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The interaction of α-human atrial natriuretic peptide (ANP) with salbutamol, sodium nitroprusside and isosorbide dinitrate in human bronchial smooth muscle

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1 Contractions in human bronchial rings evoked by methacholine (10^{-6} M) were reversed by single contractions of α-human atrial natriuretic peptide (10^{-6} M), salbutamol (10^{-6} M), sodium nitroprusside (10^{-6} M) or isosorbide dinitrate (4.2×10^{-5} M) and the extent of the relaxations compared. The activity of combinations of ANP with salbutamol, sodium nitroprusside and isosorbide dinitrate were compared with those for each agonist alone.

2 ANP and salbutamol were equipotent in reversing methacholine-evoked contraction and, in combination these agonists evoked an additive response. ANP and sodium nitroprusside also evoked similar degrees of relaxation and were additive, as were ANP and isosorbide dinitrate; however, with isosorbide dinitrate a higher concentration was required to evoke the same degree of relaxation as ANP, sodium nitroprusside or salbutamol.

3 Cumulative concentration-response curves to methacholine (10^{-9}–3×10^{-4} M) were examined in the presence and absence of the above bronchodilator substances, alone and in combination allowing their abilities to protect against contraction to be compared. ANP (10^{-6} M) and salbutamol (10^{-6} M) each attenuated subsequent contractions evoked by methacholine, an ability not shared with sodium nitroprusside (10^{-6} M) or isosorbide dinitrate (4.2×10^{-5} M). Indeed at lower concentrations of methacholine ($<3 \times 10^{-7}$ M), sodium nitroprusside evoked a paradoxical enhancement of methacholine-evoked contractions.

4 In combination, ANP and salbutamol attenuated contractions evoked by methacholine to a significantly greater degree than that seen with either agonist alone, whilst a combination of ANP and sodium nitroprusside evoked no greater effect than that seen with ANP alone. By contrast, isosorbide dinitrate and ANP together evoked a greater inhibition than ANP alone.

5 These results suggest that a combination of agents such as ANP and salbutamol evokes a greater effect than either alone, both in reversing and protecting against methacholine-evoked contractions. Such combinations may be of benefit in the treatment of patients, allowing lower doses of drug to be used. Combinations of ANP and isosorbide dinitrate may likewise be of interest; however, the mechanism underlying the enhancement of ANP responses by isosorbide dinitrate requires further study.

Keywords: Atrial natriuretic peptide; salbutamol; sodium nitroprusside; isosorbide dinitrate

Introduction

α-Human atrial natriuretic peptide (ANP) is a naturally occurring 28 amino acid peptide, released from the atria (Sagnella *et al.*, 1984) and from isolated lung tissue (Gutkowska & Nemer, 1989). Its mechanism of action has been postulated as being via activation of particulate guanylate cyclase leading to elevation of guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Ishii & Murad, 1989) and it has been shown to have vasodilator (Sagnell *et al.*, 1984) natriuretic (Gutkowska & Nemer, 1989) and diuretic (Anderson *et al.*, 1986) properties. More recently, studies *in vitro* and *in vivo* have shown that ANP may have activity in the lungs. Infused ANP evokes a bronchodilator effect in asthmatic patients (Hulks *et al.*, 1989; Angus *et al.*, 1993) and confers protection against subsequent histamine challenge (Hulks *et al.*, 1991) or the effects of nebulised distilled water (McAlpine *et al.*, 1992). Inhalation of ANP has been demonstrated to produce a small bronchodilatation (Hulks & Thomson, 1994) and to protect against subsequent histamine (Hulks & Thomson, 1992) or methacholine (Angus *et al.*, 1994a) challenge. *In vitro*, ANP evokes reversal of methacholine-evoked contraction as well as protecting against subsequent challenge with this agonist. Each of these effects was greatly enhanced by the presence of the neutral endopeptidase inhibitor, phosphoramidon, (Angus *et al.*, 1994b). As a result of the above studies, the possible therapeutic use of ANP in asthma has been postulated, possibly as an adjunct to current therapy. Its relative effectiveness compared to currently available bronchodilators has not however been established, nor has the ability of ANP to interact with such therapies been studied.

