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# Expression and Distribution of Vasoactive Intestinal Polypeptide Receptor $VPAC_2$ mRNA in Human Airways

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**SUMMARY:** Vasoactive intestinal polypeptide (VIP) is a putative neurotransmitter of the inhibitory non-adrenergic non-cholinergic nervous system and influences many aspects of mammalian airway function. VIP binds to two G-protein-coupled VPAC receptors that are highly homologous structurally but distinguished by their different affinities for peptide analogues of VIP. As VIP binding sites in the respiratory tract have only been examined by ligand binding and cytochemical techniques, we studied the distribution of the mRNA that encodes the inducible receptor subtype $VPAC_2$ in the human respiratory tract. Northern blots demonstrated the expression of $VPAC_2$ mRNA in human airways and other tissues. A human-specific $VPAC_2$ cRNA probe was used to detect $VPAC_2$ mRNA expression in human lung by nonradioactive in situ hybridization. In larger airways, positive $VPAC_2$ mRNA signals were localized to tracheal and bronchial ciliated epithelial cells. There was also marked staining of mucous and serous cells of submucosal glands. No signals were obtained in airway and vascular smooth muscle myocytes and endothelial cells. In peripheral lung tissues, $VPAC_2$ mRNA expression was localized to epithelial cells of the bronchioles. Specific staining was detected in immune cells and alveolar macrophages. In summary, $VPAC_2$ is localized in airway epithelial, glandular, and immune cells of the lung but not in airway and vascular smooth muscle. The absence of $VPAC_2$ mRNA in vascular and airway smooth muscle myocytes may indicate that the effects of VIP on vasodilation and bronchodilation are mediated by $VPAC_1$ or undefined receptors. However, a paracrine modulation of the two most prominent effects of VIP in the respiratory tract by $VPAC_2$ cannot be excluded. (*Lab Invest 2001, 81:749–755*).

The 28-amino acid vasoactive intestinal polypeptide (VIP) is a putative neurotransmitter or neuromodulator of the inhibitory non-adrenergic non-cholinergic nervous system in mammalian airways (Maggi et al, 1995). VIP-immunoreactivity (VIP-IR) is present in cells of the tracheobronchial smooth muscle layer, in the walls of pulmonary and bronchial vessels, around submucosal glands, in the lamina propria, and in pulmonary ganglia (Dey et al, 1981; Lundberg et al, 1984). VIP-IR nerve fibers are found as branching networks in the respiratory tract (Ghatei et al, 1982) They decrease in frequency as the airways become smaller but extend to peripheral bronchioles (Lundberg et al, 1984). The pattern of VIPergic nerves largely follows that of cholinergic nerves (Laitinen et al, 1985). VIP-IR is also present in sensory nerves (Lundberg et al, 1984; Luts and Sundler, 1989).

Several studies using receptor binding techniques have been performed to demonstrate the presence of VIP receptors and have shown abundant binding in different parts of the lungs (Carstairs and Barnes, 1986; Robberecht et al, 1981, 1988). Two types of VIP receptors have been cloned and characterized in the past years. They were termed the $VPAC_1$ receptor, formerly described as the $VIP_1$ receptor (Ishihara et al, 1992; Lutz et al, 1993), the VIP/PACAP type II receptor (Ciccarelli et al, 1994) or the PVR2 (Rawlings et al, 1995), and the $VPAC_2$ receptor, formerly called the $VIP_2$ receptor (Lutz et al, 1993), PACAPR-3 (Inagaki et al, 1994), or PVR3 (Rawlings et al, 1995). Several cyclic peptides and other analogues of VIP that act as agonists of high potency and selectivity distinguish $VPAC_2$ receptors from $VPAC_1$ receptors (Gourlet et al, 1997; O'Donnell et al, 1994a, 1994b; Xia et al, 1997) that have been cloned from rat, mouse, and human tissues so far (Adamou et al, 1995; Inagaki et al, 1994; Lutz et al, 1993; Svoboda et al, 1994; Usdin et al, 1994; Wei and Mojsov, 1996). In the current International Union of Pharmacology nomenclature, the receptors are classified as $VPAC_1$ and $VPAC_2$ (Harmar et al, 1998).

