Interference of WEB 2086 and BN 52021 with Paf-induced effects on guinea-pig trachea

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- 1 The thienotriazolodiazepine WEB 2086 and the ginkgolide BN52021 have been evaluated as antagonists of Paf-acether (Paf) by studying their effects on Paf-induced relaxation and Paf-induced prostaglandin E₂ (PGE₂) production in histamine-contracted guinea-pig tracheal preparations.
- 2 Relaxation induced by Paf 4 µM in histamine-contracted guinea-pig tracheal preparations was 39.67 ± 3.5% (n = 30). At the same concentration, Paf significantly increased PGE₂ production from histamine-contracted guinea-pig tracheal preparations.
- 3 WEB 2086 inhibited in a dose-related manner (IC₅₀ = 21.2 nM) the relaxant effect induced by Paf and, at 1 µM, suppressed Paf-induced release of PGE₂.
- 4 BN 52021 100 µM inhibited to about 60% Paf-induced relaxation of histamine-contracted guinea-pig tracheal preparations, but completely abolished Paf-induced increase in PGE₂.
- 5 Both antagonists had no effects on relaxations induced by arachidonic acid 10 µM or PGE₂ 0.1–1 µM in histamine-contracted guinea-pig tracheal preparations.
- 6 The results are consistent with the presence of specific Paf receptors in guinea-pig trachea and indicate that a relaxant prostanoid, namely PGE₂, at least partially mediates Paf-induced relaxation in this experimental model.

Introduction

Paf-acether (Paf, formerly platelet-activating factor; Benveniste *et al.*, 1972), identified as being 1-O-hexa/octadecyl-2-acetyl-sn-3-glycero-phosphocholine, is a potent mediator of inflammation, which induces bronchoconstriction and airway hyperreactivity in several animal species including man (Chung *et al.*, 1986; Cuss *et al.*, 1986; Anderson *et al.*, 1988). However, *in vitro*, this phosphoetherlipid does not contract bronchial and tracheal preparations from different species (Cerrina *et al.*, 1983; Stimler & O'Flaherty, 1983) and paradoxically relaxes resting guinea-pig tracheal preparations (Prancan *et al.*, 1982). We previously demonstrated that Paf relaxed, in a dose-dependent manner, guinea-pig tracheal preparations in which tone was induced by maximal effective concentrations of histamine. This effect was

observed only in the presence of an intact epithelium and was largely dependent on the production of immunoreactive relaxant prostanoids, such as prostaglandin E₂ (PGE₂, Brunelleschi *et al.*, 1987).

The presence of specific Paf receptors was previously documented in rat trachea and in human and guinea-pig lung tissue homogenates, but, as the lungs contain more than 40 different cell types, these receptors remain to be localized precisely (Hwang *et al.*, 1983; 1985). The development of specific Paf receptor antagonists has largely contributed to a better definition of the role of this mediator in several pathophysiological conditions. Among the compounds available, BN 52021 (ginkgolide B; a component purified from *Ginkgo biloba* leaves) and the thienotriazolodiazepine WEB 2086, are two chemically unrelated antagonists, whose ability to inhibit competitively Paf-induced effects is well documented in different experimental models (Casals-Stenzel *et al.*, 1987; Braquet *et al.*, 1987). We

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have therefore evaluated the effects of BN 52021 and WEB 2086 on Paf-induced relaxation of guinea-pig tracheal preparations precontracted with histamine.

A preliminary account of this work was presented at the 1st Joint Meeting of the Hungarian and Italian Pharmacological Societies at Verona, Italy (Brunelleschi *et al.*, 1988).

Methods

Preparation of tissues

Adult male guinea-pigs (300–500 g) were killed by a blow to the head and exsanguinated. The trachea was removed, carefully cleaned of adherent fat and connective tissue, cut as a spiral and divided into two paired portions. Each tracheal segment was placed in a 10 ml water jacketed organ bath at 37°C containing Tyrode solution (composition in mM: NaCl 139.2, KCl 2.7, CaCl₂ 1.8, MgCl₂ 0.49, NaH₂PO₄ 0.4 and glucose 5.5; pH 7.4) gassed with 95% O₂ and 5% CO₂.

Tissues were allowed to equilibrate for 90 min in Tyrode solution under a load of 2.5–3 g. This high initial load ensured optimal length of preparations and maximal and reproducible contractile responses. The length of the spiral was measured at the end of the equilibration period; the wet weight of each preparation was recorded at the end of the experiments.

Experimental protocols

After a 90 min equilibration period, tracheal preparations were contracted twice with a maximal effective concentration of histamine (50 µM) and extensively washed every 10–15 min with fresh Tyrode solution. When the basal tone was re-established, tracheal tissues were stimulated once more with histamine (50 µM). When the contraction reached a plateau, a fixed concentration of Paf (4 µM) was added to the organ bath in the presence or absence of specific Paf antagonists.

