Naunyn-Schmiedeberg's Arch Pharmacol (2001) 364 : 314–320
DOI 10.1007/s002100100458

ORIGINAL ARTICLE

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The effect of the vasoactive intestinal polypeptide agonist Ro 25–1553 on induced tone in isolated human airways and pulmonary artery

Received: 30 January 2001 / Accepted: 1 June 2001 / Published online: 31 July 2001

Abstract Ro 25–1553 is a metabolically stable analogue of endogenous vasoactive intestinal polypeptide (VIP). This compound is a potent bronchodilator in vitro as well as in vivo. Moreover, Ro 25–1553 has been shown to be highly selective of the $VPAC_2$ receptor. We assessed the effect of Ro 25–1553 on isolated human bronchi and pulmonary arteries in vitro. Macroscopically normal human airways and pulmonary arteries were obtained from patients undergoing surgery for lung cancer. The relaxing capability of Ro 25–1553 on bronchial and pulmonary artery tone was measured using standard techniques. Bronchial rings were pre-contracted with 0.1 mM histamine, and tone in pulmonary artery rings was induced with 10 μM PGF2α. Increasing concentrations of Ro 25–1553 within a range of 1 pM to 10 μM were added and isometric tension changes were recorded. Ro 25–1553 caused a concentration-dependent relaxation of airway and pulmonary artery preparations, with an EC_{50} of approximately 10 nM and a maximal relaxation of 70%–75% of the induced tone. The presence of $VPAC_2$ receptors in the two tissues, though low in density, was confirmed by in situ hybridization, immunocytochemistry and ligand binding. These findings indicate that the VIP analogue Ro 25–1553 may be useful in the treatment of asthma and/or chronic obstructive pulmonary diseases.

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Keywords VIP · Ro 25–1553 · $VPAC_2$ receptor · Smooth muscle relaxation · Human bronchus · Human pulmonary artery

Introduction

Vasoactive intestinal polypeptide (VIP) is believed to be one of the major neurotransmitters in the inhibitory nonadrenergic, noncholinergic (i-NANC) nervous system, the predominant inhibitory pathway in the human airways. VIP is involved in the regulation of airway tone, mucus secretion and vascular permeability (Said 1995) as well as the proliferative and synthetic function of lymphocytes (Kaltreider et al. 1997). On the basis of these regulatory functions, the observation that VIP is diminished in airways of patients with asthma (Ollerenshaw et al. 1989) and the finding that VIP relaxes human bronchus, pulmonary artery and lung parenchyma in vitro (Saga and Said 1984), a possible role for the use of VIP in the treatment of bronchial asthma has been suggested.

Since chronic obstructive airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD), are not only characterised by bronchoconstriction but may also be accompanied by an increase in the mean pressure of the pulmonary circulation, VIP might be useful both as a bronchodilator and a vasodilator. However, the first clinical studies investigating the effect of inhaled (Barnes and Dixon 1984) or infused (Morice and Sever 1986) VIP in patients with asthma were inconclusive. The lack of a bronchodilating effect of inhaled VIP was thought to be due to immediate metabolism of VIP by resident enzymes, insufficient potency and/or failure of the inhaled drug to reach its receptor (Bolin et al. 1995), and led to the development of VIP analogues with improved pharmacokinetic profiles.

One of these analogues, Ro 25–1553, was found to possess a considerably longer duration of action (Bolin et al. 1995). Moreover, Ro 25–1553 was shown to prevent antigen-induced bronchoconstriction, vasoconstriction, oedema formation and granulocyte accumulation in guinea pigs (O'Donnell et al. 1994b) and to inhibit IL-2 and IL-4 pro-

duction in mice (Tang et al. 1995). The information on the effect of Ro 25–1553 in the human lung, however, is limited to only one published report demonstrating that Ro 25–1553 is about 400 times more potent than native VIP in relaxing human isolated airways (O'Donnell et al. 1994a).

