/l glucose supplemented with 10% FCS, penicillin/streptomycin (100 IU/ml), and l-glutamine and the cultures were maintained at 37 °C in a 95% air/5% CO_2 humidified incubator. iTECs were obtained from the respective primary cells by infection with a hybrid Adeno5/SV40 virus as previously described [31-34]. These cells showed negative staining for von Willebrand factor, minimal staining for desmin, and marked staining with antibodies to cytokeratins, actin, and alkaline phosphatise [32]. Primary cultures were used within first three passages. HK-2, immortalized human proximal tubular epithelial cells from American

Type Culture Collection (Number **CRL-2190**), were cultured in DMEM containing 1 mg/l glucose supplemented with 10% FCS, penicillin/streptomycin (100 IU/ml), and l-glutamine and the cultures were maintained at 37 °C in a 95% air–5% CO_2 humidified incubator.

RT-PCR

Two µg/µl of total RNA extracted from kidney epithelial cells was processed by RevertAid™ First Strand cDNA Synthesis Kit according to the manufacturer's instruction and were subjected to RT-PCR as previously described [35]. Briefly, RT-PCR amplifications were performed in 25 µl reaction mixtures containing 2µl of cDNA, 2.5 µl of 10X buffer, 1.0 µl of 50 mM $MgCl_2$, 0.20 µl of 25 mM dNTPs mix, 0.05 U of EuroTaq DNA polymerase, and 2.5 µl of 5 µM sequence-specific oligonucleotide primers reported in Table 1. PCR amplicons were resolved in an ethidium bromide-stained agarose gel (2.5 %) by electrophoresis. GADPH gene expression was used as an internal control.

Immunocytofluorescence

Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.1% TritonX-100. Immunofluorescence was performed using the indicated antibodies against the histamine receptors, followed by incubation with Alexa-Conjugated secondary antibodies (Invitrogen). Nuclei were stained with Hoescht (Sigma). All the slides were examined at ×40 magnification using Apotome systems (Zeiss).

Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET)

The TR-FRET assay was used to evaluate cellular cAMP and IP_1 production using the LANCE® Ultra cAMP Detection Kit and the IP-One HTRF® assay kit, respectively, according to the manufacturer's instruction. Briefly, for cAMP 10,000 cells/well, pretreated with vehicle alone or with ranitidine 1 µM or 10 µM (H_2R antagonist), GSK189254 0.1 µM or 1 µM (H_3R antagonist) or JNJ7777120 1 µM or 10 µM (H_4R antagonist) for 10 min, were exposed to histamine in the range 3 pM - 10 nM for 30 min in presence

of IBMX 0.5 mM. After 1 h incubation at room temperature with Eu^{3+}-labeled antibody and allophycocyanin-labeled antibody, energy transfer was measured by exciting the Eu^{3+} at 320 nm and monitoring the allophycocyanin emission at 665 nm using the multiple plate reader Victor X (PerkinElmer Inc.). Data were expressed as TR-FRET signal over the basal one.

Similarly, for IP1, the downstream stable metabolite of IP3, 50,000 cells/well were pre-treated with vehicle alone or chlorpheniramine maleate 1 µM or 10 µM (H_1R antagonist) for 10 min and exposed to histamine in the range 3 pM - 300 nM for 1 h. After incubation for 1 h at room temperature with IP-One Tb conjugate and Lumi4™-Tb cryptate-label antibody, energy transfer was measured by exciting the Lumi4™-Tb cryptate at 320 nm and monitoring the emission at both 620 and 665 nm using the multiple plate reader Victor X. Results are expressed as the 665nm/620nm ratio over the basal one.

