Muscarinic blockade of β -adrenoceptor-stimulated adenylyl cyclase: the role of stimulatory and inhibitory guanine-nucleotide binding regulatory proteins (G_s and G_i)

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1 The functional antagonism that exists between muscarinic and β -adrenoceptor function in guinea-pig tracheal smooth muscle was investigated by assessing G_s and G_i regulated adenylyl cyclase activity in isolated membranes.

2 Membranes from guinea-pig tracheal smooth muscle contain both $G_{i\alpha}$ and $G_{i\beta}$ as assessed by Western blots with anti-G-protein antibodies.

3 GppNHp, a non-hydrolysable analogue of guanosine 5'-triphosphate (GTP), was shown to stimulate adenylyl cyclase activity at high concentrations (10^{-6} – 10^{-4} M). GppNHp also produced a concentration-dependent reduction in pertussis toxin-catalysed adenosine diphosphate (ADP)-ribosylation of $G_{i\alpha}$.

4 Pretreatment of tracheal smooth muscle slices with methacholine (10^{-6} M) provoked a blockade of isoprenaline plus GTP, GppNHp- and GTP-stimulated adenylyl cyclase.

5 Addition of methacholine to membranes did not trigger inhibition of GTP-stimulated adenylyl cyclase activity but did block the isoprenaline-mediated augmentation of GTP-stimulated adenylyl cyclase activity.

6 Pretreatment of tracheal smooth muscle with methacholine (10^{-6} M) provoked a blockade of cholera toxin-catalysed NAD^+ -dependent ADP-ribosylation of $G_{s\alpha}$.

7 Phorbol-12-myristate 13-acetate (PMA)-treatment of tracheal smooth muscle slices actually enhanced GppNHp-stimulated adenylyl cyclase activity in subsequently prepared membranes.

8 We suggest that methacholine in addition to inhibiting adenylyl cyclase via a G_i -dependent mechanism induces a functional inactivation of G_s activity. These results together may explain the functional antagonism that exists between increased muscarinic tone and the ability of β -adrenoceptor agonists to provoke excitation-contraction uncoupling.

Keywords: G-protein; protein kinase C; muscarinic agonists; adenylyl cyclase

Introduction

Contractile agonists such as acetylcholine trigger the excitation-contraction coupling of airway smooth muscle via a receptor-mediated stimulation of phospholipase C. This is a guanine nucleotide-binding regulatory protein (G-protein)-mediated event which results in the enzymatic hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂, Berridge & Irvine, 1984; Nishizuka, 1984; Chilvers *et al.*, 1989). The second messengers produced are Ins(1,4,5)P₃ (which triggers Ca²⁺ mobilization by binding to a sarcoplasmic Ins(1,4,5)P₃ receptor protein) and 1,2-*sn*-diacylglycerol (a protein kinase C activator). The receptor-triggered mobilization of Ca²⁺ leads to the formation of a Ca²⁺-calmodulin complex and the subsequent activation of myosin light chain kinase (Silver & Stull, 1982). This enzyme triggers excitation-contraction coupling by eliciting the phosphorylation of myosin light chain and thus allowing the myosin to form cross-bridges with actin. The uncoupling events governing excitation-contraction are regulated by agonists which provoke accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Katsuki & Murad, 1977). Thus, β -adrenoceptor agonists provoke relaxation of airway smooth muscle via a receptor-linked G-protein mechanism which results in the stimulation of adenylyl cyclase (Gilman, 1987). The G-protein which allows coupling between β -adrenoceptor and adenylyl cyclase

is termed G_s . This protein is a heterotrimer composed of three non-identical sub-units: α (45/52 kDa), β (35/36 kDa) and γ (8 kDa).

Several studies (Torphy *et al.*, 1986; Hall & Hill, 1988) have revealed that functional antagonism occurs between muscarinic, histamine and β -adrenoceptor agonists. Thus, increased muscarinic tone results in a progressive reduction in the ability of β -adrenoceptor agonists to elicit uncoupling of excitation-contraction. Furthermore, the extent of functional antagonism appears to be causally related to the magnitude of the agonist-stimulated Ins(1,4,5)P₃ response (Meurs *et al.*, 1988). We have suggested that this phenomenon may in part be due to 'cross-talk' between the PtdIns(4,5)P₂ signal cascade and the cyclic AMP signalling machinery (Pyne *et al.*, 1991).

In this paper, we demonstrate that methacholine can trigger an alteration in G_s function. The subsequent alteration in G_s function and G_i (inhibitory guanine-nucleotide regulatory binding protein)-mediated pathways may explain the altered responsiveness of airway smooth muscle to β -adrenoceptor agonists.