So far two receptors for VIP, the $VPAC_1$ and $VPAC_2$ receptor, have been described (Harmar et al. 1998) – the VIP analogue Ro 25–1553 being highly selective for the $VPAC_2$ receptor (Gourlet et al. 1997). However, until recently only the $VPAC_1$ receptor was described to be present in the human lung (Sreedharan et al. 1995), while the $VPAC_2$ receptor was believed to be present exclusively in areas associated with neuroendocrine function, the pituitary gland and the pancreas (Usdin et al. 1994). Only recently it has been indicated that also the $VPAC_2$ is expressed in the human lung (Busto et al. 1999), with a distribution comparable to that of the $VPAC_1$ receptor, being localised to some of the smooth muscle cells in the wall of small blood vessels as well as white blood cells (Busto et al. 2000). These studies, therefore, confirmed at least in part the localisation of the $VPAC_2$ receptor that had been indicated by functional studies in animal models (O'Donnell et al. 1994b; Tang et al. 1995).

To obtain further insight into the functional effects of the selective $VPAC_2$ receptor agonist Ro 25–1553 and the distribution $VPAC_2$ receptors in the human lung, we assessed the effect of Ro 25–1553 on human isolated pulmonary artery in addition to its effect on isolated bronchus. Secondly, in order to localise the sites of expression of $VPAC_2$ receptors and their endogenous ligands VIP and pituitary adenylate cyclase-activating peptide (PACAP), and to combine functional and morphological data, we used in situ hybridisation, immunocytochemistry and radioligand binding in adjacent sections of the bronchial and arterial preparations that were used for the functional studies.

Materials and methods

The experiments comply with the current laws of the country in which they were performed.

Functional studies in human bronchus and pulmonary artery

Tissue preparation. Macroscopically normal lung tissue was obtained from 11 patients undergoing surgery for lung cancer. Immediately after resection, peripheral airways with intact epithelium (internal diameter of 1–2 mm) and pulmonary arteries with the endothelium conserved (internal diameter of 2–4 mm) were dissected free of alveolar tissue and cut into rings of about 3 mm length.

Tension measurements. The bronchiolar rings were transferred to 10-ml organ baths containing oxygenated (95% O_2, 5% CO_2) modified Krebs buffer (pH7.4; 37°C; composition in mM: NaCl 118.4, KCl 4.7, $MgSO_4$ 0.6, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.1) and mounted for measurement of isometric contractions. Bronchiolar rings were equilibrated for about 60 min until a stable resting tension of about 4 mN was achieved. Thereafter, a single dose of the β-adrenoceptor agonist isoprenaline (1 μM) was added to determine the amount of inherent tone. After several washings and re-equilibration of the tissues, bronchial rings were pre-contracted with 0.1 mM histamine. Previous experiments have shown that this concentration induces approximately 90% of the maximal response (EC_{90}) to histamine. After 10–15 min the tone stabilised and Ro 25–1553 concentration-effect curves within a range of 1 pM–30 μM were constructed. Untreated, i.e. without Ro 25–1553, airway preparations from the same individual were run in parallel and served as tension controls. Concentration-effect curves were obtained in a cumulative manner, using incremental concentrations spaced at half-log_{10} intervals.

The pulmonary artery rings were transferred to superfusion chambers and continuously superfused with oxygenated modified Krebs buffer (see above) at a rate of 2 ml/min. Arterial rings were equilibrated for about 60 min until a stable resting tension of about 5 mN was achieved. This was found to be optimal for force development in response to contractile stimuli in human pulmonary artery preparations. Thereafter, PGF2α concentration-effect curves in a range from 10 nM to 100 μM were constructed. After washing and re-equilibration of the resting tension, pulmonary artery preparations were pre-contracted with 10 μM PGF2α, a concentration which has been shown to induce approximately 80%–90% of the maximal response (EC_{90}) to PGF2α. After 15–20 min the tone stabilised and Ro 25–1553 concentration-effect curves within a range of 1 pM–10 μM were constructed. Concentration-effect curves were obtained in a cumulative manner, using incremental concentrations spaced at log_{10} intervals.

Analysis of results. All responses were recorded as changes in isometric tension using an eight-channel thermal chart recording system (Lectromed MultiTrace 8; Lectromed, Hertfordshire, UK). The traces were evaluated manually. The relaxing effect of Ro 25–1553 on histamine- and PGF2α-induced tension was expressed as a percentage of the maximal response to either stimulus at submaximal concentrations, i.e. 0.1 mM histamine and 10 μM PGF2α (% histamine max., % PGF2α max.). The potency of Ro 25–1553 was calculated from concentration-effect curves by non-linear curve fitting using the InPlot program (GraphPad Software, San Diego, Calif., USA) for each individual tissue and expressed as the pEC_{50} value, i.e. $-log_{10}$ of the concentration of Ro 25–1553 giving a half-maximal effect. All values quoted are means ± SEM of the indicated number of experiments. Statistical comparison of the baseline characteristics of the bronchial rings between the treatment and control group and the resting tension of the time control tissues in the begin and the tension in the end of the experiments was performed using two-tailed, paired or unpaired *t*-tests, as appropriate. Concentration-effect curves were analysed using repeated-measures analysis of variance (ANOVA) followed by multiple comparisons testing. The level of statistical significance was defined as $P<0.05$.