Statistical analysis

In some cases (Fig. 3 and Fig. 5), data were best-fitted as sigmoid concentration–response curves, and analyzed with a four-parameter logistic equation by using the software Origin version 6.0 (Microcal Software, Northampton, MA). Results are shown as mean ± SEM and were analyzed by Student's t-test or one-way ANOVA with Dunnett's multiple comparison to determine significant differences between mean values: *P*-values < 0.05 were considered significant. Data analysis was performed testing for normality using the Klmogorov-Smirnov test by Prism 4 software from Graphpad (CA, USA).

Results

Histamine receptor identification in the human renal tubules

To evaluate the expression of the four histamine receptors, 12 human renal cortex specimens were subjected to immunohistochemical analysis. As showed in Fig. 1, immunolabeling for each of the antibodies tested was detected in the tubules, thus suggesting that all four receptor subtypes are present. In particular, specific H_1R, H_2R and H_4R immunoreactivities were found at the tubular level, with a robust

positivity for H_4R at the basal membrane of some tubular epithelial cells. Most intriguingly, immunolabelling sections with the anti-H_3R antibody yielded staining only on some tubule epithelium, with a stronger signal at the apical membrane. No staining was detected with specimens probed in the absence of primary antibodies (not shown).

Histamine receptor expression in human renal cells

The expression of the four histamine receptors was evaluated at both gene (Fig. 2A) and protein (Fig. 2B) levels in different human tubular epithelial cells (HK-2, iTECs and pTECs). As shown in Fig. 2A, single transcripts corresponding to the size predicted for *H1R* (403 bp), *H2R* (497 bp), *H3R* (221 bp) and *H4R* (353 bp) were obtained in both pTECs and iTECs. Consistently, both the cell cultures showed positive staining for all four receptors when the protein expression was evaluated by immunofluorescence (Fig. 2B). These results indicate that all the histamine receptor subtypes are expressed in these cells. Given the similarity between primary and immortalized cells, we used the immortalized cells for the next set of experiments. In comparison to all TECs tested, only the single transcripts corresponding to the size predicted for *H1R* and *H4R* were observed in HK-2, while the transcript corresponding to the size predicted for *H2R* was undetectable. H_3R protein was confined to some cells from tubules, but no transcript for *H3R* was detected on the HK-2 cell line (Fig. 2A).

These results were confirmed when histamine receptors were analyzed by immunofluorescence. Indeed, as shown in Fig. 2B, H_1R showed a diffuse staining in all the cells tested, with a predominant localization in the membrane of some HK-2 cells and in the perinuclear zone in iTECs. In comparison, when H_4R was considered, diffuse staining was observed with a prevalent signal at the cytoplasmic level; however, a membrane localization of H_4R signal was also found in some iTECs. A similar result was obtained for H_2R: detected in the cytoplasm; it was also clearly visible on the membrane in iTECs. Finally, H_3R was only expressed by iTECs. Similarly to iTECs, pTECs expressed all the four histamine receptor subtypes.

Second messenger production evoked by histamine in human renal cells

In order to confirm the functional expression of the histamine receptors suggested by RT-PCR and antibody labelling, we tested the activation of the histamine receptors evaluating the production of second messengers in iTECs, and HK-2 cell line challenged with histamine utilizing a TR-FRET assay.

To evaluate the effect of histamine on the activation of the H_1R, a G_q protein-coupled receptor, the levels of IP_1, the downstream more stable metabolite of IP_3, were measured. Cells exposed for 1 h to histamine 3 pM - 300 nM showed a concentration-dependent decrease in TR-FRET signal (Fig. 3), which indicates an increase of IP_1. Notably, a concentration-dependent relationship was reported for both the cell types. Different EC_{50}s for histamine were observed: 0.56 ± 0.04 nM for HK-2 (Fig. 3A) and 8.84 ± 0.89 nM for iTECs (Fig. 3B). The pre-treatment for 10 min with the selective H_1R antagonist chlorpheniramine maleate at either 1 µM or 10 µM shifted in a concentration-dependent manner the curves evoked by histamine, thus confirming the functional expression of H_1R in the renal cells. However, differences between the two cell lines were still observed, with chlorpheniramine maleate exerting a surmountable antagonism in HK-2 cells (Fig. 3A) and an apparent non-competitive antagonism in iTECs (Fig. 3B).

