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REGULAR ARTICLE

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Immunolocalisation of P2X and P2Y nucleotide receptors in the rat nasal mucosa

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Abstract Purinoceptor subtypes were localised to various tissue types present within the nasal cavity of the rat, using immunohistochemical methods. $P2X_3$ receptor immunoreactivity was localised in the primary olfactory neurones located both in the olfactory epithelium and vomeronasal organs (VNO) and also on subepithelial nerve fibres in the respiratory region. $P2X_5$ receptor immunoreactivity was found in the squamous, respiratory and olfactory epithelial cells of the rat nasal mucosa. $P2X_7$ receptor immunoreactivity was also expressed in epithelial cells and colocalised with caspase 9 (an apoptotic marker), suggesting an association with apoptosis and epithelial turnover. $P2Y_1$ receptor immunoreactivity was found within the respiratory epithelium and submucosal glandular tissue. $P2Y_2$ receptor immunoreactivity was localised to the mucus-secreting cells within the VNO. The possible functional roles of these receptors are discussed.

Keywords ATP · Purinoceptor · Olfaction · Vomeronasal · Epithelium · Rat (Sprague Dawley)

Introduction

Purinergic signalling is well known to be widespread throughout many tissues (Burnstock 1997). The "Purinergic Nerve Hypothesis" was initially proposed 30 years ago (Burnstock 1972) with the term "purinergic" coined to describe nerves that use the purine nucleotide, ATP, as a neurotransmitter. Adenosine and ATP have been demonstrated to participate in the regulation of many physiological and pathophysiological functions, and their receptors are widely distributed in mammals (Windscheif 1996). Two major types of purinergic receptors were classified by Burnstock (1978) on the basis of differential actions and properties. P1 purinoceptors are selective for the purine adenosine and act through adenylate cyclase. P2 purinoceptors are selective for nucleotides. P2 receptors have been identified in two subclasses, X and Y (Burnstock and Kennedy 1985; Abbrachio and Burnstock 1994). The P2X purinoceptor family is ligand-gated ion channel receptors and the P2Y purinoceptor family is G-protein coupled. The P2X receptors have two transmembrane domains, with a short N-terminus and C-terminus both lying intracellularly. The extracellular loop contains the ATP binding site (Jiang et al. 2000). The P2Y receptors have the typical well-conserved seven transmembrane domains, with an extracellular N-terminus and an intracellular C-terminus. Binding sites have been found on the sixth and seventh transmembrane domains (Ralevic and Burnstock 1998).

Purinergic receptors have been identified in special sensory systems including the eye (see Pintor 1998), inner ear (see Housley 1998), tongue (see Rong et al. 2000) and skin (Hamilton and McMahon 2000). Only a few studies however have been performed on the nasal system. In mammals the application of ATP and GTP to bimolecular lipid membranes modified with rat olfactory homogenates resulted in increased conductivity of the membrane in response to odorants (Vodyanoy and Vodyanoy 1987). Proteoglycan breakdown from bovine nasal cartilage is stimulated and accelerated by ATP in vitro using a process involving P2 purinoceptors (Brown et al. 1997). The effects of ATP have been described in the catfish (Kang and Caprio 1995). More recently, odour responsiveness has been demonstrated to be significantly reduced following P2Y and P2X receptor activation within mouse olfactory epithelium (Hegg et al. 2003). Alongside this, $P2X_1$, $P2X_4$ and $P2Y_2$ antibodies have shown immunoreactivity within the olfactory neuroepithelium.

The nasal organ is a complex structure making extensive use of both the vascular system, in order to perform functions with relation to the respiratory system, and the nervous system in order to perform olfactory functions. The main function with regard to the respiratory

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system is to condition inspired air by humidifying and warming it for efficient gaseous exchange at the level of the alveoli (Popp and Martin 1984; Monteiro-Riviere and Popp 1984). Glandular tissue conditions the passages by secreting mucous, providing protection by filtering unwanted particles such as potentially toxic materials and infectious agents (Bang 1961).

The nasal cavity as a whole is longitudinally divided into approximately two equal halves by the cartilaginous nasal septum and is further divided into a dorsal, middle and ventral meatus by two pairs of turbinates. Another subdivision called the ethmoid recess is found at the posterior end of the nasal cavity, and is a blind-ended cavity containing six pairs of turbinates (Young 1981). In the base of the nasal septum there is a paired tubular structure called the vomeronasal organ (VNO), which with its duct is collectively known as the vomeronasal system (VNS). It is lined with epithelium-containing neural cells on the lateral side and also has many glands, which secrete into its lumen (Salazar and Sanchez Quinteiro 1998). The VNS is considered to be a secondary olfactory system (Keverne 1978) and postulated to play a role in sexual or reproductive behaviour by detecting pheromones (Berghard et al. 1996).

There are three types of epithelia: non-keratinized stratified squamous epithelium, respiratory epithelium and olfactory epithelium, which are found lining specific regions of the nasal mucosa (Young 1981; Popp and Martin 1984). Primary olfactory neurons lie in the olfactory epithelium and function to detect odiferous substances, sending information to the olfactory cortex. Morphologically, they are elongated, columnar-like bipolar cells, whose cell bodies lie at various depths in the epithelium. Each olfactory neuron has a single axon, which passes through the lamina propria to join underlying olfactory nerve bundles, and a single dendritic process, which passes up through the layer of support cells and ends as a vesicle on the apical surface. Cilia extend radially from the vesicles, running parallel to the apical surface of the epithelium (Hosaka et al. 1998).

