Potassium channel openers, NIP-121 and cromakalim, enhance the relaxation induced by sodium nitroprusside in the guinea-pig isolated trachea

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1 The effect of the potassium channel openers, NIP-121 and cromakalim, on agonist-induced relaxation of the guinea-pig isolated trachea was investigated and the results were compared with those in the epithelium-denuded trachea.

2 Tracheal strips were incubated with a potassium channel opener or vehicle for 30 min in the presence of 5 μM indomethacin and then contracted with 30 nM leukotriene D_4 (LTD_4). Relaxant agents were added to the organ bath after the LTD_4 -elicited contraction had reached a plateau.

3 In epithelium-intact trachea, NIP-121 0.1 μM and cromakalim 1 μM , which did not modify the LTD_4 (30 nM)-induced contraction, significantly enhanced the sodium nitroprusside (SNP)-induced relaxation. This enhancement of relaxation was not seen in the case of relaxation induced by the cyclic AMP-dependent bronchodilators isoprenaline, vasoactive intestinal peptide or prostaglandin E_2 . The enhancement of SNP-induced relaxation by NIP-121 and cromakalim was abolished in the presence of the ATP-sensitive potassium channel blocker, glibenclamide (1 μM). NIP-121 and cromakalim did not produce any significant changes in the relaxation induced by 8-bromoguanosine-cyclic monophosphate (8-Br-cyclic GMP), a cyclic GMP analogue.

4 In epithelium-denuded trachea, SNP-induced relaxation alone was significantly enhanced but that induced by 8-Br-cyclic GMP was not changed. Neither NIP-121 nor cromakalim enhanced SNP-induced relaxation in denuded trachea.

5 These results suggest that in the presence of an intact epithelium the enhancement by NIP-121 and cromakalim of the relaxation of guinea-pig tracheal smooth muscle induced by SNP may be associated with the opening of glibenclamide-sensitive potassium channels.

Keywords: Potassium channel opener; NIP-121; cromakalim; sodium nitroprusside; isoprenaline; vasoactive intestinal peptide; prostaglandin E_2 ; leukotriene D_4 ; epithelium; guinea-pig trachea

Introduction

The potassium channel opener, cromakalim, relaxes guinea-pig (Allen *et al.*, 1986; Arch *et al.*, 1988), bovine (Gater, 1989; Longmore *et al.*, 1991) and human (Taylor *et al.*, 1988; Black *et al.*, 1990) airway smooth muscle as well as vascular smooth muscle (Cox, 1990). Cromakalim is effective not only in preventing histamine-induced collapse in unanaesthetized guinea-pigs (Arch *et al.*, 1988) but also cholinergic- and noncholinergic-mediated bronchoconstriction in guinea-pig isolated airways (McCaig & De Jonckheere, 1989; Ichinose & Barnes, 1990). Cromakalim stimulates the efflux of potassium ions from tracheal smooth muscle (Allen *et al.*, 1986; Gater, 1989; Longmore *et al.*, 1991) but does not affect intracellular cyclic nucleotide levels (Murray *et al.*, 1990). Since the pharmacological profile of cromakalim is different from clinically used bronchodilators, β -adrenoceptor agonists or xanthines, other potassium channel openers are being developed as bronchodilators (Paciorek *et al.*, 1990; Raeburn & Brown, 1991). NIP-121 (Figure 1) is a potent newly synthesized potassium channel opener (Masuda *et al.*, 1991), and it has about 10–20 times greater potency in relaxing guinea-pig isolated tracheal contraction than cromakalim (Shikada *et al.*, 1991b).

Bronchial epithelium has been reported to play an important role in modulating the responsiveness of airway smooth muscle to drugs. Mechanical removal of the epithelium from the isolated trachea increases the responsiveness to various bronchoconstrictors; histamine (Braunstein *et al.*, 1988), acetylcholine (Holroyde, 1986), leukotrienes (Hay *et al.*, 1987), adenosine (Advenier *et al.*, 1988), substance P (Devillier *et al.*, 1988) and endothelin (Hay, 1990), and also enhances the responsiveness to the bronchodilators

isoprenaline (Lennart Lundblad & Persson, 1988) and sodium nitroprusside (SNP) (Farmer *et al.*, 1986). These results indicate that the presence or the absence of epithelium is an important factor to be taken into account in investigating the responsiveness of guinea-pig isolated trachea to bronchoactive substances.

