Involvement of capsaicin-sensitive nerves in the bronchomotor effects of arachidonic acid and melittin: a possible role for lipoxin A₄

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1 Functional studies have been performed to evaluate the potential involvement of capsaicin-sensitive nerves in the bronchomotor responses evoked by lipid mediators produced from the metabolic breakdown of arachidonic acid (AA) in the guinea-pig bronchus.

2 In the presence of indomethacin, the exogenous administration of AA (0.01–1 mM) produced a concentration-dependent contractile response in guinea-pig isolated bronchial rings. AA-induced contractions were augmented by epithelium-removal and by thiorphan (10 µM), an inhibitor of tachykinin breakdown. A sustained downward and rightward displacement of the complete concentration-response curve to AA was observed after *in vitro* capsaicin desensitization.

3 BWA4C (1 µM), a selective inhibitor of 5-lipoxygenase, shifted the AA concentration-response curve to the right. In the presence of this inhibitor, capsaicin desensitization did not have any further inhibitory action.

4 A potent, concentration-dependent and capsaicin-sensitive bronchoconstrictor effect was also observed with the polypeptide, melittin (10 nM–1 µM), an activator of phospholipase A₂, which therefore should generate endogenous AA.

5 *In vitro* capsaicin-desensitization produced a significant reduction of the bronchomotor responses evoked by lipoxin A₄ (1–6 µM), but not of those elicited by other lipoxygenase products such as leukotriene D₄ (1–100 nM) or by 15-hydroxyeicosatetraenoic acid (15-HETE, 1–6 µM).

6 These findings indicate that lipoxin A₄ but not leukotriene D₄ or 15-HETE, might be one of the lipoxygenase mediators of excitatory effects of AA on capsaicin-sensitive sensory nerves.

Keywords: Sensory nerves; capsaicin; lipid mediators; sensory neuropeptides; asthma

Introduction

A number of mediators have been implicated in asthma pathophysiology and among them a prominent role has been suggested for lipid mediators, such as platelet-activating factor (PAF) and leukotrienes, and for neuropeptides, such as tachykinins, calcitonin gene-related peptide (CGRP) or vasoactive intestinal peptide (VIP) (Barnes, 1987; 1989a,b). Recently some evidence has been presented which suggests a possible interaction between these two classes of biological mediators. PAF has been shown to release VIP and substance P from guinea-pig isolated lung strip (Rodrigue *et al.*, 1987). In addition, some of the motor effects of leukotriene D₄ may involve the release of sensory neurotransmitters (Stewart *et al.*, 1984; Bloomquist & Kream, 1987). In contrast, CGRP and VIP inhibit the release and/or biosynthesis of peptidoleukotrienes in lung preparations (Di Marzo *et al.*, 1987; 1988). Manzini *et al.* (1989) obtained functional and neurochemical evidence that suggested in guinea-pig, the exogenous administration of arachidonic acid (AA) might produce bronchomotor effects partially through the release of sensory neuropeptides. Oxidation of arachidonic acid leads to the formation of a plethora of eicosanoids, including the cyclo-oxygenase and lipoxygenase products, that have been shown to cause bronchoconstriction, vasoconstriction and increased vascular permeability (Barnes, 1989b). Since some of our previous experiments were performed in the presence of indomethacin, we proposed the hypothesis that products of AA metabolic breakdown via the lipoxygenase pathways might activate the local efferent function of capsaicin-sensitive nerves in guinea-pig airways (Manzini *et al.*, 1989; Manzini & Meini, 1990). In this study we have investigated further the effect of capsaicin-desensitization on the motor response to AA in the presence of inhibitors of cyclo-oxygenase and/or lipoxygenases. Fur-

thermore, we have assessed whether or not the motor effects of AA are affected by epithelium removal or by inhibition of neutral endopeptidases, which increase responses to tachykinins (Maggi *et al.*, 1990). Finally, we have studied the effect of capsaicin desensitization on the bronchomotor actions produced by the polypeptide melittin, a selective activator of phospholipase A₂ (Mollay *et al.*, 1976; Shier, 1979), or by 5 and/or 15-lipoxygenase metabolites, such as leukotriene D₄, 15-hydroxyeicosatetraenoic acid (15-HETE) or lipoxin A₄ (Serhan *et al.*, 1984; Samuelsson *et al.*, 1987; Sigal & Nadel, 1988).

