Influence of epithelium on the inhibition of melittin-induced contraction of guinea-pig isolated trachea by the potassium channel opener NIP-121

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1 We have investigated the effect of the potassium channel opener, NIP-121, on contraction elicited by melittin (a phospholipase A₂ activator) in epithelium-intact and epithelium-denuded trachea isolated from guinea-pigs. The effects of NIP-121 were compared with those of isoprenaline, aminophylline and hydrocortisone.

2 In the presence of the cyclo-oxygenase inhibitor, indomethacin (5 µM), melittin (3 µg ml⁻¹) caused time-dependent contraction. The melittin-induced contractile response was significantly augmented by removal of the epithelium and was concentration-dependently and completely inhibited by the phospholipase A₂ (PLA₂) inhibitor, *p*-bromophenacyl bromide (BPB 10–100 µM), and the lipoxygenase inhibitor, phenidone (10–300 µM). Similar inhibition of the melittin response by BPB (10 µM) and phenidone (10 µM) was observed in the epithelium-denuded trachea.

3 Concentration-dependent inhibition of the melittin response was induced by preincubation with NIP-121 (0.03 and 0.1 µM), isoprenaline (0.001 and 0.01 µM), aminophylline (30 and 100 µM) and hydrocortisone (100 and 300 µM). The effect of NIP-121 was abolished by glibenclamide (1 µM).

4 The inhibitory effect of NIP-121 on the melittin response was greatly reduced by removing the epithelium while that of the isoprenaline, aminophylline or hydrocortisone was not changed.

5 The inhibition of the melittin response by these drugs was similar to their inhibition of the contraction elicited by a low concentration (3 nM) of leukotriene D₄ (LTD₄) in the epithelium-intact trachea. Inhibition of the LTD₄ response by NIP-121 was not observed in the epithelium-denuded trachea. However, higher concentrations of NIP-121 (0.3 and 1 µM) did inhibit LTD₄-induced contractions of epithelium-denuded trachea.

6 These findings suggest that melittin causes epithelium-dependent contraction of the guinea-pig isolated trachea which is mediated by products of lipoxygenase activity. NIP-121 may inhibit the melittin response by activating glibenclamide-sensitive potassium channels, which appear to be epithelium-dependent (an indirect effect of NIP-121 apart from its direct effect on the airway smooth muscle) while isoprenaline, aminophylline and hydrocortisone act directly to relax the trachealis smooth muscle.

Keywords: NIP-121; melittin; leukotriene D₄; isoprenaline; aminophylline; hydrocortisone; airway epithelium; guinea-pig trachea

Introduction

Airway hyperresponsiveness is a characteristic feature of bronchial asthma and is associated with damage to, or loss of, the epithelium lining the airways (Laitinen *et al.*, 1985; Seltzer *et al.*, 1986). Airway responsiveness to bronchoactive substances is augmented by mechanically removing the epithelium in airway preparations isolated from several animal species including man (Barnes *et al.*, 1985; Holroyde, 1986; Aizawa *et al.*, 1988; Sparrow & Mitchell, 1991). Barnes (1986) also proposed the hypothesis that the lack of epithelium sensitizes the C-fibre afferent nerve thereby promoting the release of bronchoconstrictor neuropeptides, such as substance P. The epithelium, therefore, may play an important role in the regulation of airway smooth muscle responsiveness.

