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Thromboxane prostanoid receptor in human airway smooth muscle cells: a relevant role in proliferation

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Abstract

Thromboxane A_2 has been implicated as a mediator of bronchial hyperresponsiveness in asthma. Modulating agents are currently marketed in Japan and under clinical evaluation in the US, but full characterization of the thromboxane A_2 receptor and the signaling pathways that link it to the proliferative events taking place during airways structural remodeling has not been achieved. Here, we report that the presence of mRNA for both α and β isoforms of the thromboxane A_2 receptor in smooth muscle cells from human bronchi correlates with protein expression evaluated by radioligand binding of the antagonist, SQ29,548 ([1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic-acid) ($K_d = 3.4$ nM ± 44%CV, coefficient of variation, $B_{max} = 41$ fmol/mg prot ± 38%CV). The receptor is functional, as the agonist, U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic-acid), induced a concentration-dependent Ca^{2+} transient ($EC_{50} = 0.12$ μM ± 27%CV). Furthermore, U46619 concentration dependently increased DNA synthesis and markedly potentiated the epidermal growth factor mitogenic effect. Both events were specifically inhibited by SQ29,548, independently from transactivation of the epidermal growth factor receptor and partially sensitive to pertussis toxin.
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Keywords: Asthma; Thromboxane A_2; Smooth muscle cell; Human; Proliferation; Airway

1. Introduction

Asthma is a chronic inflammatory disease characterized by reversible airway obstruction and nonspecific bronchial hyperresponsiveness. Both endobronchial biopsy and pathological findings in postmortem tissues have demonstrated the presence of underlying inflammation even in subjects with mild asthma or who were asymptomatic (McFadden and Gilbert, 1992). The principal features of this chronic state are eosinophil infiltration in the submucosa and epithelium, thickening of the airway wall by collagen deposition in the basement membrane and hypertrophy and hyperplasia of the bronchial smooth muscle cells (Hossain, 1973; Jeffery et al., 1989).

Leukotrienes, prostaglandins and thromboxane A_2, all arachidonic acid metabolites, have been demonstrated to be crucial in the pathogenesis of asthma. Thromboxane A_2's role in asthma has recently been regarded with new emphasis because, in addition to being a well-known bronchoconstrictor, it is also believed to be involved in both the late asthmatic response and bronchial hyperresponsiveness (Devillier and Bessard, 1997). It has also been suggested to stimulate proliferation of human airway smooth muscle cells (Belvisi et al., 1998).

Pharmacological heterogeneity of TP receptors has been suggested from the results obtained in several studies with different ligands, cells or tissues (Halushka et al., 1995), but a single gene encoding a G protein-coupled receptor protein has been cloned (Nusing et al., 1993). However, alternative splicing of the carboxyl-terminal tail leads to two variants that share the first 328 amino acids: TPα was cloned from a cDNA library derived from placenta (Hirata

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[2] This paper is specially dedicated to the memory of our mentor Simonetta Nicosia. Her open-minded and critical approach to experimental work will always remain a guiding principle for us.

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et al., 1991) and TPβ from a human umbilical endothelial cell cDNA library (Raychowdhury et al., 1994). The precise biological functions subserved by the two isoforms are still unknown.

Considerable effort was recently assigned to the identification of more specific pharmacological tools to inhibit or antagonize the actions of thromboxane A_2 in asthma (Dogne et al., 2002). Unfortunately, thromboxane synthase inhibitors have limited possibilities, because activation of TP receptors is still possible in the presence of high levels of other eicosanoids such as prostaglandin D_2, which has recently been implicated as a mediator of allergic asthma (Matsuoka et al., 2000), or prostaglandin H_2 and $F_2\alpha$. In fact, it is well known that although each prostanoid is at least one order of magnitude more potent for its specific receptor than for any of the other prostanoids, all show some degree of cross-reactivity (Coleman et al., 1994). Furthermore, cyclooxygenase inhibitors do not appear to be of any benefit for asthmatic patients and can even precipitate asthmatic attacks in a subset of individuals (aspirin-induced asthma). It is debated whether or not this is due to an imbalance of arachidonic acid metabolism toward the production of leukotrienes (Szczeklik and Stevenson, 1999), which in turn have a clear role in asthmatic disease (Nicosia et al., 2001) and/or to the simultaneous inhibition of multiple prostanoids (Narumiya and FitzGerald, 2001), since antiinflammatory prostanoids are also produced during inflammation (Gilroy et al., 1999). Finally, also the isoprostane 8-isoprostaglandin $F_2\alpha$, a chemically stable product of oxidative stress, is also known to activate TP receptors, albeit as a partial agonist (Janssen et al., 2000; Kinsella et al., 1997). This leads to the conclusion that the identification of selective TP receptor antagonists seems to be a more useful strategy than that of upstream inhibitors.

Among several TP receptor antagonists and thromboxane synthase inhibitors that have been studied in double-blind, placebo-controlled clinical trials, some have been proven to be effective for treating asthma: the thromboxane A_2 synthase inhibitor, ozagrel hydrochloride, or OKY-046 (Kurosawa, 1995; Nakazawa et al., 1994) and the TP receptor antagonist, seratrodast, or AA-2414 (Hoshino et al., 1999; Muramatsu et al., 2001; Terao et al., 1999), have been available as anti-asthmatic agents in Japan since 1992 and 1997, respectively. Moreover, seratrodast and another TP receptor antagonist, ramatroban or BAY-U3405 (Aizawa et al., 1996; McKenniff et al., 1991), are currently under phase III clinical evaluation in the US for the treatment of asthma (Dogne et al., 2002). Furthermore, recent work has revealed a positive association of a single nucleotide polymorphism in the $TBXA_2R$ gene with bronchial asthma in adults (Unoki et al., 2000) and children (Leung et al., 2002), although the TP receptor and its signaling pathways have not been fully characterized in human airways smooth muscle cells. We therefore investigated this issue by measuring gene expression, binding affinity and capacity, and by performing functional studies in a model of human airway smooth muscle cells in culture and we demonstrated the presence of a functional TP receptor which could substantially contribute to the proliferative events taking place during structural remodeling of the airways.