To evaluate the effect of histamine on the activation of the G_s protein, preferentially coupled with H_2R, and G_i protein, coupled with both H_3R and H_4R, protein, the levels of cAMP were measured. As shown in Fig. 4, iTECs exposed for 30 min to histamine 3 pM - 10 nM produced a double bell-shaped dose-response curve: the first in the 3 pM – 0.1 nM range, with the maximum increase in TR-FRET signal at 0.02 nM, the second in the 0.1 – 10 nM range, with the maximum increase achieved at 0.4 nM (Fig. 4A). When singly-administrated, ranitidine 1 µM or 10 µM modified both of the two peaks of cAMP production at 0.1 nM and 10 nM, respectively (Fig. 4B), GSK189254 0.1 µM or 1 µM suppressed the second bell-shaped curve (Fig. 4C), while JNJ7777120 (1 µM or 10 µM) ablated the first high-affinity histamine response (Fig. 4D). Notably, the co-administration of all three antagonists completely abolished the effect evoked by histamine (Fig. 4E). Thus, the data herein obtained are consistent with the presence of H_2R, H_3R and H_4R. In comparison to iTECs, the HK-2 cell line (Fig. 5) displayed a single sigmoid increase in TR-FRET signal

(EC_{50} 1.04 ± 0.15 nM), suggesting that only a G_i protein coupled receptor is expressed. The antagonism produced by JNJ7777120 10 μM indicates that the contributing receptor to this latter effect is the H_4R.

Discussion

This study provides the first comparative evaluation of the four histamine receptor expression in the human renal tubules. Whether functional histamine receptors are expressed by human renal cells has been scantly investigated, with studies identifying H_1R and H_2R based on the use of pharmacological tools [32, 33]. Herein, by using a combined molecular, anatomical and pharmacological strategies, we provide convergent lines of evidence strongly supporting the conclusions that all histamine receptors are present in the kidney. In particular, our study was focused on tubules, where we demonstrate that all of the histamine receptors are expressed on epithelial cells.

Our *in vitro* observations were performed on two epithelial tubular cell lines iTECs and HK-2. iTECs were isolated from human renal cortex and represent a mixed population of tubular epithelial cells, while HK-2 is a commercially available pure proximal tubular epithelial cell line, selected for study based on the assumption that the most abundant epithelial tubular cells within the cortex are proximally located. Moreover, to avoid bias due to the cell immortalization process, we also compared primary cells pTECs to the corresponding immortalized human cells.

H_1R, H_2R and H_4R were found in all the cell types tested, thus demonstrating an even distribution along the tubules; while H_3R was confined to a minor subpopulation of renal cells as expressed only by TECs. These data extend the previous findings on the localization of H_1R and H_2R in mammalian glomeruli [2, 13, 36] to other nephron components, such as tubular epithelial cells. Moreover, we extended our recent observation on H_4R expression in the kidney of rats [14], now demonstrating its expression on human proximal tubular cells. Although we previously reported that in the kidney of rats the H_4R is expressed by resident renal cells of the loop of Henlé, and not by proximal tubular cells as observed in humans, these two apparently contrasting observations are in keeping with the intra-species differences of H_4R expression already widely

reported [16]. Moreover, the present study was performed only on renal cortex derived specimens and cells, thus a wider distribution also in the medulla and on the epithelial cells of the human loop of Henlé could not be ruled out.