The potassium channel opener, cromakalim, induces not only relaxation of guinea-pig (Arch *et al.*, 1988) and human (Black *et al.*, 1990) airway smooth muscle but also prevention of bronchoconstriction mediated by cholinergic (McCaig & De Jonckheere, 1989) and non-adrenergic non-cholinergic (Ichinose & Barnes, 1990) nerve stimulation. These data suggest that a potassium channel opener may be a useful drug for the airway hyperreactivity evoked by neurogenic inflammation mediated by activation of non-adrenergic, non-cholinergic nerves. NIP-121, a potassium channel opener

(Masuda *et al.*, 1991), has potent bronchodilator activity in the guinea-pig isolated trachea (Shikada *et al.*, 1991). We reported recently that NIP-121, as well as cromakalim, inhibited neurally-mediated contraction (Yamamoto *et al.*, 1992) and epithelium-dependent potentiation of sodium nitroprusside-induced relaxation in the guinea-pig isolated trachea (Shikada & Tanaka, 1992). Wessler *et al.* (1992) found that the inhibitory effect of cromakalim on parasympathetic nerve-induced acetylcholine release is epithelium-dependent. These data postulate that a potassium channel opener may act on the epithelium in airway tissues. Since epithelium releases putative broncho-relaxing substances that regulate the airway smooth muscle responsiveness (Hay *et al.*, 1987; Fernandes & Goldie, 1990), it is important to study the interaction between a potassium channel opener and airway epithelium.

In the present study we have further investigated the mode of action of NIP-121 on airway tissues, by examining the influence of the epithelium on the inhibition by NIP-121 of contractions induced by a phospholipase A₂ (PLA₂) activator, melittin, in the guinea-pig isolated trachea. The effects of NIP-121 have been compared with those of the anti-asthma drugs isoprenaline, aminophylline and hydrocortisone. We have previously reported that melittin caused contraction of the isolated guinea-pig trachea and that the response was similar to that of exogenously applied arachidonic acid (AA) (Yamamoto *et al.*, 1990).

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Methods

Tracheae were removed from male Hartley guinea-pigs (280–350 g) which had been killed by stunning and bleeding. Each trachea was cut spirally and divided into two or three segments (one was used as a control). In some experiments, the epithelium was removed mechanically by gently rubbing the luminal surface with a cotton-tipped applicator. Individual tissues were suspended under an applied load of 1 g in a 10 ml organ bath containing 8 ml of modified Tyrode solution at 37°C and gassed continuously with 95% O₂ + 5% CO₂. The composition of the modified Tyrode solution was (mM): NaCl 137, KCl 2.7, CaCl₂ 1.8, MgCl₂ 1.0, NaHPO₄ 0.3, NaHCO₃ 20 and dextrose 11. Trachealis length changes were measured isotonicity (transducer type TD-112S, Nihon Koden). After the tissues had equilibrated for 50–60 min, the maximal response to histamine (100 µM) was evoked. Subsequent contractile responses were expressed as percentages of the maximal response to histamine. The tissues were washed several times for 30 min to re-establish the baseline and were then incubated for an additional 30 min with the test drug or vehicle in the presence of 5 µM indomethacin. Melittin at a concentration of 3 µg ml⁻¹ and leukotriene D₄ (LTD₄) at concentrations from 0.1–30 nM were singly and cumulatively added to the organ bath respectively. The area under the time contraction curve (AUC) in the melittin response was calculated by a computer programme and compared with that of the paired control.

In a separate experiment, the potencies of the test drugs in suppressing spontaneous tone of the guinea-pig isolated trachea were measured. Cumulative concentration-relaxation curves for test drugs were generated by successive increases in the organ bath concentration of these drugs. These experiments were performed on epithelium-intact trachea in the absence of indomethacin. The relaxant response was expressed as a percentage of the maximum relaxation obtained with 1 mM aminophylline added to the organ bath at the end of the experiment.

In all experiments, only one concentration-response curve for a relaxant drug was constructed in a given tissue. All results are expressed as means ± s.e.mean, and the statistical significance ($P < 0.05$) was determined by Student's one-tailed *t* test for paired observations.