2. Materials and methods

2.1. Materials

Cell culture supplies (media, serum, trypsin, amino acids and antibiotics) and TriZol Reagent for total RNA isolation were purchased from Gibco BRL (Now Invitrogen Life Technologies). 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5*Z*,13*E*-dien-1-oic acid (U46619), and [1*S*-[1α,2α(*Z*),3α,4α]]-7-[3-[[2-[(phenylamino)carbonyl]-hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid (SQ29,548) were obtained from Cayman Chemical [5,6-^{3}H]SQ29,548 was from NEN Life Science Products, Stock solutions of these compounds were stored at −20 °C. All salts for the preparation of saline solutions, EDTA, EGTA, epidermal growth factor and anti-smooth muscle α-actin antibody, were purchased from Sigma. All other antibodies were purchased from Santa Cruz Biotechnology, except for anti-epidermal growth factor receptor, which was a generous gift of Dr. Bice Chini. 4-(3-Chloroanilino)-6,7-dimethoxyquinazoline (AG1478) and pertussis toxin were from Calbiochem. Fura 2/AM (2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-acetyloxy methyl ester) and pluronic F-127 were purchased from Molecular Probes. Ultima Gold scintillation liquid was from Packard Instruments. 1st Strand cDNA Synthesis Kit for reverse transcriptase polymerase chain reaction (RT-PCR) was from Boehringer Mannheim and the protease inhibitor complex, Complete™, from Roche. Reagents and films for chemoluminescence and [6-^{3}H]thymidine were from Amersham Biotech. All the reagents and supplies for electrophoresis and DC™ Protein assay (Lowry quantitation assay) were purchased from Bio-Rad Laboratories.

2.2. Human airway smooth muscle cells culture

Smooth muscle cells from human bronchi were purchased from Cambrex (former Clonetics) or isolated in our laboratory as previously described (Accomazzo et al., 2001; Viganò et al., 1997). Briefly, macroscopically normal lung fragments were obtained at thoracotomy. Third-order bronchi were removed under sterile conditions, the connective tissue and the epithelium were removed and the smooth muscle was cut into pieces approximately 10 mg each. The explants were grown at 37 °C in a humidified atmosphere of 5% CO_2 in Medium 199, with the addition of 20% (v/v) fetal bovine serum, 100 U/ml penicillin and 100 μg/ml

streptomycin in 25 cm^2 culture flask. The primary isolates were positively stained with an anti-smooth muscle α-actin antibody to assess the identity of the cultures. Thereafter, cells were routinely grown in monolayers in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin, passaged at a 1:3 ratio in 75 cm^2 culture flasks and used between the 3rd and 8th passages (our isolates) or between the 3rd and the 12th passages (purchased cells). For this investigation, we established three lines and purchased two, and cell lines from these two fonts were used to address each topic so that the data shown are the means ± S.E.M., indicating variability between cell lines.

2.3. RT-PCR and Southern blot analysis

Total RNA was isolated from human airway smooth muscle cells grown in monolayers by means of the phenol and guanidine isothiocyanate method (TRIzol® Reagent) and cDNA was generated using 1 μg of total RNA (1st Strand cDNA Synthesis Kit for RT-PCR, Boehringer Mannheim). Further, 100 ng of cDNA was amplified in vitro by PCR, using AmpliTaq® DNA Polymerase (Perkin Elmer). The primers were selected on the basis of the previously published sequences (Hirata et al., 1991): one pair of primers included F3 (5′-CTGCTCATCTACTTGCGCGT-3) corresponding to nucleotides 868–887 and R4 (5′-CAGGGTCAAAGAGCATGCAA-3′) corresponding to nucleotides 1787–1806; another pair included F55 (5′-GTCGCTACACCGTGCAATACC-3′) corresponding to nucleotides 515–535 and R53 (5′-CTACTGCAGCCCGGAGCGCTG-3′) corresponding to nucleotides 1013–1033. After revelation and identification of the PCR products by ethidium bromide staining, Southern blot analysis of PCR products was performed by hybridization (QuickHyb®, Sratagene) with oligonucleotide probes, under high stringency conditions. Probes were previously labelled with ^{32}P (Ready-To-Go™ T4 Polynucleotide Kinase, Pharmacia Biotech) and purified (ProbeQuant™ G-50 Micro Columns, Pharmacia Biotech) following the manufacturers' instructions.

2.4. Equilibrium binding assays

Radioligand binding was performed at equilibrium on subconfluent human airway smooth muscle adherent cells ($5–6 \times 10^4$ cells/cm^2) in a final volume of 500 μl of MEM, containing 20 mM HEPES buffer pH 7.4 and 0.2% bovine serum albumin, for 30 min at 25 °C. We used a mixed type binding protocol, obtained by combining both saturation (0.3–5 nM of [^3H]SQ29548) and competition (10 nM–10 μM of SQ29548) protocols in a single curve (Capra et al., 1998; Rovati, 1998). At the end of the incubation (30 min at 25 °C), the cells were washed with ice-cold phosphate-buffered saline containing 0.2% bovin serum albumin and lysed in 0.25 N NaOH. Radioactivity was then measured by liquid scintillation. Analysis of binding data was performed by means of the computer program LIGAND (Munson and Rodbard, 1980) (see Section 2.8). The protein content was determined with the Lowry quantitation assay. Nonspecific binding ranged between 40% and 50% of the total binding.