The aim of this study was to explore and analyse the location of P2 purinoceptor subtypes in this complex system using immunohistochemistry. The choice of P2X and P2Y receptors for investigation was influenced by the results of previous studies investigating the purinergic expression of epithelium and nervous tissue (Gröschel-Stewart et al. 1999a,b; Lee et al. 2000; Burnstock 2002; Shin et al. 2000).

Materials and methods

Animals and tissue preparation

Five adult female Sprague-Dawley rats from in-house breeding in the comparative biological unit at the Royal Free and University College Medical School were killed by asphyxiation using a rising concentration of CO_2 followed by cervical dislocation, according to the UK Animal Scientific Procedures Act, 1986. Animals were decapitated and dissected to the bone surrounding the nasal cavity. Tissue was fixed immediately after dissection with 10% formalin in phosphate-buffered saline (PBS). This solution was gently flushed retrogradely through the pharygeal duct and discharged through the nostrils. Whole tissue sections were placed in 10% formalin in PBS for at least 24 h at 4°C and then transferred to 10% formic acid solution for 4 days at 4°C to allow decalcification. Following this, tissue was rinsed under running tap water for at least 4 h. To provide cryoprotection, sections were then placed in concentration gradient of 10, 20 and finally left overnight in 30% sucrose prepared in PBS, at 4°C.

Tissue was sectioned according to Young's method (Young 1981), which allows thorough examination of the nasal cavity with no destructive invasion at the time of necropsy. Briefly, each section was embedded in OCT compound (Agar Scientific, Stansted, UK) and then frozen in isopentane in liquid nitrogen. Serial slide sections were collected on a Leica CM3050 Cryostat (Leica, Nussloch,

Table 1 List of antisera used for immunocytochemistry

Antigen	Host	Dilution	Source
Primary antisera			
$P2X_3$ receptor	Rabbit	1:200 (Single labelling) 1:400 (Double labelling)	Roche Bioscience, CA
$P2X_5$ receptor	Rabbit	1:200	Roche Bioscience, CA
$P2X_7$ receptor	Rabbit	1:200	Roche Bioscience, CA
$P2Y_1$ receptor	Rabbit	1:200	Roche Bioscience, CA
$P2Y_2$ receptor	Rabbit	1:200	Roche Bioscience, CA
Protein gene product (PGP) 9.5	Rabbit	1:400	Ultraclone Ltd, UK
Caspase 9	Rabbit	1:200	Cell Signal Technology, Beverley, USA
Secondary antisera and streptavidin complex			
Biotinylated donkey anti-rabbit IgG		1:500	Jackson ImmunoResearch Lab, PA
Donkey anti-rabbit Cy3		1:250	Jackson ImmunoResearch Lab, PA
Streptavidin-fluorescein isothiocyanate (FITC-green fluorophore)		1:200	Amersham Life Sciences, UK

Germany) at −28°C, at a thickness of 11 μm, and mounted on gelatin-coated slides. Slides were air-dried at room temperature for at least 30 min and stored at −20°C. Prior to use, slides were kept at room temperature for at least 15 min.

Immunofluorescent localisation of purinoreceptors, with TSA amplification for $P2X_3$ receptor studies

The antibodies used in this study along with their respective dilution can be found in Table 1. Mounted sections were air-dried and washed in PBS for 15 min at room temperature then pre-incubated in 10% normal horse serum (NHS; Gibco, CA) in PBS containing 0.05% merthiolate (Sigma, Poole, UK) for 20 min at room temperature. Sections were incubated in primary antibody (1:200) diluted with 10% NHS (Gibco, CA)-PBS-merthiolate overnight at room temperature, or for 3 days at 4°C. The $P2X_3$ antibody was diluted in 10% NHS containing 0.2% Triton X-100 (Sigma, Poole, UK).

Slides were washed for 15 min and then incubated in biotinylated donkey anti-rabbit IgG, diluted 1:500 in 1% NHS-PBS-merthiolate for 1 h at room temperature. Slides were again washed and incubated in streptavidin-fluorescein isothiocyanate (FITC-green fluorophore) diluted 1:200 in PBS-merthiolate for 1 h, at room temperature. Finally sections were washed and incubated for 5 min in Pontamine sky blue, washed again in PBS for 15 min and immediately mounted in Citiflour (Citifluor Ltd, London).

For all $P2X_3$ sections, TSA amplification was performed. Sections were incubated in 0.4% hydrogen peroxide and 50% methanol for 10 min after incubation in secondary antibody. Sections were washed with PBS-thimerosal (Sigma, Poole, UK) for 15 min at room temperature, then incubated in ExtrAvidin-horseradish peroxidase (Sigma, Poole, UK) diluted 1:1,500 in PBS-thimerosal for 30 min at room temperature. The sections were washed for 30 min and incubated in tyramide diluted 1:50 in amplification solution (NEN Life Science Products, Boston, MA). Following another 30 min wash, sections were incubated in streptavidin-FITC for 8 min in an opaque incubation chamber at room temperature. Finally the sections were washed for 30 min before being mounted in Citifluor.