An original and unexpected result of our study is the H_3R identification on renal tubular epithelial cells. It has been largely demonstrated that H_3R is mostly expressed in the central and, to a lesser extent, in the peripheral nervous system. Only a small number of experimental observations have showed its presence on non-neuronal cell types such as rodent fundic mucosa endocrine cells [26], cholangiocytes [37], pancreatic β-cells [38], and in the human bronchial epithelial cell line BEAS-2B [39]. Therefore, our data add a new non-neuronal localization of the H_3R, contributing to the hypothesis that the H_3R could also mediate non-neuronal histamine effects. It is not sufficient to claim the presence of histamine receptors in the renal tubules based purely on PCR and immunological techniques, thus a pharmacological approach was adopted herein to provide stronger evidence for the presence of functional histamine receptors.

The canonical H_1R-mediated response (second messenger IP_3, evaluated by IP_1) was measured in both HK-2 and iTECs, consistent with the observed H_1R mRNA and protein expression. The involvement of histamine-mediated cAMP response was also demonstrated in both of the cell types tested. In HK-2, although both H_2R and H_4R were identified at both the gene and protein level, histamine evoked only a reduction in cAMP, consistent with a G_i-protein coupled receptor such as H_4R. Indeed, JNJ7777120 completely ablated the histamine-evoked effect in this cell line. This finding, in keeping with the apparent largely cytoplasmatic localization of H_2R, suggests that in HK-2, this receptor is non-functional. Perhaps, the presence of nM histamine in the media (results not shown) has down-regulated this receptor.

The complex non-sigmoid shape of the concentration-response curve observed in iTECs for cAMP is likely a composite of the response evoked by histamine through H_2R, H_3R and H_4R. Indeed, only the co-administration of ranitidine, GSK189254 and JNJ7777120 completely blunted the effect evoked by histamine in iTECs. The EC_{50}s observed for H_1R-mediated response ranged from 0.56 ± 0.04 nM for HK-2 to 8.84 ± 0.89 nM for iTECs. For H_4R in HK-2 cell line, the EC_{50} was 0.97 ± 0.02 nM; in iTECs the effect

of histamine through H_4R was displayed at picomolar concentrations. These differences in the EC_{50}s between the two cell lines could be ascribed to the different intracellular localization of the histamine receptors: H_1R and H_4R displayed a predominant membrane localization in the HK-2 and iTECs, respectively. Notably, the values of EC_{50} herein determined are lower than the Kd values reported for each receptor challenged by the natural ligand. It is known that H_1R and H_2R have an affinity in the micromolar range, whereas H_3R and H_4R are high-affinity receptors with Kd values of 5–10 nM [40]. The discrepancy between both the measured EC_{50}s and the reported Kd values, and the discrepancies among the tested cell types could be explained by differences in receptor reserves [41], species variance and/or the presence of isoforms, already reported for H_3R and H_4R [27]. It has been reported that the H_4R splice variants are able to retain H_4R 390 (the full length variant) intracellularly [27]. The possible presence of different dominant negative H_4R variants is in keeping with the cytoplasmatic staining observed when cells were immunolabelled with the anti-full length H_4R isoform antibody. Furthermore, the presence of nM histamine (results not shown) in the media may also increase the apparent potencies of histamine in these functional studies. The apparent non-competitive behavior displayed by chlorpheniramine maleate in iTECs, compared to the surmountable antagonism showed in HK-2, is in keeping with previous observations of a pronounced non-competitive antagonism of first generation H_1R antagonists in some cellular system such as HL-60 and HeLa cells, and could be explained by non-equilibrium conditions, as already reported [42].

In conclusion, our results provide the first dual anatomical and functional identification of the histamine receptor family in human kidney tubules. This new knowledge will foster a better understanding of the roles of histamine in renal pathophysiological conditions and may contribute to new therapeutic approaches.

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The specific contributions to the work are listed as follows: E.V., A.C.R. and P.L.C. designed the research study; E.V., C.G., A.M., C.L. and A.C.R. performed the research; and wrote the paper; E.V., C.G., A.P., and A.C.R. analyzed the data; P.L.C. and G.C. revised the paper. The authors declare that they have no competing interests.