Drugs used. Ro 25–1553 was obtained from Hoffmann-La Roche (Nutley, N.Y., USA). ^{125}I-labelled Ro 25–1553 was prepared according to instructions given by Dr. Stefan Bergquist (AstraZeneca R&D Lund). Isoprenaline hemisulfate and histamine dihydrochloride were obtained from Sigma Chemical (St. Louis, Mo., USA). PGF2α (solution) was purchased from Pharmacia and Upjohn (Erlangen, Germany). Ro 25–1553, isoprenaline and histamine were dissolved in 0.9% NaCl. The peptides used for the in situ hybridization and radioligand binding experiments (VIP, PACAP-27) were obtained from Sigma.

Distribution of the $VPAC_2$-receptor

Tissue handling. Specimens of human bronchioli (sixth to seventh generation, diameter 1–2 mm) were obtained at surgery and fixed by immersion in Stefanini's fixative (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2), repeatedly rinsed in Tyrode buffer supplemented with 10% sucrose, and then frozen in mounting medium (Tissue-Tek; Miles, Elkhart, Ind., USA). Alternatively, specimens were fresh frozen. Specimens were cut (10 μm thickness) in a cryostat and mounted on slides coated with chrome alum.

In situ hybridization. We used oligodeoxyribonucleotide probes, synthesised and purified at the Biomedical Resource Facility, Lund

University, Sweden. One probe is a "humanized" rat $VPAC_2$ probe, which has been successfully used previously in the rat (Filipsson et al. 1998). It is a 30mer and spans the nucleotide sequence 1045–1074 of human $VPAC_2$ cDNA. The other probe (also 30mer) spans the sequence 277–306. The probes were checked in GenBank database (using the NCBI BLAST e-mail server) for complementarity to other genes, but with negative result. The probes were used as a mix to give maximal sensitivity.

Cryostat sections from Stefanini-fixed specimens were used. The probes were ^{35}S-labelled and the protocol used has been described in detail (Myrsen-Axcrona et al. 1997). Sections hybridized either after incubation in RNAse A, or in the presence of a 100-fold molar excess of unlabelled probe as described served as controls. Sections were viewed in both bright and dark field microscopy.

Immunocytochemistry. $VPAC_2$ receptors were demonstrated using mouse monoclonal antibodies raised against a peptide fragment (aa 105–122) of the human receptor (Antibody Solutions, Palo Alto, Calif., USA) and used in dilution 1:40. This antibody was recently used in immunocytochemistry (Smith et al. 2000). For VIP and PACAP immunolocalization we used previously characterised rabbit polyclonal antibodies (anti-VIP from EuroDiagnostica, Malmö, Sweden, and anti-PACAP, kind gift from Prof. A. Arimura, U.S.–Japan Biomedical Research Labs, Tulane University, Belle Chasse, La., USA; for details see Sundler et al. 1992). For control purposes, primary antibodies were either omitted, or replaced by rabbit non-immune serum. Preabsorption controls were run as described (Sundler et al. 1992).

Cryostat sections from Stefanini-fixed specimens and indirect immunofluorescence were used. Briefly, sections (10 μm thickness) were incubated with primary antibodies overnight and, after rinsing, in FITC-labelled secondary antibodies for 1 h at room temperature.