In the present study we investigated the effect of the potassium channel openers, NIP-121 and cromakalim, on the responsiveness of guinea-pig isolated trachea to a range of bronchodilators and the results were compared with those obtained with epithelium-denuded trachea.

Methods

Tracheae were removed from male Hartley guinea-pigs (250–400 g) stunned by a blow to the head. Each trachea

Figure 1 Chemical structure of NIP-121.

was cut spirally and divided into two or three segments (one was the 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. The contractility was measured isotonically (Type TD-112 S, Nihon Kohden). After the tissues had equilibrated for 50–60 min, maximal response to histamine (100 µM) was obtained. 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 baseline tension and were then incubated for an additional 30 min with a potassium channel opener (either NIP-121, 0.1 µM or cromakalim, 1 µM), its blocker (glibenclamide 1 µM) or with the combination of either NIP-121 or cromakalim and glibenclamide in the presence of 5 µM indomethacin. Leukotriene D₄ (LTD₄) at a concentration of 30 nM was added to the organ bath. Contractile responses induced by LTD₄ were not attenuated by either NIP-121 or cromakalim (at the above concentrations) in either epithelium-intact or epithelium-denuded tracheas. After the contractile response elicited by LTD₄ had reached a plateau, one concentration-relaxation curve was obtained for a relaxant in each tissue. 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. The negative logarithms of EC₅₀ values (concentration producing 50% of the maximal relaxant response) were calculated by linear regression analysis applied to the linear portion of each concentration-response curve. All results were expressed as means ± s.e.mean, and statistical significance ($P < 0.05$) was determined by Student's one-tailed *t* test for paired observations.

Drugs

The following agents were used: LTD₄ (Ultrafine Chemicals, England); vasoactive intestinal peptide (VIP), (–)-isoprenaline hydrochloride, aminophylline, indomethacin, 8-bromoguanosine 3':5'-cyclic monophosphate (8-Br-cyclic GMP), glibenclamide, pyrilamine maleate, nordihydroguaiaretic acid (NDGA) (Sigma Chemical Co., USA); histamine dihydrochloride, SNP (Wako Pure Chemical Industries, Japan); atropine sulphate (E. Merck, Germany); prostaglandin E₂ (PGE₂) (Nacalai Tesque, Japan); phosphoramidon (Peptide Institute, Japan); NIP-121 and cromakalim were synthesized by the Nissan Chemical Industries Ltd. (Central Research Laboratory, Japan).

Indomethacin was dissolved in 100% ethanol, glibenclamide, NDGA, PGE₂, NIP-121 and cromakalim were dissolved in 100% dimethylsulphoxide. Other drugs were dissolved in distilled water. Drug vehicles did not cause any change in the tracheal response.

Results

Effect of NIP-121 and cromakalim on agonist-induced relaxation of the guinea-pig isolated trachea contracted with 30 nM leukotriene D₄

We previously showed that in guinea-pig isolated trachea the relaxant effect of a bronchodilator was greater when the magnitude of LTD₄-induced contraction was smaller (Shikada *et al.*, 1991a). For this reason, concentrations of NIP-121 and cromakalim that did not affect the LTD₄-induced contraction were used in the present study. At these concentrations NIP-121, 0.1 µM and cromakalim, 1 µM significantly enhanced SNP-induced relaxation as compared

with paired controls (Figure 2, a,b) seen as a leftward shift in the respective concentration-response curves for SNP. Neither NIP-121 nor cromakalim affected either the isoprenaline-induced relaxation (Figure 2, c,d) or that induced by VIP or PGE₂ (Table 1). SNP, isoprenaline and VIP caused complete relaxation while PGE₂ produced partial relaxation (76 ± 4%). Neither NIP-121 nor cromakalim caused a significant change in the maximal relaxation induced by PGE₂ (NIP-121, 73 ± 8%; cromakalim, 77 ± 6%, $P > 0.05$). Higher concentrations of NIP-121 (> 0.1 µM) and cromakalim (> 1 µM) were not used in the present study because they strongly inhibited the 30 nM LTD₄-induced contraction (data not shown).