2.5. Measurements of the concentration of cytosolic-free Ca^{2+} ion

Human airway smooth muscle cells were seeded onto 12-mm diameter glass coverslips and used when 90–100% confluence was reached. Cells were incubated for 60 min at 30 °C in the dark with 5 μM fura 2/AM in MEM plus 0.03% pluronic F-127, 2.5 mM probenecid and 10 mM HEPES. After loading, the cells were washed twice with a saline solution (NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1.8 mM, HEPES 10 mM, glucose 10 mM; pH 7.4) plus 2.5 mM probenecid. The coverslips were transferred to the spectrofluorimeter (Perkin Elmer LS50) cuvette and fluorescence was monitored at 30 °C (505 nm emission, 340 and 380 nm excitation). In order to evaluate the concentration of cytosolic-free Ca^{2+} ion ($[Ca^{2+}]_i$) from fluorescence recording, calibration was performed as follows: F_{max} (maximal fluorescence of the system) was obtained by adding 2.7 μM ionomycin and 100 μM digitonin, F_{min} was obtained by adding 5 mM EGTA and 60 mM Tris base. $[Ca^{2+}]_i$ was calculated as described by Grynkiewicz et al. (1985) with a $K_d = 224$ nM. $[Ca^{2+}]_i$ increase has been expressed as the ratio of the stimulated over the basal level (s/b).

2.6. Proliferation assay

Cells were subcultured into 24-well plates at a 1:3 split ratio and allowed to grow to sub-confluence. Forty-eight hours before stimulation, cells were placed in serum-free MEM (with all other additions present) to synchronize the entire population cell cycle. The medium was then replaced by "serum-deficient" MEM containing 1% fetal bovine serum, which represents our control. The agonists, U46619 and epidermal growth factor at the concentration indicated, the antagonists and inhibitors were always added in "serum-deficient" medium. Prior to stimulation, there were a 30-min preincubation with SQ29,548 and 60-min preincubation with AG1478 where indicated. The duration of stimulation was 48 h and [^3H]thymidine was added at 44 h, for 4 h, at a final concentration of 1 μCi ml^{-1}. The cells were then washed twice with ice-cold phosphate-buffered saline to rinse loosely associated radioactive tracer. Acid-soluble radioactivity was removed by 20-min treatment with 5% trichloroacetic acid at 4 °C followed by a two-step wash with 95% ethanol. The acid-insoluble portion was recovered by 60-min solubilization with 2% Na_2CO_3 in 0.1 M NaOH. Radioactivity was then measured by liquid scintillation counting.