Human $VPAC_2$ receptor mRNA was prominent in the central nervous system (CNS) and several other peripheral tissues, including lung (Adamou et al, 1995; Svoboda et al, 1994; Wei and Mojsov, 1996). As VIP binding sites in the airways have only been characterized by autoradiographic ligand binding studies and receptor immunoreactivity studies leading to controversial results, the present study was performed to define the localization of mRNA encoding the $VPAC_2$ receptor in the human respiratory tract.

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## Results

### *Detection of $VPAC_2$ Receptor mRNA by Northern Blot*

Northern blots were performed to demonstrate the expression of the $VPAC_2$ receptor mRNA in human

tissues. Using a radiolabeled human specific cRNA probe, $VPAC_2$ receptor mRNA was detected at different levels in extracts of human lung and other tissues. The decreasing order of intensity of $VPAC_2$ mRNA expression was as follows: CNS, heart, pancreas, skeletal muscle, kidney, lung, stomach, and liver (Fig. 1).

### *Distribution of $VPAC_2$ Receptor mRNA in Human Respiratory Tract*

The expression of $VPAC_2$ mRNA was detected by nonradioactive in situ hybridization and high-resolution interference contrast microscopy. Abundant staining of different cell types was present in all areas of larger airways and peripheral lung. Positive staining was reproducibly detected after hybridization with antisense probe. Control hybridizations on alternate sections with equivalent amounts of sense-probe using the same hybridization and washing stringency were unstained and demonstrated the specificity of antisense hybridization signals. Omission of labeled cRNA probes from the hybridization mixture also resulted in unstained sections being identical to results obtained when RNA was digested before hybridization.

In sections of trachea and extrapulmonary and intrapulmonary bronchi, positive $VPAC_2$ mRNA signals were localized to the cytoplasm of basal and ciliated cells (Fig. 2A). Goblet cells did not show $VPAC_2$ mRNA expression (Fig. 2A). There were also no hybridization signals present in airway and vascular smooth muscle myocytes (Fig. 3), connective and cartilaginous tissue, and endothelial cells (Fig. 2A). Control slides that were treated with the sense probe did not show specific staining (Fig. 2B).

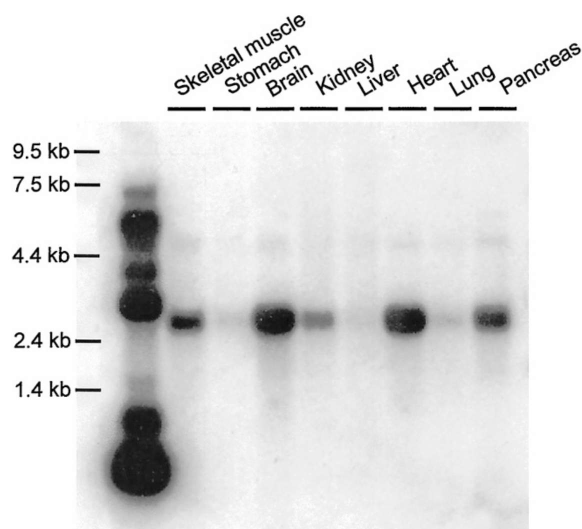



**Figure 1.**
Detection of $VPAC_2$ receptor mRNA in human lung by Northern Blot. Two micrograms of poly ($A^+$) RNA prepared from human lung and other tissues was hybridized with a [$^{32}P$]-labeled human-specific $VPAC_2$ receptor probe. $VPAC_2$ receptor mRNA levels are detected with decreasing intensity in central nervous system, heart, pancreas, skeletal muscle, kidney, lung, stomach, and liver.