Methods

Dissection of tracheal smooth muscle

Trachealis was removed from the guinea-pig and rinsed in Krebs-Henseleit solution, pH 7.4, and a longitudinal cut made at the front, opposite the smooth muscle strip. Connec-

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tive tissue was removed and the smooth muscle strip cleaned by dissection under a microscope.

Preparation of tracheal smooth muscle membrane

Tracheal smooth muscle strips were homogenized in 2 ml of buffer A containing (mM): Tris/HCl (pH 7.4) 20, ethylenediaminetetraacetic acid 1, sucrose 250, phenylmethylsulphonyl-fluoride 0.1 and benzamidine 2, at 4°C, with an ultraturrex homogenizer (4–5 up and down strokes). The resulting homogenate was centrifuged at 50000 g for 20 min at 4°C, in a Beckman 55.2 Ti rotor. The supernatant was discarded and the pellet resuspended in 2 ml of buffer A and recentrifuged as before. The final pellet fraction was resuspended with 0.2 ml of buffer A (0.4 mg ml⁻¹ of protein). Membranes were used immediately in the adenylyl cyclase assay.

Incubation of trachealis smooth muscle strips

Tracheal smooth muscle strips (3 mg wet weight) were placed into 10 ml of constantly gassed (O₂/CO₂, 95:5%) Krebs-Hensleit, pH 7.4 and allowed to preincubate for 10 min at 37°C. After this time methacholine (10⁻⁶ M) or phorbol-12-myristate 13-acetate (PMA, 0.16 × 10⁻⁶ M) was added to some of the samples and these were allowed to incubate for a further 10–30 min at 37°C. Incubations with atropine (10⁻⁶ M) involved a 10 min incubation, prior to the addition of methacholine. In all cases, tissue strips were removed and a membrane fraction prepared.

Adenylyl cyclase assay

Adenylyl cyclase activity was assayed according to Salomon *et al.* (1974). The membranes (20–30 µg) were combined with a reaction cocktail containing, final concentrations (mM): Tris/HCl (pH 7.8) 12.5, ATP 0.1, MgSO₄ 2.5, cyclic AMP 0.4, creatine phosphate 2.5, creatine kinase 1.5 units, KCl 7.5 and sucrose 30. Routinely, 1 µCi of [α-³²P]-ATP was added to each assay. In some instances, additions were made to the incubation and these included: isoprenaline (10⁻⁶ M), GTP (10⁻⁴ M), GppNHp (10⁻¹⁰–10⁻⁴ M) and methacholine (10⁻⁶ M). The total assay volume was 100 µl and the incubations were performed at 37°C for 30 min and were terminated by the addition of ATP 40 mM, followed by boiling for 2 min. Samples were centrifuged at 15,000 g for 10 min and the recovered supernatants 'spiked' with [³H]-cyclic AMP (5000 d.p.m.), which allowed the assessment of recovered [α-³²P]-cyclic AMP from the Dowex WH⁺ and alumina column chromatography.

Preactivation of G_i by GppNHp

The tracheal smooth muscle membranes (100 µg) were resuspended in final concentrations (mM) Tris/HCl (pH 7.4) 10, MgCl₂ 5, dithiothreitol (DTT) 1. To this was added an equal volume of GppNHp (final concentration 100 µM), and the samples were incubated at 37°C for 30 min. After this time the samples were centrifuged for 10 min at 15000 g at 4°C. The pellet was resuspended in buffer A and taken for cholera toxin-catalysed ADP-ribosylation. Under these conditions, activated ADP-ribosylation factor will accumulate.

Toxin-catalysed ADP-ribosylation of G_i and G_s

Cholera toxin-catalysed ADP-ribosylation of tracheal membrane protein was performed according to Heyworth *et al.* (1985). Membranes (10 µg) were combined with an ADP-ribosylation cocktail containing final concentrations (mM): potassium phosphate, (pH 7.6) 150, DTT 3, CaCl₂ 0.005, MgCl₂ 1, thymidine 7.5 and to this was added 2 µCi [³²P]-NAD⁺ 0.02. It is not necessary to buffer the Ca²⁺ in the incubation with EGTA, since isolated membranes are used, and therefore Ca²⁺ is unlikely to vary markedly in the assay.