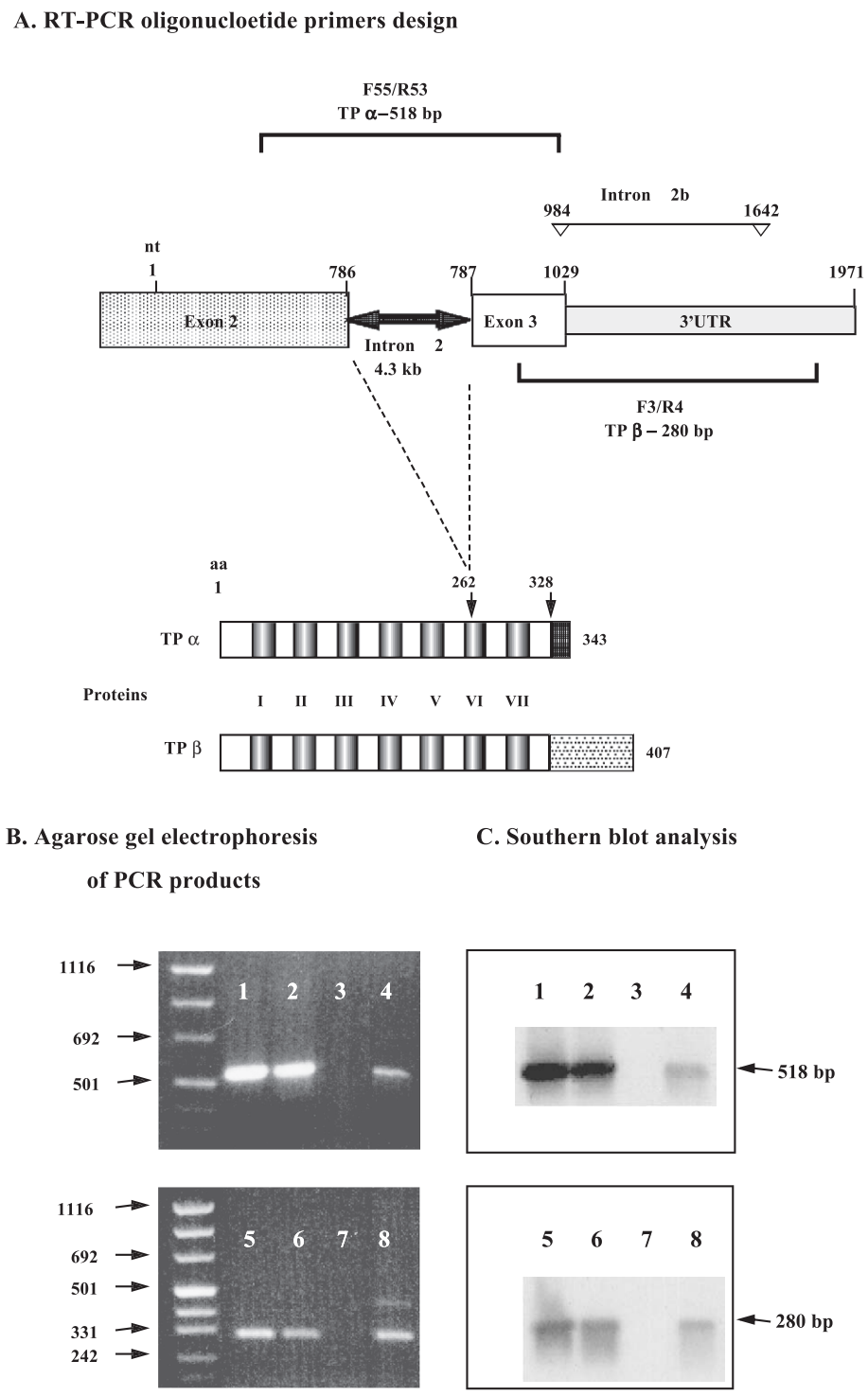

Fig. 1. (A) Thromboxane A_2 gene and receptor protein organization. The general organization of exons and introns encoding for the different C-terminal tail of TPα and TPβ are depicted. In particular, TPα does not utilizes the splice site present in exon 3, whereas TPβ originates from its use. In fact, nucleotides 984–1642 behave as an intron (inverted triangles), and splicing results in a different mRNA with an open reading frame for a protein of 407 amino acids. This results in two protein isoforms that share the first 328 amino acids (putative transmembrane domains I–VII, closed boxes), but have peculiar C-terminal tails (hatched box for peptide-α and dotted box for peptide-β). The position of primer pairs, F3/R4 and F55/R53, and the expected sizes of PCR products are also shown. (B) Analysis of the expression of the mRNA for TP receptors: agarose gel electrophoresis of RT-PCR products derived from human airway smooth muscle cells (hAMSC) 1st cDNA template. Lanes 1 and 2, TPα; lanes 5 and 6, TPβ; lanes 3 and 7, blank; lanes 4 and 8, hASMC (C) Southern blot analysis of TPα and TPβ RT-PCR products, generated using primer pairs F55/R53 and F3/R4, screened using a [^{32}P]-radiolabelled probe. Lanes 1 and 2, TPα; lanes 5 and 6, TPβ; lanes 3 and 7, blank; lanes 4 and 8, hASMC.

2.7. Phosphorylation of epidermal growth factor receptor

Subconfluent cells in 60-mm dishes were serum-starved for 48 h, and 2 h before stimulation the medium was replaced by MEM containing 0.1% fetal bovine serum. The cells were then stimulated at 37 °C with U46619 or epidermal growth factor at the concentrations indicated for 5 min. AG1478 was preincubated for 60 min before the addition of epidermal growth factor. Monolayers were then placed on ice, washed twice with phosphate-buffered saline, treated with lysis buffer (20 mM Tris–HCl pH 7.5, 1 mM dithiotreitol, 2 mM EGTA, 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and the protease inhibitor complex Complete™) and sonicated four times for 15 s. The samples were subsequently diluted in Laemmli buffer, resolved by sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE, 20 μg/lane–6% gel) and transferred to a nitrocellulose membrane. Immunoblotting was performed with a polyclonal anti-phosphotyrosine antibody at a concentration of 0.2 μg/ml (Santa Cruz Biotechnology) for 18 h at 4 °C and immune complex was detected by chemoluminescence (Amersham Biotech) using horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) as a secondary antibody. The membrane was stripped with stripping buffer and reblotted with polyclonal anti-epidermal growth factor receptor antibody.

2.8. Computer analysis of equilibrium binding data and concentration–response curves

Analysis of binding data was performed using the program LIGAND (Munson and Rodbard, 1980), analyzing several experiments simultaneously. A statistical level of significance of $P<0.05$ was accepted. Nonspecific binding was calculated by LIGAND as one of the unknown parameters of the model. Binding is expressed as the ratio of bound ligand concentration over total ligand concentration, (B/T, dimensionless), vs. the logarithm of total ligand concentration (log T). B (in M) is the sum of "hot", "cold" and nonspecific binding; T (in M) is the sum of "hot" and "cold" ligand incubated.

Analysis of concentration–response curves was performed with the computer program ALLFIT (De Lean et al., 1978), which calculates the lower and upper plateaux, the slope and the EC_{50}. Selection of the best-fitting model and evaluation of the statistical significance of the parameter difference was based on the F-test for the extra sum of square principle (Draper and Smith, 1966), both for binding and concentration–response curves. Parameter errors are always expressed as % coefficient of variation (%CV). All the curves shown were computer-generated.

2.9. Statistical analysis

When indicated, t-test or analysis of variance (ANOVA) followed by Bonferroni's or Fisher's least square differences (LSD) "post hoc test" for multiple comparisons were performed. A statistical level of significance of $P<0.05$ was accepted. Data are presented as means ± S.E.M.

3. Results

3.1. RT-PCR of the thromboxane A_2 receptor

The general organization of the human TP gene in the region where the differential splicing event gives rise to the two receptor isoforms is outlined in Fig. 1A. The relative positions of the oligonucleotide primer pairs which were used in the RT-PCR reaction to amplify either TPα (F55/R53) or TPβ (F3/R4) specific sequences are also shown. To validate data obtained from ethidium bromide-stained gels (Fig. 1B), PCR fragments were subjected to Southern blotting analysis using a ^{32}P-oligonucleotide probe X (5′-ACCTGGAACCAGATCCTGGAC-3′ corresponding to

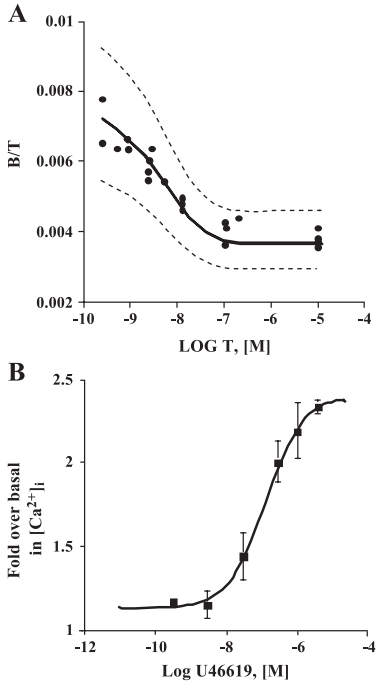

Fig. 2. (A) Equilibrium binding curve of [^{3}H]SQ29,548 in intact human airway smooth muscle cells 4. Mixed type binding curves were made using 0.3–0.5 nM [^{3}H]SQ29,548 (saturation part of the curve) and 10 nM–10 μM of unlabelled SQ29548 (competition part of the curve). Binding is expressed as the ratio of bound ligand concentration over total ligand concentration, (B/T, dimensionless), vs. the logarithm of total ligand concentration (log T). B (in M) is the sum of "hot", "cold" and nonspecific binding; T (in M) is the sum of "hot" and "cold" ligand incubated. Dotted lines, ± 95% confidence limits. Data are means of five experiments, each performed in triplicate on three different cell lines, simultaneously analyzed with LIGAND. (B) Concentration–response curve of the $[Ca^{2+}]_i$ transient induced by U46619 in human airway smooth muscle cells. Data are presented as means of three to six replicates from four experiments performed on three different cell lines ± S.E.M., analyzed with ALLFIT.