In this present study, a direct comparison of ANP with other bronchodilators has been undertaken *in vitro*, to examine further the possible usefulness of this peptide in asthma. The initial bronchodilator chosen for comparison was the β_2-adrenoceptor agonist salbutamol, which is thought to act via K^+channel opening (Miura *et al.*, 1992) as well as via adenosine 3':5'-cyclic monophosphate (cyclic AMP) activation (Rinard *et al.*, 1983). In addition, the effects of sodium nitroprusside and isosorbide dinitrate, each of which is thought to act as a nitric oxide donator (Feelisch & Noack, 1987), resulting in activation of soluble, rather than particulate guanylate cyclase, have been compared to ANP. The extent to which ANP interacts with each of these dilators has been evaluated.

Methods

Tissue collection and preparation

Macroscopically normal human bronchi (3rd to 6th order) were obtained from patients undergoing thoracic surgery.

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Tissues were dissected free of connective tissue and fat and stored overnight at 4°C in oxygenated Krebs-Henseleit solution of the following composition (mM): NaCl 118.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2 and glucose 11.1. Published data (Brink *et al.*, 1980) have shown that overnight storage of this tissue does not alter its reactivity.

Measurement of contractile responses

Contractile responses were measured from rings of human bronchi (3–5 mm) in vertical organ baths (10 ml) at 37 ± 0.5°C in oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution. Tension (2 g wt.) was applied by inserting two platinum wires into the lumen. One wire was anchored and the other attached to a force displacement transducer (Grass FT03T). After pre-incubation of tissues with methacholine (10^{-6} M), cumulative concentration-response curves were constructed to ANP (10^{-8}–3×10^{-6} M), salbutamol (10^{-8}–10^{-5} M), sodium nitroprusside (10^{-8}–10^{-5} M) and isosorbide dinitrate (4.2×10^{-7}–4.2×10^{-4} M) in order to establish pD_2 values for these dilators. These concentrations of agonists were subsequently used to reverse contractions evoked by methacholine (3×10^{-6} M). In this and in all subsequent experiments where ANP was used, the neutral endopeptidase inhibitor phosphoramidon (3.67×10^{-5} M) was present to prevent ANP's breakdown. Combinations of ANP with each of these agonists were then compared with the agonists alone.

In a second set of experiments, cumulative concentration-response curves were constructed to methacholine in the presence and absence of ANP, salbutamol, sodium nitroprusside and isosorbide dinitrate, alone and in combination. Drugs were added directly to the organ bath.

Materials

The following chemicals were used; α-human atrial natriuretic peptide 28 amino acid (ANP, Bachem), isosorbide dinitrate (Isoket, Schwarz Pharma Ltd.), methacholine chloride (Sigma), N-(α-rhamnopyranosyloxyhydroxy-phosphinyl)-L-leucyl-L-tryptophan (Phosphoramidon, Sigma), salbutamol (Sigma), sodium nitroprusside (Sigma). Concentration in text refers to the salts, with the exception of salbutamol which is expressed as the base. Stock solutions of drugs were prepared in distilled water and subsequent dilutions made in Krebs-Henseleit solution.

Analysis of results

Results are expressed as mean ± s.e. mean. Statistical significance between data samples was tested by two-way analysis of variance. Significance between pD_2 values (the negative log of the concentration evoking 50% of the maximum response) was calculated by Student's *t* test. A probability level of $P<0.05$ was considered significant. Number of observations (*n*) refers to the number of animals used.