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Fig. 1. Histamine receptor expression in the human renal tubules

Representative micrographs of 12 different human renal cortex section from apparently healthy tissue of patients underwent to elective nephrectomy, immunolabelled with specific anti-H_1 anti-H_2, anti-H_3, or anti-H_4 receptor antibodies, respectively.

Fig. 2. Histamine receptor expression in renal epithelial cell lines from human cortex

A: Agarose gels representative of three different RT-PCR assays for cDNA from tubular derived cells 1= HK-2 cell line, 2= pTECs and 3= iTECs. Single transcript corresponding to the size predicted for *H1R* (403 bp), *H2R* (497 bp), *H3R* (221 bp), and *H4R* (353 bp) were detected. The housekeeping gene *GAPDH* was used as control. B: Representative merged immunofluorescence images (original magnification 400X) labelled with specific anti-H_1 anti-H_2, anti-H_3, or anti-H_4 receptor antibodies (green), respectively, and Hoescht (blue). The micrographs are representative of at least three independent experiments with similar results.

Fig. 3. IP_1 production in different renal epithelial cell lines from human cortex

The levels of IP_1, downstream metabolite of IP_3, were measured, according to the manufacturer's instruction, by IP-One HTRF® assay kit (Cisbio) in HK-2 (A) and iTECs (B): 50,000 cells/well pretreated for 10 min with vehicle alone (black square, solid line) or the selective H_1R antagonist, chlorpheniramine maleate 1 µM (black circle, dash-dot line) or 10 µM (white circle, dash line), were exposed to histamine (3 pM – 300 nM) for 1 h. Results, calculated from the 665nm/620nm ratio, are expressed as increase over the

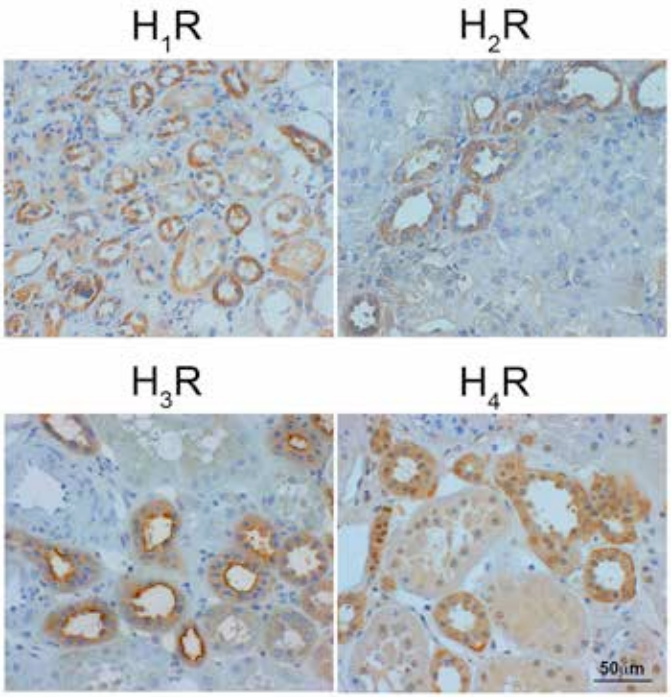
basal and are the mean ± SEM of 3 independent experiments run in duplicate. Statistical analysis was performed by one-way ANOVA and Dunnett test.

