ELSEVIER

European Journal of Pharmacology 274 (1995) 7–15

ejp

β_2-Adrenoceptors mediate a reduction in endothelial permeability in vitro

Michael J. Allen *, Robert A. Coleman

Department of Pharmacology 1, Glaxo Research & Development Ltd., Park Road, Ware, Herts, SG12 0DP, UK

Received 14 July 1994; revised MS received 3 November 1994; accepted 4 November 1994

Abstract

The permeability of bovine pulmonary artery endothelial (CPAE) monolayers to Evans blue-labelled albumin (Evans blue-albumin) has been measured in vitro. Thrombin caused a concentration-dependent increase in Evans blue-albumin clearance across endothelial monolayers. Isoprenaline inhibited thrombin-induced Evans blue-albumin clearance in a concentration-dependent manner (EC_{50} 21 nM). This effect was mimicked by the selective β_2-adrenoceptor agonists salbutamol (EC_{50} 64 nM) and salmeterol (EC_{50} 2.7 nM), but not by the selective β_1-adrenoceptor agonist, RO-363 ((1-[3',4'-dihydroxyphenoxy]-2-hydroxy-[3'',4''-dimethoxyphenethylamino]-propane)oxalate), nor by the selective β_3-adrenoceptor agonist, CL-316,243 (disodium (*R*,*R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate). Isoprenaline, salbutamol and salmeterol, but not RO-363 or CL-316,243 produced small, but significant reductions in Evans blue-albumin clearance across unstimulated endothelial monolayers. Inhibition of the response to thrombin by isoprenaline was antagonised by the selective β_2-adrenoceptor antagonist, ICI-118,551 ((*erythro*-DL-1(7-methylindan-4-yloxy)3-isopropylaminobutan-2-ol), pK_B 8.4). Salmeterol also inhibited hydrogen peroxide-stimulated Evans blue-albumin clearance. Hence, the widely used β_2-adrenoceptor agonists, salbutamol and salmeterol, are able to reduce endothelial permeability at nanomolar concentrations.

Keywords: β-Adrenoceptor; Endothelium; Vascular permeability; Protein extravasation; Salbutamol; Salmeterol

1. Introduction

The vascular endothelium is responsible for maintaining a permeability barrier between the vascular lumen and the extravascular space. Alterations in the permeability of venular endothelium lead to changes in vascular permeability (Majno and Palade, 1961; Wu and Baldwin, 1992), and are of critical importance in inflammation. Increased permeability of arterial endothelium may play an important role in the development of atherosclerosis (Ross, 1986; DeMichele and Minnear, 1992).

As the endothelium plays an important role in both inflammatory and vascular diseases, in vitro models have been developed that allow direct measurement of endothelial permeability by culturing endothelial monolayers on filters (Cooper et al., 1987). The endothelium-coated filter is then used to separate two chambers and the passage of macromolecules between these chambers, across the filter, is measured.

β-Adrenoceptor agonists have been widely reported to reduce vascular permeability in vivo (e.g. Mizus et al., 1985; Adamski et al., 1987; Whelan and Johnson, 1992). However, while in vivo observations are essential to establish physiological or pharmacological relevance, it has been questioned whether these reductions in vascular permeability are due to direct or indirect effects (Persson, 1993). Vascular permeability in vivo depends on both true changes in vascular permeability and changes in regional haemodynamics (Williams and Peck, 1977). Additionally, indirect alteration of vascular permeability may also be brought about by the involvement of inflammatory cells (Wedmore and Williams, 1981), and it has previously been shown that β-adrenoceptor agonists can reduce the release of inflammatory mediators from human lung (Butchers et al., 1980). These complicating factors make assessment of direct effects on endothelial permeability difficult.

* Corresponding author. Tel. +44 0920 884311, fax +44 0920 882263.

SSDI 0014-2999(94)00689-X

Osmotic, oncotic or hydrostatic pressure gradients (parameters which depend on both vascular and perivascular conditions) may also affect plasma exudation. Characterisation of receptors in vivo is also hampered by non-equilibrium conditions, and by unknown drug concentrations at the site of action. While these *complicating* factors are all essential components of the response in vivo, measurement of endothelial permeability in vitro allows the investigation of agents acting directly on this cell type. We were interested in examining effects mediated via β-adrenoceptors directly on endothelial permeability in isolation, and whether these effects could be brought about by pharmacologically relevant concentrations of therapeutically used β-adrenoceptor agonists.

β-Adrenoceptor agonists have previously been shown to reduce the permeability of endothelial monolayers in vitro (Minnear et al., 1989, 1993; Gudgeon and Martin, 1989; Langeler and Van Hinsbergh, 1991), suggesting a direct action on endothelial permeability. However, most of these studies have only used high concentrations of β-adrenoceptor agonists (1 μM or above) where pharmacological selectivity cannot be guaranteed. The only paper to report concentration-response characteristics (Zink et al., 1993) reported the curious finding that while the β_2-adrenoceptor agonist, formoterol, reduced endothelial permeability at subnanomolar concentrations, isoprenaline only produced responses at supra-micromolar concentrations. There are no published reports on the possible involvement of β_3-adrenoceptors.