A concentration-effect curve for Paf was obtained in preliminary experiments, yielding the ED₅₀ value of 0.4 µM. At this concentration, the amount of relaxation was less than 25% (data not shown); therefore, to evaluate better the effects of Paf antagonists and to avoid mistakes attributable to the small relaxant effect observed, we decided to use a higher concentration of Paf, 4 µM, which ensured about 40% relaxation (see Results).

BN 52021 and WEB 2086 were administered at the peak of histamine contraction (7 min), 3 min before the addition of the phospholipid. Preliminary experiments, in which antagonists were put into the organ bath 3–15 min before the addition of

Paf, showed no differences in the amount of relaxation observed. Hence, a 3 min contact time with antagonists was used.

Because tissues were highly tachyphylactic to Paf, each tracheal segment was treated only once with this agent, that is, one tracheal segment was challenged with Paf and its pair with the antagonist + Paf. BN 52021 and WEB 2086 did not affect by themselves the amplitude of histamine-induced contraction. The specificity of these antagonists was also evaluated in guinea-pig tracheal preparations contracted with histamine and then relaxed by arachidonic acid or PGE₂.

Determination of PGE₂ in tracheal effluents

The measurement of PGE₂ was performed on samples of bath fluid derived from preparations at resting tone and after challenge with histamine, histamine + Paf or histamine + antagonist + Paf. The following protocol was used. A bath fluid sample was collected at the end of 20 min at basal tone. Preparations were re-equilibrated for 5 min and contracted with histamine (50 µM). A second sample was obtained 15 min after the addition of histamine. Thereafter, tissues were washed until they returned to resting tone and a third sample was collected after another 20 min period. The tissues were then re-equilibrated for 5 min, challenged with histamine (7 min), Paf antagonist or vehicle (3 more min) and Paf (5 min more) and the fourth sample was obtained. The samples were immediately frozen and kept at –20°C until use; aliquots (100 µl) of the sample were assayed without extraction.

PGE₂ content in guinea-pig tracheal effluents was determined by an enzyme-immunological assay according to Pradelles *et al.* (1985) and expressed as pg mg⁻¹ tissue. The minimal detectable concentration of PGE₂ was 10 pg ml⁻¹. Basal release of PGE₂ was subtracted from the values obtained after agonist challenge.

Calculation of results

Changes in force were measured from isometric recordings, by using isometric force transducers (MARB, mod 83F) and Hewlett Packard (HP66) physiographs. The relaxation induced by Paf, in the presence or absence of a specific antagonist, was expressed as a % reversal of histamine-induced contractile response. IC₅₀ value for WEB 2086 was calculated using the Cheng-Prusoff equation: $IC_{50} = K_B(1 + A)/ED_{50}$, where A is Paf 4 µM and the K_B was estimated from Paf concentration-effect curves according to the method of Arunlakshana & Schild (1959).

Statistical analysis of the results obtained was performed by use of Student's *t* test for paired variables. A probability value of less than 0.05 was considered to be statistically significant. All results are expressed as means $\pm$ s.e.mean.

Materials

The compounds used and their sources were: histamine dihydrochloride, PGE₂, fatty acid-free bovine serum albumin (BSA), arachidonic acid (Sigma, St. Louis, MO, USA); Paf (1-octadecyl-2-O-acetyl-sn-glycero-3-phosphocholine; Bachem, Bubendorf, Switzerland).

Kits for performing enzyme-immunological assay of PGE₂ were purchased from Laboratoires des Stalergenes (Fresnes, France). BN 52021 (3-[1,1-dimethylethyl]hexahydro-1,4,7b-trihydroxy-8-methyl-9H-1,7a(epoxymethanol)-1H,6aH-cyclopenta[c]furo[2,3-b]furo[3',2':3,4]cyclopenta[1,2-d]furan-5,9,12-[4H]-trione) and WEB 2086 (3-(4-(2-chlorophenyl)-9-methyl-6H-thieno-(3,2-f)(1,2,4)-triazolo-(4,3-a)(1,4)-diazepine-2-yl)-1-(4-morpholinyl)-1-propanone) were kindly supplied by Institut Henri Beaufour (Le Plessis-Robinson, France) and by Boehringer Ingelheim Ltd. (Ingelheim am Rhein, West Germany), respectively. Paf was dissolved in Tyrode solution containing 0.25% BSA, stored as a stock solution (10 mM) at -20°C and added to the organ bath as aliquots of 100 μl , so that the BSA final concentration was 25 $\mu\text{g ml}^{-1}$. WEB 2086 was dissolved in water, BN 52021 was dissolved in dimethylsulphoxide (DMSO) 50%. Final concentration of DMSO was not more than 0.5%.