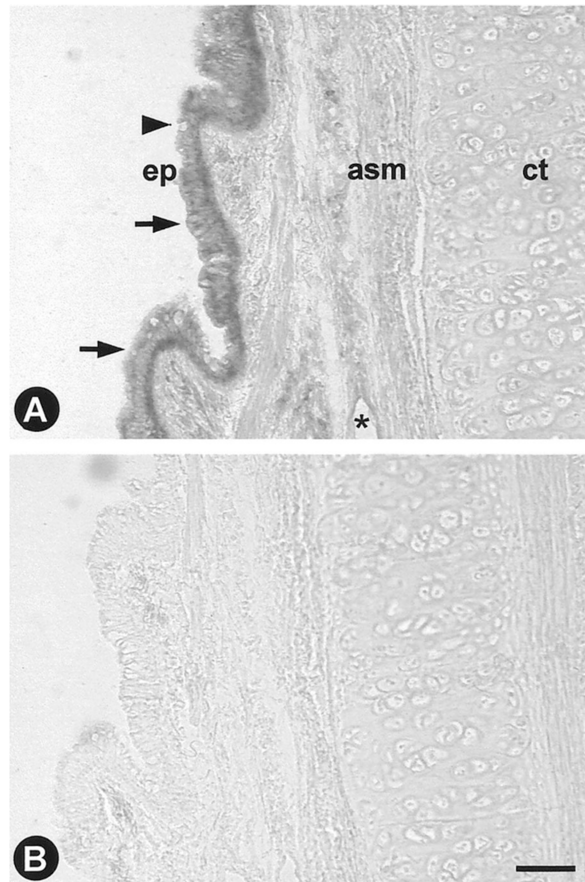



**Figure 2.**
Localization of $VPAC_2$ receptor mRNA in larger airways by in situ hybridization. Eight-microgram cryostat sections of human main bronchus were subjected to nonradioactive in situ hybridization for $VPAC_2$ mRNA. $VPAC_2$ mRNA hybridization signals are localized to basal and ciliated cells of bronchial epithelium (*ep*, arrows in A). No signals are present in goblet cells and airway smooth muscle (*asm*) or connective and cartilaginous (*ct*) tissue. Control slides that were treated with the sense probe did not show specific staining (B). Bar = 40 $\mu$m.

$VPAC_2$ mRNA signals were also present in submucosal gland cells that surrounded trachea and bronchi (Fig. 4A). In these submucosal glands, both serous and mucous cells were stained for $VPAC_2$ mRNA (Fig. 4, A and C). Sense probes did not stain these cells (Fig. 4B).

Differences in the distributional pattern of $VPAC_2$ mRNA between trachea and extrapulmonary and intrapulmonary bronchi were not evident. In peripheral lung, the epithelium of small bronchioles displayed positive hybridization signals (Fig. 5A). In alveolar space, signals were found in cells that were identified as alveolar macrophages by their characteristic morphology in the alveolar lumen (Fig. 5c).

Also, positive $VPAC_2$ mRNA hybridization signals were localized to peribronchiolar and peribronchial clusters of immune cells (Fig. 5D). Controls using the sense-probe were unstained (Fig. 5, B and E).

## Discussion

The present study was performed to localize the mRNA of the VIP receptor $VPAC_2$ in human lung

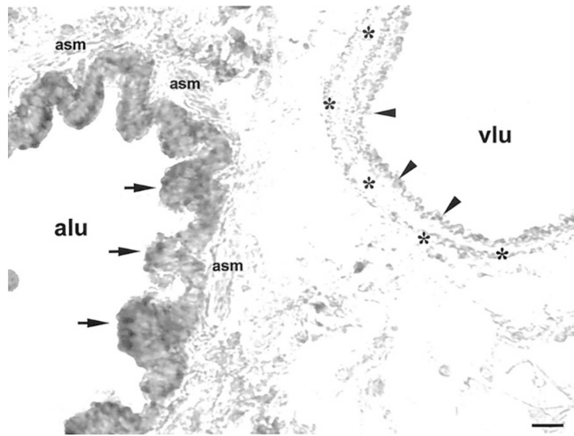
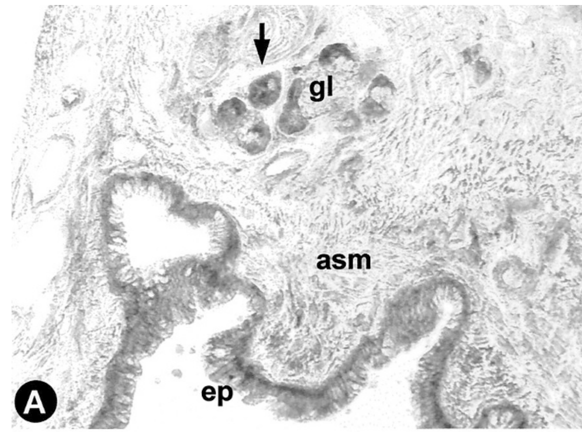
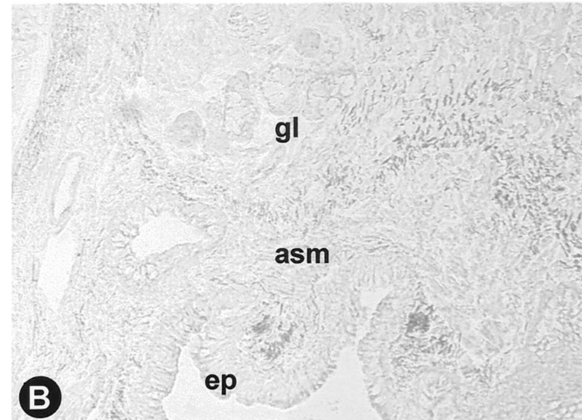