Radioligand binding. Sections from fresh frozen specimens were preincubated with binding buffer [50 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$, 2% serum albumin (BSA), and 0.5 μg/ml bacitracin] for 30 min at room temperature. The preincubation buffer was aspirated and sections were incubated with binding buffer containing 20 pM ^{125}I-labelled Ro 25–1553 with or without 1 μM unlabelled Ro 25–1553, VIP or PACAP-27 for 1 h at room temperature. The concentration of labelled Ro 25–1553 was chosen on the basis of pilot experiments and on previous experience (Moller et al. 1997). The sections were then washed 3×5 min with ice-cold binding buffer with reduced (0.1%) BSA content. Afterwards, the sections were quickly rinsed in water and air-dried. Autoradiography was performed for 5–7 days on Hyperfilm MP. After the first exposure, the sections were dipped in Ilford K5 liquid autoradiographic emulsion (diluted 1:1 with distilled water and exposed for 7–10 days. The films and sections were developed in Kodac D 19. The sections were counterstained with Ehrlich's hematoxylin. Light microscopy was used to evaluate the binding of ^{125}I-labelled Ro 25–1553. As positive controls for the radioligand binding we used fresh frozen tissues known to express $VPAC_2$ receptors (rat stomach and guinea-pig trachea).

Results

Baseline characteristics of the bronchial and pulmonary artery rings

There were no differences in the functional characteristics of bronchiolar ring preparations between the control (n=5) and treatment group (n=6). The overall mean (± SEM) wet weight of the bronchiolar rings was 13.9±1.38 mg, mean resting tension was 3.97±0.41 mN and mean inherent tone (i.e. the magnitude of relaxation after a single dose of isoprenaline) was 2.05±0.44 mN.

There were no differences in the characteristics of pulmonary artery preparations between the control (n=5) and treatment group (n=5). The overall mean (± SEM) resting tension of the artery rings was 4.69±0.23 mN and the mean tension change after a maximal concentration (0.1 mM) of PGF2α was 4.37±0.54 mN.

Effect of Ro 25–1553 on induced bronchial tone

Histamine 0.1 mM increased human bronchiolar tone by about 6.38±0.72 mN to a total tension of 9.24±0.95 mN. Ro25–1553 relaxed the pre-contracted bronchi concentra-

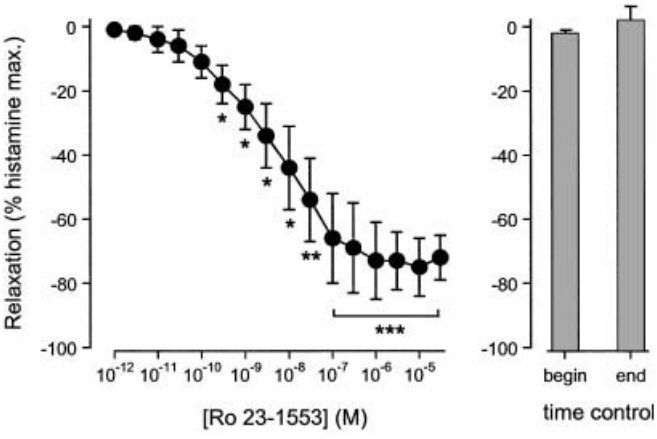

Fig. 1 *Left panel:* relaxation of histamine-induced tone by Ro 25–1553 in human bronchial rings (●); the data are means ± SEM of six experiments. *Right panel:* there were no significant tension changes over time in control preparations that were run parallel; shown is the mean (± SEM) tension at the begin and the end of the experiment (shaded bars). *P<0.001 (ANOVA) denotes the comparison of responses in Ro 25–1553-treated tissues and the respective time controls

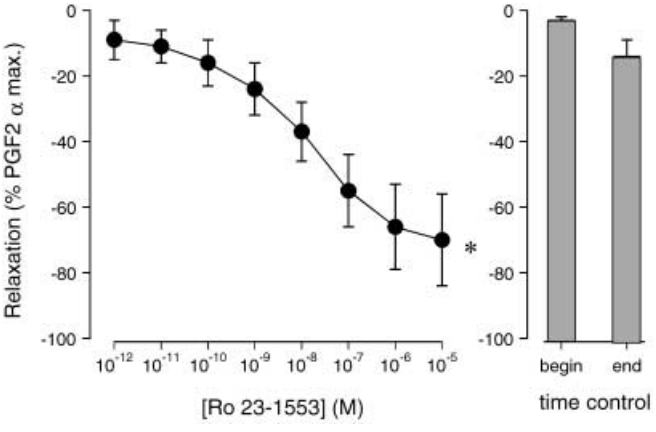

Fig. 2 *Left panel:* relaxation of histamine-induced tone by Ro 25–1553 in human pulmonary artery rings (●); the data are means ± SEM of five experiments. *Right panel:* there were no significant tension changes over time in control preparations that were run parallel; shown is the mean (± SEM) tension at the begin and the end of the experiment (shaded bars). *P<0.001 (ANOVA) denotes the comparison of responses in Ro 25–1553-treated tissues and the respective time controls