Results

After pre-constriction of tissues with methacholine (10^{-6} M, the EC_{50} for methacholine in this tissue), cumulative concentration-response curves were constructed to ANP (10^{-8}–3×10^{-6} M, in the presence of phosphoramidon (3.67×10^{-5} M)) (Figure 1), to salbutamol (10^{-8}–10^{-5} M) (Figure 1), to sodium nitroprusside (10^{-8}–10^{-5} M) and to isosorbide dinitrate (4.2×10^{-7}–4.2×10^{-4} M) from which pD_2 values of 5.91 ± 0.1, 6.27 ± 0.18, 6.05 ± 0.2 and 4.8 ± 0.3 respectively were calculated.

In order to examine additive effects of bronchodilators, without reaching maximal dilatation in these tissues, methacholine 3×10^{-6} M was used to pre-constrict tissues in subsequent experiments rather than the 10^{-6} M used in the calculation of pD_2 values. The use of the higher concentrations of methacholine resulted in small relaxations being evoked by the dilators, since there is an inverse relationship between the level of airway tone and the potency of relaxant agonists (Van den Brink, 1973; Angus *et al.*, 1994b). Contractions of the bronchial rings evoked by single concentrations of methacholine (3×10^{-6} M) were reversed by ANP (16.4 ± 2.8% inhibition, $n=6$) and salbutamol (23.8 ± 13.4% inhibition, $n=6$) each at 10^{-6} M. A combination of ANP and salbutamol (each at 10^{-6} M) evoked an additive (43.3 ± 8.7%, $n=6$) inhibition of methacholine-evoked tone compared with these agonists alone (Figure 2a). Phosphoramidon alone (3.67×10^{-5} M) did not evoke any reversal of methacholine-evoked tone in these tissues. ANP (10^{-6} M) alone and in combination with either sodium nitroprusside (10^{-6} M) or isosorbide dinitrate (4.2×10^{-5} M) was also examined. A combination of ANP and sodium nitroprusside similarly evoked a relaxation (36.9 ± 8.6%, $n=6$) which was significantly greater than that evoked by sodium nitroprusside (16.4 ± 7.6%, $n=6$) or ANP (22.6 ± 4.7%, $n=6$) alone (Figure 2b) as did a combination of ANP and isosorbide dinitrate (40.1 ± 6, compared with 15.6 ± 2.8 and 19.3 ± 2.1% respectively, Figure 2c, $n=6$).

Cumulative concentration-response curves were constructed to methacholine (10^{-9}–3×10^{-4} M) in the presence and absence of phosphoramidon (3.67×10^{-5} M) alone and ANP (10^{-6} M, in the presence of phosphoramidon as previously described), salbutamol (10^{-6} M), sodium nitroprusside, (10^{-6} M) and isosorbide dinitrate (4.2×10^{-5} M), either alone or in combination. Phosphoramidon alone did not alter subsequent methacholine challenge; however, the presence of either ANP or salbutamol attenuated (each $P<0.001$) the contractions evoked by methacholine (pD_2 values; methacholine alone; 6.01 ± 0.09, $n=6$; methacholine plus ANP; 5.68 ± 0.15, $n=6$; methacholine plus salbutamol; 5.43 ± 0.17, $n=6$, $P<0.05$ compared to control in each case. Figure 3). ANP, but not salbutamol evoked a significant ($P<0.005$) decrease in maximum response. Neither the presence of sodium nitroprusside ($n=9$, Figure 4) nor of isosorbide dinitrate ($n=6$, Figure 5) alone significantly altered the contractions to methacholine. ANP and salbutamol in combination evoked a significant attenuation ($P<0.001$) of the methacholine responses compared with either agonist alone (Figure 3). In this case, the pD_2 value in