Our aim was to demonstrate that β-adrenoceptor agonists can modulate endothelial permeability in vitro at pharmacologically relevant concentrations, and to characterise the β-adrenoceptors involved. We have measured clearance of albumin labelled with Evans blue, an albumin-binding dye (Patterson et al., 1992), across bovine pulmonary artery endothelial cell monolayers. These cells are well documented to respond to thrombin with an increase in permeability (Garcia et al., 1986; Minnear et al., 1989; DeMichele et al., 1990; Patterson et al., 1992). We have therefore used thrombin as our primary stimulus, though we have also investigated other stimuli. To investigate β-adrenoceptors, we have examined responses to isoprenaline, a non-selective β-adrenoceptor agonist (Ball et al., 1991), RO-363 ((1-[3′,4′-dihydroxyphenoxy]-2-hydroxy-[3″,4″-dimethoxyphenethylamino]-propane)oxalate), a selective β_1-adrenoceptor agonist (McPherson et al., 1984), salbutamol and salmeterol, selective β_2-adrenoceptor agonists (Ball et al., 1991), CL-316,243 (disodium (*R*,*R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]-propyl]-1,3-benzodioxole-2,2-dicarboxylate), a selective β_3-adrenoceptor agonist (Bloom et al., 1992), and ICI-118,551 (*erythro*-DL-1(7-methylindan-4-yloxy)3-isopropylaminobutan-2-ol), a selective, competitive, β_2-adrenoceptor antagonist (O'Donnell and Wanstall, 1980), and have drawn some conclusions regarding the nature of the β-adrenoceptor mediating changes in endothelial permeability.

2. Materials and methods

2.1. Cells

Bovine pulmonary artery endothelial cells (CPAE) were obtained from the European Collection of Animal Cell Cultures (Salisbury, catalogued as CPAE; alternatively catalogued by the American Type Culture Collection, Rockville, USA, as CCL-209). This cell line has been shown to be endothelial in origin by angiotensin-converting enzyme activity, factor VIII-related antigen and the presence of Weibel-Palade bodies (Del Vecchio and Lincoln, 1983). Passages 28–34 were used for this study.

2.2. Measurement of Evans blue-labelled albumin clearance across endothelial monolayers

CPAE were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 17% foetal calf serum. Cells were cultured in 75 cm^2 tissue culture flasks until confluent. The cells were detached from the flask with trypsin/EDTA and resuspended in 12 ml DMEM/foetal calf serum. Cell suspension was seeded in plates of polycarbonate filter assemblies (Costar Transwell, 12 assemblies per plate, 1 cm^2 filter, 0.4 μm pore size, pre-soaked in DMEM/foetal calf serum for at least 1 h, 0.5 ml cell suspension per filter), and cultured for 7 days (medium replaced every 1–3 days). The filter assemblies consisted of an inner, *luminal*, chamber and an outer *abluminal*, chamber, separated by the filter.

The filter assemblies were rinsed with phosphate-buffered saline (PBS). Bathing medium (DMEM containing 25 mM Hepes, 1.2 mM $NaHCO_3$, 40 $mg \cdot ml^{-1}$ bovine serum albumin, 0.001% silicone anti-foaming agent, but without foetal calf serum or phenol red, pH adjusted to 7.4, 37°C, pre-gassed with air) was added to the luminal chamber (0.5 ml bathing medium added) and the abluminal chamber (1.4 ml bathing medium added). Evans blue (50 μl, 10 $mg \cdot ml^{-1}$ in bathing medium) was added to the luminal chamber and was shown to be > 99.9% albumin-bound as measured by acid precipitation. Under the conditions of the experiment, there were no hydrostatic or oncotic pressure gradients. Samples (50 μl) were removed from the luminal chambers and diluted 1:10 for absorbance measurement. The plate was placed in a water bath (37°C, exposed to air) and left for 20 min (*equilibration* period). The plate was gently swirled and samples (100

μl) removed from the abluminal chambers; this volume was replaced with fresh bathing medium. The plate was left for 1 h (*basal* period) before samples (100 μl) were again removed from the abluminal chambers and replaced with fresh bathing medium. Drug (or vehicle) was then added to the luminal chamber. Antagonists, where investigated, were added to both luminal and abluminal chambers 30 min prior to addition of the other drugs. The plate was left for 1 h (*treatment* period) before samples (100 μl) were removed from the abluminal chambers. Absorbance (600 nm) of the samples was measured in a 96-well plate reader (Molecular Devices). The absorbance of bathing medium was subtracted from all readings, which were then corrected for dilution and replacement of medium. The Evans blue-labelled albumin (Evans blue-albumin) clearance rate (theoretical volume of the luminal chamber cleared of Evans blue-albumin per unit time) was calculated for the basal and treatment periods as described below:

$$\text{Clearance } (\mu\text{l/h}) = \frac{\text{Abluminal volume } (\mu\text{l}) \times \text{Increase in abluminal absorbance}}{\text{Luminal absorbance} \times \text{Time (h)}}$$

The difference in Evans blue-albumin clearance rates between basal and treatment periods was calculated. Positive numbers indicate an increase in Evans blue-albumin clearance.

2.3. Chemicals

Hepes (1 M, cell culture grade), bovine serum albumin, thrombin (bovine plasma), (±)-isoprenaline hemisulphate, lipopolysaccharide and sodium nitroprusside were obtained from Sigma. Salbutamol, salmeterol, CL-316,243, RO-363 and ICI-118,551 were synthesised in-house. U46619 (9,11-dideoxy-11α,9α-epoxymethano prostaglandin $F_{2\alpha}$) was obtained from Upjohn. Trypsin/EDTA (1 × solution), DMEM, foetal calf serum and PBS were obtained from Gibco.

Isoprenaline was dissolved and diluted in bathing medium containing 100 μM ascorbic acid. Salbutamol, CL-316,243, RO-363, ICI-118,551, sodium nitroprusside, lipopolysaccharide and U46619 were dissolved and diluted in bathing medium. Salmeterol was dissolved at 1 M in dimethylacetamide, and diluted in bathing medium. None of the vehicles had a significant effect on Evans blue-albumin clearance.