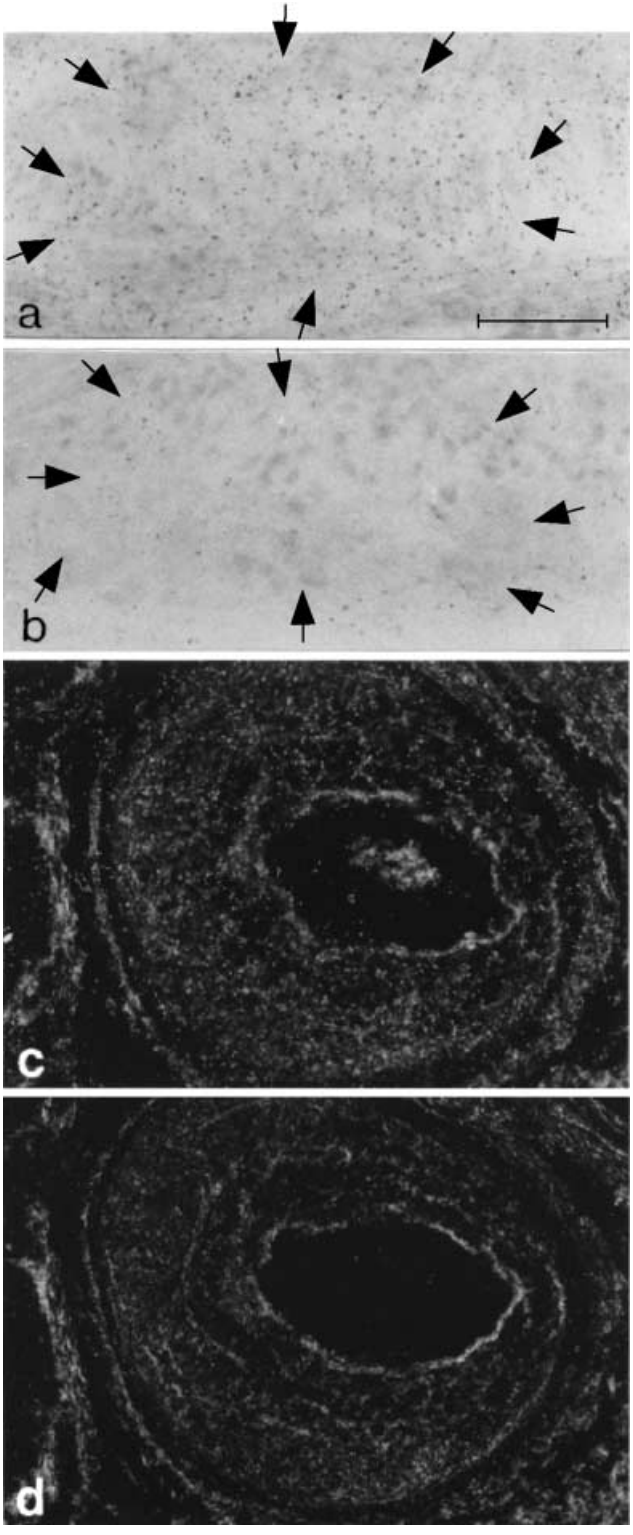

Fig. 3 In situ hybridisation for $VPAC_2$ receptor mRNA in **a** bronchial and **c** vascular smooth muscle; **b** and **d** show corresponding control sections which were obtained in the presence of excess unlabelled probe. Low levels of specific probe labelling of both muscles. **a,b** Bright field, **c,d** dark field microscopy, ×300. The (obliquely cut) muscle bundles in a and b are delineated by *arrows*. *Inserted bar* =50 μm

tion-dependently (*n*=6; Fig. 1), with a maximal relaxation of 75±9% of the histamine-induced contraction and a pEC_{50} of 8.30±0.19, while only slight changes could be observed in the tension-control tissues during the same period of time (Fig. 1).