**Figure 3.**
Localization of $VPAC_2$ receptor mRNA in airways by in situ hybridization. Positive $VPAC_2$ mRNA signals are localized to epithelial cells of subsegmentary bronchi (*arrows*). No hybridization signals in endothelial cells (*arrowheads*), vascular smooth muscle cells (*asterisks*), and airway smooth muscle cells (*asm*). *alu* = airway lumen, *vlu* = vessel lumen. Bar = 20 $\mu$m.



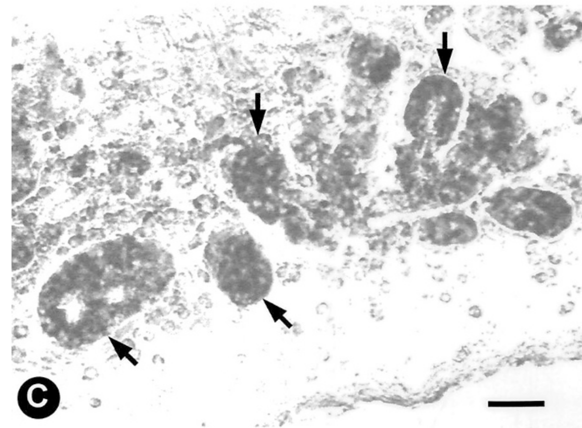




**Figure 4.**
Localization of $VPAC_2$ receptor mRNA in bronchial glands by in situ hybridization. $VPAC_2$ mRNA signals are present in acinar and ductal submucosal gland cells (*gl*) (A, C) of 8 $\mu$m human trachea crystate sections. Also, there is abundant staining of epithelial cells (*ep*) but not goblet cells (A). Negative airway smooth muscle (*asm*). Sense probes did show specific signals. Bar = 40 $\mu$m (A, B); Bar = 16 $\mu$m (C).

tissues. Although there is a large body of evidence on the functional role of the neuropeptide VIP in normal and pathological airway tissues, a detailed analysis of the expression of distinct VIP receptors does not exist so far.

VIP binding sites have been shown in airway tissues of several species by autoradiographic ligand binding studies using ($^{125}$I)-VIP (Carstairs and Barnes, 1986; Robberecht et al, 1981, 1988) and by an immunocytochemical reporter for increases in intracellular cyclic AMP (Lazarus et al, 1986) that is formed when VIP stimulates adenylyl cyclase (Frandsen et al, 1978). Ligand binding sites were initially reported in airway smooth muscle of large but not small airways and in pulmonary vascular smooth muscle. Binding sites were also found in submucosal glands and in the alveolar wall (Carstairs and Barnes, 1986). Immunohistochemical studies using an antibody against cyclic AMP after stimulation by VIP confirmed the autoradiographic findings in different species (Lazarus et al, 1986).

Autoradiography with high-affinity radioligands allows a characterization of binding sites but is limited by a poor morphological resolution. Therefore, it does not reveal the exact cellular sites of the ligand binding. As two distinct receptors for VIP were identified and cloned (Adamou et al, 1995; Ciccarelli et al, 1994; Inagaki et al, 1994; Ishihara et al, 1992; Lutz et al, 1993; Rawlings et al, 1995; Svoboda et al, 1994; Wei and Mojsov, 1996), a localization of a specific receptor mRNA could distinguish the cellular distribution and molecular basis of VIP binding sites.