To initiate the incubation, cholera toxin (66 µg ml⁻¹) was added. This was preactivated by combining an equal volume of DTT 50 mM, with cholera toxin and incubating for 1 h at room temperature. Ribosylation incubations were performed at 37°C for 45 min after which they were terminated by the addition of trichloroacetic acid (TCA final concentration 6%, w/v) and deoxycholate (0.01%, w/v). The samples were centrifuged at 15,000 g for 10 min at 4°C in a Beckman microfuge. The resulting pellet was resuspended in 10 µl of Tris/HCl 1 M, and 20 µl of the Laemmli buffer and these were boiled for 5 min after which they were subjected to SDS-PAGE (10% acrylamide).

Pertussis toxin-catalysed ADP-ribosylation was performed according to Pyne *et al.* (1989). Membranes (10 µg) were combined with a final concentration (mM): thymidine 30, potassium phosphate 80 (pH 7.5), arginine 20, MgCl₂ 1 and to this was added 2 µCi [³²P]-NAD⁺ 0.02, and these were then combined with preactivated pertussis toxin (0.64 µg/assay) and incubated at 37°C for 30 min. Preactivation of pertussis toxin and the incubation of samples was performed in an identical manner to those treated with cholera toxin.

After electrophoresis, the gels were fixed in 10% TCA (w/v) for 30 min and dried prior to autoradiography at -20°C for 72 h. Location of the ADP-ribosylated peptides was followed by the densitometric analysis of the intensity of the radioactive peptides.

Western blotting

Membranes (200–300 µg protein) were subjected to SDS-PAGE, and transferred to nitrocellulose. After transfer, the sheets were blocked in 3% (w/v) gelatin and buffer B containing Tris/HCl (pH 7.4) 10 mM, NaCl 0.9%, at 37°C for 2 h. After washing, the sheets were incubated with either an anti-G_s antisera or an anti-G_i antisera (SG1), for 12 h at 30°C. Routinely, a 1/200 dilution of antisera was used. After this incubation, the sheets were washed in buffer B containing Tween-20 (0.05% v/v) and then in buffer B alone. The sheets were then incubated with a horse radish peroxidase-linked anti-rabbit antibody (1/200 dilution) for 2 h at room temperature and then washed as before. The sheets were then reacted with 1 ml of O-dianisidine (10 mg ml⁻¹), 10 µl H₂O₂ (30%, v/v) and 40 ml Tris/HCl, pH 7.4 (10 mM) in order to locate the immunoreactive peptide bands.

Materials

All biochemicals such as GppNHp, GTP and ATP were from Boehringer Mannheim (Germany). All chemicals and toxins were from Sigma Chemical Company (England). [³²P]-NAD was purchased from Dupont (U.S.A.) and [³H]-cyclic AMP and [α-³²P]-ATP were obtained from Amersham International plc (UK). Horse-radish peroxidase linked antibody was a gift from the Scottish antibody production unit (Carlisle, Scotland). SG1 was a kind gift from Dr G. Milligan (University of Glasgow). Anti-G_s anti-sera was produced to the C-terminal decapeptide of G_{sα} (RMHLRGYELL).

Results

Immunoblotting of G_i and G_s with peptide-directed antibodies

Western blotting of membranes with anti-G_{sα} anti-sera (which detected recombinant G_{sα}, data not shown) revealed a single immunologically reactive peptide with an M_r = 45 kDa (Figure 1). This polypeptide co-migrates with the 45 kDa recombinant spliced variant of G_{sα}. These data suggest that tracheal smooth muscle membranes contain only the small version of G_{sα}, since no immunoreactive polypeptide was identified with a molecular mass of 52 kDa. Furthermore, no immunoreactive polypeptides were identified in the molecular

Figure 1 Immunological identification of G_{ss} : this figure shows a typical western blot of an SDS PAGE of resolved proteins from tracheal smooth muscle. Blotting was against the anti- G_i antisera. The location of G_{ss} sub-unit is shown. Molecular mass markers are shown. This is a typical result from the experiment performed at least 10 times on different membrane preparations.

mass range of 39–42 kDa corresponding to the α sub-units of G_i , G_o and G_q .

Western blotting of membranes with SG1 revealed a single immunoreactive peptide with an $M_r = 41$ kDa corresponding to G_{ss} (Figure 2). However, the exact identity of the G_i is unknown, since SG1 immunoreacts with both $G_{\text{ss}1}$ and $G_{\text{ss}2}$ as both share the common C-terminal decapeptide.

Effect of GppNHp on pertussis toxin-catalysed ADP-ribosylation of G_{ss} in tracheal smooth muscle

GppNHp produced a concentration-dependent reduction in the ability of pertussis toxin to catalyse ADP-ribosylation of G_{ss} (Figures 3 and 4). The maximal effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of G_{ss} was observed at 10^{-6} M GppNHp.