oligonucloetides 892–912 of the TPα and TPβ mRNA) and Y (5′-ATGGTGGTGGCCAGCGTGTGTT-3′ corresponding to oligonucleotides 751–772 of the TPα and TPβ mRNA) under high-stringency conditions (Fig. 1C). In each case, data generated by autoradiographic analysis of the Southern blots were consistent with those generated by ethidium bromide-stained gels and no cross-reacting bands were detected. The extent of hybridization measured by autoradiography indicated that both α and β isoforms of the TP receptor could potentially be expressed in human airway smooth muscle cells.

3.2. Equilibrium binding of [^{3}H]SQ29,548

Equilibrium binding was assayed to verify the expression of the TP receptor at a protein level also and to obtain the parameters characteristic of the ligand–receptor interaction. Fig. 2A shows the mixed type binding curve (see Materials and methods) of the TP receptor antagonist, [^{3}H]SQ29,548, that appeared to be monophasic, spanning two orders of magnitude. Computer analysis of the binding data indicates that SQ29,548 binds in a specific and saturable way with a K_d of 3.4 nM ± 44%CV. TP receptor was expressed in this tissue with a B_{max} of 41 fmol/mg prot ± 38%CV.

3.3. Mechanisms responsible for U46619-induced Ca^{2+} rise

To demonstrate that the TP receptor proteins expressed in human airway smooth muscle cells are functional and specific, we performed signaling studies measuring the classical intracellular Ca^{2+} variations evoked by a thromboxane A_2 stable analogue, further characterizing the mechanisms leading to this response. The TP receptor agonist, U46619, was able to trigger a dose-dependent increase in $[Ca^{2+}]_i$ with an EC_{50} value of 0.12 μM ± 27%CV (Fig. 2B). Fig. 3A shows a representative trace of the $[Ca^{2+}]_i$ transient induced by 1 μM U46619. The rise in cytosolic calcium was specifically inhibited by a 5-min pretreatment with the antagonist, SQ29,548 (Fig. 3B). In addition, 5-min pretreatment of human airway smooth muscle cells with 1 μM U46619 almost completely abolished the calcium mobilization induced by successive stimulation with the same agonist concentration (Fig. 3C).

Changes in $[Ca^{2+}]_i$ in the presence of 1 mM EGTA are shown in Fig. 3D. Removal of external Ca^{2+} reduced the response to a simple spike that returned to baseline within 1 min. The addition of 2.5 mM $CaCl_2$ induced an increase in $[Ca^{2+}]_i$ that was higher in the presence (Fig. 3D) than in the absence (Fig. 3E) of agonist stimulation (2.26 ± 0.47 S.D. vs. 1.69 ± 0.29 S.D.-fold over basal, respectively, $P < 0.05$). Furthermore, pretreatment with 300 nM thapsigargin, an inhibitor of sarcoplasmic reticulum calcium ATPase, induced a massive release of Ca^{2+} ion from intracellular stores that did not increase in response to the subsequent stimulation with U46619 (Fig. 3F).

3.4. Measurement of DNA synthesis by [^{3}H]thymidine incorporation assay

One of the principal features of chronic inflammation in asthma is the development and progression of structural changes referred to as airway remodeling and consisting of

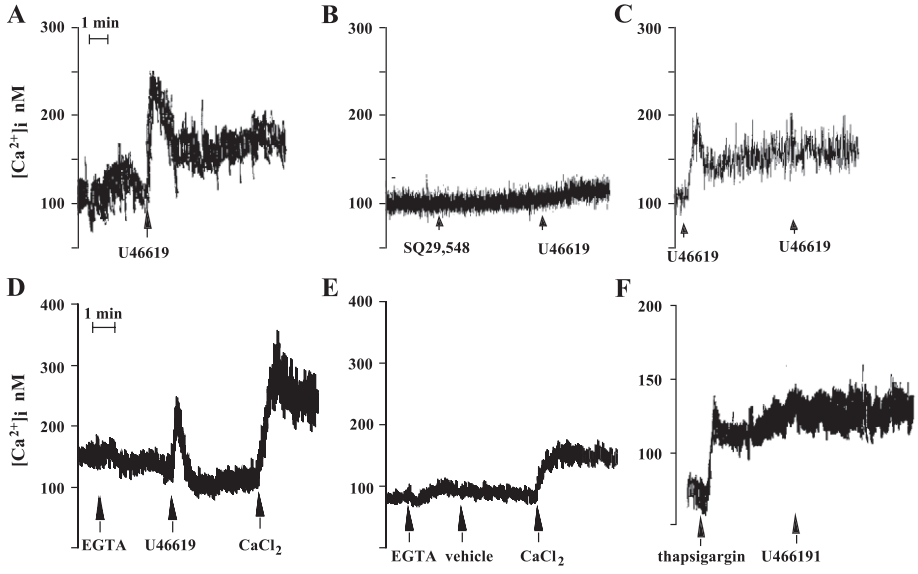

Fig. 3. Typical tracing of agonist-induced free Ca^{2+} rise in a population of human airway smooth muscle cells. (A) $[Ca^{2+}]_i$ transient induced by 1 μM U46619. (B) Effect of 5-min pretreatment with 25 μM SQ29548 on 3 μM U46619-induced calcium mobilization. (C) Effect of 5-min pretreatment with 1 μM U46619 on 1 μM U46619-induced calcium mobilization. (D) 1 μM U46619-induced Ca^{2+} release from intracellular stores in the presence of 1 mM EGTA, where Ca^{2+} influx was restored in the presence of 2.5 mM $CaCl_2$. (E) The corresponding tracing in the presence of the vehicle. (F) Typical tracing after treatment with 300 nM thapsigargin. Data are representative of at least three independent experiments performed in duplicate on at least two different cell lines.