Double immunofluorescence of $P2X_3$ and PGP9.5

Slides were air-dried and pre-incubated in 10% NHS in PBS containing 0.05% merthiolate for 1 h at room temperature, then in primary antibodies (1:400) diluted with 10% NHS (Gibco, CA) overnight at room temperature. TSA amplification was performed up until the streptavidin-FITC incubation as described above. Sections were then incubated in 10% NHS in PBS containing 0.05% merthiolate for 1 h at room temperature. Sections were then incubated in the secondary antisera diluted to 1:400, in 10% NHS in PBS with 0.2% Triton X-100 overnight at room temperature. Sections were washed in PBS for 15 min and then incubated in donkey anti-rabbit Cy3 in PBS-merthiolate (1:250) for 1 h at room temperature. Finally sections were washed for 15 min in PBS and mounted in Citifluor.

Double immunofluorescence of $P2X_7$ and caspase 9

Slides were air-dried and washed in TRIS-buffered saline containing 0.1% Triton X-100 (TBS-T) for 15 min. Non-specific binding sites were blocked with 3% bovine serum albumin (BSA) (Sigma, Poole, UK) in TBS-T for 30 min. Sections were incubated with caspase 9 antibody (1:200) diluted in 3% BSA in TBS-T overnight at 4°C. Sections were washed in TBS-T for 30 min and incubated in biotinylated donkey anti-rabbit IgG, diluted 1:500 in 1% NHS-PBS-merthiolate for 1 h at room temperature. Sections were washed for 15 min in TBS-T and incubated in 0.4% hydrogen peroxide and 50% methanol for 15 min to block endogenous peroxidase activity. Following another wash, sections were incubated in Extravidin Peroxidase (Sigma, Poole, UK) diluted to 1:1,000 in PBS-merthiolate for 30 min. Sections were washed and incubated in tyramide diluted 1:50 in amplification solution (NEN Life Science Products, Boston, MA) for 8 min. Following this, sections were washed for 30 min and incubated in FITC, diluted to 1:200 in PBS-merthiolate for 10 min at room temperature. Sections were washed and incubated in 3% BSA in TBS-T for 1 h. Sections were then incubated in $P2X_7$ antibody diluted to 1:200 in 1% BSA in TBS-T overnight. Sections were washed and incubated in donkey anti-rabbit Cy3 in PBS-merthiolate (1:250) for 1 h at room temperature. Finally, sections were washed in TBS before being mounted in Citifluor.

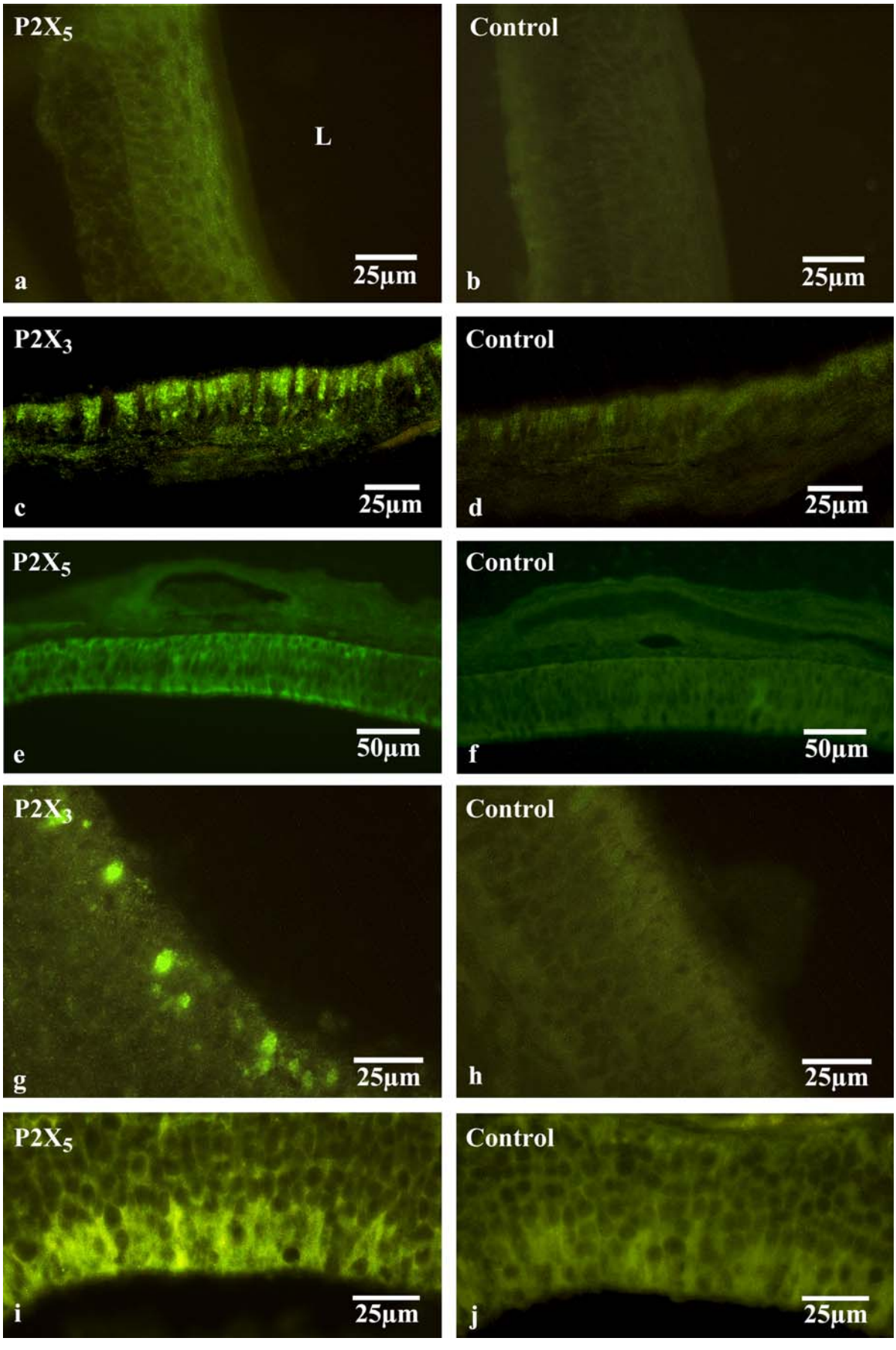
All sections were visualised under a Zeiss Axioplan microscope (Jena, Germany). Images were captured using a Leica DC200 digital camera (Leica, Heenbrugg, Switzerland)