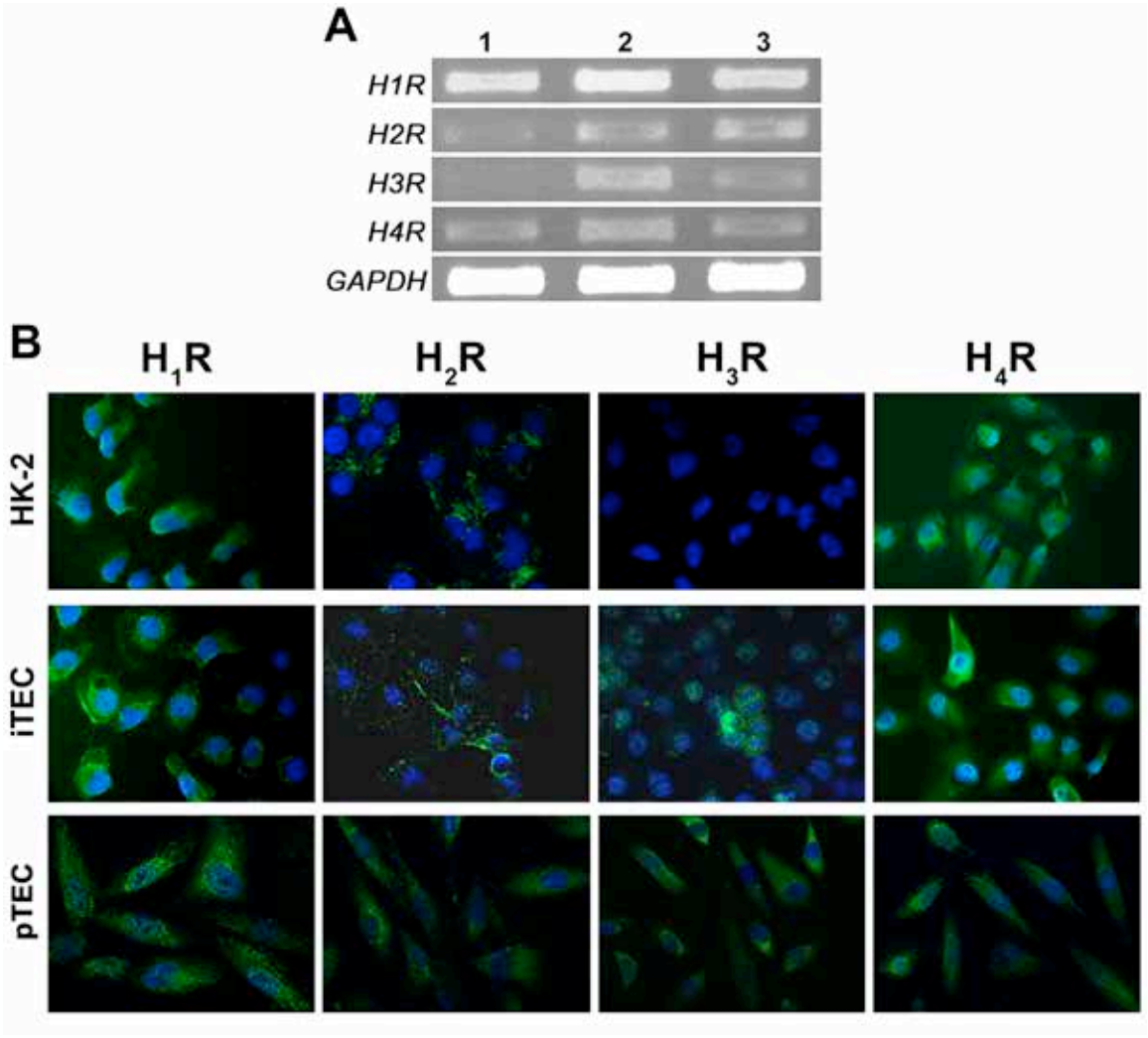
Fig. 4. cAMP production in tubular epithelial cells