Drugs

The following agents were used: histamine hydrochloride (Wako Pure Chemical Industries, Japan); LTD₄ (Ultrafine

Chemicals, England); indomethacin, melittin (bee venom, purity 85%), (–)-isoprenaline hydrochloride, aminophylline hydrate, hydrocortisone, 1-phenyl-1-pyrazolidone (phenidone), glibenclamide, pyrilamine maleate (Sigma Chemical Co., USA); atropine sulphate (E. Merck, Germany); *p*-bromophenacyl bromide (BPB, Nacalai tesque, Japan); NIP-121 ((+)-7,8-dihydro-6,6-dimethyl-7-hydroxy-8-(2-oxo-piperidin-1-yl)6H-pyrano[2,3-f]benz-2,1,3-oxadiazole) and cromakalim were synthesized by Nissan Chemical Industries Ltd., Central Research Laboratory, Japan. Indomethacin was dissolved in 100% ethanol, and hydrocortisone, BPB, phenidone, glibenclamide, NIP-121 and cromakalim were dissolved in 100% dimethylsulphoxide. Other drugs were dissolved in distilled water. At the final bath concentrations used, the drug vehicles did not cause any trachealis length changes.

Results

Melittin-induced contraction of guinea-pig isolated trachea and influence of epithelium removal on the effect of inhibitors of the arachidonic acid (AA) lipoxigenase pathway on the melittin response

In the presence of indomethacin (5 µM), melittin at a concentration of 3 µg ml⁻¹ induced a slowly developing contraction of the guinea-pig tracheal strip. The peak response usually occurred within 10–15 min and the magnitude of the contraction was about 50% of the histamine maximum (Figure 1). When the tracheal luminal surface was gently rubbed with cotton to remove the epithelium, the melittin-induced contractile response was significantly enhanced (Figure 1). The extent of the contraction judged by the AUC was also significantly increased (Table 1).

The PLA₂ inhibitor, BPB and the lipoxigenase inhibitor, phenidone concentration-dependently and completely, inhibited the melittin response observed in epithelium-intact trachea (Table 1). Similar inhibition of the melittin response by these inhibitors was also observed in the epithelium-denuded trachea. Pyrilamine (1 µM) and atropine (1 µM) each had no inhibitory effect on the melittin response in intact tissue.

Effect of NIP-121, isoprenaline, aminophylline and hydrocortisone on melittin response in epithelium-intact and epithelium-denuded trachea

To investigate the effect of the test drugs on the melittin response, we determined concentrations of the test drugs that caused equivalent suppression of the spontaneous tone of the

Figure 1 Effect of epithelium removal on melittin (3 µg ml⁻¹)-induced contraction of the guinea-pig isolated trachea. Each point represents the mean ± s.e.mean of 5 preparations: (O) epithelium-intact tissues and (●) epithelium-denuded tissue. *Indicates that the data points from the epithelium-denuded tissues were significantly greater ($P < 0.05$) than the corresponding data points from the epithelium-intact tissues.

Table 1 Effect of *p*-bromophenacyl bromide (BPB), phenidone, pyrilamine and atropine on melittin-induced contraction of epithelium-intact and epithelium-denuded trachea isolated from guinea-pigs

Inhibitor	concentration (µM)	Epithelium	% of paired control AUC
None		denuded	129 ± 5
BPB	10	intact	41 ± 4
	100	intact	0
	10	denuded	38 ± 7
Phenidone	10	intact	50 ± 7
	100	intact	23 ± 4
	300	intact	5 ± 4
	10	denuded	62 ± 5
Pyrilamine	1	intact	100 ± 9
Atropine	1	intact	109 ± 14

Values are expressed as the mean ± s.e.mean of 4–6 preparations. The AUC was calculated by a computer programme and is expressed as % of the paired control AUC.

trachea. All test drugs concentration-dependently suppressed the spontaneous tone of the epithelium-intact trachea in the absence of indomethacin. In these experiments we determined two concentrations of each drug that caused about 30–40% and 80–90% decreases in spontaneous tone, respectively. Table 2 shows the effect of two concentrations of the test compounds against the spontaneous tone of the trachea. We

Table 2 Effect of NIP-121 and reference drugs on spontaneous tone in the epithelium-intact trachea isolated from guinea-pig