Part of these data have been presented in a preliminary report (Manzini *et al.*, 1989) and communicated at the XIth International Congress of Pharmacology (Manzini & Meini, 1990).

Methods

Male albino guinea-pigs (Rodentia, Torre Pallavicina, Bergamo, Italy) weighing 300–350 g were used throughout the study. Animals were stunned, exsanguinated and the lungs with attached bronchi and trachea were removed quickly. Main bronchi were dissected from the surrounding parenchyma and two (2 mm long) epithelium-free rings were prepared by gently rubbing the luminal surface with a moistened pipe cleaner. In some preparations removal of epithelium was confirmed histologically. Bronchial rings were mounted on two 'L'-shaped stainless steel holders and placed in 5 ml organ baths containing physiological salt solution, (at 37°C and oxygenated with 95% O₂ and 5% CO₂), of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂·2H₂O 2.5, MgCl₂·6H₂O 0.5, NaH₂PO₄·H₂O 1.0, NaHCO₃ 25 and glucose 10. Contractile responses were recorded with an isometric force transducer and displayed on a Basile Unirecord 7050 polygraph. An optimal resting tension of 750 mg was applied to the preparations. Each preparation was allowed to

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equilibrate for at least 45 min before the start of the experiments. Inhibitors of cyclo-oxygenase and/or lipoxygenase were added to the organ bath at least 45 min before challenge with AA.

In some experiments concentration-response curves (CRC) to AA, melittin and lipoxygenase products were obtained in a cumulative manner, the next concentration being added when the response to the previous one had reached a steady state. Two main bronchi obtained from the same animal were set up in parallel. One was used for the control CRC, the other for CRC after capsaicin-desensitization. Capsaicin-desensitization was performed by incubating the bronchial rings with capsaicin ($10\text{ }\mu\text{M}$) for 15 min followed by several washes before constructing the CRC to the bronchoconstrictor agent.

Analysis and statistical evaluation of results

All data in the text are mean $\pm$ standard error of the mean (s.e.mean). Motor effects were measured either as mg of contraction or normalized to the maximal motor response induced, in the same preparation, by KCl (60 mM).

Statistical analyses were performed by Student's *t* test for paired or unpaired data, when appropriate, with a chosen probability value of 0.05 for significance.

Drugs

Drugs used were the following: arachidonic acid (Sigma), capsaicin, indomethacin and leukotriene D_4 (Serva), carbachol (Merck), lipoxin A_4 and 15(RS)-hydroxyeicosatetraenoic acid (15(RS)-HETE) (Cascade Biochem), thiorphan (Peninsula), melittin (Bachem) and BW A4C (Wellcome, U.K.).

Solutions of peptides and eicosanoids were made daily in distilled water from stock solutions (kept frozen) and kept on ice during the experiment. Capsaicin (10 mM) and indomethacin (2 mM) were dissolved in absolute ethanol; sodium arachidonate (10 mM) was dissolved as a 10 mM stock solution in water and stored under N_2 at -20°C . Further drug dilutions were made in distilled water. Ethanol did not affect tissue responses at the concentrations used.

Results

Effect of capsaicin desensitization on arachidonic acid-induced bronchomotor response in the presence of indomethacin or BW A4C