Figure 2 Effect of NIP-121, 0.1 µM (●; a,c) and cromakalim, 1 µM (●; b,d) on dose-response curve to sodium nitroprusside (a,b) or isoprenaline (c,d) in the guinea-pig isolated trachea contracted with leukotriene D₄ (30 nM). Each point represents the mean of 5–7 preparations in paired control tissues and in test tissues; (± s.e.mean) shown by vertical lines. *Indicates a significant difference from the corresponding values in the paired control (○).

Table 1 Effect of NIP-121 (0.1 μ M) and cromakalim (1 μ M) on agonist-induced relaxation of the isolated guinea-pig trachea contracted with leukotriene D₄ (LTD₄, 30 nM)

Condition	NIP-121 (0.1 μ M)		Cromakalim (1 μ M)	
	% contraction	$-\log EC_{50}$	% contraction	$-\log EC_{50}$
Sodium nitroprusside				
Control	74 $\pm$ 3	6.72 $\pm$ 0.08	69 $\pm$ 4	6.74 $\pm$ 0.10
Test	74 $\pm$ 3	6.96 $\pm$ 0.08*	64 $\pm$ 3	7.02 $\pm$ 0.10*
Isoprenaline				
Control	75 $\pm$ 3	8.42 $\pm$ 0.06	71 $\pm$ 4	8.28 $\pm$ 0.11
Test	78 $\pm$ 1	8.46 $\pm$ 0.08	72 $\pm$ 3	8.35 $\pm$ 0.14
VIP				
Control	75 $\pm$ 4	8.25 $\pm$ 0.02	75 $\pm$ 3	8.44 $\pm$ 0.05
Test	78 $\pm$ 2	8.24 $\pm$ 0.01	70 $\pm$ 6	8.48 $\pm$ 0.05
PGE ₂				
Control	80 $\pm$ 3	6.05 $\pm$ 0.06	86 $\pm$ 4	6.23 $\pm$ 0.12
Test	78 $\pm$ 3	6.09 $\pm$ 0.12	80 $\pm$ 7	6.18 $\pm$ 0.17

Data are the means $\pm$ s.e.mean of 4–7 preparations in paired control tissues and in test tissues. % contraction refers to the % of that induced by histamine. VIP: vasoactive intestinal peptide; PGE₂: prostaglandin E₂. *Indicates a significant difference from the corresponding values in the paired control.

Table 2 shows that the ATP-sensitive potassium channel blocker, glibenclamide (1 μ M), had little effect on SNP-induced relaxation but it prevented the enhancement of SNP relaxation induced by NIP-121 or cromakalim. This result suggests that the enhancement of SNP-induced relaxation by NIP-121 and cromakalim is dependent upon the activation of ATP-sensitive potassium channels.

SNP is known to be a guanylate cyclase stimulator and to increase intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) levels. 8-Br-cyclic GMP, which is an analogue of cyclic GMP, produced concentration-dependent and maximum relaxation of the LTD₄ contraction. Neither NIP-121 nor cromakalim caused any significant change in 8-Br-cyclic GMP-induced relaxation (data not shown).

Effect of NIP-121 and cromakalim on sodium nitroprusside-induced relaxation in epithelium-denuded trachea

When the tracheal lumen was gently rubbed with cotton, the LTD₄ concentration-response curve was significantly shifted to the left as compared with the paired control ($-\log EC_{50}$: rubbed trachea 8.49 $\pm$ 0.09; control 8.26 $\pm$ 0.10, $P < 0.05$) without affecting the maximum response at a concentration of 30 nM LTD₄. The relaxation induced by SNP in rubbed

trachea elicited by 30 nM LTD₄ was significantly greater than that in the paired control, but that induced by 8-Br-cyclic GMP was not changed (Table 3). Neither NIP-121 nor cromakalim caused a significant leftward shift of the concentration relaxation curve in response to SNP in the rubbed trachea (Figure 3). The maximal relaxation was unchanged by these compounds (data not shown).