2.4. Statistical analysis

All data are expressed as mean ± standard error of the mean (S.E.M.) unless otherwise stated. n represents the number of individual experiments. Data were tested for normalcy and homogeneity of variance. If these criteria were met, then statistical significance was tested for using either Student's t-test, or analysis of variance (followed by Dunnett's test or Duncan's test) as appropriate, otherwise Kruskal-Wallace non-parametric statistics were used. A probability (P) of 0.05 or less was considered significant. EC_{50} values were calculated by logarithmic interpolation of concentration-response data. Individual EC_{50} values were meaned geometrically and are expressed with 95% confidence intervals.

pK_B was calculated according to the following equation (Kenakin, 1987):

$$pK_B = \log_{10}(\text{concentration ratio} - 1) - \log_{10}(\text{antagonist molar concentration})$$

3. Results

3.1. Effect of cells on Evans blue-albumin clearance across filters

Evans blue-albumin clearance was measured across cell-free filters after incubation in DMEM/foetal calf serum for 24 h, and a value of 72.3 $\mu\text{l} \cdot \text{h}^{-1}$ ($\sigma_{n-1} = 9.8$ $\mu\text{l} \cdot \text{h}^{-1}$, $n = 24$) was obtained. In contrast, basal clearance across CPAE-coated filters was 11.4 $\mu\text{l} \cdot \text{h}^{-1}$ ($\sigma_{n-1} = 5.0$ $\mu\text{l} \cdot \text{h}^{-1}$, $n = 422$). Hence the CPAE monolayer markedly restricts the passage of Evans blue-albumin across the filter.

3.2. Effect of thrombin, lipopolysaccharide, U46619, hydrogen peroxide and sodium nitroprusside on Evans blue-albumin clearance across endothelial monolayers

We investigated a range of mediators that could possibly increase Evans blue-albumin clearance across endothelium. Thrombin (10 $\text{U} \cdot \mu\text{l}^{-1}$) and hydrogen peroxide (1 mM) both caused a significant increase in Evans blue-albumin clearance (Fig. 1a). However, in contrast, lipopolysaccharide (50 mg/μl), the thromboxane mimetic, U46619 (1 μM), and sodium nitroprusside (10 μM) all failed to affect Evans blue-albumin clearance across endothelial monolayers. As high concentrations of hydrogen peroxide could affect the absorbance of Evans blue, luminal absorbance was also monitored throughout the experiment. Hydrogen peroxide (1 mM) had no significant effect on luminal absorbance.

The effect of thrombin was further investigated; thrombin (0.1–100 $\text{U} \cdot \text{ml}^{-1}$) caused a concentration-dependent increase in Evans blue-albumin clearance across the endothelial monolayers (Fig. 1b). At 100 $\text{U} \cdot \text{ml}^{-1}$, thrombin increased Evans blue-albumin clearance by 8.0 $\mu\text{l} \cdot \text{h}^{-1}$ over basal clearance (an increase of 0.6 $\mu\text{l} \cdot \text{h}^{-1}$ was observed in unstimulated

monolayers). 10 U · ml^{-1} thrombin caused a clear, yet sub-maximal, response, and was therefore chosen as the concentration against which the β-adrenoceptor agonists would be tested.

3.3. Effect of β-adrenoceptor agonists on thrombin-stimulated Evans blue-albumin clearance

β-Adrenoceptor agonists were tested for inhibitory activity against thrombin (10 U · ml^{-1})-stimulated Evans blue-albumin clearance. Isoprenaline, up to 100 nM, caused apparently concentration-related inhibition (EC_{50} 21 nM, 95% c.i. 7.4–59 nM, $n = 5$), but increasing the concentration to 1 μM caused no greater inhibition (maximum 64% inhibition, Fig. 2a). Salbutamol (10 nM–1 μM) caused a concentration-related inhibition of thrombin-stimulated Evans blue-albumin clearance (Fig. 2b) with an EC_{50} of 62 nM (95% c.i. 23–162 nM, $n = 4$). At 1 μM, salbutamol reduced clearance to below control levels, and there was no greater reduction in Evans blue-albumin clearance at 10 μM salbutamol. Salmeterol (10 nM–1 μM) caused a concentration-related inhibition of thrombin-stimulated Evans blue-albumin clearance (Fig. 2c) with an EC_{50} of 2.7 nM (95% c.i. 0.50–14 nM, $n = 6$). At 1 μM, salmeterol reduced clearance to below control levels, and there was no greater reduction in Evans blue-albumin clearance at 10 μM.

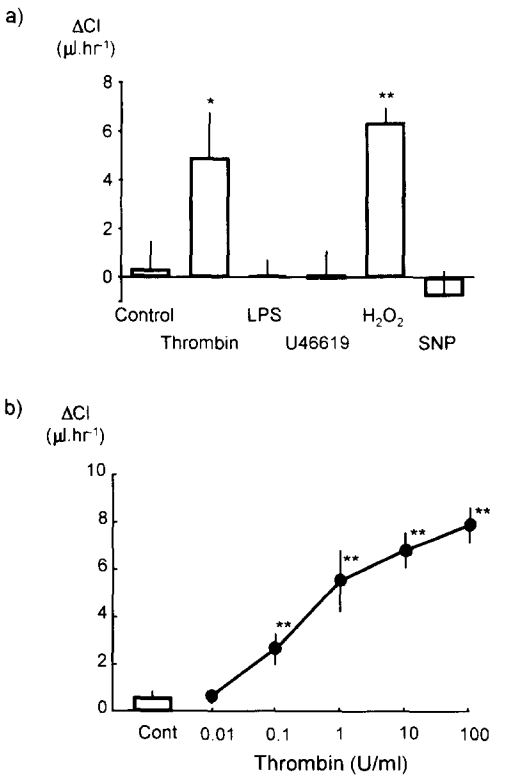

Fig. 1. (a) The effect of thrombin (10 U·ml^{-1}), lipopolysaccharide (LPS, 50 μg·ml^{-1}), U46619 (1 μM), hydrogen peroxide (H_2O_2, 1 mM) and sodium nitroprusside (SNP, 10 μM) on Evans blue-albumin clearance across CPAE monolayers. (b) The concentration-response relationship of the effect of thrombin on albumin clearance. Ordinate shows change in clearance rate above basal clearance (μl·h^{-1}). Data are represented as mean ± S.E.M. ($n = 5$–8). * $P < 0.05$, ** $P < 0.01$, denotes significant differences from the vehicle control (1a: Dunnett, 1b: Kruskal-Wallace).