Drug	Concentration (μM)	% relaxation
NIP-121	0.03	31 $\pm$ 13
	0.1	81 $\pm$ 7
Isoprenaline	0.001	36 $\pm$ 8
	0.01	93 $\pm$ 1
Aminophylline	30	42 $\pm$ 3
	100	88 $\pm$ 3
Hydrocortisone	100	33 $\pm$ 6
	300	48 $\pm$ 6

% relaxation is expressed as the maximum response induced by 1 mM aminophylline added at the end of the experiment. Values are expressed as the mean $\pm$ s.e.mean of 4–7 preparations.

used 300 μM hydrocortisone as the upper tested concentration of hydrocortisone. Further increase in the tested concentration of hydrocortisone was limited by its solubility in dimethylsulphoxide.

In the epithelium-intact trachea, preincubation with the low concentration of NIP-121 (0.03 μM) markedly suppressed the melittin response while low concentrations of isoprenaline, aminophylline and hydrocortisone had no significant effect (Figure 2). At the higher concentration, isoprenaline completely blocked the melittin response while NIP-121, aminophylline and hydrocortisone moderately inhibited it to about the same extent (Figures 2 and 3a). Reduction of the NIP-121 concentration to 0.01 μM , which did not suppress the spontaneous tone, had little effect on the melittin response (116 $\pm$ 7% of the paired control AUC, $n = 4$). The combination of the ATP-sensitive potassium channel blocker

Figure 2 Effect of NIP-121 (a (●) 0.03 μM ; (▲) 0.1 μM), isoprenaline (b (●) 0.001 μM ; (▲) 0.01 μM), aminophylline (c (●) 30 μM ; (▲) 100 μM) and hydrocortisone (d (●) 100 μM ; (▲) 300 μM) on melittin-induced contraction of the epithelium-intact guinea-pig isolated trachea. Each point represents the mean $\pm$ s.e.mean of 4–5 preparations in paired control (○) and in test tissues.

Figure 3 Effect of NIP-121, isoprenaline (Iso), aminophylline (AP) and hydrocortisone (HC) on melittin-induced contraction of the epithelium-intact (a) and the epithelium-denuded (b) guinea-pig isolated trachea. The AUC in the test tissue is expressed as % of the paired control AUC. Each value shows the mean $\pm$ s.e.mean of 4–5 preparations.

Figure 5 Effect of NIP-121 (a (●) 0.03 μM; (▲) 0.1 μM), (b (■) 0.3 μM; (▼) 1 μM) on leukotriene D₄ (LTD₄)-induced contraction of the epithelium-denuded guinea-pig isolated trachea. Each point represents the mean ± s.e.mean of 5 preparations in paired control (○) and in test tissues. *Indicates that the data points of the test tissues were significantly different ($P < 0.05$) from the corresponding data points of the control tissues.

glibenclamide (1 μM) with 0.1 μM NIP-121 failed to inhibit the melittin response ($102 \pm 14\%$ of the paired control AUC). This finding suggests that the inhibition of the melittin response by NIP-121 is dependent upon the activation of ATP-sensitive potassium channels.

In the epithelium-denuded trachea, the inhibition of the melittin response by NIP-121 was greatly reduced but inhibition by isoprenaline, aminophylline and hydrocortisone was not different from that in the epithelium-intact trachea (Figure 3b). A similar reduction of the inhibition of the melittin response was also observed in the case of cromakalim. Cromakalim at a concentration (0.1 μM) which caused appreciable suppression of spontaneous tracheal tone ($68 \pm 12\%$, $n = 5$) inhibited the melittin response in the epithelium-intact trachea ($70 \pm 23\%$ of the paired control AUC, $n = 5$) but the inhibition disappeared in the epithelium-denuded trachea ($106 \pm 8\%$ of the paired control AUC, $n = 5$).