hyperplasia of the airway smooth muscle cells, besides other alterations, leading us to start a characterization of the involvement of the thromboxane prostanoid receptor in this event. U46619 alone (0.01–1 μM) was able to produce concentration-dependent ($P<0.01$) synthesis of DNA which was inhibited by pretreatment with 1 μM of the specific antagonist, SQ29,548, $P<0.01$ (Fig. 4A). In addition, 1 μM of the agonist potentiated the mitogenic effect induced by 20 ng/ml epidermal growth factor, $P<0.01$, and pretreatment with SQ29,548 abolished the potentiating effect, restoring the proliferative effect back to that of epidermal growth factor alone $P<0.01$ (Fig. 4B). Finally,

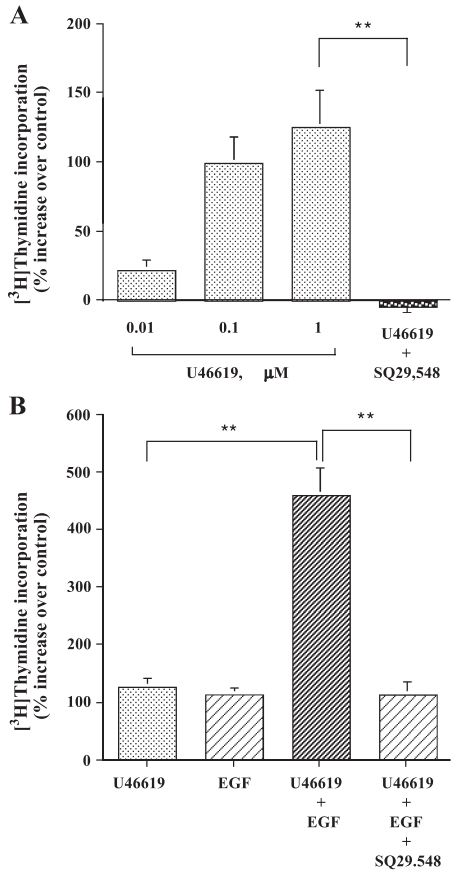

Fig. 4. Effect of the exogenously added U46619, EGF and SQ29,548 on [^{3}H]thymidine incorporation in human airway smooth muscle cells. (A) Concentration-dependent ($P<0.01$, linear regression) increase of [^{3}H]thymidine incorporation induced by U46619 and effect of 30-min pretreatment with 1 μM of the antagonist, SQ29,548, on the maximal concentration of the agonist. Control is represented by MEM plus 1% fetal bovine serum ([^{3}H]thymidine levels, 3825 ± 885 d.p.m./well). The results are presented as means ± S.E.M. of three experiments performed in triplicate on two different cell lines. Data are expressed as percent increase over control. (B) Potentiating effect of the simultaneous addition of U46619 and epidermal growth factor on [^{3}H]thymidine incorporation in human airway smooth muscle cells. Increase of [^{3}H]thymidine incorporation induced by 1 μM U46619 and 20 ng/ml EGF alone and in combination, in the absence and presence of 1 μM SQ29,548 (30-min pretreatment). Control is represented by MEM plus 1% fetal bovine serum ([^{3}H]thymidine levels, 3763 ± 655 d.p.m./well). The results are presented as means ± S.E.M. of six experiments performed in triplicate on three different cell lines. Data are expressed as percent increase over control. **$P<0.01$ (one-way ANOVA).

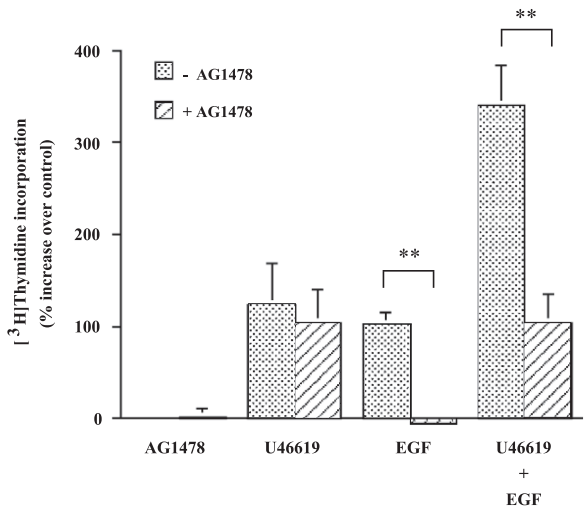

Fig. 5. Effect of AG1478 on [^{3}H]thymidine incorporation induced by exogenously added U46619, epidermal growth factor, or the combination of both in human airway smooth muscle cells. Increase of [^{3}H]thymidine incorporation induced by 1 μM U46619 and 20 ng/ml EGF alone and effect of the simultaneous addition of U46619 and EGF in the absence and presence of 250 nM AG1478 (30-min pretreatment). Control is represented by MEM plus 1% fetal bovine serum ([^{3}H]thymidine levels, 3105 ± 391 d.p.m./well). The results are presented as mean ± S.E.M. of three experiments performed in triplicate on two different cell lines. Data are expressed as percent increase over control. **$P<0.01$ (one-way ANOVA).