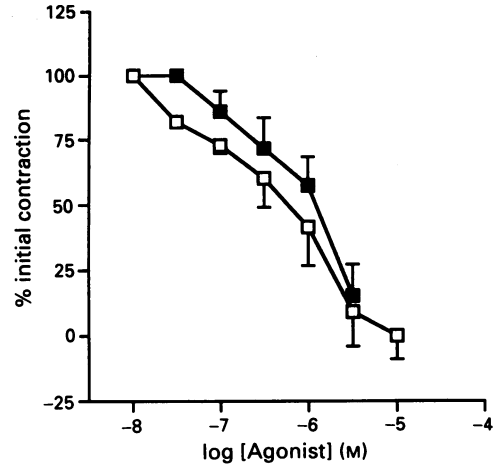

Figure 1 Concentration-response curves (CRCs) evoked by atrial natriuretic peptide (ANP 3×10^{-8}–3×10^{-6} M), in the presence of phosphoramidon (3.67×10^{-5} M, ■) and salbutamol (10^{-8}–10^{-5} M, □) after pre-constriction of human bronchial rings with methacholine (10^{-6} M). ANP (+phosphoramidon) and salbutamol each evoked concentration-dependent relaxations of evoked tone. Number of observations (n) = 6 in each case.

the presence of a combination of these agonists (5.48 ± 0.2), was not significantly different from that evoked by either agonist alone, however there was a decrease in the magnitude of contraction at each methacholine concentration, with the decrease in maximum response being 43%

ANP and sodium nitroprusside combined were no more effective than ANP alone (pD_2 for methacholine alone, 5.99 ± 0.1, plus ANP, 5.59 ± 0.18 ($P<0.05$ compared with control), plus sodium nitroprusside, 6.07 ± 0.23 plus both, 5.71 ± 0.17, $n = 9$, Figure 4).

ANP and isosorbide dinitrate in combination evoked a greater attenuation of the methacholine-evoked curve than did ANP alone ($P<0.005$). This was not reflected in a rightward shift in the concentration-response curves (pD_2 for methacholine alone, 5.97 ± 0.09, plus ANP, 5.68 ± 0.22, plus isosorbide dinitrate 5.82 ± 0.18 and plus both, 5.78 ± 0.11), but rather in suppression of the magnitude of the responses evoked by methacholine at concentrations above 10^{-5} M. The maximum response in the presence of such a combination was decreased by 48% compared with control (Figure 5).