Results

Physical and mechanical characteristics of guinea-pig tracheal preparations

The length and wet weight of the guinea-pig tracheal preparations used were 20.5 ± 0.15 mm and 47.18 ± 1.5 mg, respectively ($n = 70$). Histamine (50 μM)-induced contraction was 0.93 ± 0.03 g ($n = 70$); the relaxation induced by Paf (4 μM) was $39.67 \pm 3.5\%$ reversal of histamine-induced contraction ($n = 30$).

Effects of antagonists on Paf-induced relaxation in guinea-pig tracheal preparations

WEB 2086 (1 nM–1 μM) dose-dependently inhibited the relaxation induced by Paf 4 μM ($40 \pm 3.2\%$; $n = 20$) and, at 1 μM , completely blocked it (Figure 1a). By taking as 100% the Paf-induced relaxation, a concentration-effect curve of antagonism could be drawn in the presence of increasing concentrations of

Figure 1 Effect of WEB 2086 on Paf-induced relaxation of histamine (50 μM)-contracted guinea-pig tracheal preparations. (a) Paf-induced relaxation in the absence or presence of WEB 2086. (b) Reversal by WEB 2086 of Paf-induced relaxation. Relaxation induced by Paf was taken as 100%. Each tracheal strip was divided into two paired segments, one was treated with Paf and the other with WEB 2086 + Paf. The number of experiments is indicated in parentheses. Means are shown with vertical lines indicating s.e.mean. *0.05 > *P* > 0.01.

WEB 2086 (1 nM–1 μM) (Figure 1b). In these conditions, the IC₅₀ value for WEB 2086 was 21.2 nM.

As shown in Figure 2, BN 52021 100 μM blocked by 58% the mechanical effect induced by Paf. In this set of experiments, the relaxation evoked by Paf 4 μM was $38.5 \pm 4.5\%$ in the absence and $16.4 \pm 4.7\%$ ($n = 6$) in the presence of BN 52021 100 μM . At 10 μM BN 52021 did not significantly antagonize the effect of Paf (Figure 2).

Effects of Paf antagonists on arachidonic acid- and PGE₂-induced relaxation in guinea-pig tracheal preparations

As previously demonstrated, arachidonic acid or PGE₂ were able to relax histamine-contracted

Figure 2 Effect of BN 52021 on Paf-induced relaxation of histamine-contracted guinea-pig tracheal preparations. Each tracheal strip was divided into two paired segments, one of which was treated with Paf and the other with BN 52021 + Paf. The number of experiments is indicated in parentheses. Means are shown with vertical lines indicating s.e.mean. * $P < 0.05$.

guinea-pig tracheal spirals (Braunstein *et al.*, 1988). Therefore, we decided to verify the specificity of WEB 2086 and BN 52021 as Paf receptor antagonists, by evaluating their effects on relaxation induced by fixed concentrations of arachidonic acid or PGE_2 .

Arachidonic acid $10 \mu\text{M}$ and PGE_2 $1 \mu\text{M}$ relaxed to $59.6 \pm 3.5\%$ ($n = 8$) and 76.2% ($n = 6$), respectively, histamine-contracted guinea-pig tracheal preparations (data not shown). In the presence of WEB 2086 $1 \mu\text{M}$ the relaxations induced by the two agents were $55.7 \pm 5.8\%$ ($n = 4$) for arachidonic acid, and $79 \pm 3\%$ ($n = 3$) for PGE_2 . Similar results were obtained by evaluating the effects of BN 52021. In the presence of this antagonist, arachidonic acid-induced relaxation was $60.2 \pm 4\%$ ($n = 4$), while PGE_2 -induced relaxation was $80 \pm 5.2\%$ ($n = 3$). Moreover, WEB 2086 and BN 52021 were devoid of any antagonistic effect on relaxation evoked by lower concentrations (0.1 and $0.3 \mu\text{M}$) of PGE_2 , which induced $21.98 \pm 3\%$ ($n = 5$) and $44.75 \pm 3.1\%$ ($n = 4$), respectively (data not shown).

Effects of Paf antagonists on Paf-induced PGE_2 production in guinea-pig tracheal spirals

As previously found, Paf $4 \mu\text{M}$ caused a significant increase in detectable levels of PGE_2 in effluents from histamine-contracted guinea-pig tracheal preparations (Brunelleschi *et al.*, 1987). In the presence of WEB 2086 $1 \mu\text{M}$ or BN 52021 $100 \mu\text{M}$, the increase in PGE_2 levels observed when Paf was added to

histamine-contracted guinea-pig tracheal preparations was completely abolished and the actual levels of PGE_2 were similar to those measured in the presence of histamine alone (Figure 3a and b).