Effect of methacholine-pretreatment of tracheal smooth muscle on isoprenaline and GTP-stimulated adenylyl cyclase

GTP (10^{-4} M) elicited a 2.3 fold stimulation of adenylyl cyclase in isolated membranes (Figure 5). Isoprenaline (10^{-6} M) alone was without effect, but, in combination with GTP elicited a 3.9 fold stimulation of adenylyl cyclase activity. In contrast, methacholine pretreatment of tracheal smooth

muscle slices abolished the GTP-stimulated adenylyl cyclase and reduced the ability of isoprenaline to augment GTP-stimulated adenylyl cyclase in isolated membranes to 1.6 fold. Atropine (10^{-6} M) pretreatment of tracheal strips reversed the methacholine-induced blockade of GTP-stimulated adenylyl cyclase activity (data not shown).

Addition of methacholine directly to membranes did not significantly inhibit either basal or GTP-stimulated adenylyl cyclase, but did inhibit the augmentation of GTP-stimulated adenylyl cyclase by isoprenaline (Figure 6).

Effect of methacholine-pretreatment of tracheal smooth muscle upon GppNHp-stimulated adenylyl cyclase

GppNHp (10^{-7} – 10^{-4} M) produced a concentration-dependent stimulation of adenylyl cyclase activity (Figure 7) with a 7 fold maximal activation at 10^{-4} M GppNHp. The pretreatment of tracheal smooth muscle with methacholine (10^{-6} M) triggered a blockade of this activity with an approximate 75% reduction in the maximal stimulation induced by GppNHp.

Effect of methacholine-pretreatment upon cholera toxin-catalysed ADP-ribosylation of G_{ss}

Methacholine-pretreatment of tracheal smooth muscle slices triggered a 90% reduction in the ability of cholera toxin to

Figure 2 Immunological identification of G_{ss} : this figure shows a typical western blot of an SDS PAGE of resolved proteins from tracheal smooth muscle. Blotting was against the anti- G_i antisera. The location of G_i α sub-unit is shown. Molecular mass markers are shown. This is a typical result from the experiment performed at least 10 times on different membrane preparations.

Figure 3 The effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of G_i in tracheal smooth muscle. A concentration-dependent effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of G_i in isolated tracheal smooth muscle membranes. This is a typical autoradiogram of an SDS PAGE of the resolved [32 P]-ADP-ribosylated peptides. Lane 1, no GppNHp; Lane 2, 100 μ M GppNHp; Lane 3, 10 μ M GppNHp; Lane 4, 1 μ M GppNHp; Lane 5, 0.1 μ M GppNHp; Lane 6, 0.01 μ M GppNHp. The location of G_i α sub-units are shown. Molecular mass markers are shown. This is a typical result from an experiment performed at least 3 times on different membrane preparations.

Figure 4 The quantification of the effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of G_i in tracheal smooth muscle. A concentration-dependent effect of GppNHp upon pertussis toxin-catalysed ADP-ribosylation of G_i in isolated tracheal smooth muscle membranes. These are means ($\pm$ s.e.mean, vertical bars) of the [32 P]-ADP-ribose incorporated into G_i at different concentration of GppNHp. These are averaged from results of an experiment performed at least 3 times on different membrane preparations.

catalyse ADP-ribosylation of $G_{\alpha s}$ (Figure 8). GppNHp (100 μ M) stimulated the ability of $G_{\alpha s}$ to act as a substrate for cholera toxin-catalysed ADP-ribosylation in membranes from untreated tracheal smooth muscle.

Effect of PMA treatment of tracheal smooth muscle upon adenylyl cyclase activity

Prior treatment of tracheal smooth muscle slices with PMA had no effect upon basal adenylyl cyclase activity. GppNHp (10 $^{-6}$ M) triggered a 1.8 fold activation of adenylyl cyclase activity in membranes isolated from untreated smooth muscle slices, yet in membranes from PMA-treated cells GppNHp was more effective at stimulating adenylyl cyclase, yielding a 2.8 fold activation of activity (Table 1).

Discussion

The functional antagonism between muscarinic and β -adrenoceptor agonists, appears to be dependent upon the magnitude of the agonist-stimulated Ins(1,4,5) P_3 (Meurs *et al.*, 1988). In addition to Ins(1,4,5) P_3 , muscarinic agonists promote the accumulation of Ca^{2+} and diacylglycerol, a potent activator of protein kinase C, and it is this enzyme which has been implicated as being responsible for the development of latch-bridges between actin and myosin (Rasmussen *et al.*, 1984) and in the underlying mechanistic antagonism. Protein kinase C has also been shown to phosphorylate and alter the functional activity state of various G-proteins (Katada *et al.*, 1985; Zick *et al.*, 1986; O'Brien *et al.*, 1987; Pyne *et al.*, 1989; 1992a) and adenylyl cyclase (Yoshimasa *et al.*, 1987).