High resolution interference contrast microscopy revealed positive $VPAC_2$ mRNA signals in tracheal and bronchial epithelial cells and glandular cells that corresponded to earlier binding studies for VIP (Carstairs and Barnes, 1986). These results demonstrate that the reported ligand binding of VIP in these cells is at least partly represented by the $VPAC_2$ receptor.

In peripheral lung, there was staining for $VPAC_2$ mRNA of clusters of immune cells and alveolar macrophages. Thus, some of the VIP binding sites reported for the alveolar wall (Carstairs and Barnes, 1986) may consist of $VPAC_2$ receptor expression in macrophages and of expression of the more widespread $VPAC_1$ as reported previously (Kaltreider et al, 1997).

$VPAC_1$ expression has been previously detected in the lung by RT-PCR, Northern blotting, in situ hybrid-

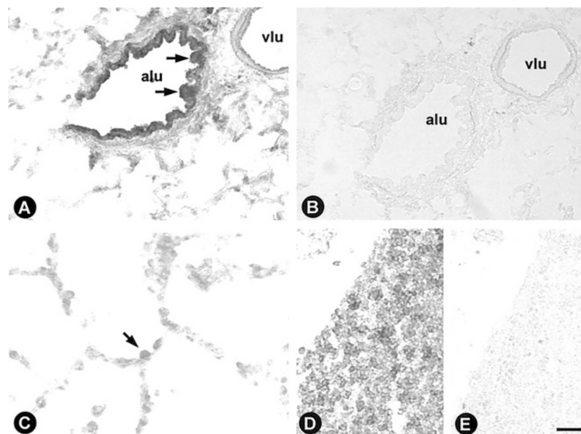



**Figure 5.**

Localization of $VPAC_2$ receptor mRNA in peripheral lung by in situ hybridization. Positive staining for $VPAC_2$ mRNA in epithelial cells of small bronchioli and alveolar macrophages (*arrows* in A and C). Marked staining of clusters of immune cells (D). No specific staining in control sense probes (B, E). Bar = 40 $\mu$m (A, B, D, E); Bar = 25 $\mu$m (C). *alu* = airway lumen, *vlu* = vessel lumen.

ization (Usdin et al, 1994), and immunohistochemistry (Busto et al, 2000; Ichikawa et al, 1995). Localization studies lead to controversial results. Autoradiographic in situ hybridization revealed staining of the epithelium in large and moderate size bronchi as well as vascular endothelium (Usdin et al, 1994). One study in rat airways using immunohistochemistry for $VPAC_1$ led to staining of macrophages and airway epithelium from trachea to terminal bronchioles, but not of bronchial smooth muscle or vascular smooth muscle other than infrequent staining of some intermediate-sized pulmonary veins (Ichikawa et al, 1995). A second study reported $VPAC_1$-like immunoreactivity in leukocytes and vascular smooth muscle myocytes of some vessels but not in epithelial cells (Busto et al, 2000).

VIP potently relaxes the vessels that supply the upper airways (Lundberg et al, 1981; Lung and Widdicombe, 1987; Malm et al, 1980), trachea, bronchi (Laitinen et al, 1987), and pulmonary vessels (Hamasaki et al, 1983a, 1983b; Nandiwada et al, 1985; Obara et al, 1989), and VIP-induced vasodilation is more potent in tracheal circulation than in bronchial circulation (Matran et al, 1989a, 1989b). The vasodilation is reported to be independent of endothelial cells (Barnes et al, 1986; Greenberg et al, 1987). By demonstrating the absence of $VPAC_2$ receptor mRNA in the vascular smooth muscle layer, our findings indicate that VIP-induced vasodilation has to be mediated by a different VIP receptor or by a paracrine $VPAC_2$-dependent pathway.

A recent study reported the presence of $VPAC_2$-like immunoreactivity in myocytes of peripheral arteries but not airway smooth muscle cells. However, these signals were extremely weak and restricted to a minority of smooth muscle cells. The specificity of the reaction was not confirmed by preabsorption techniques and, therefore, not proven as specific for the $VPAC_2$ protein (Busto et al, 2000).

A dense network of VIP-IR nerve fibers (Lundberg et al, 1984; Luts and Sundler, 1989), VIP binding sites (Carstairs and Barnes, 1986; Elgavish et al, 1989), and VIP-receptor immunoreactivity (Fischer et al, 1992) has been reported around and in submucosal glands, and a complex action of VIP on mucus secretion has been suggested (Maggi et al, 1995). Our findings support these suggestions by directly localizing $VPAC_2$ mRNA to serous and mucous cells of submucosal glands.