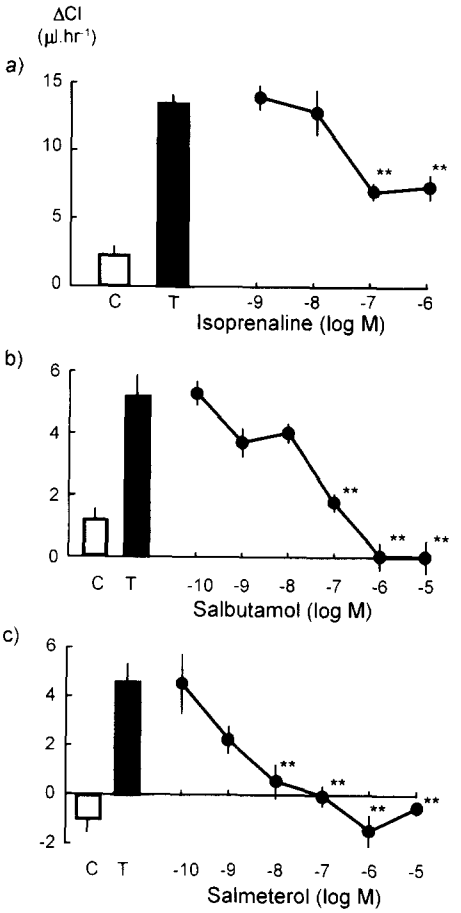

Fig. 2. The inhibitory concentration-effect curves for (a) isoprenaline, (b) salbutamol or, (c) salmeterol against thrombin (10 U·ml^{-1})-stimulated Evans blue-albumin clearance across CPAE monolayers. The ordinate shows change in clearance rate above basal clearance (μl·h^{-1}). The open and solid columns (C and T) show unstimulated and thrombin-stimulated controls respectively. The graph shows clearance stimulated by thrombin in the presence of isoprenaline, salbutamol or salmeterol (log M). Data are represented as mean ± S.E.M. ($n = 4$–6). ** $P < 0.01$, denotes significant difference from the thrombin-stimulated control (Dunnett).

Neither the β_1-adrenoceptor agonist, RO-363 (1 nM–10 μM), nor the β_3-adrenoceptor agonist, CL-316,243 (0.1 nM–10 μM), significantly affected thrombin-stimulated Evans blue-albumin clearance across CPAE monolayers (Fig. 3).

As the maximal inhibition obtained with isoprenaline, salbutamol and salmeterol appeared to differ markedly, the effects of a maximally-effective concentration of each agent (1 μM) on thrombin (10 U · ml^{-1})-stimulated Evans blue-albumin clearance were compared within a single series of experiments (all

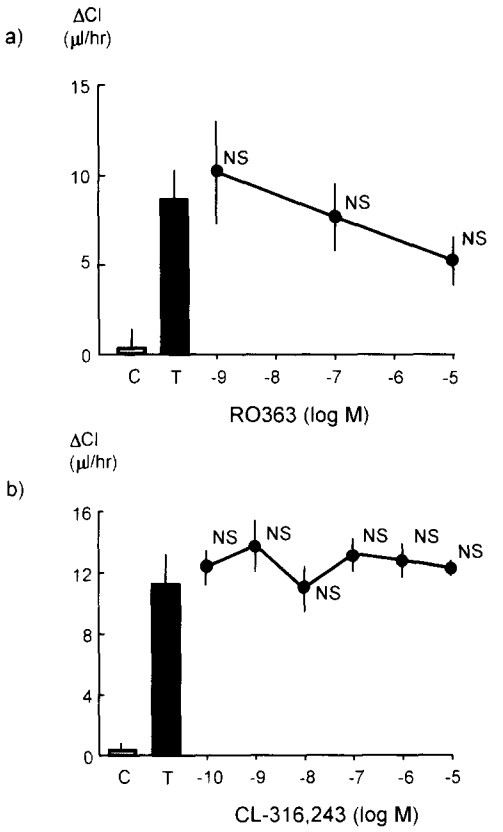

Fig. 3. The effects of (a) RO-363 and (b) CL-316,243 on thrombin (10 U·ml^{-1})-stimulated Evans blue-albumin clearance across CPAE monolayers. The ordinate shows change in clearance rate above basal clearance (μl·h^{-1}). The open and solid columns (C and T) show unstimulated and thrombin-stimulated controls respectively. The graphs show clearance stimulated by thrombin in the presence of RO-363 or CL-316,243 (log M). Data are represented as mean ± S.E.M. ($n = 6$). NS, denotes no significant difference ($P > 0.05$) from the thrombin-stimulated control (Dunnett).

drugs compared on the same plate). In these experiments, isoprenaline, salbutamol and salmeterol caused 49%, 81% and 72% inhibition of the response to

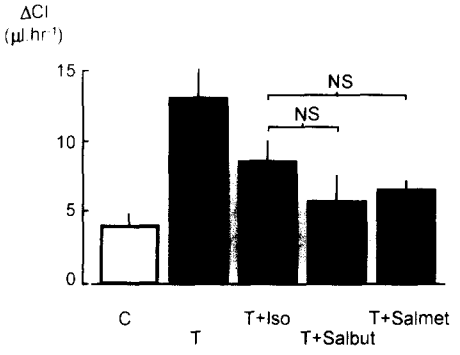

Fig. 4. A comparison of maximally effective concentrations (1 μM) of isoprenaline, salbutamol and salmeterol against thrombin (10 U·ml^{-1})-stimulated Evans blue-albumin clearance across CPAE monolayers. The open column (C) shows unstimulated control. The solid columns (T) show change in clearance stimulated with thrombin. NS, denotes no significant difference ($P > 0.05$) from the isoprenaline-treated monolayers (Duncan).