Control experiments were performed on adjacent slide sections by omission of the primary antibody from the immunostaining process and by using primary antibody pre-absorbed with cognate peptide (Roche Bioscience, CA) to the same concentration of its respective primary antibody. The specificity of antibodies has been previously established (Oglesby et al. 1999).

Results

Localisation of purinoceptors on stratified squamous epithelium

Stratified squamous epithelium only represents a small proportion of the total epithelial lining of the rat nasal cavity. Immunoreactivity to $P2X_5$ receptors was observed in the stratified squamous epithelium of the rat nasal mucosa (Fig. 1a). Stratified squamous epithelium by its nature contains layers of cells of different shapes from the


P2X5
L
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◀ **Fig. 1** $P2X_3$ and $P2X_5$ immunoreactivity (IR) throughout the rat nasal mucosa. **a** $P2X_5$ IR on stratified squamous epithelium. **b** Cognate peptide pre-absorption. Lumen of nasal cavity (*L*). **c** $P2X_3$ IR of the respiratory epithelium. **d** Peptide pre-absorption of $P2X_3$ antibody on respiratory epithelium. **e** $P2X_5$ IR on respiratory epithelium. **f** Cognate peptide pre-absorption. **g** $P2X_3$ IR of the olfactory receptor neurons in olfactory epithelium. **h** Peptide pre-absorption of $P2X_3$ antibody on olfactory epithelium. **i** $P2X_5$ IR on olfactory epithelium. **j** Cognate peptide pre-absorption

cuboidal cells at the base of the layer to the superficial squamous epithelial cells. The whole epithelial layer was immunoreactive.

Immunoreactivity to $P2X_7$ receptors was also seen and was particularly highlighted upon colocalisation with caspase 9 (Fig. 2a–c). The tissue appears to show prominent immunoreactive cells. Upon colocalisation $P2X_7$ receptor immunoreactivity is nearly always congruous with caspase 9 labelling; however, there is also much caspase labelling that does not colocalise with the $P2X_7$ antibody. This is shown in Fig. 2c where green and yellow immunofluorescence is observed, but no red immunofluorescence, showing that there is a population of stratified squamous epithelial cells that reacted to caspase 9 antibodies but not to $P2X_7$ antibodies. No immunoreactivity to $P2Y_1$ and $P2Y_2$ was observed in the stratified squamous epithelium.