Effect of various inhibitors on sodium nitroprusside-induced relaxation of intact tracheal preparation

Farmer *et al.*, (1986) suggested that SNP may cause the tracheal epithelium to release a smooth muscle excitatory factor. It could therefore be hypothesized that NIP-121 and cromakalim might either modulate the release of, or antagonize the action of, such a factor. In an attempt to verify this supposition we examined whether SNP-induced relaxation was influenced by either selective antagonists or inhibitors of putative candidates for such a factor, namely acetylcholine, substance P, histamine and peptide leukotrienes. SNP-induced relaxation of the intact trachea contracted with 30 nM LTD₄ was not influenced by the anticholinergic agent, atropine at 1 μ M, the neutral endopeptidase inhibitor, phosphoramidon at 10 μ M, the antihistamine,

Table 2 Effect of NIP-121 (0.1 μ M) and cromakalim (1 μ M) in the presence of glibenclamide (GBC, 1 μ M) on sodium nitroprusside-induced relaxation of the guinea-pig isolated trachea contracted with leukotriene D₄ (30 nM)

Condition	% contraction	$-\log EC_{50}$
Control	75 $\pm$ 3	6.87 $\pm$ 0.08
GBC	78 $\pm$ 3	6.80 $\pm$ 0.05
Control	78 $\pm$ 4	6.72 $\pm$ 0.11
NIP-121 + GBC	80 $\pm$ 3	6.76 $\pm$ 0.08
Control	77 $\pm$ 3	6.63 $\pm$ 0.08
Cromakalim + GBC	80 $\pm$ 3	6.55 $\pm$ 0.08

Data are the means $\pm$ s.e.mean of 5 preparations in paired control tissues and in test tissues. % contraction refers to the % of that induced by histamine.

Table 3 Effect of epithelium removal on sodium nitroprusside- and 8-Br-cyclic GMP-induced relaxation of the guinea-pig isolated trachea contracted with leukotriene D₄ (30 nM)

Condition	% contraction	$-\log EC_{50}$
Sodium nitroprusside		
Control	81 $\pm$ 3	6.73 $\pm$ 0.09
Rubbed	82 $\pm$ 4	7.01 $\pm$ 0.08*
8-Br-cyclic GMP		
Control	81 $\pm$ 4	4.96 $\pm$ 0.03
Rubbed	83 $\pm$ 3	4.92 $\pm$ 0.06

Data are the means $\pm$ s.e.mean of 5 preparations in paired control tissues and in tested tissues. % contraction refers to the % of that induced by histamine.

*Indicates a significant difference from the corresponding values in the paired control.

Figure 3 Lack of effect of NIP-121, 0.1 μM (●, a) and cromakalim, 1 μM (●, b) on dose-response curve to sodium nitroprusside in epithelium-rubbed guinea-pig trachea contracted with leukotriene D_4 (30 nM). Each point represents the mean of 5 preparations in paired control tissues (○) and in test tissues (●); ($\pm$ s.e.mean) shown by vertical lines.

pyrilamine at 1 μM , or the lipoxygenase inhibitor, NDGA at 10 μM (Table 4).

Discussion

NIP-121 at 0.1 μM and cromakalim at 1 μM did not inhibit the LTD_4 (30 nM)-induced contraction but significantly enhanced the SNP-induced relaxation to the same extent in the guinea-pig isolated trachea. We previously showed that the relaxant effect of a bronchodilator in the guinea-pig isolated trachea increased as the magnitude of the contractile response decreased (Shikada *et al.*, 1991a). Because higher concentrations of NIP-121 ($>0.1 \mu\text{M}$) and cromakalim ($>1 \mu\text{M}$) markedly inhibited LTD_4 (30 nM)-induced contraction, we used 0.1 μM NIP-121 and 1 μM cromakalim, which did not affect the LTD_4 -induced contraction, for investigating the effect of these potassium channel openers on agonist-induced relaxation. Since these concentrations of NIP-121 and cromakalim caused a marked and similar reduction in the spontaneous tone in the guinea-pig isolated trachea (Shikada *et al.*, 1991b), it seems reasonable to assume that

Table 4 Effect of modulators affecting bronchoactive substances on sodium nitroprusside-induced relaxation of the guinea-pig trachea contracted with leukotriene D_4 (30 nM)

Condition	% contraction	$-\log EC_{50}$
Control	77 ± 3	6.73 ± 0.14
Atropine, 1 μM	77 ± 6	6.66 ± 0.19
Phosphoramidon, 10 μM	68 ± 3	6.64 ± 0.10
Control	81 ± 5	6.67 ± 0.05
Pyrilamine, 1 μM	85 ± 5	6.57 ± 0.05
NDGA, 10 μM	88 ± 8	6.56 ± 0.13