The molecular events that govern the functional antagonism between the muscarinic receptor and the adrenoceptor are unknown. In addressing this question, it is important to note that the protein kinase C-mediated phosphorylation of G_i leads to its inactivation and uncoupling from agonist-bound receptors (Katada *et al.*, 1985; Pyne *et al.*, 1989). We have also previously shown that recombinant $G_{\alpha s}$ is a substrate for

Figure 5 The effect of methacholine pretreatment of tracheal smooth muscle strips upon adenylyl cyclase activity. Histograms showing an assessment of the effect of various agents upon adenylyl cyclase activity in isolated membranes from tracheal smooth muscle strips that were either untreated (open columns) or treated with methacholine (10 $^{-6}$ M, stippled columns). Additions to membranes were: (1) no additions; (2) isoprenaline (10 $^{-6}$ M); (3) isoprenaline (10 $^{-6}$ M) plus GTP (10 $^{-4}$ M); (4) GTP (10 $^{-4}$ M) alone. These results are expressed as means $\pm$ SEM, for 3 different experiments on different membrane preparations. All columns have $P < 0.005$.

Figure 6 The effect of adding methacholine to membranes: adenylyl cyclase activity. Histograms showing an assessment of the effect of various agents upon adenylyl cyclase activity in isolated membranes from tracheal smooth muscle strips in the presence (stippled columns) and absence (open columns) of methacholine (10 $^{-6}$ M). (1) No additions; (2) isoprenaline (10 $^{-6}$ M); (3) GTP (10 $^{-4}$ M); (4) isoprenaline (10 $^{-6}$ M) plus GTP (10 $^{-4}$ M). These results are expressed as means with s.e.mean (vertical bars), for 3 different experiments on different membrane preparations.

*Indicates that isoprenaline + GTP stimulated adenylyl cyclase activity in membranes from methacholine-treated smooth muscle is significantly lower than in membranes from untreated tissue, $P < 0.025$ Student's t test.

Figure 7 Effect of GppNHp upon adenylyl cyclase activity in membranes from untreated and methacholine-treated smooth muscle strips. A concentration-dependent effect of GppNHp upon adenylyl cyclase in isolated tracheal smooth muscle membranes, from methacholine-treated (10^{-6} M)-treated (●) and untreated (○) tracheal smooth muscle strips. These are means ($\pm$ s.e.mean, vertical lines), an experiment performed at least 3 times on different membrane preparations.

*Indicates that GppNHp-stimulated adenylyl cyclase activity at 10^{-4} M in membranes from methacholine-treated smooth muscle is significantly lower than in membranes from untreated tissue, $P < 0.025$, Student's *t* test. All other points have P significance < 0.005 .

Figure 8 Effect of methacholine upon cholera toxin catalysed-ribosylation. Tracheal smooth muscle strips were either treated with methacholine (10^{-6} M) or untreated. Membranes were then taken for cholera toxin-catalysed ADP-ribosylation and then subjected to SDS PAGE. This is an autoradiogram of the resolved [32 P]-ADP-ribosylated peptides. Lane 1, untreated membranes; Lane 2, GppNHp (0.1×10^{-3} M)-treated membranes; Lane 3, membranes from methacholine-treated tissue; Lane 4, GppNHp (0.1×10^{-3} M) treated membranes from methacholine-treated tissue; Lane 5, membranes incubated in the ADP-ribosylation cocktail but without cholera toxin. This is a representative result from an experiment performed at least 3 times. G_s is marked and the molecular wt markers are shown.

protein kinase C- and protein kinase A-catalysed phosphorylation (Pyne *et al.*, 1992a,b). We have shown that PMA-pretreatment of tracheal smooth muscle slices leads to enhanced GppNHp-stimulated adenylyl cyclase activity in subsequently prepared membranes. This might be as a consequence of an enhanced responsiveness of G_s to GppNHp or a reduced responsiveness of G_i . Since basal adenylyl cyclase remains unaltered, it is probable that adenylyl cyclase is not modified by protein kinase C in these cells, although alterations in G-protein and phosphorylated adenylyl cyclase coupling cannot be excluded.