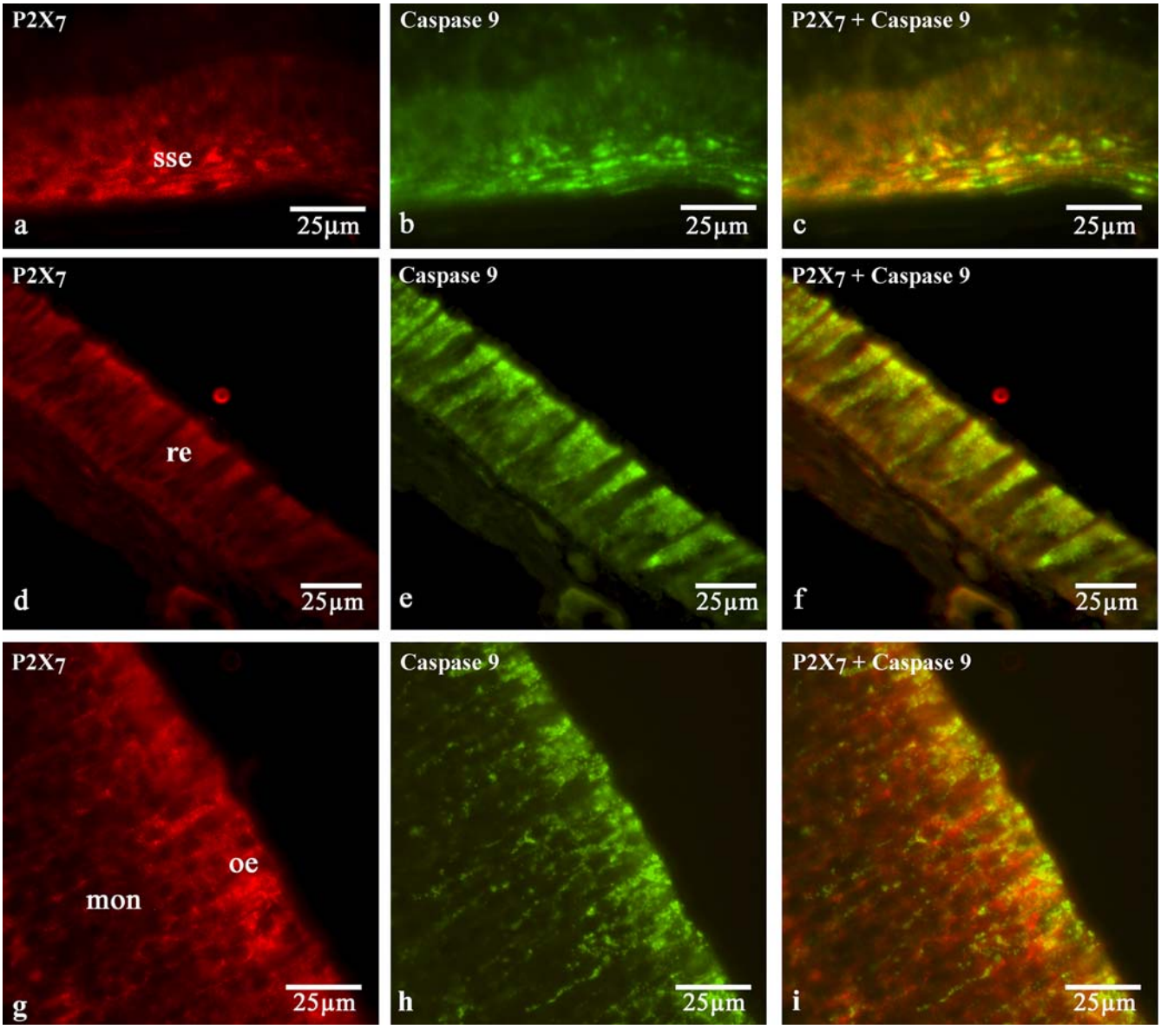

Fig. 2 Colocalisation of $P2X_7$ and caspase 9 in the rat nasal mucosa **a**–**c** on stratified squamous epithelium (*sse*). **a** $P2X_7$ IR **b** caspase 9 IR. **c** $P2X_7$ and caspase 9 colocalisation (shown in *yellow*). Note areas only showing caspase 9 IR (shown in *green*). **d**–**f** On respiratory epithelium (*re*). **d** $P2X_7$ IR, **e** caspase 9 IR. **f** $P2X_7$ and caspase 9 colocalisation. **g**–**i** On olfactory epithelium (*oe*). **g** $P2X_7$ IR. **h** Caspase 9 IR. **i** $P2X_7$ and caspase 9 colocalisation (shown in *yellow*)

Localisation of purinoceptors on respiratory epithelium

The respiratory epithelium clearly showed immunoreactivity to the $P2X_5$ receptor antibody (Fig. 1e). Positive immunostaining was intense and uniform. There appeared to be no difference in the staining of the cuboidal epithelial cells and the goblet cells. Immunoreactivity to $P2X_3$ receptor antibodies was also seen in the cells of the respiratory epithelium (Fig. 1c). Throughout the respiratory epithelium background non-specific staining was fairly widespread with these antibodies. Pre-absorption studies are important here, highlighting that the specific immunofluorescence is present in the epithelial tissue above the background. Deep to the respiratory epithelium, nerve fibres probably originating from the trigeminal nerve that are likely to supply the epithelium and surrounding structures showed colocalisation of the $P2X_3$ receptor and PGP9.5, a nerve stain (Fig. 3a–c).

The $P2X_7$ receptor antibody also showed immunoreactivity throughout the respiratory epithelium (Fig. 2d–f). The staining was diffuse throughout the epithelium, but stronger towards the apical surface. In comparison to the other P2X antibodies, staining for $P2X_7$ receptors was fairly weak throughout the whole study.

The $P2Y_1$ receptor antibody produced dense immunostaining, some of which was clearly associated with the respiratory epithelium throughout the nasal tissue (Fig. 4a). The staining did not differentiate between cuboidal epithelial cells and goblet cells, showing equal intensity of immunofluorescence in both. The $P2Y_2$ receptor antibody only weakly stained the respiratory epithelium.

Localisation of purinoceptors on olfactory epithelium

Upon single immunofluorescence with the $P2X_3$ receptor antibody, strong immunofluorescence of individual cells was seen in olfactory epithelium (Fig. 1g) on a background of diffuse weak staining. Olfactory neuroepithelium by its nature contains olfactory neurones at different stages of development. It may be that a low basal level of $P2X_3$ is constitutively expressed, which is markedly upregulated within individual olfactory neurones once they become functional, detecting odiferous stimuli. Colocalisation studies of the $P2X_3$ receptor with the nerve stain PGP9.5 suggested that $P2X_3$ receptors are colocalised on primary olfactory neurones within the olfactory epithelium (see Figs. 1g, 3d–f). Staining with PGP9.5 highlighted the vast amount of nervous tissue that underlies the olfactory epithelium. This was seen as numerous large red bundles under the olfactory epithelium within the endoturbinates and ectoturbinates, nasal septum and lateral walls of the nasal cavity.

The olfactory epithelium showed immunoreactivity to the $P2X_5$ receptor antibody that localised pre-dominantly to the cell layer containing olfactory receptor neurons and support cells. The underlying layer of maturing olfactory receptor neurons showed no significant immunofluorescence (Fig. 1i). The $P2X_7$ receptor antibody showed sparse but moderate immunoreactivity within the olfactory epithelium, likely to be reflecting the population of cells in the process of apoptosis. Upon colocalisation of $P2X_7$ receptor antibody and caspase 9 antibody, immunoreactivity was observed throughout the epithelium (olfactory receptor neurons, support cells and basal cells) and the maturing olfactory receptor neuron layer (Fig. 2g–i).