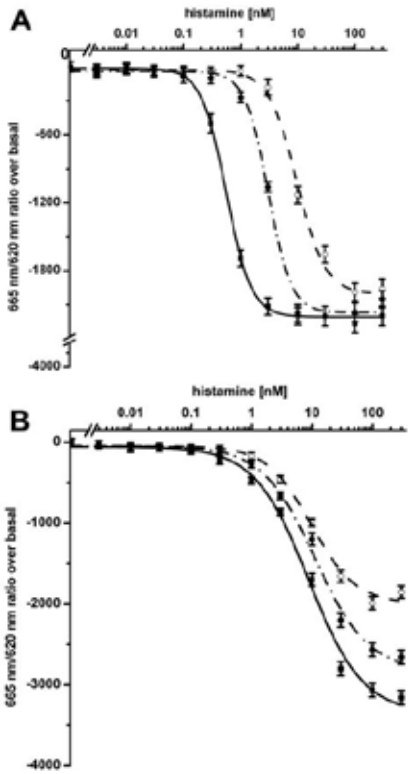
cAMP was measured, according to the manufacturer's instruction, by LANCE Ultra cAMP assay (PerkinElmer) in iTECs: 10,000 cells/well, pretreated for 10 min with vehicle alone (A) or the selective H_2R antagonist, ranitidine 1 µM (black turbot) or 10 µM (white turbot) (B), the selective H_3R antagonist, GSK189254 0,1 µM (black triangle) or 1 µM (white triangle) (C), or the selective H_4R antagonist, JNJ7777120 1 µM (black star) or 10 µM (white star) (D) alone, or co-administrated (E) were exposed to histamine (3 pM – 10 nM) for 30 min. Results, expressed as TR-FRET signal at 665 nm over the basal, are the mean ± SEM of 3 independent experiments run in duplicate. Statistical analysis was performed by one-way ANOVA and Dunnett test. **P* <0.05; ***P* <0.01; ****P* <0.001 *vs* 0 and #*P* <0.05; ##*P* <0.01 *vs* higher dose.

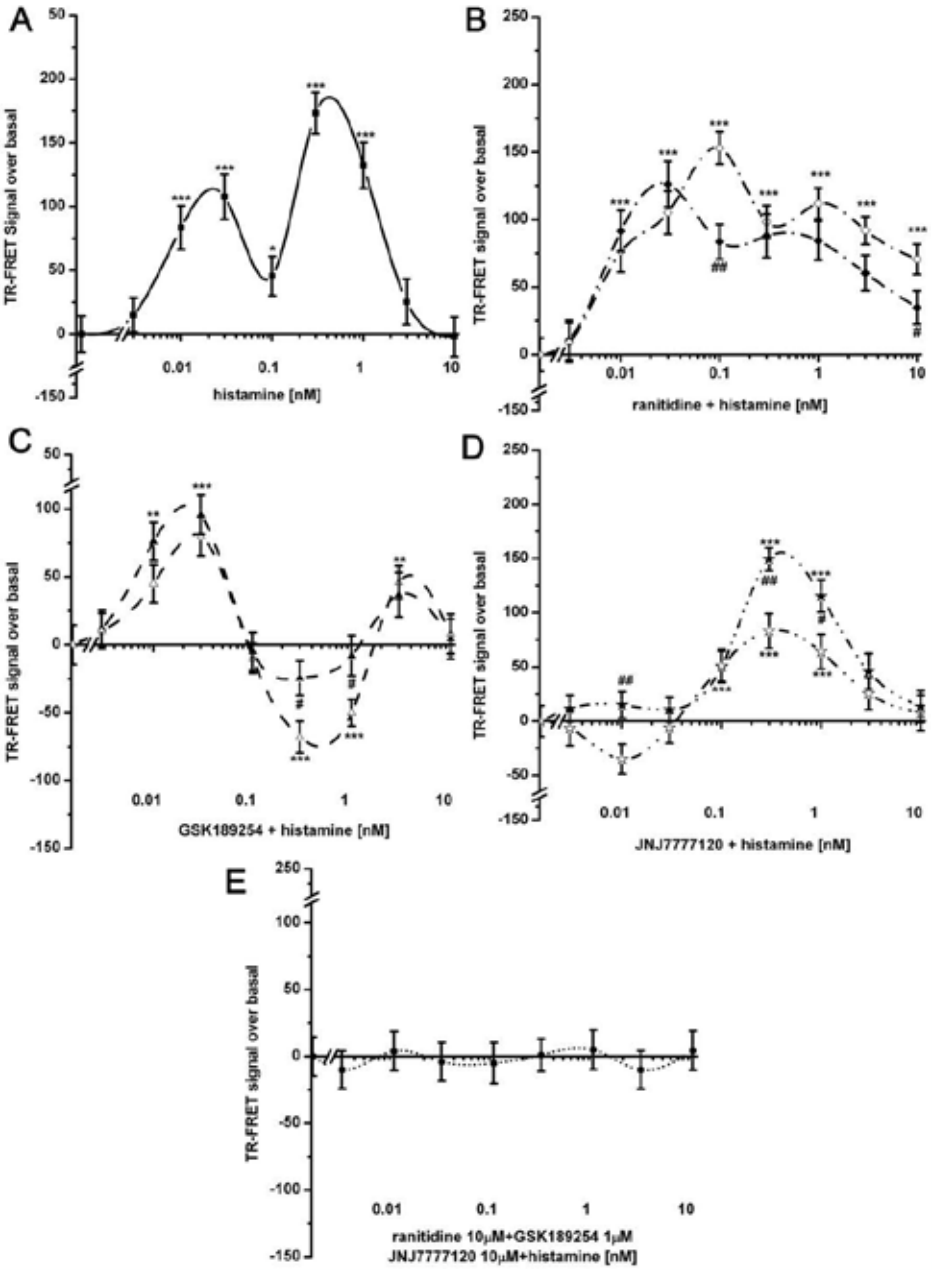
Fig. 5. cAMP production in HK-2 cell line