Table 1 Effect of phorbol-12-myristate 13-acetate (PMA)-treatment upon adenylyl cyclase activity

Treatment	Adenylyl cyclase activity (pmol min ⁻¹ mg ⁻¹)
Untreated smooth muscle	
Basal	13.3 $\pm$ 1.3
GppNHp (10^{-6} M)	24 $\pm$ 1
PMA-treated	
Basal	11.4 $\pm$ 3
GppNHp (10^{-6} M)	37 $\pm$ 7

Experiments were performed according to the methods. This is a representative result (mean $\pm$ s.e.mean) of an experiment performed at least three times.

Pretreatment of tracheal smooth muscle with methacholine substantially blocked GTP-, isoprenaline and GTP-, and GppNHp-stimulated adenylyl cyclase activity in subsequently prepared membranes. The sustained blockade of adenylyl cyclase activity throughout a procedure involving the disruption of the cells and preparation of membranes was the first indication that this phenomenon might not be entirely due to receptor-mediated G_i activation as this is likely to be a transient process. However, the possibility that the muscarinic receptors remain occupied with methacholine cannot be ignored. An alternative explanation for the inhibitory action of methacholine involves a modification of G_s activity, although this is unlikely to occur via a protein kinase C-mediated phosphorylation of G_s , since methacholine does not mimic PMA. The interpretation that G_s is modified, is based upon the observation that the addition of methacholine directly to isolated membranes does not trigger inhibition of GTP-stimulated adenylyl cyclase activity, whilst in methacholine pretreated tissue, subsequently prepared membranes exhibit markedly inhibited GTP-stimulated adenylyl cyclase activity. Furthermore, G_i appears to be functionally active in isolated membranes, since methacholine added directly to membranes was capable of inhibiting the ability of isoprenaline to augment GTP-stimulated adenylyl cyclase and thus the lack of effect upon GTP-stimulated adenylyl cyclase is not a consequence of an inability to measure G_i function. The selective inhibition of isoprenaline-stimulated adenylyl cyclase activity when methacholine is added directly to membranes is entirely consistent with previous observations (Hildibrand & Kohnken, 1990) where only hormonal stimulation of adenylyl cyclase appeared susceptible to inhibition by G_i .

Our results also suggest that the α sub-unit of G_i is unlikely to be involved in the inhibition of GppNHp-stimulated adenylyl cyclase at 10^{-4} M GppNHp, since $G_{i\alpha}$ is already maximally activated at this concentration and the equilibrium binding of this guanine nucleotide will be very rapid indeed (Gilman, 1987). This was supported by the observation that GppNHp provoked a reduction in pertussis toxin-catalysed ADP-ribosylation, indicative of GppNHp-stimulated dissociation of the G_i holomer, and this was maximal at 10^{-6} M GppNHp. Our results, therefore, suggest that at the very least, G_i is probably not involved in inhibiting guanine nucleotide-stimulated adenylyl cyclase. Adenosine released in response to methacholine is unlikely to contribute to the observed effects, since adenosine is a bronchodilator acting via A_2 receptors in guinea-pig trachea (Brown & Collis, 1982).

A role for altered G_s function is also indicated from studies using cholera toxin to catalyse ADP-ribosylation of the α sub-unit of this G-protein. In this case, $G_{s\alpha}$ in membranes from methacholine pretreated cells was a markedly poorer substrate for ADP-ribosylation than $G_{s\alpha}$ in untreated cells. The inclusion of GppNHp with membranes from untreated tissue resulted in a marked increase in cholera toxin-catalysed

ADP-ribosylation of $G_{\alpha s}$, and is consistent with the observation of Kahn & Gilman (1986), where GppNHp activates a membrane-bound ADP-ribosyl transferase factor (ARF) which in turn enhances cholera toxin activity.

Free $G_{\alpha s}$ is an exceedingly poor substrate for cholera toxin-catalysed ADP-ribosylation (Graziano *et al.*, 1989), whilst the addition of $\beta\gamma$ sub-units enhances the ability of $G_{\alpha s}$ to act as a substrate for cholera toxin-catalysed ADP-ribosylation. It is possible that $G_{\alpha s}$ is functionally dissociated from the $\beta\gamma$ sub-units in methacholine-treated smooth muscle, and therefore unable to couple to β -adrenoceptors, whilst modification of the free $G_{\alpha s}$ -GDP sub-unit may well affect subsequent GTP binding and effector recognition. If this is the case then the reduced amount of ADP-ribosylated $G_{\alpha s}$ may reflect lower amounts of available functionally active G_s . In fact, this is consistent with the reduced maximal activation of adenylyl cyclase by GppNHp, that is observed in membranes from methacholine pretreated smooth muscle. However, the extent of reduced ADP-ribosylation and reduced activation of adenylyl cyclase activity in methacholine-treated cells does not correlate absolutely, although this may be the consequence of the possibility that only a small fraction of G_s may be required for full activation of adenylyl cyclase.