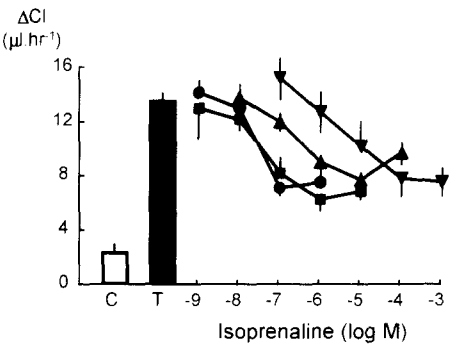

Fig. 5. Inhibitory effect of isoprenaline on thrombin (10 U·ml^{-1})-stimulated Evans blue-albumin clearance across CPAE monolayers in the presence and absence of ICI-118,551. The ordinate shows change in clearance rate above basal clearance (μl·h^{-1}). The open and solid columns (C and T) show unstimulated and thrombin-stimulated controls respectively. The graphs show change in clearance stimulated by thrombin in the presence of isoprenaline (●) alone, and in the presence of (■) 0.01 μM ICI-118,551, (▲) 0.1 μM ICI-118,551 and (▼) 1 μM ICI-118,551. Data are represented as mean ± S.E.M. ($n = 5$).

thrombin respectively (Fig. 4). However, there were no significant differences between the changes in clearance rates induced by these three β-adrenoceptor agonists.

3.4. Effect of ICI-118,551 on the inhibition of thrombin-stimulated Evans blue-albumin clearance by isoprenaline

ICI-118,551 (0.1 and 1 μM) antagonised the response to isoprenaline in a concentration-related manner (Fig. 5), causing a rightward shift of the concentration-response curve, with no significant change in the maximal response to isoprenaline. The pK_B for ICI-118,551 was 8.40 (95% c.i. 7.87–8.93). There was no significant difference in pK_B values calculated at 0.1 μM or 1 μM ICI-118,551. ICI-118,551 (1 μM) alone had no significant effect on either basal or thrombin (10 U·ml^{-1})-stimulated Evans blue-albumin clearance (control change in clearance = -0.6 ± 0.3 μl·h^{-1},

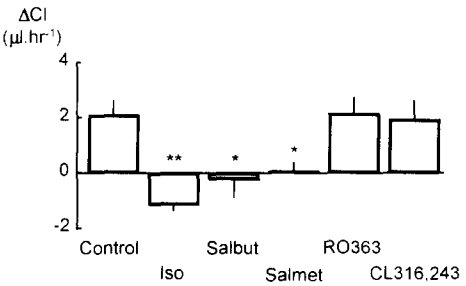

Fig. 6. The effect of β-adrenoceptor agonists (1 μM) on Evans blue-albumin clearance across CPAE monolayers in the absence of thrombin. The ordinate shows change in clearance rate above basal clearance (μl·h^{-1}). * $P < 0.05$, ** $P < 0.01$, denotes significant difference from the control (Dunnett).

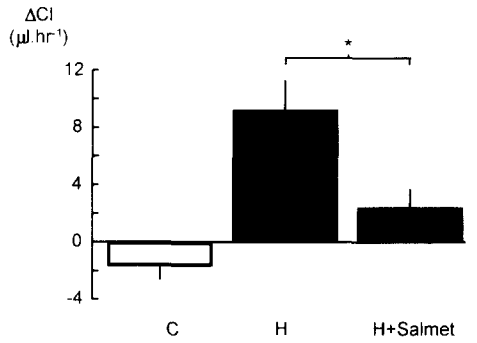

Fig. 7. The effect of salmeterol (1 μM) on hydrogen peroxide (H_2O_2,1 mM)-stimulated Evans blue-albumin clearance across CPAE monolayers. The ordinate shows change in clearance rate above basal clearance ($\mu l \cdot h^{-1}$). The open column (C) shows unstimulated control. The solid columns (H) show change in clearance stimulated with hydrogen peroxide. * $P < 0.05$, denotes significant difference from the hydrogen peroxide-stimulated control (Student t).

ICI-118,551 = 0.2 ± 0.6 $\mu l \cdot h^{-1}$, thrombin = 10.2 ± 1.7 $\mu l \cdot h^{-1}$, thrombin + ICI-118,551 = 10.1 ± 0.8 $\mu l \cdot h^{-1}$, $n = 6$).

3.5. Effect of β-adrenoceptor agonists on unstimulated Evans blue-albumin clearance

We investigated whether any of the β-adrenoceptor agonists were capable of increasing Evans blue-albumin clearance, by measuring their effects in the absence of thrombin. Isoprenaline (1 μM), salbutamol (1 μM) and salmeterol (1 μM) all caused small, but significant ($P < 0.05$), reductions in the Evans blue-albumin clearance rate in the absence of thrombin. In contrast, neither RO-363 (1 μM) nor CL-316,243 (1 μM) had any effect on unstimulated Evans blue-albumin clearance (Fig. 6).