Fig. 5 shows that the EGF receptor tyrosine kinase inhibitor, AG1478, at a concentration of 250 nM, had no effect on the [^{3}H]thymidine incorporation induced by U46619 whereas,

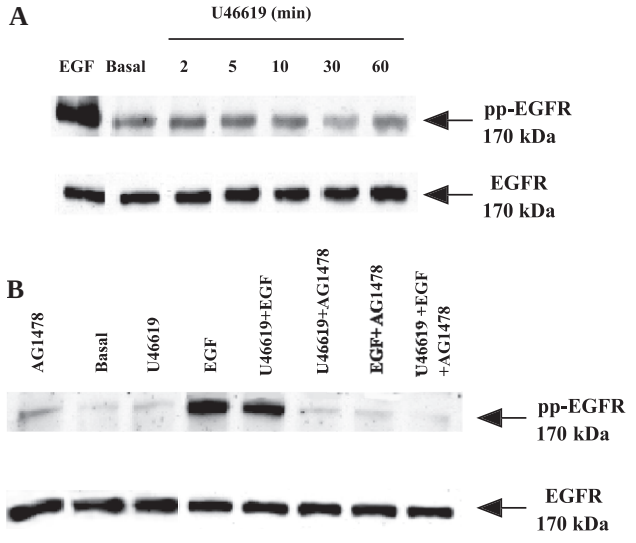

Fig. 6. Analysis of tyrosine phosphorylation of epidermal growth factor receptor in human airway smooth muscle cells. (A) Time course of U46619-stimulated epidermal growth factor receptor tyrosine phosphory lation. (B) Effect of 300 nM U46619, 1 ng/ml epidermal growth factor, or the combination of both, for 5 min at 37 °C, in the absence and presence of 250 nM AG1478 (1-h preincubation). Lysates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine or anti-epidermal growth factor receptor. The position of the epidermal growth factor receptor is indicated by an arrow. The results presented are representative of two other experiments performed on two different cell lines.

as expected, it completely abolished the mitogenic effect of epidermal growth factor alone. When tested on the combination of U46619 plus epidermal growth factor, AG1478 restored the [^{3}H]thymidine uptake levels induced by U46619 alone.

3.5. Phosphorylation of the epidermal growth factor receptor

Since the mitogenic potential of U46619 seems to be independent of transactivation of the epidermal growth factor receptor, we further addressed this question by examining the ability of U46619 to promote direct tyrosine phosphorylation of the epidermal growth factor receptor. As shown in Fig. 6B, epidermal growth factor stimulation (1 ng/ml, 5 min) of human airway smooth muscle cells resulted in strong tyrosine phosphorylation of epidermal growth factor receptor that was completely prevented by 250 nM of the specific inhibitor, AG1478. On the contrary, TP receptor activation by 300 nM U46619 up to 60 min failed to promote epidermal growth factor receptor tyrosine phosphorylation (Fig. 6A and B) nor did it potentiate epidermal growth factor-stimulated autophosphorylation (Fig. 6B).

3.6. Effect of pertussis toxin on $[Ca^{2+}]_i$ transient and DNA synthesis

To examine whether the effect of the thromboxane A_2 mimetic on $[Ca^{2+}]_i$ transient and cell growth is mediated by pertussis toxin-sensitive G proteins, we investigated the influence of pertussis toxin on U46619-induced Ca^{2+} signaling and DNA synthesis.

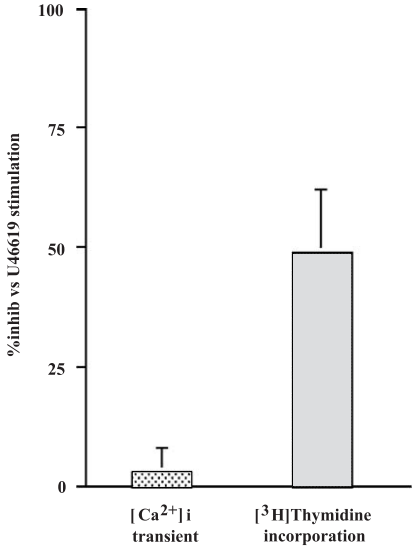

Fig. 7. Effect of pertussis toxin on U46619-induced $[Ca^{2+}]_i$ transient and DNA synthesis in human airway smooth muscle cells. Cells were preincubated with 300 ng/ml pertussis toxin for 18 h prior to stimulation with 1 μM U46619. Data are presented as means ± S.E.M. of at least three experiments performed in triplicate on two different cell lines. Data are expressed as % inhibition vs. U46619 stimulation.

Preincubation of human airway smooth muscle cells with 300 ng/ml pertussis toxin for 18 h did not significantly alter Ca^{2+} responses evoked by 3 μM U46619 (Fig. 7). Cells were tested with other stimuli to confirm the ability of pertussis toxin to produce inhibition in $[Ca^{2+}]_i$ transient (data not shown).

On the contrary, the same treatment inhibited by almost 50% the mitogenic response to U46619 assessed by [^{3}H]thymidine incorporation. The toxin was quite ineffective on the epidermal growth factor mitogenic response and produced 30% inhibition of the mitogenic effect due to co-stimulation of TP receptor agonist and growth factor (data not shown).

4. Discussion

Bronchoconstriction, inflammatory cell recruitment, mucus hypersecretion, bronchial hyperresponsiveness and structural remodeling of the airways (hyperplasia and hypertrophy of the smooth muscle cells) are all hallmarks of bronchial asthma. Thromboxane A_2 is known to be a potent constrictor of respiratory smooth muscles, but it has also been implicated as a mediator of bronchial hyperresponsiveness in asthma (Devillier and Bessard, 1997). Antagonists of the TP receptor were found to be effective to control asthma symptoms and lower airway hyperresponsiveness (Fujimura et al., 1995) although to date, full molecular and functional characterization of the thromboxane A_2 receptor in smooth muscle cells from human bronchi is still lacking.

Thromboxane A_2 exerts its actions by interacting with a seven transmembrane spanning receptor coupled to G proteins, officially termed TP (Coleman et al., 1994), of which two isoforms (α and β) have been identified arising from the alternative splicing from a single gene, and differing only in their carboxyl terminal tail.