Resolution of the roles of G_i and G_s might be more fully addressed by assessing the effect of pertussis toxin upon the methacholine-induced blockade of isoprenaline stimulated adenylyl cyclase. However, this requires extended incubation times (10–20 h) of tracheal smooth muscle strips with pertussis toxin at 37°C, and after this time the β -adrenoceptor responsiveness of the tissue is severely compromised (data not shown).

Previous studies (Meurs *et al.*, 1987) have shown that protein kinase C can perturb β -adrenoceptor adenylyl cyclase in lymphocytes via an alteration in receptor-G-protein coupling. However, our studies indicate that methacholine treatment of tracheal smooth muscle also perturbs the interaction of G_s with adenylyl cyclase. Sankary *et al.* (1988) have shown that the methacholine-mediated inhibition of adenylyl cyclase activity in canine airway smooth muscle was mediated by G_i . However, the use of pertussis toxin to block this response and the subsequent interpretation has to be speculative, since this toxin can block receptor-mediated activation of phospholipase C (Issakani *et al.*, 1990), and it is unknown whether canine trachea contains a pertussis toxin-insensitive phospholipase C. Our results suggest that G_i may well be involved in inhibiting isoprenaline-stimulated adenylyl cyclase, although G_s inactivation also appears to be an additional mechanism that is important. We suggest, therefore, that the antagonism that exists between increased muscarinic tone and reduced β -adrenoceptor-induced relaxation occurs via a pathway involving modulation of both G_i and G_s .

We are currently investigating whether G_s is a substrate for phosphorylation in primary cultures of tracheal smooth muscle, since this methodology allows more successful incorporation of ^{32}P into the intracellular ATP pool and will enable studies to be performed in the absence of contaminating epithelial cells.