3.6. Effect of salmeterol on hydrogen peroxide-stimulated albumin clearance

To investigate whether the action of salmeterol was specific to thrombin, we examined the effect of salmeterol (1 μM) against hydrogen peroxide (1 μM). Salmeterol significantly inhibited the increase in Evans blue-albumin clearance stimulated by hydrogen peroxide (Fig. 7).

4. Discussion

In this study, we have investigated endothelial albumin permeability in vitro in the absence of complicating factors such as haemodynamic effects and the involvement of cellular inflammatory processes, and in the absence of osmotic, oncotic or hydrostatic pressure gradients. In agreement with previous reports, thrombin increased the permeability of CPAE monolayers to albumin. The action of thrombin is dependent on inter-endothelial cell gap formation without actual loss of the endothelial cells (Laposata et al., 1983). Thrombin's action appears to be receptor-mediated, as its effect may be mimicked by thrombin receptor activating peptide (Minnear et al., 1993). In addition to changes in endothelial permeability being involved in atherosclerosis (Ross, 1986), thrombin-induced changes in endothelial permeability have been associated with inflammatory reactions involving increased vascular leakage (Malik and Fenton, 1992). Thrombin, therefore, provides a good stimulus against which permeability-reducing agents may be tested. Ideally, other mediators such as bradykinin and substance P would also have been investigated; however, we have previously shown that CPAE monolayers do not respond to these mediators (Allen and Cox, 1993). The lack of response to some of the *classical* inflammatory mediators may be due to kinetics; endothelial gap formation in vivo in response to histamine, for example, is transient (Wu and Baldwin, 1992) and could possibly be missed when measuring albumin clearance over a 1 h period. Quantification of transient changes in endothelial permeability in vitro is difficult, as changes in absorbance of the abluminal fluid over short periods cannot be measured exactly. We did, however, also observe an increase in permeability induced by hydrogen peroxide (see below), an inflammatory mediator released from neutrophils which is known to cause disruption of the endothelium (Weiss et al., 1981). The failure to show an effect of lipopolysaccharide may, again, be due to the time-course of our experiments as Berman et al. (1993) showed that lipopolysaccharide increased albumin permeability of bovine aortic endothelial cells in vitro, but only after at least 4 h exposure. The lack of response to classical inflammatory mediators could, however, possibly reflect a true distinction in endothelial response between the pulmonary vascular bed and other vascular beds.

There have been previous reports on β-adrenoceptor mediated reduction in endothelial permeability, but most have only used high concentrations of agonist. Langeler and Van Hinsbergh (1991) reported that isoprenaline (10 μM) reduced the basal passage of horseradish peroxidase across monolayers of human umbilical vein endothelial cells, an effect reversed by propranolol (10 μM). Minnear et al. (1989) demonstrated that isoprenaline (2 μM) reduced both basal and thrombin-stimulated albumin clearance across bovine pulmonary artery endothelial cells, an effect also inhibited by propranolol (20 μM). Similar results were obtained by Gudgeon and Martin (1989), who demonstrated that isoprenaline (20 μM) reduced phorbol ester-stimulated albumin clearance across pig aortic endothelial monolayers, an effect reversed by pro-

pranolol (20 μM). Using these high concentrations of isoprenaline, both Minnear et al. (1989) and Gudgeon and Martin (1989) showed a similar degree of inhibition of mediator-stimulated albumin clearance as the maximal inhibition by isoprenaline observed in the present study. Minnear et al. (1993) also found that the β_2-adrenoceptor agonist, terbutaline, reduced endothelial permeability, though this was only tested at 1 μM. Minnear et al. (1993) showed that ICI 118,551, but not the β_1-antagonist ICI 89406, antagonised the effect of isoprenaline, and therefore suggested that this provided evidence for the involvement of β_2-adrenoceptors. However, the antagonists were used at 10 μM, where selectivity may not be assured. In radioligand binding studies, Minnear et al. (1993) showed that the majority of the receptors on bovine pulmonary artery endothelial cells were of the β_2 subtype, though there was a small population of β_1-adrenoceptors (11% of total β-adrenoceptors). Though we have produced clear evidence that it is the β_2-subtype that mediates a reduction of endothelial permeability, we found no evidence that β_1-adrenoceptors modulate the permeability of the endothelium.

Zink et al. (1993) are the only workers to have previously reported concentration-response relationships for β-adrenoceptor agonists. Our work differs from that previous report in several important aspects: We have shown that agonists at β_2-adrenoceptors are capable of reducing not only basal permeability, but also of antagonising the increased permeability induced by agents such as thrombin. Zink et al. found that, while formoterol had the expected potency consistent with effects at β_2-adrenoceptors, isoprenaline had only weak potency. In our study, we found that the relative potencies of isoprenaline, salbutamol and salmeterol are as expected for an action through β_2-adrenoceptors. Additionally, though Zink et al. suggested that, because formoterol reduced endothelial permeability, it is the β_2-adrenoceptors that mediate the reduction in permeability, they did not rule out additional involvement of either β_1 or β_3 receptors.

We have extended previous observations by demonstrating that isoprenaline and the selective β_2-adrenoceptor agonists, salbutamol and salmeterol, can inhibit thrombin-stimulated Evans blue-albumin clearance even at sub-micromolar concentrations. The potency of isoprenaline is consistent with an action through either β_1-, β_2- or β_3-adrenoceptors. The high potencies of salbutamol and salmeterol are consistent with effects at β_2-adrenoceptors. Salmeterol was the most potent of the β-adrenoceptor agonists tested.