In isolated bronchial rings (with epithelium) the administration of exogenous AA (1 mM) produced weak and erratic contractions ($n = 8$). On the other hand, in the presence of the cyclo-oxygenase inhibitor indomethacin ($5\text{ }\mu\text{M}$), AA (1 mM) consistently elicited a sustained contraction with an amplitude, at steady state, of $323 \pm 17\text{ mg}$ ($n = 33$) (Figure 1). In the same preparation, such responses were reproducible at 90 min intervals ($n = 6$). Exposure of bronchial rings to capsaicin ($10\text{ }\mu\text{M}$) elicited a substantial contraction ($536 \pm 46\text{ mg}$) which could be evoked only once in each preparation, indicating desensitization ($n = 6$). Following capsaicin challenge the response to AA was significantly reduced to $108 \pm 34\text{ mg}$ ($n = 6$; $P < 0.05$; Figure 1) (see also Manzini *et al.*, 1989). A similar reduction was not observed when bronchial rings were previously challenged with a KCl concentration (60 mM) equi-effective to capsaicin ($n = 3$; data not shown). Furthermore, capsaicin desensitization had no effect on the motor response to carbachol ($0.3\text{ }\mu\text{M}$) ($n = 3$, Figure 1).

In the presence of indomethacin, it is also possible to obtain a cumulative CRC to AA (0.01–1 mM). Previous capsaicin challenge produced a rightward and downward shift of the CRC to AA (Figure 2). The EC_{50} for AA increased from $196\text{ }\mu\text{M}$ (172–229) to $508\text{ }\mu\text{M}$ (435–610) ($n = 6$).

In a further set of experiments we have assessed the effect of capsaicin desensitization on the CRC to AA obtained in the

Figure 1 Typical tracings showing the motor response (mg) elicited by arachidonic acid (AA) or carbachol (CCh) in guinea-pig isolated bronchus before or after capsaicin (Caps) challenge. In the same preparations a second application of capsaicin did not exert any contractile effect. Experiments were performed in the presence of indomethacin ($5\text{ }\mu\text{M}$).

presence of indomethacin and BW A4C, a selective inhibitor of 5-lipoxygenase (Tateson *et al.*, 1988). In the presence of BW A4C ($1\text{ }\mu\text{M}$, incubated 45 min before and during AA administration) the CRC to AA was shifted to the right

Figure 2 Concentration-response curves to arachidonic acid (AA, 0.01–1 mM) in guinea-pig epithelium-denuded bronchial rings in control conditions (○) and in preparations previously challenged with a desensitizing concentration ($10\text{ }\mu\text{M}$ incubated for 15 min) of capsaicin (■). Experiments were performed in the presence of indomethacin ($5\text{ }\mu\text{M}$). Statistical evaluation was performed with Student's *t* test for unpaired data (* $P < 0.05$; ** $P < 0.01$). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: arachidonic acid concentration.

Figure 3 Concentration-response curves to arachidonic acid (AA) in guinea-pig epithelium-denuded bronchial rings in control conditions (circles) and in preparations previously challenged with a desensitizing concentration of capsaicin ($10\mu\text{M}$ incubated for 15 min) (squares). Experiments were performed in presence of only indomethacin ($5\mu\text{M}$) (open symbols) and either indomethacin and BW A4C ($1\mu\text{M}$) (solid symbols). Statistical evaluation was performed with Student's *t* test for unpaired data (* $P < 0.05$; ** $P < 0.01$ versus BW A4C). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: arachidonic acid concentration.

(Figure 3). The EC_{50} increased from $196\mu\text{M}$ (172–229) to $389\mu\text{M}$ (361–422) ($n = 6$). Interestingly, when 5-lipoxygenase was inhibited, capsaicin-desensitization no longer affected the motor response to exogenous AA [EC_{50} : $389\mu\text{M}$ (344–447)] (Figure 3). BW A4C ($1\mu\text{M}$) itself had no effect on baseline tone ($n = 12$).