Figure 4 Effect of NIP-121 (a (●) 0.03 μM; (▲) 0.1 μM), isoprenaline (b (●) 0.001 μM; (▲) 0.01 μM), aminophylline (c (●) 30 μM; (▲) 100 μM) and hydrocortisone (d (●) 100 μM; (▲) 300 μM) on the leukotriene D₄ (LTD₄) log concentration-contraction curve in the epithelium-intact, guinea-pig isolated trachea. Each point represents the mean ± s.e.mean of 4 preparations in paired control (○) and in test tissues. *Indicates that the data points of the test tissues were significantly different ($P < 0.05$) from the corresponding data points of the control tissues.

Effect of NIP-121 and reference drugs on the LTD₄ concentration-response curve in epithelium-intact and epithelium-denuded tracheae

In the epithelium-intact trachea, the low concentration of NIP-121 (0.03 μ M) caused significant inhibition of the contractions induced by low concentrations of LTD₄ (1 and 3 nM). The low concentration (100 μ M) of hydrocortisone also had a slight but significant inhibitory effect on LTD₄ responses. The low concentrations of isoprenaline and aminophylline did not have any significant effect on the LTD₄ response (Figure 4). The high concentrations of these drugs induced marked inhibition of the LTD₄ response. The magnitude of inhibition by these drugs of contraction induced by 3 nM LTD₄ (approximately 40–50% of the histamine maximum response) was similar to that observed against the melittin response in the epithelium-intact trachea, except that the low dose of hydrocortisone had a slight but significant effect on the LTD₄ response.

We have previously reported that epithelium removal causes a significant leftward shift of the LTD₄ concentration-response curve without affecting its maximal response (Shikada & Tanaka, 1992). In the epithelium-denuded trachea, the inhibitory effect of NIP-121 on the LTD₄ contraction was greatly reduced (Figure 5). The reduction of the effect of NIP-121 on the LTD₄ response by removing the epithelium was similar to that obtained with the melittin response. However, higher concentrations of NIP-121 (0.3 and 1 μ M) did inhibit LTD₄-induced contractions of epithelium-denuded trachea (Figure 5).

Discussion

Melittin caused a contraction that was slow in onset of the tracheal strip isolated from a guinea-pig in the presence of the cyclo-oxygenase inhibitor, indomethacin. Our previous study showed that the melittin-induced contractile response was significantly inhibited by both the peptide-leukotriene receptor antagonist, FPL55712, and the lipoxygenase inhibitor, nordihydroguaiaretic acid; similar results were obtained for AA-induced contraction (Yamamoto *et al.*, 1990). We therefore suggested that melittin could contract the guinea-pig isolated trachea by promoting the formation of AA lipoxygenase products. The present data support this hypothesis since not only the PLA₂ inhibitor BPB but also another lipoxygenase inhibitor, phenidone, concentration-dependently and completely, inhibited the melittin response. The inhibitory activity of BPB and phenidone against the melittin response in the epithelium-intact trachea is about the same as that obtained in the epithelium-denuded trachea. This result suggests that when melittin is applied to the trachea, lipoxygenase products are generated in trachealis smooth muscle and thereby induce contraction. It is conceivable that peptide leukotrienes may mediate the melittin response since they are more potent bronchoconstrictors in man (Dahlen *et al.*, 1980; Jones *et al.*, 1982) and guinea-pigs (Drazen *et al.*, 1980; Weichman *et al.*, 1982) than other lipoxygenase products such as LTB₄, which induce cyclo-oxygenase-dependent contraction (Lawson *et al.*, 1986) and lipoxins, which are inactive in tracheal preparations (Jacques *et al.*, 1988).