The $P2Y_1$ receptor antibody exhibited immunoreactivity within the olfactory epithelium and showed particularly intense staining at the apical edge. Staining may have been slightly stronger in support cells within the epithelium. The $P2Y_2$ receptor antibody only weakly stained the olfactory epithelium.

Localisation of purinoceptors on glandular tissue

Goblet cells within the respiratory epithelium showed no difference in staining patterns when staining of the epithelium was present. Alongside the goblet cells, mucus is also produced by submucosal glandular tissue, which exists as both glands and ducts under the respiratory epithelium of the rat nasal mucosa.

Submucosal glandular tissue was only immunoreactive to the $P2Y_1$ receptor antibody. This could be seen in numerous ducts and collections of glandular tissue. Staining was just as intense in the epithelium as in the glandular tissue. Within the nasal septum (Fig. 4) two populations of tubular structures exist, and these showed different staining patterns. The blood vessel walls stained weakly in comparison to the surrounding submucosal ducts.

Localisation of purinoceptors on the vomeronasal organ

The VNO contains epithelium with sensory elements. This organ showed immunoreactivity to the $P2Y_1$ receptor antibody. Staining was particularly strong at the luminal face of the organ but also extended back through the whole epithelium. Immunostaining of the VNO with PGP9.5 illustrated the discretely contained neural content of this organ, which only appears on one side of the lumen (Fig. 3b). Colocalisation studies of $P2X_3$ receptors with PGP9.5 showed that $P2X_3$ receptors exist on the sensory neurones of the VNO (Fig. 3d–f). This colocalisation is only seen on one side of the VNO. On the side without neurosensory epithelium, it appears that $P2X_3$ immunoreactivity is also localised alone. Sparse cells within the VNO showed positive staining for $P2Y_2$ within the neurosensory epithelium of the organ, and peptide pre-absorption resulted in no immunoreactivity in the same area of tissue.