Effect of epithelium removal and inhibition of neutral endopeptidases on arachidonic acid-induced bronchomotor responses

AA (1mM)-induced motor responses were different in control and in epithelium-denuded preparations (always in the presence of indomethacin). Epithelium removal significantly increased the amplitude of AA-induced bronchomotor responses from $255 \pm 27\text{mg}$ to $368 \pm 40\text{mg}$ ($n = 6$; $P < 0.05$). In a further set of experiments we have investigated the effect of the neutral endopeptidase inhibitor, thiorphan ($10\mu\text{M}$) on the motor response elicited by AA (1mM , in the presence of indomethacin). Thiorphan was used at a concentration previously demonstrated to enhance markedly the motor response to capsaicin and tachykinins (Maggi *et al.*, 1990). Figure 4 shows typical tracings where in the presence of thiorphan, AA-induced contractions were more rapid in onset, similar in amplitude ($258 \pm 29\text{mg}$ and $399 \pm 94\text{mg}$ in absence or in presence of thiorphan respectively, $n = 4$, not significant) and were significantly sustained for longer periods. In control preparations the motor responses to AA declined by $44 \pm 5\%$ after 80 min, whereas, in presence of thiorphan, responses declined by only $23 \pm 5\%$ after 80 min ($n = 4$, $P < 0.05$, Figure 4).

Effect of capsaicin desensitization on bronchomotor responses elicited by melittin

In the presence of indomethacin ($5\mu\text{M}$), thiorphan ($10\mu\text{M}$) and in the absence of epithelium, melittin (10nM – $1\mu\text{M}$) was an extremely potent and effective bronchoconstrictor agent (Figure 5). It induced contractions starting at a concentration as low as 10nM and, at the maximal concentration tested ($1\mu\text{M}$), it produced a contraction of $688 \pm 123\text{mg}$ ($n = 6$). After capsaicin pretreatment a marked rightward and downward displacement of the CRC to melittin was observed

Figure 4 Effect of thiorphan ($10\mu\text{M}$) on arachidonic acid (AA, 1mM)-induced contraction in guinea-pig bronchial rings. Time decay of tonic contraction in control conditions (○) and in presence of thiorphan (●) is shown in (a). Vertical axis: contractile effect expressed as % of relaxation of obtained tone. Horizontal axis: time (min). In (b) the tracings represent two typical motor responses (mg) obtained in two bronchi excised from the same animal. All experiments were carried out in presence of indomethacin ($5\mu\text{M}$). Statistical evaluation was performed with Student's *t* test for unpaired data (* $P < 0.05$; ** $P < 0.01$).

Figure 5 Cumulative concentration-response curves of the bronchomotor responses elicited on guinea-pig bronchial rings by melittin (0.01 – $1\mu\text{M}$) in control conditions (□) and in preparations pretreated with a desensitizing concentration of capsaicin ($10\mu\text{M}$ incubated for 15 min) (■). All experiments were performed in epithelium-denuded preparations and in the presence of indomethacin ($5\mu\text{M}$) and thiorphan ($10\mu\text{M}$). Statistical evaluation was performed with Student's *t* test for unpaired data (* $P < 0.05$). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: melittin concentration.

(Figure 5). After capsaicin challenge a concentration of melittin as high as $1 \mu\text{M}$ was required to produce an appreciable contraction (Figure 5).

Effect of capsaicin desensitization on bronchomotor responses elicited by leukotriene D_4 , 15-HETE or lipoxin A_4

In this set of experiments we studied the effect of capsaicin desensitization on the motor response produced by three eicosanoids previously found to exert bronchomotor responses in guinea-pigs, i.e. leukotriene D_4 (LTD $_4$) (Dahlen *et al.*, 1980), 15-HETE (Copas *et al.*, 1982) and lipoxin A_4 (Serhan *et al.*, 1986; Jacques *et al.*, 1988).

LTD $_4$ (1–100 nM) was a potent and effective bronchospastic agent ($n = 4$; Figure 6). Its EC $_{50}$ was similar in control and in capsaicin-pretreated bronchial preparations being 24 nM (20–29) and 15 nM (12–20), respectively ($n = 4$; Figure 6).

15-HETE was (at least up to the maximum concentration tested) a rather weak contractile agent. At the maximum concentration tested ($6 \mu\text{M}$) it produced a contraction with an amplitude of $293 \pm 103 \text{ mg}$ in control and $220 \pm 46 \text{ mg}$ in capsaicin-pretreated preparations (Figure 7a; $n = 4$; not significant).