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References

- BERRIDGE, M.J. & IRVINE, R.F. (1981). Inositol trisphosphate, a novel second messenger in cellular signal transduction. *Nature*, **312**, 315–321.
- BROWN, C.M. & COLLIS, M.G. (1982). Evidence for an A2/Ra adenosine receptor in guinea-pig trachea. *Br. J. Pharmacol.*, **76**, 381–387.
- CHILVERS, E.R., CHALLIS, J., BARNES, P.J. & NAHORSKI, S.R. (1989). Mass changes in inositol (1,4,5) trisphosphate in tracheal muscle following agonist stimulation. *Eur. J. Pharmacol.*, **614**, 587–590.
- GILMAN, A.G. (1987). G-proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.
- GRAZIANO, M.P., FREISSMUTH, M.P. & GILMAN, A.G. (1989). Expression of $G_{\alpha s}$ in *E. coli*. Purification and properties of the two forms of the protein. *J. Biol. Chem.*, **264**, 409–418.
- HALL, I.P. & HILL, S.J. (1988). β_2 -Adrenoceptor stimulation inhibits histamine-induced inositol phospholipid hydrolysis in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **95**, 1204–1212.
- HEYWORTH, C.M., WHETTON, A.D., WONG, S., MARTIN, R. & HOUSLAY, M.D. (1985). Insulin inhibits the cholera toxin-catalysed ADP-ribosylation of a Mr = 25 kDa protein. *Biochem. J.*, **228**, 593–603.
- HILDBRANDT, J.D. & KOHNEN, R.E. (1990). Different mechanisms for the inhibition of adenylyl cyclase by hormones and G-protein $\beta\gamma$. *J. Biol. Chem.*, **265**, 9825–9830.
- ISSAKANI, S.D., SPIEGEL, A.M. & STRULOVIC, B. (1990). Lipopolysaccharide response is linked to the GTP-binding protein GiII in the premonocytic cell, U937. *J. Biol. Chem.*, **264**, 20240–20247.
- KAHN, R.A. & GILMAN, A.G. (1986). The protein cofactor necessary for ADP-ribosylation is itself a GTP-binding protein. *J. Biol. Chem.*, **261**, 7906–7911.
- KATADA, T., GILMAN, A.G., WATANABE, Y., BAUHR, S. & JAKOBS, K.H. (1985). Protein kinase C phosphorylates the inhibitory guanine-nucleotide binding regulatory component and apparently suppresses its function in the inhibition of adenylyl cyclase. *Eur. J. Biochem.*, **151**, 431–437.
- KATSUKI, S. & MURAD, F. (1977). Regulation of adenosine 3'5' monophosphate and guanosine 3'5' monophosphate levels and contractility in bovine tracheal smooth muscle. *Mol. Pharmacol.*, **13**, 330–341.
- MEURS, H., KAUFFMAN, H.F., KOETER, G.H., TIMMERMANS, A. & DE VRIES, K. (1987). Regulation of β -receptor adenylyl cyclase in lymphocytes of allergic patients with asthma: possible role for protein kinase C in allergen induced, non-specific refractoriness of adenylyl cyclase. *J. Allergy. Clin. Immunol.*, **80**, 326–339.
- MEURS, H., ROFFEL, A.F., PASTEMA, J.B., TIMMERMANS, A., ELZINGAN, C.R.A., KAUFFMAN, H.F. & ZAAGSMA, J. (1988). Evidence for a direct relationship between phosphoinositide metabolism and airway smooth muscle contraction induced by muscarinic agonists. *Eur. J. Pharmacol.*, **156**, 271–274.
- NISHIZUKA, Y. (1984). Molecular heterogeneity of protein kinase C and its implication for cellular signalling. *Nature*, **308**, 693–698.
- O'BRIEN, R.M., HOUSLAY, M.D., MILLIGAN, G. & SIDDLE, K. (1987). The insulin receptor tyrosyl kinase phosphorylates holomeric forms of the guanine-nucleotide regulatory protein G_i and G_s . *FEBS Lett.*, **212**, 281–288.
- PYNE, N.J., MURPHY, G.J., MILLIGAN, G. & HOUSLAY, M.D. (1989). Treatment of intact hepatocytes with either the phorbol ester TPA or glucagon elicits the phosphorylation and functional inactivation of the inhibitory guanine-nucleotide regulatory protein G_i . *FEBS Lett.*, **243**, 77–82.
- PYNE, N.J., SHEHNAZ, D. & RODGER, I.W. (1991). Cholinergically mediated inhibition of GTP-stimulated adenylyl cyclase in airway smooth muscle. *Am. Rev. Resp. Dis.*, **143**, 549.
- PYNE, N.J., FREISSMUTH, M. & PALMER, S. (1992a). Phosphorylation of the recombinant spliced variant forms of the stimulatory G-protein (G_s) by protein kinase C. *Biochem. J.*, **285**, 333–338.
- PYNE, N.J., FREISSMUTH, M. & PYNE, S. (1992b). Phosphorylation of the recombinant spliced variant forms of the stimulatory G-protein (G_s) by the catalytic sub-unit of protein kinase A. *Biochem. Biophys. Res. Commun.*, **186**, 1081–1086.
- RASMUSSEN, H., KOJIMA, I., ZAWALICH, W. & APPELDORF, W. (1984). Calcium as an intracellular messenger, sensitivity modulation, C-kinase pathway and sustained cellular response. *Adv. Cyclic. Protein. Phosphor. Res.*, **18**, 159–193.
- SALOMAN, Y., LONDOS, C. & ROBBELL, M. (1974). A highly sensitive adenylyl cyclase assay. *Anal. Biochem.*, **56**, 502–514.

- SANKARY, R.M., JONES, C.A., MADISON, J.M. & BROWN, J.K. (1988). Muscarinic cholinergic inhibition of cyclic AMP accumulation in airway smooth muscle. Role of a pertussis toxin-sensitive protein. *Ann. Rev. Resp. Dis.*, **138**, 145–150.
- SILVER, P.J. & STRULL, J.T. (1982). Regulation of myosin light chain and phosphorylation of tracheal smooth muscle proteins. *J. Biol. Chem.*, **257**, 6145–6150.
- TORPHY, T.J., BURMAN, M., SCHWARTZ, L.W. & WASSERMAN, M.A. (1986). Differential effects of methacholine and LTD₄ on cyclic nucleotide content and isoprenaline induced relaxation in the opossum trachea. *J. Pharmacol. Exp. Ther.*, **237**, 332–340.
- YOSHIMASA, T., SIBLEY, D.R., BOUVIER, M., LEFKOWITZ, R.J. & CARON, M.G. (1987). Cross-talk between cellular signalling pathways suggested by phorbol ester-induced adenylyl cyclase phosphorylation. *Nature*, **327**, 67–70.
- ZICK, Y., SAGIT-EISENBERG, R., PINES, M., GIERSECHIK, P. & SPIEGEL, A.M. (1986). Multi-site phosphorylation of the α sub-unit of transducin by the insulin receptor kinase and by protein kinase C. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 9294–9297.

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