Here, we report that mRNAs for both TPα and TPβ isoforms are co-expressed in airway smooth muscle cells from human bronchi. This is not surprising, as it has been demonstrated by Miggin and Kinsella (1998) that a number of tissues and cells co-express both isoforms with few exceptions, e.g. HepG2 cells. In order to confirm TP receptor expression at the protein level also and to demonstrate its functionality, we performed binding studies with the selective radiolabelled TP receptor antagonist, [^{3}H]SQ29,548, and analysed $[Ca^{2+}]_i$ transients induced by the stable agonist, U46619. Analysis of the binding data allowed us to determine a value for the antagonist ligand affinity in agreement with the K_d's previously obtained with other tissues or cells, ranging between 1 and 25 nM (Armstrong and Wilson, 1995; Narumiya et al., 1999). The events that follow TP receptor activation have been extensively studied in human platelets and vascular smooth muscle cells, where receptor stimulation results in a pertus-

sis toxin-insensitive activation of phospholipase C and hydrolysis of membrane phosphoinositides phospholipids with an increase of intracellular free Ca^{2+} ion (Halushka et al., 1995; Kinsella et al., 1997). In human airway smooth muscle cells, we found that agonist stimulation of the TP receptor evokes the classical signaling pathway of calcium transient in a concentration-dependent manner, with an EC_{50} value of 0.12 μM, consistent with values given in the literature for human myometrial smooth muscle cells (Moore et al., 2002). Furthermore, we observed that $[Ca^{2+}]_i$ transient appears to be due to both release from intracellular storages and influx through calcium channels, since removal of external Ca^{2+} with EGTA clearly highlighted the rapid discharge of intracellular stores, which is also demonstrated by the agonist inability to trigger a further $[Ca^{2+}]_i$ rise following depletion with thapsigargin. Likewise, the presence of a concentration of $CaCl_2$ exceeding the chelating properties of EGTA restores Ca^{2+} influx, inducing an increase in $[Ca^{2+}]_i$ that is significantly greater in the presence than in the absence of agonist stimulation. Finally, TP receptors present in these cells undergo agonist-induced desensitization, as a subsequent dose of U46619 is not able to elicit any further $[Ca^{2+}]_i$ transient. These data are in good agreement with the demonstration that, in HEK293 cells at least, TPβ isoform undergoes agonist-induced internalization (Parent et al., 1999).

Numerous contractile agents, such as histamine, thrombin, substance P and endothelin (Ammit and Panettieri, 2001), have been shown to induce proliferation of airway smooth muscle cells in culture and, thus might play an important role in stimulating the smooth muscle hypertrophy and hyperplasia associated with asthma (Johnson et al., 2001). We demonstrated that in human airway smooth muscle cells the stable analogue of thromboxane A_2, U46619 is able to stimulate proliferation in a concentration-dependent and specific manner. Furthermore, the addition of U46619 to EGF elicited a clear-cut increase of [^{3}H]thymidine uptake into DNA, which is completely prevented by SQ29,548, indicating that this potentiation is specifically mediated by TP receptors. Considering that the effect of the combined doses of U46619 and epidermal growth factor is significantly greater (*t*-test, $P<0.01$) than the sum of the effect of each single agonist, we can suggest that U46619 acts synergistically with epidermal growth factor. This implies that, when the local hormonal or inflammatory state is disrupted in diseases such as asthma, even a minimal activation of TP receptor could aggravate the growth factor-regulated proliferation of human airway smooth muscle cells, narrowing the bronchial lumen and greatly contributing to the more chronic features of the disease.

Many G protein-coupled receptors acts as mitogens transactivating receptor tyrosine kinases such as epidermal growth factor receptor (Daub et al., 1997) and there are data in the literature indicating that the events that lead to mitogen-activated protein kinase activation following TP receptor activation are dependent upon epidermal growth factor receptor transactivation (Gao et al., 2001; Miggin and Kinsella, 2001). At variance with these data we demonstrated that, in human airway smooth muscle cells, U46619 has a mitogenic potential independent of transactivation of the epidermal growth factor receptor, as it was completely unable to promote direct tyrosine phosphorylation of the epidermal growth factor receptor or to potentiate epidermal growth factor-stimulated autophosphorylation. Consistent with these results, the specific inhibitor of epidermal growth factor receptor tyrosine kinase, AG1478, failed to inhibit U46619-induced DNA synthesis and was able to prevent the synergy between epidermal growth factor and U46619 in the proliferative response. However, our data are in agreement with other results obtained in human airway smooth muscle cells for distinct inflammatory stimuli, i.e. thrombin, histamine and carbachol, for which a different synergic mechanism has been suggested (Krymskaya et al., 2000).

Hirata et al. (1996) have demonstrated that the TPα and TPβ, at least in platelets, showed the same agonist-induced activation of phospholipase C, but the opposite effect on adenylate cyclase activity, suggesting that TPα activates adenylate cyclase, whereas TPβ inhibits it. Therefore, it seems that the different isoforms of the TP receptor couple to more than one G protein (TPα to G_q and G_s whereas TPβ to G_q and G_i), some sensitive to pertussis toxin and some not. To better define which G protein is involved in the U46619-induced $[Ca^{2+}]_i$ increase observed in human airways smooth muscle cells, pertussis toxin, that is known to inhibit activation of $G_{i/o}$ protein, was utilized in both proliferation and $[Ca^{2+}]_i$ measurement. As a result, we observed that the U46619-induced $[Ca^{2+}]_i$ transient was unaffected by pertussis toxin treatment, whereas U46619-induced DNA synthesis was partially abolished, indicating that a G_i-mediated signaling pathway connects the TP receptor to the nucleus for the control of human smooth muscle cell proliferation, but that at least one other pathway is involved, which is pertussis toxin insensitive.

In conclusion, we demonstrated that smooth muscle cells from human bronchi express specific receptors for thromboxane A_2, that are positively coupled to a $[Ca^{2+}]_i$ rise in a pertussis toxin-insensitive manner and to cell proliferation in both a pertussis toxin-sensitive and -insensitive manner. Furthermore, we demonstrated that TP receptor activation leads to a marked potentiation of the epidermal growth factor mitogenic response, yet independently of epidermal growth factor transactivation. This control of smooth muscle cell proliferation could be important during proliferative events taking place during structural remodeling of the airways, one of the main characteristics of chronic asthma. The complete characterization of the signaling pathway linking TP receptor activation to cell proliferation will be important to define the precise role of thromboxane A_2 and its receptor in asthma and is the target of ongoing investigation in our laboratory.

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