Data are the means $\pm$ s.e.mean of 4–5 preparations in paired control tissues and in test tissues. % contraction refers to the % of that induced by histamine.

they are potassium channel opening concentrations, although sub-maximal in this respect. The enhancement of SNP-induced relaxation by NIP-121 or cromakalim was abolished when the ATP-sensitive potassium channel blocker, glibenclamide, was present with NIP-121 or cromakalim. These results suggest that both NIP-121 and cromakalim enhance SNP-induced relaxation by stimulating ATP-sensitive potassium channels.

NIP-121 and cromakalim enhanced SNP-induced relaxation but did not cause a significant change in the relaxation induced by the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent bronchodilators isoprenaline, VIP and PGE_2 . Since SNP has been used as a guanylate cyclase stimulator and to increase intracellular cyclic GMP levels, a possible interpretation is that NIP-121 and cromakalim selectively enhance cyclic GMP-dependent relaxation in the guinea-pig trachea. However, neither NIP-121 nor cromakalim enhanced the relaxation induced by 8-Br-cyclic GMP, a cyclic GMP analogue. This result suggests that both NIP-121 and cromakalim enhance SNP-induced relaxation without affecting cyclic GMP-induced relaxation of the tracheal smooth muscle.

In epithelium-rubbed trachea, NIP-121 and cromakalim failed to enhance SNP-induced relaxation. These results suggest that NIP-121 and cromakalim enhance SNP-induced relaxation in the tracheal smooth muscle by stimulating ATP-sensitive potassium channels present in the epithelium. Farmer *et al.*, (1986) stated that nitroprusside may cause the epithelium to release a smooth muscle excitatory factor. In this study the dose-relaxation curve in response to SNP in epithelium-rubbed trachea was significantly shifted to the left, but that in response to 8-Br-cyclic GMP was not changed. If putative smooth muscle excitatory factors are released from the epithelium during SNP-induced relaxation, an alternative interpretation is that NIP-121 and cromakalim counteract the release of these factors from the epithelium or inhibit the action of these factors on smooth muscle. However, the anti-cholinoceptor agent, atropine, the anti-histamine, pyrilamine, the 5-lipoxygenase-inhibitor, NDGA and the neutral endopeptidase inhibitor, phosphoramidon had no effect on SNP-induced relaxation of epithelium-intact trachea. These results suggest that acetylcholine, histamine, peptide-leukotrienes and substance P are not involved in SNP-induced relaxation. We previously found that NIP-121 and cromakalim strongly relax prostanoind-induced contraction of the isolated trachea (Shikada *et al.*, 1991b). It is extremely unlikely that prostaglandins are released and modulate SNP-induced relaxation, because our experiments were carried out in the presence of the cyclo-oxygenase inhibitor, indomethacin.

Another possible interpretation is that both NIP-121 and cromakalim act as selective inhibitors of cyclic GMP-dependent phosphodiesterase and cause a synergistic effect on SNP-induced relaxation. Since NIP-121 and cromakalim failed to enhance SNP-induced relaxation in the epithelium-rubbed trachea, neither compound may be able to act as an inhibitor of cyclic GMP-dependent phosphodiesterase in the tracheal smooth muscle. However, it may still be possible for NIP-121 and cromakalim to act as inhibitors of cyclic GMP-dependent phosphodiesterase in the guinea-pig tracheal epithelium.

In conclusion, the present results suggest that in the presence of an intact epithelium, the enhancement by NIP-121 and cromakalim of the relaxation of the guinea-pig trachea induced by SNP may be associated with the opening of glibenclamide-sensitive potassium channels. The present findings indicate that potassium channel openers may regulate the airway responsiveness via an indirect, epithelium-dependent pathway, in addition to providing direct bronchodilatation. The reason for the inability of either NIP-121 or cromakalim to enhance the relaxant effects of the other bronchodilators used in this study is unclear. This finding may be related to these bronchodilators possibly

already opening potassium channels to the extent that the sub-maximal potassium channel-opening concentrations of

NIP-121 and cromakalim used had no additional relaxant effect.

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