It has been shown that VIP relaxes airways size-dependently and dilates only bronchi but not smaller bronchioles (Altiere and Diamond, 1984). There was no expression of $VPAC_2$ receptor mRNA in airway smooth muscle myocytes. Therefore, the bronchodilation may be mediated by a paracrine transcellular $VPAC_2$ signal pathway or a different receptor.

The absence of $VPAC_2$ mRNA in vascular and airway smooth muscle cells may imply that this receptor subtype does not directly mediate vasodilatory and bronchodilatory effects in the respiratory tract. However, a paracrine modulation of the two most prominent effects of VIP in the respiratory tract by $VPAC_2$ cannot be excluded.

The demonstration of $VPAC_2$ mRNA in airway immune cells is a finding that affects the significance of VIP in immunomodulation. So far, various functional studies have reported the ability of VIP to influence complex cellular and subcellular immunologic events such as migration, antigen-induced proliferation, and cytokine production (Bellinger et al, 1996). Together with the recent demonstration of $VPAC_2$-like immunoreactivity in macrophages and leukocytes (Kaltreider et al, 1997), our finding provides evidence for the participation of VIP in immunomodulation via the $VPAC_2$ receptor subtype.

We have demonstrated that $VPAC_2$ receptor mRNA is widely distributed in the human respiratory tract and have identified the cellular sites of expression. The $VPAC_2$ receptor mRNA distribution does not correspond with all cellular sites reported by previous ligand binding studies. The failure to detect $VPAC_2$ transcripts in both airway and vascular smooth muscle myocytes is a novel aspect of VIP signaling in the lung, indicating that the previously reported functional VIP binding sites are represented by different receptor types.

Our findings are the first report on the distribution of $VPAC_2$ receptor mRNA in the human respiratory tract, and they support the view that VIP is an important neuromodulator that acts by molecular distinct receptor pathways.

## Materials and Methods

### *Tissues and Materials*

Surgically resected human lung tissues ($n = 6$) from patients with central carcinoma were provided by the Departments of Surgery and Pathology, University of Giessen. The tissues were examined for malignant cells, and only areas with normal histology and cytol-

ogy were subjected to the study protocol. If not stated otherwise, all solutions were supplied by Sigma, Deisenhofen, Germany.

#### *Northern Blot*

Two micrograms of poly ($A^+$) RNA from human CNS, lung, heart, kidney, stomach, pancreas, skeletal muscle, and liver were electrophoresed and blotted onto a nylon membrane. $VPAC_2$ receptor mRNA was identified by overnight hybridization at 42° C with a [$^{32}$P]-labeled PCR-derived 0.6-kbp probe representing the transmembrane domains 3 to 6 of $VPAC_2$. After hybridization, membranes were washed twice for 15 minutes at 65° C in 2× SSC containing 0.1% SDS and twice for 15 minutes at 65° C in 0.5× SSC containing 0.1% SDS and examined after development.

#### *$VPAC_2$ receptor cRNA Probes*

Digoxigenin-labeled $VPAC_2$ receptor-specific cRNA probes were produced with restriction and in vitro transcription kits (Boehringer Mannheim, Mannheim, Germany) as follows: a human-specific $VPAC_2$ receptor PCR fragment (sequence of the region spanning from TM 3 to 7 of the $VPAC_2$ receptor from $T_{SUP1}$ human T lymphoblast cDNA) was subcloned into the pGEM-T vector (Promega, Mannheim, Germany). The plasmid was transfected into *Escherichia coli* and amplified. After linearization of the plasmid with *Nco*I (for sense probe) or *Spe*I (for anti-sense probe) the nonradioactive cRNA probes were synthesized and labeled by in vitro transcription using digoxigenin-11-UTP. For generation of anti-sense probes, the *Spe*I-linearized plasmid was transcribed with T7 polymerase. For the sense probe, the *Nco*I-linearized plasmid was transcribed with SP6 polymerase. For transcription, 1 μg of linearized plasmid was incubated in transcription buffer with 150 μmole of UTP, 600 μmole of digoxigenin-labeled UTP, 750 μmole of ATP, CTP, and GTP, 1 U/μl RNase inhibitor, and 2 U/μl polymerase for 2 hours at 37° C. A DNase I step at 37° C for 15 minutes was followed by the addition of 0.2 M EDTA (pH 8). The transcripts were isolated by LiCl and ethanol precipitation and checked for probe length by TAE-agarose gel electrophoresis (Seakem-ME Agarose, Biozym, Oldendorf, Germany) and ethidium bromide staining.