Isoprenaline, salbutamol and salmeterol all caused small, but significant, reductions in the permeability of unstimulated monolayers, suggesting that there is a degree of permeability *tone* in this system that may be reversed by β-adrenoceptor agonists. This is consistent with the findings of Minnear et al. (1989) and Langeler and Van Hinsbergh (1991). However, the reduction in permeability in the absence of any permeability-enhancing agent was smaller than that observed when permeability had been enhanced by thrombin.

RO-363 is a potent and selective β_1-adrenoceptor agonist (McPherson et al., 1984) and CL-316,243 is a potent and selective β_3-adrenoceptor agonist (Bloom et al., 1992). However, neither RO-363 nor CL-316,243 had any effect on thrombin-stimulated Evans blue-albumin clearance, suggesting that neither β_1- nor β_3-adrenoceptors reduce the permeability of bovine pulmonary artery endothelium. Additionally, neither agent affected permeability in the absence of thrombin, suggesting neither β_1- nor β_3-adrenoceptors cause any increase in permeability of this endothelium.

The rank order of agonist potencies: salmeterol > isoprenaline > salbutamol $\gg$ RO-363 = CL316,243, is clearly consistent with a key role for β_2-adrenoceptors in the reduction of endothelial permeability by these agonists.

Further support for the involvement of β_2-adrenoceptors was provided by ICI-118,551, a potent and selective antagonist. ICI-118,551 antagonised the inhibitory effect of isoprenaline on thrombin-induced Evans blue-albumin clearance, with a pK_B consistent with isoprenaline acting exclusively at β_2-adrenoceptors. Testing a selective antagonist against a non-selective agonist should reveal if there is more than one receptor subtype involved; we saw no evidence to suggest the involvement of any subtype other than the β_2-adrenoceptor. These observations extend those of Minnear et al. (1993), and demonstrate that ICI-118,551 antagonises the effect of isoprenaline with a potency indicative of action at β_2-adrenoceptors.

In individual series of experiments, salbutamol and salmeterol appeared to produce a greater degree of inhibition than isoprenaline. However, when compared within a single series of experiments, we observed no significant difference in the degree of inhibition. The initial difference observed in the individual series of experiments may be due to the difference in the magnitude of the response to thrombin between experimental series – a variable excluded when comparing the agonists within a single series of experiments.

We investigated whether agents other than thrombin could increase permeability of CPAE monolayers. Hydrogen peroxide, which increases permeability of porcine pulmonary artery endothelial monolayers (Suttorp et al., 1993), increased permeability to a similar degree to that by thrombin. However, U46619, lipopolysaccharide and sodium nitroprusside were all without an affect on permeability. Salmeterol inhibited the response to hydrogen peroxide, showing that its effects are not specific to thrombin-stimulated increases in endothelial permeability.

Studies have shown that selective β_2-adrenoceptor agonists may reduce plasma protein extravasation in vivo (Svensjö et al., 1977; Whelan and Johnson, 1992). As described earlier, the mechanisms underlying in vivo observations are difficult to interpret. Our results suggest that, in the bovine pulmonary circulation, the β_2-adrenoceptor subtype alone directly modulates endothelial permeability, with no apparent role for either the β_1- or β_3-adrenoceptors. It remains to be seen whether this also applies to human vascular endothelial permeability. One caveat that must be applied to experiments performed on endothelial permeability is that they are generally performed on endothelial cells from large vessels, due to the difficulty in the isolation and culture of sufficient quantities of endothelial cells from post-capillary venules. While this is not a problem in modelling permeability of large vessels, there is not yet sufficient evidence to indicate how far these results can be extrapolated to the post-capillary venule.

In summary, these results clearly demonstrate that activation of β_2-adrenoceptors, but not β_1- or β_3-adrenoceptors, directly reduces the permeability of bovine pulmonary artery endothelial monolayers. Submicromolar concentrations of isoprenaline, salbutamol and salmeterol are capable of significantly attenuating thrombin-induced increases in endothelial Evans blue-albumin permeability.

References

Adamski, S.W., J.L. Langone and G.J. Grega, 1987, Modulation of macromolecular permeability by immune complexes and a β-adrenoceptor stimulant, Am. J. Physiol. 253, H1586.

Allen, M.J. and J.C. Cox, 1993, Clearance across cultured endothelial monolayers: an in vitro model of plasma protein extravasation?, Neuropeptides 24, 247.

Ball, D.I., R.T. Brittain, R.A. Coleman, L.H. Denyer, D. Jack, M. Johnson, L.H.C. Lunts, A.T. Nials, K.E. Sheldrick and I.F. Skidmore, 1991, Salmeterol, a novel, long-acting β_2-adrenoceptor agonist: characterisation of pharmacological activity in vitro and in vivo, Br. J. Pharmacol. 104, 665.

Berman, R.S., J.D Frew and W. Martin, 1993. Endotoxin-induced arterial endothelial barrier dysfunction assessed by an in vitro model, Br. J. Pharmacol. 110, 1282.

Bloom, J.D., M.D. Dutia, B.D. Johnson, A. Wissner, M.G. Burns, E.E. Largis, J.A. Dolan and T.H. Claus, 1992. Disodium (*R,R*)-5-[2-[[2-(3-chlorophenyl)-2-hydroxyethyl]-amino]propyl]-1,3-benzodioxole-2,2-dicarboxylate (CL 316,243). A potent β-adrenergic agonists virtually specific for β_3 receptors. A promising antidiabetic and antiobesity agent, J. Med. Chem. 35, 3081.

Butchers, P.R., I.F. Skidmore, C.J. Bardey and A. Wheeldon, 1980, Characterisation of the receptor mediating the anti-anaphylactic effects of β-adrenoceptor agonists in human lung tissue in vitro, Br. J. Pharamcol. 71, 663.