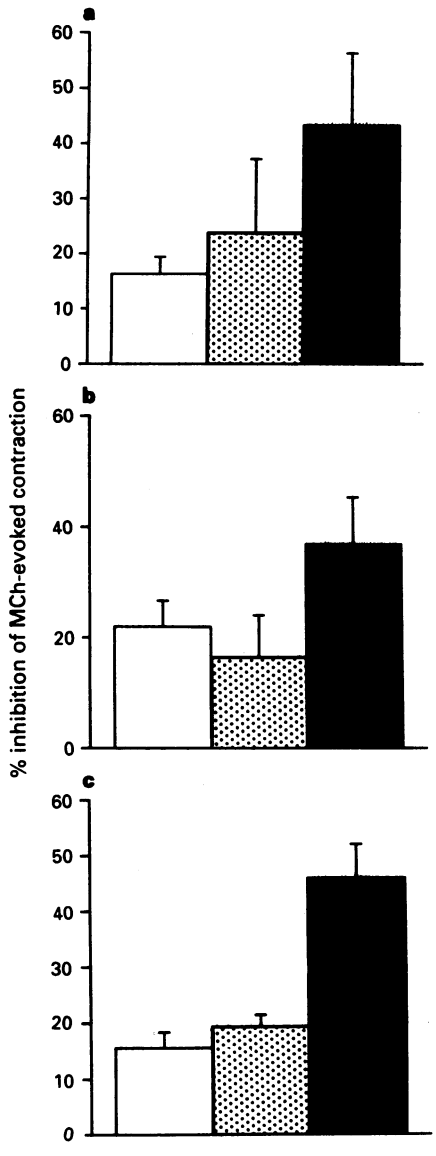

Figure 2 Relaxations of methacholine-evoked contractions (3×10^{-6} M), evoked by (a) atrial natriuretic peptide (ANP 10^{-6} M, open column) and salbutamol (10^{-6} M, stippled column) alone and in combination (solid column); (b) ANP (10^{-6} M, open column) and sodium nitroprusside (10^{-6} M, stippled column) alone and in combination (solid column) and (c) ANP (10^{-6} M, clear column) and isosorbide dinitrate (4.2×10^{-5} M, stippled column) alone and in combination (solid column). In each case where ANP was used, phosphoramidon (3.67×10^{-5} M) was present to inhibit its degradation. Alone, each of these agonists evoked similar relaxations at the concentrations used, while combinations of ANP with salbutamol, sodium nitroprusside or isosorbide dinitrate evoked additive responses. Number of observations (n) = 6 in each case.

Discussion

These results suggest for the first time, that, at the concentration chosen, ANP and salbutamol can evoke similar relaxa-

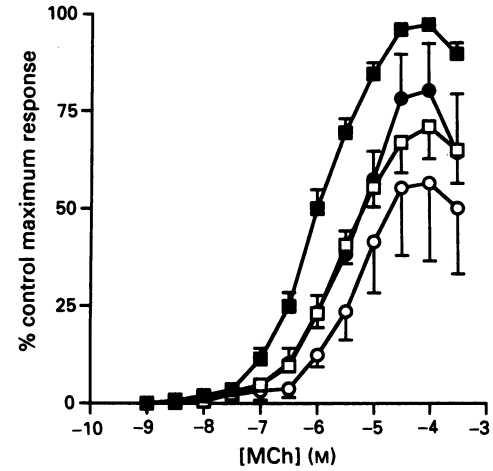

Figure 3 Concentration-response curves (CRCs) evoked by methacholine (MCh, 10^{-9}-3×10^{-4} M) were constructed in human bronchial rings in the absence (■, $n = 12$) and in the presence of atrial natriuretic peptide (ANP) (□, 10^{-6} M, $n = 6$) or salbutamol (●, 10^{-6} M, $n = 6$) alone and (○) in combination ($n = 6$). Where ANP was used, phosphoramidon (3.67×10^{-5} M) was present to inhibit its degradation. ANP and salbutamol each evoked similar rightward shifts of the MCh-evoked concentration-response curve. A combination of these agonists evoked a significantly greater attenuation than that evoked by either alone.

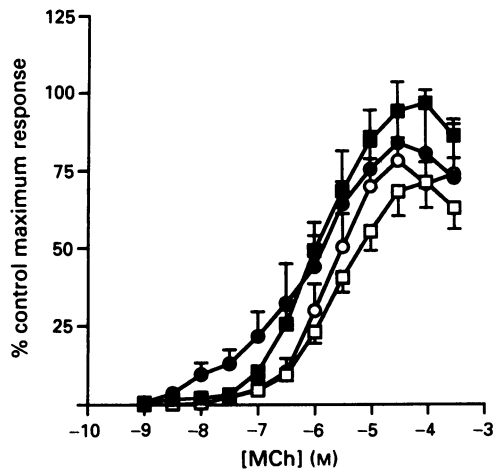

Figure 4 Concentration-response curves (CRCs) evoked by methacholine (10^{-9}-3×10^{-4} M) were constructed in human bronchial rings in the absence (■, $n = 15$) and in the presence of atrial natriuretic peptide (ANP, □, 10^{-6} M, $n = 6$) or sodium nitroprusside (●, 10^{-6} M, $n = 9$) alone and (○) in combination ($n = 6$). Where ANP was used, phosphoramidon (3.67×10^{-5} M) was present to inhibit its degradation. In contrast to ANP, sodium nitroprusside did not evoke any rightward shift of the methacholine-evoked concentration-response curve and indeed enhanced lower concentrations of methacholine. A combination of these agonists evoked no greater attenuation than that evoked by ANP alone.