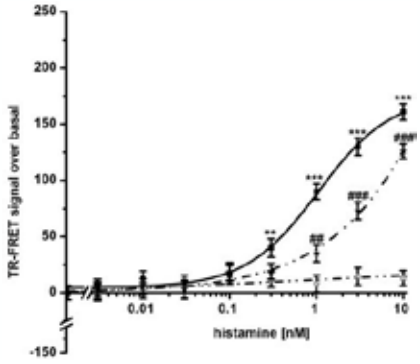
cAMP was measured, according to the manufacturer's instruction, by LANCE Ultra cAMP assay (PerkinElmer) in HK-2 cells: 10,000 cells/well, pretreated for 10 min with vehicle alone (black square, solid line) or the selective H_4R antagonist, JNJ7777120 1 µM (black star) or 10 µM (white star), were exposed to histamine (3 pM – 10 nM) for 30 min. Results, expressed as TR-FRET signal at 665 nm over the basal, are the mean ± SEM of 3 independent experiments run in duplicate. Statistical analysis was performed by one-way ANOVA and Dunnett test. ***P* <0.01; ****P* <0.001 *vs* 0 and #*P* <0.05; ##*P* <0.01; ###*P* <0.001 *vs* higher dose.


H1R
H2R
H3R
H4R
50μm


A
1
2
3
H1R
H2R
H3R
H4R
GAPDH
B
H1R
H2R
H3R
H4R
HK-2
iTEC
pTEC


A
histamine [nM]
0.01
0.1
1
10
100
0
-500
-1200
-1800
-4000
665 nm/620 nm ratio over basal
B
histamine [nM]
0.01
0.1
1
10
100
0
-1000
-2000
-3000
-4000
665 nm/620 nm ratio over basal


A
TR-FRET Signal over basal
histamine [nM]
B
TR-FRET signal over basal
ranitidine + histamine [nM]
C
TR-FRET signal over basal
GSK189254 + histamine [nM]
D
TR-FRET signal over basal
JNJ7777120 + histamine [nM]
E
TR-FRET signal over basal
ranitidine 10μM+GSK189254 1μM
JNJ7777120 10μM+histamine [nM]


TR-FRET signal over basal
250
200
150
100
50
0
-150
0.01
0.1
1
10
histamine [nM]
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