Lipoxin A_4 produced a fairly rapid and sustained contraction (Figure 8). Albeit less potent than LTD $_4$ this lipid produced, at the maximum concentration tested ($6 \mu\text{M}$), a contraction with an amplitude of $512 \pm 33 \text{ mg}$ ($n = 4$), similar to that produced by LTD $_4$ (100 nM) or by KCl (60 mM). Lipoxin A_4 -induced contractions were significantly (Figure 7b) reduced in capsaicin-pretreated bronchial rings (Figures 7 and 8). After capsaicin desensitization lipoxin A_4 produced a small response ($172 \pm 40 \text{ mg}$, $n = 4$) only at the maximum concentration tested ($6 \mu\text{M}$).

Discussion

Local release of sensory neuropeptides from capsaicin-sensitive sensory nerve endings can promote plasma protein extravasation, mucus secretion and bronchoconstriction. Such a neurogenic mechanism may play a relevant role in asthma pathophysiology (Barnes, 1986). A number of endogenous

Figure 6 Cumulative concentration-response curves of the bronchomotor responses elicited on guinea-pig bronchial rings by leukotriene D_4 (LTD $_4$, 1–100 nM) in control conditions ($\square$) and in preparations pretreated with a desensitizing concentration of capsaicin ($10 \mu\text{M}$ incubated for 15 min) ($\blacksquare$). All experiments were performed in epithelium-denuded preparations and in the presence of indomethacin ($5 \mu\text{M}$) and thiorphan ($10 \mu\text{M}$). No significant statistical difference was observed. Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: LTD $_4$ concentration.

Figure 7 Cumulative concentration-response curves of the bronchomotor responses elicited on guinea-pig bronchial rings by 15-eicosatetraenoic acid (15-HETE, 1, 3 and $6 \mu\text{M}$) (a) or lipoxin A_4 (1, 3, $6 \mu\text{M}$) (b) in control conditions ($\square$) and in preparations pretreated with a desensitizing concentration of capsaicin ($10 \mu\text{M}$ incubated for 15 min) ($\blacksquare$). All experiments were performed in epithelium-denuded preparations and in the presence of indomethacin ($5 \mu\text{M}$) and thiorphan ($10 \mu\text{M}$). Statistical evaluation was performed with Student's t test for unpaired data (* $P < 0.05$; ** $P < 0.01$). Vertical axis: contractile effect expressed as % of maximum contraction produced by KCl (60 mM). Horizontal axis: 15-HETE or lipoxin A_4 concentration.

Figure 8 Typical tracings of the motor responses (mg) elicited on guinea-pig bronchial ring by lipoxin A_4 (1, 3 and $6 \mu\text{M}$) in control (a) and in a preparation previously desensitized with capsaicin (Caps $10 \mu\text{M}$ incubated for 15 min) (b). The two bronchial preparations were taken from the same animal. Veh = vehicle.

substances (bradykinin, histamine, nicotinic agonists) induce at least part of their pulmonary effects through activation of the local efferent function of capsaicin-sensitive nerves (Saria *et al.*, 1988; Ichinose *et al.*, 1990). We have presented evidence

that some lipid mediators generated by the oxidation (via the lipoxygenase pathways) of arachidonic acid might also act through such a mechanism (Manzini *et al.*, 1989; Manzini & Meini, 1990; this study). This is supported by the following evidence: (a) AA-induced bronchomotor responses are reduced both *in vitro* and *in vivo* by previous capsaicin desensitization; (b) AA induces a capsaicin-sensitive release of sensory neuropeptides from isolated rubbed bronchial rings; (c) AA-induced motor responses are enhanced by interventions, such as epithelium removal or neutral endopeptidase inhibition, which amplify biological responses elicited by local release of sensory neuropeptides (Maggi *et al.*, 1990). It is interesting to note that capsaicin desensitization markedly reduced not only the motor responses produced by exogenously added AA, but also those evoked by melittin, a proposed selective activator of phospholipase A₂ and/or releaser of endogenous AA (Mollay *et al.*, 1976; Shier, 1979). Melittin is a polypeptide extracted from bee venom (Habermann, 1972) which produces endothelium-dependent vasorelaxation (Rapoport *et al.*, 1989), an effect that is prevented by inhibition of phospholipase A₂ and by lipoxygenase, but not by cyclo-oxygenase, inhibitors (Forstermann & Neufang, 1985). It is clear that melittin-induced bronchoconstriction might be attributed not only to AA-derived eicosanoids but also to other lipids the synthesis of which might follow after activation of phospholipase A₂. In this regard it is interesting to note that it has recently been shown that PAF-induced airway hyperresponsiveness in rabbits is abolished by previous *in vivo* treatment with capsaicin (Coyle *et al.*, 1990).