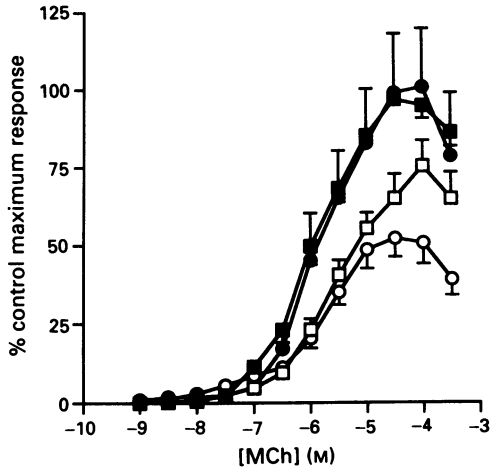

Figure 5 Concentration-response curves (CRCs) evoked by methacholine (10^{-9}–3×10^{-4} M) were constructed in human bronchial rings in the absence (■, $n = 12$) and in the presence of atrial natriuretic peptide (ANP, □, 10^{-6} M, $n = 6$) or isosorbide dinitrate (●, 4.2×10^{-5} M, $n = 6$) alone and (○) in combination ($n = 6$). Where ANP was used, phosphoramidon (3.67×10^{-5} M) was present to inhibit its degradation. In contrast to ANP, isosorbide dinitrate did not evoke any rightward shift of the methacholine-evoked concentration-response curve, however, a combination of these agonists evoked a greater reduction of the maximum response than that evoked by ANP alone.

tion of methacholine-evoked contraction, with a combination of these agonists evoking an approximately additive effect in human bronchi. The possibility exists that this represents synergy between cyclic GMP and cyclic AMP elevation, or K^+ channel opening, however further study would be required to confirm that this is the mechanism. A similar pattern of interaction is seen when ANP is compared with the nitric oxide donor, sodium nitroprusside, however, with isosorbide dinitrate a higher concentration was required to evoke the same degree of relaxation as ANP. Isosorbide dinitrate and ANP nevertheless had an additive effect when used in combination. Previous studies in bovine airway smooth muscle (Gruetter *et al.*, 1989) suggested that isosorbide dinitrate was ineffective in bovine bronchi up to a concentration of 10^{-5} M, and indeed, in this present study, a higher concentration was used. This might be expected to limit its usefulness *in vivo*; however it has been shown to protect against some forms of asthma (e.g. exercise-induced asthma; Tullet & Patel, 1983). Isosorbide dinitrate is a compound currently used in the treatment of angina and it is thought to release nitric oxide after first forming an intermediary complex (Ignarro *et al.*, 1981). The requirement to form such an intermediary may explain the difference in potency between isosorbide dinitrate and sodium nitroprusside.

When these bronchodilator substances were pre-incubated, ANP (in the presence of phosphoramidon) showed an ability to confer protection against methacholine-induced contraction, which is in keeping with our previous studies in human and bovine bronchi (Angus *et al.*, 1994b). Salbutamol likewise attenuated subsequent methacholine-challenge, which is at variance with a number of studies which have shown little (Advenier *et al.*, 1988) or no (Gustaffson & Persson, 1991; Advenier *et al.*, 1991) pre-protectant effect of this drug. Clinical studies (e.g. Tattersfield, 1987; Britton *et al.*, 1988) have however shown an ability of salbutamol to inhibit bronchial reactivity and our studies appear to indicate a similar effect against methacholine-challenge. The combination of these two agonists evoked an additive effect, again suggesting a possible role for the use of these agonists in combination.