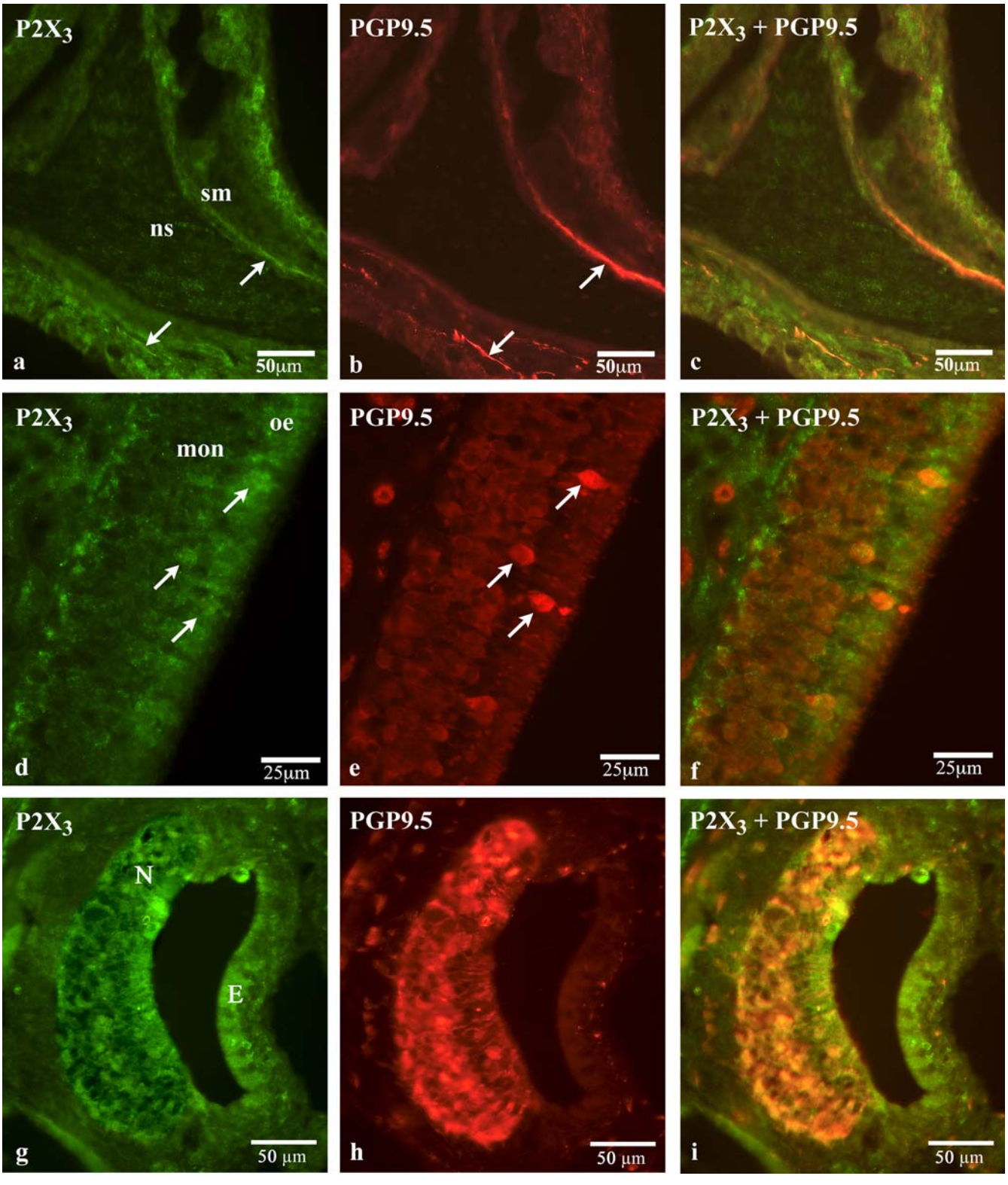

Fig. 3 Colocalisation of $P2X_3$ and PGP9.5 in the rat nasal mucosa **a**–**c** in the base of the nasal septum (*ns*). **a** $P2X_3$ IR, on submucosal (*sm*) nerve fibres and respiratory epithelium with *arrows* to submucosal nerve fibres. **b** PGP9.5 IR on submucosal nerve fibres (*arrows*). **c** $P2X_3$ and PGP9.5 colocalisation on submucosal nerve fibres. **d**–**f** In the olfactory epithelium (*oe*). **d** $P2X_3$ IR, maturing olfactory neurons (*mon*). **e** PGP9.5 IR of olfactory receptor neurones (*arrowed*) and underlying maturing olfactory neurons. **f** $P2X_3$ and PGP9.5 colocalisation on olfactory receptor neurons. **g**–**i** In the vomeronasal organ. **g** $P2X_3$ IR of neural (*N*) and epithelial (*E*) component of the VNO. **h** PGP9.5 IR of the neural tissue in the VNO. **i** $P2X_3$ and PGP9.5 colocalisation on the neural component of the VNO (shown in *yellow*)

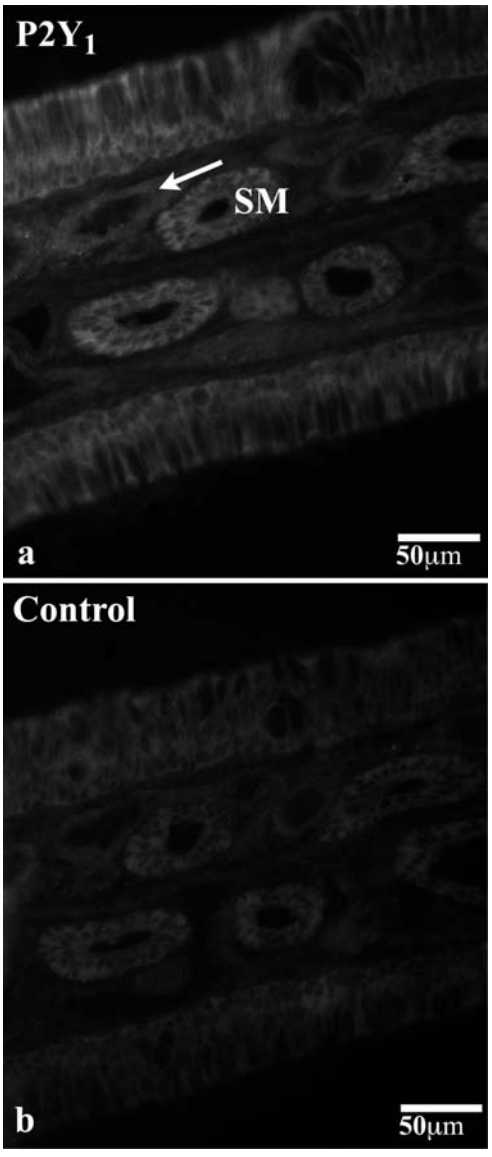

Fig. 4 $P2Y_1$ Immunoreactivity of the nasal septum in the respiratory region. **a** $P2Y_1$ IR on respiratory epithelium, submucosal glands (*SM*) and blood vessel (*arrowed*) walls. **b** Cognate peptide preabsorption

Discussion

These results demonstrate the distribution of different purinoceptor subtypes throughout the rat nasal mucosa, suggesting a role for purinergic signalling in these tissues.

Some receptors are associated with cell turnover in the epithelium of the skin. In particular $P2X_5$ and $P2X_7$ receptors are associated with cell proliferation and differentiation and death, respectively (Gröschel-Stewart et al. 1999a). This has been demonstrated in mucosal epithelium of the gut (Gröschel-Stewart et al. 1999b) and of the bladder and ureters (Lee et al. 2000). Immunoreactivity to the $P2X_5$ receptor was found in all three epithelial types, i.e., stratified squamous, respiratory and olfactory epithelium, throughout the nasal mucosa. The regulation of proliferation within the nasal epithelium may be mediated by $P2X_5$ receptors in the sense that it mediates cell differentiation, which in effect leads to inhibition of proliferation. The $P2X_5$ receptor may however play a part in the control of proliferation rates throughout differing regions, producing areas of variable epithelial thickness.

Immunostaining with the $P2X_7$ receptor antibody can be seen along the epithelium, especially the outer most edge of most if not all cells. Colocalisation studies with caspase 9 (an apoptotic marker) helped to extend our results. Immunoreactivity for the $P2X_7$ receptor antibody frequently colocalised with that of caspase 9, laying further support for the claim that the $P2X_7$ receptor is associated with the process of apoptosis (Chow et al. 1997). It should also be noted here that under certain circumstances the $P2X_7$ receptor is internalised, and therefore rendered non-functional (Burnstock 2001).