Effects of Ro 25–1553 on induced vascular tone

PGF2α 0.1 mM increased human pulmonary artery tone by about 3.55±0.44 mN to a total tension of 8.14±0.52 mN. Ro 25–1553 relaxed the pre-contracted arteries concentration-dependently (*n*=5; Fig. 2) with a maximal relaxation of 70±14% of the PGF2α-induced contraction and a pEC_{50} of 7.74±0.20.

Distribution of the $VPAC_2$ receptor

In situ hybridisation revealed a weak, specific labelling for $VPAC_2$ mRNA in both bronchial and vascular smooth muscle (Fig. 3a–d). Immunoreactivity for $VPAC_2$ receptors was localised to smooth muscle of the bronchial wall, as well as smooth muscle of peribronchial blood vessels (Fig. 4a–d). In addition, scattered cells in the interstitial tissue were immunoreactive. VIP and PACAP occurred in nerve fibres, which were quite numerous in bronchial smooth muscle, and more sparse around blood vessels (Fig. 4e,f). Radioligand binding with ^{125}I-labelled Ro 25–1553 also revealed weak, specific labelling of vascular and bronchial smooth muscle (Fig. 5). In the reference tissues, the smooth muscle of the rat stomach was strongly labelled (Fig. 5) while guinea pig tracheal smooth muscle was moderately labelled. Both VIP and PACAP27 (Sigma, St. Louis, Mo., USA) at concentrations of 1 μM replaced radiolabelled Ro 25–1553, in the same way as Ro 25–1553 did.

Discussion

The present study demonstrates that the long-acting and $VPAC_2$-selective VIP analogue Ro 25–1553 produces a significant relaxation of human bronchioli pre-contracted with histamine. In this respect, Ro 25–1553 was almost as potent as the long-acting β_2-adrenoceptor agonist formoterol as compared with previous data obtained in this laboratory under similar experimental conditions (EC_{50}=5.0 nM and 1.3 nM for Ro 25–1553 and formoterol, respectively; Schmidt et al. 2000). The maximum relaxation achieved by Ro 25–1553 appeared to be slightly less than that produced by formoterol, suggesting a somewhat lower efficacy. In another study on human bronchus (up to the second generation) pre-contracted with histamine, Ro 25–1553 (EC_{50}=20 nM) was about 30 times more potent than salbutamol and was at least as efficacious as this standard reference for bronchodilation (O'Donnell et al. 1994a). Thus Ro 25–1553 is able to efficiently relax human airway smooth muscle over a wide range of the bronchial tree.