Interactions between these drugs have not, to the best of our knowledge, been studied in airway smooth muscle before, although interactions between other bronchodilators has been evaluated. For example, theophylline and the β-adrenoceptor agonist, isoprenaline (Karlsson & Persson, 1981) demonstrated no additive effect in dilating pre-constricted guinea-pig trachea, whilst in human isolated bronchus (Advenier *et al.*, 1988), a combination of fenoterol and the muscarinic antagonist, ipratropium, produced an additive effect in attenuating subsequent challenge. In rat blood vessels a synergistic interaction in inhibiting phenylephrine-induced challenge has been demonstrated between the atrial natriuretic peptide, atriopeptin II and the non-selective β-agonist, isoprenaline (Jang *et al.*, 1993). This result was attributed to an inhibitory action of cyclic GMP on cyclic nucleotide phosphodiesterase type III and, while this is one possible explanation of our results, it is not possible from the present study to evaluate whether this mechanism underlies the interaction seen here between ANP and salbutamol, either in reversing or preventing methacholine-evoked contraction. Our results do however suggest that a combination of agonists, such as ANP with salbutamol, might be of benefit in the treatment of such conditions as acute severe asthma, allowing lower doses of β-agonist to be given.

The combinations of ANP with the other two agonists, sodium nitroprusside and isosorbide dinitrate, demonstrated some interesting dissimilarities between these two putative nitric oxide donor compounds. Sodium nitroprusside, at the concentration used, appears to have no ability to protect against subsequent methacholine-evoked contraction, despite evoking bronchodilatation at this level. Indeed at concentrations of methacholine less than 3×10^{-7} M, the presence of sodium nitroprusside evoked a significant enhancement of contractions, for which we have no explanation. This is in contrast with studies in vascular tissue, (e.g. rat aorta; Maurice *et al.*, 1991) where sodium nitroprusside inhibited subsequent contractions. Sodium nitroprusside likewise did not alter the ability of ANP to protect against subsequent contraction, suggesting perhaps that only activation of the particulate form of guanylate cyclase, as is thought to occur with ANP, leads to protection of these tissues against methacholine-evoked contraction. Isosorbide dinitrate did not enhance any methacholine-evoked contractions in the manner of sodium nitroprusside and, although it was ineffectual alone, it did enhance the ANP effect at higher concentrations of methacholine. Sodium nitroprusside and isosorbide dinitrate are proposed to (Feelisch & Noack, 1987) share a common mechanism, nitric oxide release stimulating intracellular cyclic GMP elevation, albeit with an intermediary step involved in the case of isosorbide dinitrate. It is thus tempting to speculate that nitric oxide production is not the mechanism by which isosorbide dinitrate is exerting its pre-protective effect when combined with ANP in human bronchial smooth muscle. Alternatively, the higher concentration of isosorbide dinitrate used, although required to evoke the same relaxation as that evoked by lower concentrations of sodium nitroprusside, might explain the differences seen between these two compounds in the present study.

The inability of sodium nitroprusside and isosorbide dinitrate to protect against contractions, despite evoking bronchodilatation, is in keeping with other studies. Gustafsson & Persson (1991), for example, found similar results using β_2-agonists and xanthines in guinea-pig isolated trachea. These findings suggest that 'functional antagonism' might not simply be the mechanism by which bronchodilators exert a pre-protective effect. It has been proposed (Persson *et al.*, 1982), that β_2-agonists, for example, have effects on cells other than airway smooth muscle and that this may contribute to their protective effects in human asthmatic subjects (Britton *et al.*, 1988).

In conclusion, in their capacities as both bronchodilators and as agents which, in the present study, attenuate subse-

quent challenge, ANP and salbutamol have an additive effect which may suggest their concurrent use may be of benefit in the treatment of asthma. Sodium nitroprusside and isosorbide dinitrate similarly evoke a bronchodilator effect; however, this is not accompanied by a pre-protecting ability. Nevertheless, in the case of isosorbide dinitrate, enhancement of the ANP response can be demonstrated and the differences in activity between sodium nitroprusside and isosorbide dinitrate remain to be fully explained.

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