When the epithelium was removed by rubbing the tracheal luminal surface with cotton, the melittin response was significantly increased. The enhancement of the melittin response by removal of epithelium may be due to the epithelium being a metabolic site of loss for melittin. This interpretation is supported by reports that the epithelium is a site of metabolism for some agents including catecholamines (Farmer *et al.*, 1986) and neurokinins (Devillier *et al.*, 1988). Some inhibitory substances released from epithelium have been reported to suppress tracheal responsiveness against bronchoactive substances (Hay *et al.*, 1987; Fernandes & Goldie, 1990). It is possible that such epithelium-derived putative

inhibitory substances may also modulate the melittin-induced contraction. Streck *et al.* (1992) reported that PLA₂-induced, epithelium-dependent substances increased muscarinic responsiveness and the effect was attenuated by eicosanoid blockage. If melittin-induced epithelium-dependent substances increase airway hyperresponsiveness, the present study further supports the theory that the activation of endogenous PLA₂ is an important step in the development of airway hyperresponsiveness.

Two concentrations of NIP-121, isoprenaline, aminophylline and hydrocortisone which have the ability to suppress the spontaneous tone of the epithelium-intact trachea by about 30–40% and 80–90% respectively, were used to investigate the effect on melittin-induced contraction. Although the low concentration of NIP-121 greatly inhibited the melittin response as compared to the reference drugs, lowering the dose of NIP-121 to 0.01 μ M affected neither the spontaneous tone nor the melittin response. These results suggest that the effects of concentrations of NIP-121 that reduce the melittin contraction are related to the direct inhibitory effects of the drug on the tracheal responses. Furthermore, of the various agents tested, isoprenaline was the most potent inhibitor of the melittin response. The reason for the difference in the potency among these test drugs in inhibiting the melittin response may be the ability of these drugs to inhibit LTD₄-induced contraction. The extent of inhibition of 3 nM-LTD₄ induced contraction (about 40–50% of the histamine maximum) by these drugs was very similar to that of inhibition of the melittin response. These results suggest that the effect of NIP-121 as well as reference drugs on the melittin response may be closely associated with the inhibition of LTD₄-induced contraction by these drugs, and also strongly support the hypothesis that peptide-leukotrienes contribute to the melittin-induced tracheal contraction. The finding that the inhibition of the melittin response by NIP-121 was completely inhibited by the presence of the ATP-sensitive potassium channel blocker, glibenclamide, suggests that the effect of NIP-121 on the melittin response is dependent on activation of the glibenclamide-sensitive potassium channels.

The inhibitory effect of NIP-121 on the melittin response was greatly reduced in the epithelium-denuded trachea while that of the reference drugs was not changed. A similar reduction was also observed in the effect of the standard potassium channel opener, cromakalim, on the melittin response in the epithelium-denuded trachea. We reported recently that NIP-121 and cromakalim augmented the relaxation induced by sodium nitroprusside and that the effect was epithelium-dependent in the guinea-pig isolated trachea (Shikada & Tanaka, 1992). Moreover, Wessler *et al.* (1992) found that potassium channel openers inhibit acetylcholine release in the airway via an indirect, epithelium-dependent pathway. We therefore propose that the potassium channel openers NIP-121 and cromakalim may inhibit the melittin response via an indirect, epithelium-dependent mechanism. This interpretation is supported by reduction of the effect of NIP-121 on the LTD₄ response in the epithelium-denuded trachea. However, since higher concentrations of NIP-121 inhibited LTD₄-induced contractions of epithelium-denuded trachea, we feel that NIP-121 may have at least two mechanisms in the guinea-pig tracheal preparation; a direct relaxation of smooth muscle and an indirect effect via an epithelium-dependent pathway. The mechanism of the action of NIP-121 on the tracheal epithelium is not clear but may be related to an increase in either the release of epithelium-dependent inhibitory substances or their activity.

In conclusion, melittin causes epithelium-dependent contraction of the guinea-pig isolated trachea which is mediated by products of lipoxygenase activity. NIP-121 may inhibit the melittin response by activating glibenclamide-sensitive potassium channels, which appears to be an epithelium-dependent, indirect effect of NIP-121 apart from its direct effect on the airway smooth muscle, while isoprenaline, aminophylline and hydrocortisone primarily act directly to relax the trachealis smooth muscle.

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