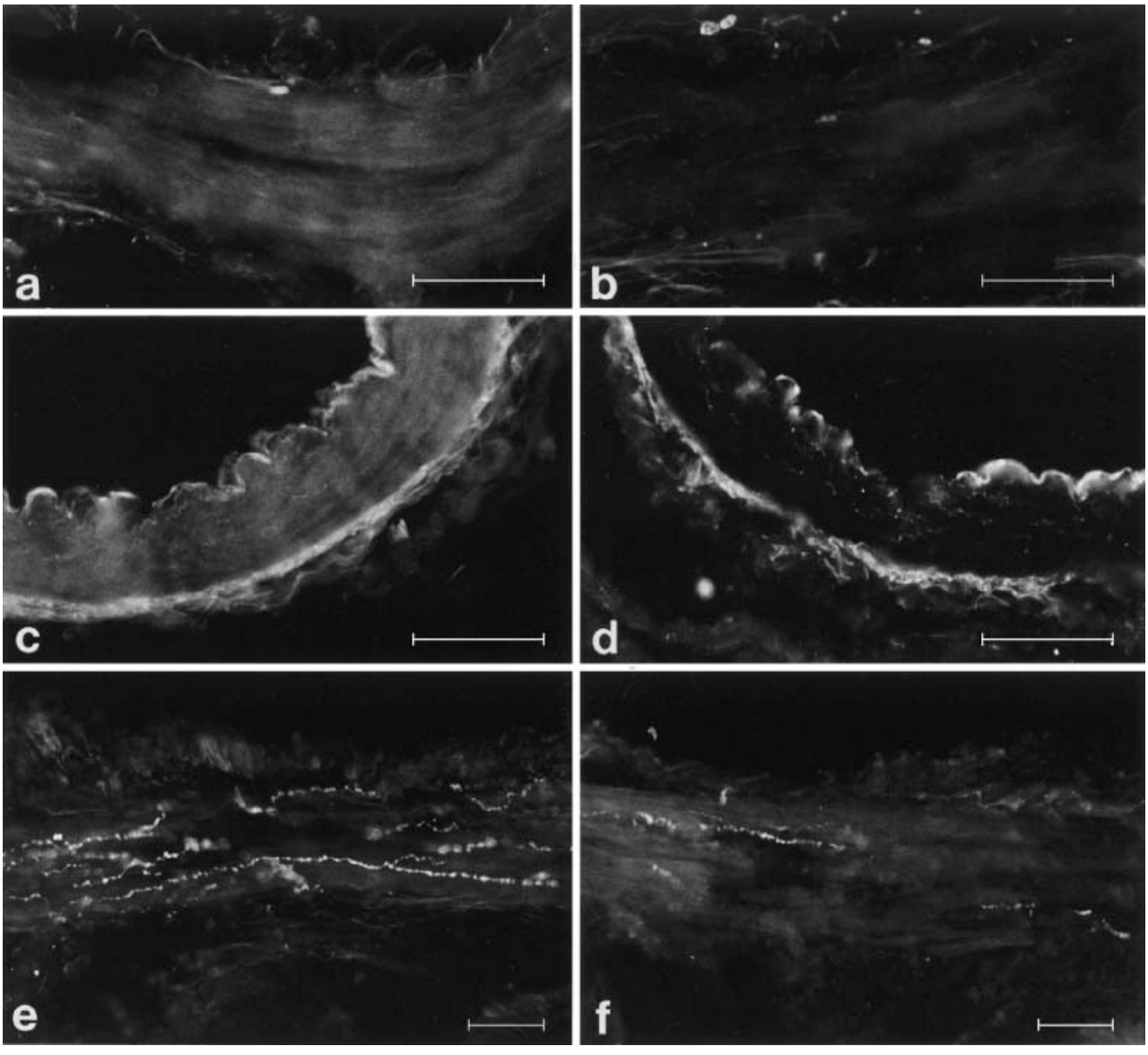

Fig. 4 $VPAC_2$ receptor immunofluorescence in **a** bronchial and **c** vascular smooth muscle. **b,d** Immunonegative control sections. Autofluorescence in elastic membranes of vessel wall. Bronchial smooth muscle containing **e** numerous VIP and **f** less numerous PACAP immunofluorescent nerve fibers (**a–d** ×350, **e,f** ×200). *Inserted bar* =50 μm

Ro 25–1553 also significantly relaxed human pulmonary artery pre-contracted with PGF2α. This is a novel observation and it occurred in the same concentration range as the relaxation of the bronchi. The relaxing capability of Ro 25–1553 was comparable with the effect of the non-selective phosphodiesterase inhibitor, theophylline, reported previously (Rabe et al. 1995) although Ro 25–1553 was about 1000 times more potent. This, together with previous observations on the isolated and perfused guinea-pig lung (O'Donnell et al. 1994b), underlines the action of Ro 25–1553 as a potent vasodilator in the lung.

These functional effects on human airways and pulmonary arteries presented here together with the observation that Ro 23–1553 prevented antigen-induced bronchoconstriction, vasoconstriction, oedema formation and granulocyte accumulation in guinea pigs (O'Donnell et al. 1994b) suggests a possible role for Ro 23–1553 for the treatment of asthma and COPD, i.e. lung diseases that are characterised by chronic airway obstruction and in some cases also pulmonary hypertension, in addition to pronounced airway inflammation. Therefore, treatment of asthma and COPD with a VIP analogue might bear a spectrum of effects exceeding even those of β_2-adrenoceptor agonists by including vasodilation and anti-inflammatory effects.

While the functional results presented in this study suggest the presence of the $VPAC_2$ receptor in airway as well as in vascular smooth muscle, we found only weak mRNA expression in the airway and vascular preparations taken from adjacent sections. This is in agreement with a previous study that found $VPAC_2$ mRNA to be highly expressed