Discussion

These results confirm our previous observations that Paf relaxes histamine-contracted guinea-pig tracheal preparations and indicate that this effect can be significantly reduced, not only by cyclo-oxygenase

Figure 3 Effects of Paf antagonists on Paf-induced production of prostaglandin E_2 (PGE_2) in histamine-contracted guinea-pig tracheal preparations. (a) Effects of WEB 2086 on Paf-induced PGE_2 production. (b) Effects of BN 52021 on Paf-induced PGE_2 production. Each tracheal strip was divided into two segments, one of which was treated with Paf and the other by antagonist + Paf. Column A: PGE_2 production evoked in the presence of histamine $50 \mu\text{M}$; column B: histamine $50 \mu\text{M}$ + Paf $4 \mu\text{M}$; Column C: histamine $50 \mu\text{M}$ + WEB 2086 $1 \mu\text{M}$ + Paf $4 \mu\text{M}$ (a) or histamine $50 \mu\text{M}$ + BN 52021 $100 \mu\text{M}$ + Paf $4 \mu\text{M}$ (b). Means are shown with vertical lines indicating s.e.mean. The number of experiments is indicated in parentheses. * $P < 0.05$. See Methods for further details.

inhibitors (Brunelleschi *et al.*, 1987), but also by Paf receptor antagonists. In fact, BN 52021 and WEB 2086 inhibited, with different potencies, Paf-induced relaxation of guinea-pig trachea and this antagonism was specific, since relaxation induced by either arachidonic acid or PGE₂ was unchanged. WEB 2086 was the more active compound: at 1 μ M it completely blocked the relaxant effect induced by Paf, IC₅₀ value being 21.2 nM, while BN 52021 was less potent (about 60% inhibition at 100 μ M). The greater potency of WEB 2086 has been previously documented in different experimental models; for instance, O'Donnell & Barnett (1988) obtained pA₂ values of 7.31 and 6.38 for WEB 2086 and BN 52021, respectively, on Paf-induced aggregation in rabbit platelets. According to Evans *et al.* (1988) the dose of WEB 2086 required to abolish completely Paf-induced microvascular leakage of guinea-pig airways *in vivo* was 500 fold lower than that of BN 52063, a standardized mixture of ginkgolides A, B and C.

A role for Paf as an important regulator of eicosanoid generation in pulmonary tissues may be claimed, based on a variety of experimental evidence (Stimler & O'Flaherty, 1983; Lefort *et al.*, 1984; Hamasaki *et al.*, 1984; Desquand *et al.*, 1986; Chung *et al.*, 1986; Brunelleschi *et al.*, 1987; this study). For example, intra-arterial injection of Paf to the perfused guinea-pig lungs elicits a dose-related liberation of thromboxane A₂ (Lefort *et al.*, 1984). Moreover, Desquand *et al.* (1986) showed that the contractile activity of Paf in guinea-pig lung parenchymal strips is accompanied by thromboxane A₂ (TXA₂) release: BN 52021 100 μ M inhibited to about 60% the phosphoetherlipid-induced contraction, but totally suppressed the concomitant thromboxane A₂ release (Desquand *et al.*, 1986). The fact that the activation of the cyclo-oxygenase pathway preferentially leads to production of TXA₂ in guinea-pig parenchyma and PGE₂ in guinea-pig trachea (Grodzinska *et al.*, 1975; Lefort *et al.*, 1984) may help to explain the opposite functional effects (contraction

or relaxation) elicited by Paf. However, the ability of BN 52021 to reduce Paf-induced mechanical events and Paf-induced eicosanoid generation was observed to the same extent in both tissues (Desquand *et al.*, 1986; this study).

In conclusion, our results indicate that WEB 2086 and BN 52021 are able to reduce Paf-induced effects (relaxation and PGE₂ generation) in histamine-contracted guinea-pig trachea and are consistent with the presence of specific Paf receptors in this tissue.

A role for PGE₂ as mediator of Paf-induced relaxation is strongly indicated, since both antagonists, besides reducing the relaxation, also prevent the phosphoetherlipid-induced increase of PGE₂ in tracheal effluents.

The observation that BN 52021 only partially reduced the mechanical event, while suppressing Paf-induced PGE₂ production, warrants two possible explanations. The first, and more obvious one, is that the concentration of BN 52021 we used was not high enough to block Paf-induced relaxation. However, 100 μ M BN 52021 was the maximal concentration we could use in our experimental model, due to the concentration of DMSO in which the compound was dissolved. The second explanation which could be drawn from our results with BN 52021 is that PGE₂ is not the sole mediator of the relaxation elicited by Paf.

Participation of other mediators and/or a direct effect of Paf could be envisaged; as a matter of fact, we previously showed that pretreatment of guinea-pig tracheal spirals with indomethacin strongly reduced, but did not completely abolish, Paf-induced relaxation (Brunelleschi *et al.*, 1987). However it is noteworthy that a potent Paf receptor antagonist, namely WEB 2086, is able to block totally Paf-induced tracheal effects.

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