#### *Nonradioactive mRNA In Situ Hybridization*

Localization of $VPAC_2$ receptor mRNA was performed by nonradioactive in situ hybridization. Cryostat (Jung Frigocut 2800E, Leica, Bensheim, Germany) sections of human lung between 8 μm and 12 μm were thawed onto precoated glass slides (Hybri Slips, Sigma, Deisenhofen, Germany) and air-dried for 15 minutes. The sections were then fixed by immersion for 30 minutes in 4% paraformaldehyde containing PBS (pH 7.4). After rinsing in PBS 3× 10 minutes and in distilled water for 1 minute the slides were permeablized with 0.1 M HCl for 10 minutes and acetylated with 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 10 minutes. After washes in PBS (10 minutes) and distilled water (1 minute), the slides were dehydrated in 70%, 80%, and 96% ethanol. Each section was air-dried for 15 minutes and covered with 100 μl of prehybridization buffer [50% formamide, 1× Denhardt's, 10 mM triethanolamine, 5 mM EDTA, 6.25% dextransulfate, 0.3 M NaCl, 1 mg/ml tRNA (Roche, Mannheim, Germany)] for 1 to 2 hours at 40° C in a moist chamber. After removal of the prehybridization buffer, the slides were covered with 50 μl of hybridization buffer (containing 10 ng/μl $VPAC_2$-specific digoxigenin-labeled sense or antisense probe in 50% formamide, 1× Denhardt's, 10 mM triethanolamine, 5 mM EDTA, 6.25% dextransulfate, 0.3 M NaCl, 1 mg/ml tRNA) and protected with coverslips (Hybrislips, Sigma). Hybridization was performed at 40° C for 14 to 16 hours in a moist chamber.

After hybridization, several washing steps were performed, including the following: 20 minutes in 2× SSC and 20 minutes in 1× SSC at room temperature (rt); RNase treatment for 30 minutes at 37° C; 1× SSC for 20 minutes, 0.5× SSC for 20 minutes, and 0.2× SSC for 20 minutes at rt; 0.2× SSC at 50° C for 1 hour; and 0.2× SSC for 15 minutes and distilled water for 15 minutes at rt.

To detect hybridization signals with the digoxigenin detection kit (Boehringer), sections were rinsed in buffer I (100 mM Tris-HCl; 150 mM NaCl; pH 7, 5) for 10 minutes at rt, and unspecific binding sites were blocked with 1% bovine serum albumin and 10% normal swine serum in 1× PBS in buffer I for 30 minutes at rt. After removal of the blocking medium, the sections were incubated with 40 μl of sheep-anti-digoxigenin-alkaline-phosphatase conjugate (1:500) diluted in blocking medium for 12 hours at 4° C in a moist chamber. Sections were then rinsed twice in Buffer I for 15 minutes and equilibrated in Buffer III (100 mM Tris-HCl; 100 mM NaCl; 50 mM MgCl2; pH 9, 5) for 2 minutes at rt. Detection of the signal was performed by incubation with 150 μl of chromogen solution per slide [45 μl of nitroblue tetrazolium (75 mg/ml) in 70% dimethylformamide], 35 μl of 5-bromo-4-chloro-3-indolyl-phosphate (50 μg/ml in 100% dimethylformamide), and levamisole in 10 ml of Buffer II in a moist chamber at 4° C. Slides were checked for color development after 1 to 15 hours. The reaction was stopped by rinsing the slides in 10 mM Tris HCl with 1 mM EDTA (pH 8.0) for 15 minutes. After washing in PBS twice for 15 minutes the slides were coverslipped with Kaisers glycerine gelatin (Merck, Darmstadt, Germany) and examined with an Olympus BX 60 microscope (Olympus, Hamburg, Germany).

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