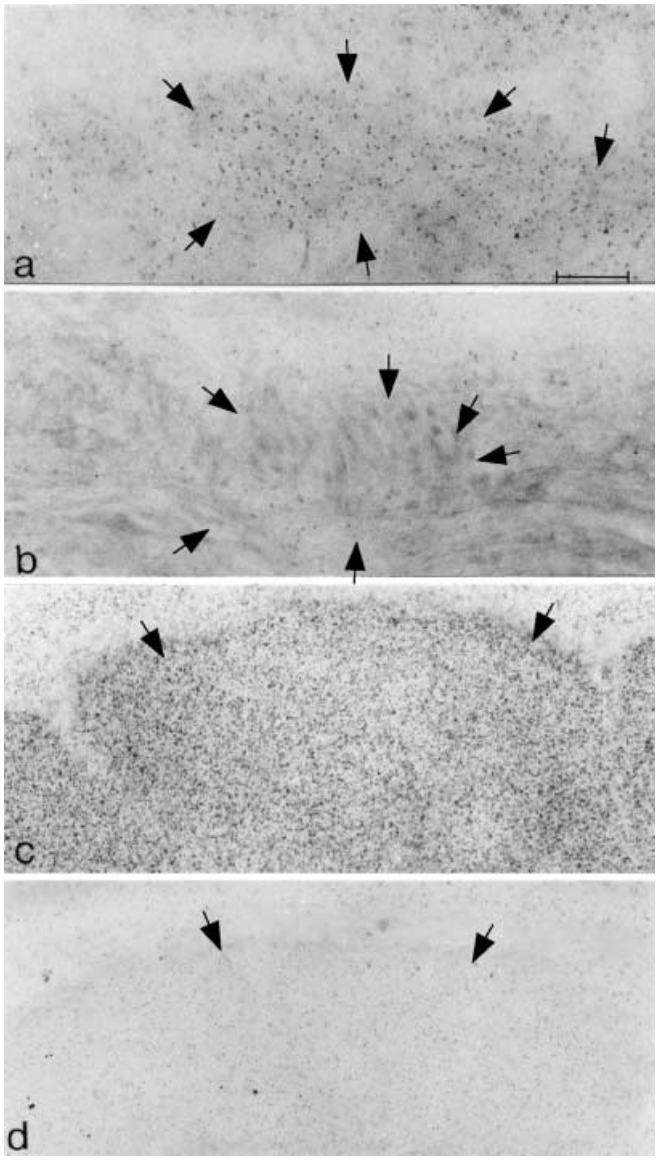

Fig. 5 Binding of ^{125}I-labelled Ro 25–1553 **a** to bronchial smooth muscle, and **c** to rat gastric smooth muscle used as reference tissue; **b** and **d** show corresponding control sections (presence of excess unlabelled Ro 25–1553). Weak labelling of bronchial muscle, while the reference tissue is strongly labelled, ×200. Smooth muscle margins are indicated by *arrows*. *Inserted bar* =50 μm

in skeletal muscle, and moderately expressed in the heart, brain and kidney, while its expression in the human lung was only sparse (Adamou et al. 1995). Recently, the $VPAC_2$ receptor was localised by immunohistochemistry to vascular smooth muscle and white blood cells in the human lung (Busto et al. 2000). However, the weak expression of the $VPAC_2$ receptor in our preparations was confirmed by both immunohistochemistry and direct binding of ^{125}I-labelled Ro 25–1553. Obviously, only a few receptors need to be occupied by the agonist to exert a marked functional response. There are other examples to show that the functional significance of a G-protein-coupled receptor is difficult to assess from the level of expression. Thus for the CCK-2 (CCK-B/gastrin) receptor in the human oxyntic mucosa of the stomach, in situ RT-PCR, a highly sensitive method, is needed for visualisation of the cellular sites of mRNA expression (Kulaksiz et al. 2000).

The concentration-effect curve for Ro 23–1553 obtained in the airways as well as in the pulmonary artery was rather shallow in comparison to that of the β_2-adrenoceptor agonist formoterol, which has been shown earlier for human tissue preparations (Linden et al. 1999) and might indicate receptor heterogeneity. However, more detailed information on this hypothesis is not available so far and studies that look further into this point are currently under way.

In conclusion, the results of the present study indicate that stimulation of the $VPAC_2$ receptor leads to relaxation of the smooth muscle of the human bronchi and pulmonary artery. Considering the potential anti-inflammatory properties of Ro 25–1553 previously described this compound might be useful in the treatment of bronchial asthma and, possibly, COPD. However, clinical studies will have to evaluate the clinical implications of Ro 25–1553, possible side-effects and therapeutic value in comparison with drugs already established such as β-adrenoceptor agonists or theophylline.

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