Cooper, J.A., P.J. Del Vecchio, F.L. Minnear, K.E. Burhop, W.M. Selig, J.G.N. Garcia and A.B. Malik, 1987, Measurement of albumin permeability across endothelial monolayers in vitro, J. Appl. Physiol. 62, 1076.

Del Vecchio, P.J. and D.W. Lincoln, 1983, Phenotypes of cultured endothelium expression of angiotensin-converting enzyme activity, in: The Endothelial Cell: A Pluripotent Control Cell of the Vessel Wall, eds. D.G.S. Thilo-Korner and R.I. Freshney (Karger, Basel) p. 673.

DeMichele, M.A.A. and F.L. Minnear, 1992, Modulation of vascular endothelial permeability by thrombin, Semin. Thromb. Hemost. 18, 287.

DeMichele, M.A.A., D.G. Moon, J.W. Fenton and F.L. Minnear, 1990, Thrombin's enzymatic activity increases permeability of endothelial cell monolayers, J. Appl. Physiol. 69, 1599.

Garcia, J.G., A. Solfinger-Birnboim, R. Bizios, P.J. Del Vecchio, J.W. Fenton and A.B. Malik, 1986, Thrombin-induced increase in albumin permeability across the endothelium, J. Cell. Physiol. 128, 96.

Gudgeon, J.R. and W. Martin, 1989, Modulation of arterial endothelial permeability: studies on an in vitro model, Br. J. Pharamcol. 98, 1267.

Kenakin, T.P., 1987, Pharmacologic Analysis of Drug-Receptor Interaction (Raven Press, New York).

Langeler, E.G. and W.M. Van Hinsbergh, 1991, Norepinephrine and iloprost improve barrier function of human endothelial cell monolayers: role of cAMP, Am. J. Physiol. 260, C1052.

Laposata, M.L., D.K. Dovnarsky and H.S. Shin, 1983, Thrombin-induced gap formation in confluent endothelial cell monolayers in vitro, Blood 62, 549.

Majno, G. and G.E. Palade, 1961, Studies on inflammation. I: Effect of histamine and serotonin on vascular permeability: an electron-microscopic study, J. Biophys. Biochem. Cytol. 11, 571.

Malik, A.B. and J.W. Fenton, 1992, Thrombin-mediated increase in vascular endothelial permeability, Semin. Thromb. Hemost. 18, 193.

McPherson, G.A., E. Malta, P. Molenaar and C. Raper, 1984, The affinity and efficacy of the selective β_1-adrenoceptor stimulant RO-363 at β_1- and β_2-adrenoceptor sites, Br. J. Pharmacol. 82, 897.

Minnear, F.L., M.A.A. DeMichele, D.G. Moon, C.L. Reider and J.W. Fenton, 1989, Isoproterenol reduces thrombin-induced pulmonary endothelial permeability in vitro, Am. J. Physiol. 257, H1613.

Minnear, F.L., M.A.A. DeMichelle, S. Leonhardt, T.T. Anderson and M. Teitler, 1993, Isoprenaline antagonises endothelial permeability induced by thrombin and thrombin receptor peptide, J. Appl. Physiol. 75, 1171.

Mizus, I., W. Summer, I. Farrukh, J.R. Michael and G.H. Gutner, 1985, Isoproteronol or aminophylline attenuate pulmonary edema after acid lung injury, Am. Rev. Respir. Dis. 131, 256.

O'Donnell, S.R. and J.C. Wanstall, 1980, Evidence that ICI 118,551 is a potent, highly β_2-selective adrenoceptor antagonist and can be used to characterise β adrenoceptor populations in tissues, Life Sci. 27, 671.

Patterson, C.E., R.A. Rhoades and J.G.N. Garcia, 1992, Evans blue dye as a marker of albumin clearance in cultured endothelial monolayer and isolated lung, J. Appl. Physiol. 72, 865.

Persson, C.G.A., 1993, The action of β-receptors on microvascular endothelium or: is airways plasma exudation inhibited by β-agonists?, Life Sci. 52, 2111.

Ross, R., 1986, The pathogenesis of atherosclerosis – an update, New Engl. J. Med. 314, 488.

Suttorp, N., U. Weber, U. Welsch and C. Schudt, 1993, Role of phosphodiesterase in the regulation of endothelial permeability in vitro, J. Clin. Invest. 91, 1421.

Svenjö, E., C.G.A. Persson and G. Rutili, 1977, Inhibition of bradykinin-induced macromolecular leakage from post-capillary venules by a β_2-receptor stimulant, terbutaline, Acta Physiol. Scand. 101, 504.

Wedmore, C.V. and T.J. Williams, 1981, Control of vascular permeability by polymorphonuclear leukocytes in inflammation, Nature 289, 646.

Weiss, S.J., J. Young, A.F. LoBuglio, A. Slivka and N.F. Nimeh,

1981, Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells, J. Clin. Invest. 68, 714.

Whelan, C.J. and M. Johnson, 1992, Inhibition by salmeterol of increased vascular permeability and granulocyte accumulation in guinea-pig lung and skin, Br. J. Pharamcol. 105, 831.

Williams, T.J. and M.J. Peck, 1977, Role of prostaglandin-mediated vasodilation in inflammation, Nature 270, 530.

Wu, N.Z. and A.L. Baldwin, 1992, Transient venular permeability increase and endothelial gap formation induced by histamine, Am. J. Physiol. 262, H1238.

Zink, S., P. Rösen, B. Sackmann and H. Lemoine, 1993, Regulation of endothelial permeability by β-adrenoceptor agonists: contribution of β_1- and β_2-adrenoceptors, Biochim. Biophys. Acta 1178, 286.