The $P2Y_1$ receptor has been associated with the process of cell growth and proliferation (Abbrachio 1996; Burnstock 2002). The $P2Y_1$ antibody showed strong immunoreactivity in the respiratory and olfactory epithelium of the nasal mucosa. The $P2Y_1$ receptor is metabotrophic, and if involved in the proliferation of epithelial cells, it would provide a separate mechanism to that of the $P2X_5$ receptor, possibly responding to nucleotides acting as trophic factors, causing second messenger activation, and may mediate the release of secondary trophic factors. ATP and other nucleotides and nucleosides may act in synergy with growth factors to regulate mitosis, and therefore proliferation in epithelial cells (Huang et al. 1989; Burnstock 2002).

Glandular tissue within the submucosal glands of the nasal cavity was found to be strongly immunoreactive to the $P2Y_1$ receptor antibody. The $P2Y_1$ receptor may mediate induction of glandular phenotype within the mucosa following the cue of a specific growth factor. It may also contribute to the control of the rate of proliferation of the actual glandular cells within the glandular units, in response to the need for mucous production. The $P2Y_1$ receptor is not likely to mediate the same process in goblet cells, as there was no differentiation of immunoreactivity between the goblet cells and the cuboidal epithelial cells of the respiratory epithelium. This may be due to the different origins for the two cell types (Tos and Mogenson 1978; Norlander et al. 1998). Goblet cells in intestinal mucosa have also been found to label with the $P2X_5$ receptor antibody (Gröschel-Stewart et al. 1999b).

The VNO exhibited immunoreactivity to the $P2Y_2$ receptor in discrete loci throughout the neural side of the epithelium. The pattern of immunoreactivity observed suggests localisation of $P2Y_2$ receptors on the secretory cells of the VNO. ATP has been shown to act as a potent mediator of goblet cell mucin secretion in the respiratory epithelia (Shin et al. 2000). $P2Y_2$ receptors been have shown to regulate exocytosis in SPOC1 cells, a mucin-secreting cell line from the airways (Scott et al. 1998). As previously mentioned the VNO also has many glands, which secrete into its lumen (Salazar and Sanchez Quinteiro 1998). This result supports the hypothesis that

$P2Y_2$ receptors may play a role in mediating mucin secretion.

Colocalisation studies within the VNO have shown that the $P2X_3$ receptor is likely to be expressed in the neural constituent of this organ, in keeping with the evidence that $P2X_3$ receptors have a role in the function of sensory neurons (Burnstock 2000). The receptor may play a role in the detection of pheromones, which are non-volatile molecules capable of influencing the sexual and social behaviours and interactions between members of the same species (Tirindelli et al. 1998). $P2X_3$ receptor immunoreactivity was also present on the respiratory epithelium of the VNO (the opposed luminal epithelium to the neural bundle), but this did not colocalise with PGP9.5, showing that this receptor subtype can also be expressed in non-neuronal tissues.

In addition to nerve fibres in the VNO, the $P2X_3$ receptor has been colocalised to the primary olfactory neurones of the olfactory epithelium and submucosal nerve fibres innervating the respiratory system. With respect to olfaction, the $P2X_3$ receptor may act as part of a receptor-mediated transcytosis mechanism responding to odiferous substances, activating further receptors because of changes in ion currents. This would account for an upregulation of $P2X_3$ receptor expression when the olfactory neurone becomes responsive to odiferous stimuli. The support cells, possibly stimulated by odiferous substances, may act as a source of ATP to act on these $P2X_3$ receptors localised on olfactory neurones.

The respiratory epithelium receives sensory innervation from the trigeminal nerve (Grunditz et al. 1994). The $P2X_3$ receptor has been colocalised to nerve fibres in the submucosa that may represent branches of the trigeminal nerve. Previous studies have investigated the neuropeptide content of this nerve localising substance P (SP) and calcitonin gene-related peptide (CGRP), and additionally concluded that CGRP neurons preferentially innervate the lamina propria and SP neurons preferentially innervate the epithelium (Hunter and Dey 1998). There is a reduction in SP and CGRP following capsaicin treatment, suggesting that the neuropeptides are present on sensory C-fibres (Lundblad 1984). The results of the present study support the idea that there may be a separate subpopulation of non-peptidergic nociceptors supplying the nasal cavity and epithelium in particular. Previous studies have localised $P2X_3$ receptor immunoreactivity to the small and large nerve cell bodies within the trigeminal ganglia (Bradbury et al. 1998). Stimulation of these nerves may be involved in mechanisms that regulate nasal responses such as neurogenic inflammation (Baranuik and Kaliner 1990; Solway and Leff 1991) and hyper-responsiveness (Kennedy 1992), which help to protect lower respiratory airways from potentially damaging particles and gases (Lundblad 1984) by triggering mucous secretion, plasma extravasion and vasodilatation.

The results presented in this study show that purinergic signalling may occur throughout the nasal mucosa, associating with many processes such as cell proliferation, apoptosis, olfaction, mucous secretion and sensory reception. These receptors may represent targets for the development of pharmacological agents for administration via the intranasal route. The intranasal route of drug delivery provides an alternative to systemic drug administration and has the advantage that it bypasses both the tightly regulated blood-brain barrier and the process of hepatic first-pass metabolism, which pose problems with systemic drug delivery (Mathison et al. 1998).

Future studies of colocalisation between purinoceptors implicated in the process of proliferation with Olfactory Marker Protein (Margolis 1985), a marker of immature primary olfactory neurons (Biffo et al. 1990), and GAP-43 may provide further information on the roles of purinoceptors in this unique neural tissue.

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