In this paper we have utilized the *in vitro* technique of capsaicin desensitization (Bartho *et al.*, 1987; Maggi *et al.*, 1987) to investigate the capsaicin-sensitive component of AA-induced bronchomotor responses. *In vitro* exposure to high concentrations of capsaicin desensitized a particular subset of primary afferents rendering them resistant to subsequent challenge with capsaicin itself or other stimuli (Maggi & Meli, 1988). Therefore, this procedure permits the identification of agents with actions which might rely upon activation of the efferent function of these nerves. The involvement of capsaicin-sensitive nerves in the response to eicosanoids appears to be rather specific, in fact capsaicin desensitization is effective in the presence of indomethacin but not when the lipoxygenase pathways or even only the 5-lipoxygenase pathway are pharmacologically blocked with BW755C (Manzini & Meini, 1990) or BW A4C (this study). Furthermore, among various eicosanoids, the synthesis of which involves the 5-lipoxygenase pathway, and which have been shown to exert bronchomotor effects, only the motor response to lipoxin A₄, and not those exerted by LTD₄ or 15-HETE, is reduced by previous capsaicin challenge.

These data suggest that lipoxin A₄ might be relevant in lung pathophysiology not only for its direct inflammatory, vasodilator and motor effects (Dahlen *et al.*, 1988) but also as an endogenous activator of sensory nerves. Lipoxin A₄ contracts lung parenchymal strips of various species, including man (Dahlen *et al.*, 1988; Jacques *et al.*, 1988; Sigal & Nadel, 1988). In guinea-pig lung part of its contractile actions are mediated by generation of thromboxane A₂ (Dahlen *et al.*, 1988), but in the present study this is unlikely, since indomethacin was present throughout. Lipoxin A₄ also activates directly protein kinase C (Hansson *et al.*, 1986), and this biochemical action can produce depolarization of nonmyelinated C fibres (Rang & Ritchie, 1988). Further studies are necessary to clarify whether or not lipoxins activate sensory nerves by stimulation of specific receptors on the outer membrane and/or by activation of some intracellular biochemical mechanisms. Interestingly, lipoxin A₄ can be generated by neutrophils (Serhan, 1989), alveolar macrophages, eosinophils and, possibly, epithelial cells (Sigal & Nadel, 1988). Significant amounts of lipoxin A₄ have been detected in bronchoalveolar lavage fluids obtained from patients with lung diseases but not from healthy volunteers (Lee *et al.*, 1990). In view of the above, it is reasonable to speculate that eosinophilia and epithelial shedding, typical hallmarks of airway hyperreactivity and asthmatic bronchial biopsies (Barnes, 1989a), can lead to an increased local production of lipoxins which in turn contributes to the activation of sensory nerves, thus producing neurogenic inflammation.

In conclusion our studies indicate that, in guinea-pig bronchus, some lipid mediators (the synthesis of which involves the 5-lipoxygenase pathway) can interact with capsaicin-sensitive sensory nerves, and therefore, that at least part of the motor effects produced by AA might rely indirectly upon the local release of sensory neuropeptides. Among the eicosanoids, lipoxin A₄ appears to be one of the most likely candidates which activates capsaicin-sensitive nerves in guinea-pig bronchial tissues. The pharmacological control of the interaction between inflammatory mediators and sensory nerves may represent a relevant target for new strategies in treatment of asthma.

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