Evidence of the Existence of G_i-Coupled Inhibitory Modulaton of Adenylyl Cyclase by Muscarinic Receptor in Guinea-Pig Trachealis

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Summary: The present study involved functional investigation of the signalling pathway of adenylyl cyclase inhibition by the muscarinic receptor. Methacholine pre-treatment of tracheal smooth muscle resulted in inhibition of isoprenaline- and guanine nucleotide-stimulated adenylyl cyclase. The effect could be reversed by pre-incubation of the tissue with the antibody that identifies the C-terminal decapeptide of inhibitory G proteins 1 and 2 (G_i1 and G_i2) indiscriminately. Methacholine also abated the pertussis toxin-catalysed ADP-ribosylation of $G_{i\alpha}$. These results suggest the existence of M_2 and/or M_4 subtype of the muscarinic receptor in the tracheal smooth muscle of guinea-pig.

Introduction

The inhibitory regulation of adenylyl cyclase by muscarinic acetylcholine receptors (mAChR) has been reported in various tissues and species. For example in cardiac muscle [1,2], in tracheal smooth muscle of dog [3] and in two different cell lines *i.e.*, 1321 NI human astrocytoma and NG 108-15 rat neuroblastoma glioma [4]. The diversity of functions is in part connected with the multiplicity of receptor subtypes. Generally, it is believed that M_1, M_3 and M_5 receptors are linked to the breakdown of phosphoinositide membrane lipids, whereas, M_2 and M_4 are coupled to the inhibition of adenylyl cyclase (reviewed by Peralta *et al.*, 1988, Hulme *et al.*, 1990, Richards, 1991) [5-7]. However, the M_2 receptors present on the parasympathetic nerves innervating the airways in rabbit have been implicated in the inhibition of acetylcholine release [8-10]. Actually, the M_2 site has further been classified into two subtypes, $M_{2\alpha}$ in the cardiac and vascular tissue and $M_{2\beta}$ in the smooth muscle and glandular tissue [11]. Hamer *et al.* (1986) have discussed the heterogeneity of M_2 receptor subtypes and their specificity with regards to the tissue [12].

In guinea-pig and rabbit airways, the contractions of the smooth muscle have been associated sequentially with the receptor-triggered mobilization of calcium, formation of the calcium-calmodulin complex and phosphorylation of the myosin light chain kinase [13]. Subsequently, myosin forms cross-bridges with actin and allows the coupling of excitation-contraction in the smooth muscle. This is a guanine nucleotide-binding regulatory protein (G protein)-mediated event that results in the activation of phospholipase C leading to the hydrolysis of membrane phospholipid, phosphatidylinositol 4,5-bisphosphate [14-16]. The coupling of M_3 mAChR with the phospholipase C explains the repeatedly reported implication of this subtype in the contractions of the airway smooth muscle [8-10]. However, it is still debatable whether or not the activation of adenylyl cyclase-linked mAChR subtypes (M_2, M_4) contributes towards the contractile response of smooth muscle. The likelihood of their implication is possible. Particularly, when it is believed that stimulation of β-adrenoceptor leading to the accumulation of 3`:5`-cyclic monophosphate (cyclic AMP) provokes the uncoupling of excitation-contraction [17].

We have previously demonstrated the functional antagonism between muscarinic and β-adrenoceptor agonists in airway smooth muscle [18]. The purpose of the present study was to determine if G_i-coupled subtype of muscarinic receptor, played a role in the inhibition of adenylyl cyclase in tracheal smooth muscle of guinea-pig. The selective antibodies raised against the carboxyl terminus of

the α subunit of the G proteins have been widely used for the investigation of receptor/G protein coupling [19,20]. We have also employed the same method to study the coupling of muscarinic receptor with adenylyl cyclase through G_i. The results suggests that adenylyl cyclase activity is directly modulated by the acetylcholine-bound muscarinic receptor-mediated activation of G_i.

Results and Discussion

Pertussis Toxin-Catalysed ADP-Ribosylation

The non-hydrolysable analogue of GTP, GppNHp elicited a reduction in the pertussis toxin-catalysed adenosine 5`-diphosphate (ADP)-ribosylation of $G_{i\alpha}$ (Fig. 1). This is because the toxin preferably ribosylates the holomeric G protein and GppNHp promotes the release of α from βγ units. The inhibition of ADP-ribosylation in the presence of [^{32}p]-labelled NAD^+ was reflected in the reduced intensity of the bands on auto-radiograph of the gel (Fig. 1). It was also observed that GppNHp-induced inhibition was directly proportional to the concentration of GppNHp. The pre-treatment of the tissue with methacholine resulted into an enhancement of the GppNHp effect. However, the basal ADP-ribosylation remained unchanged. Since receptor mediated activation of G proteins promotes the release of α subunit, the decrease in ribosylation by methacholine treatment may indicate increased activation of G_i.

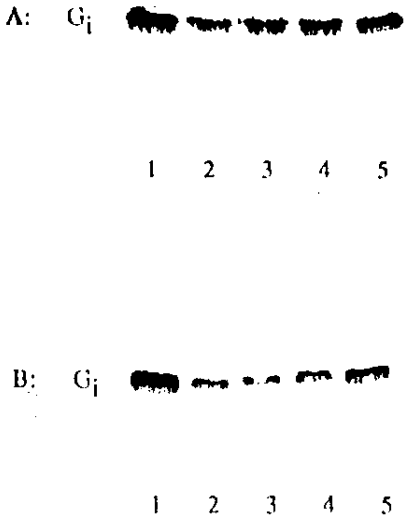

Fig. 1: **The pertussis toxin-mediated [32P]NAD+dependent ADP-ribosylation of the membrances prepared from guinea-pig trachealis, pre-treated with methacholine:GppNHp dose-response:** Panel A and b show control and methacholine-treated membrances respectively. In each pannel, 1 is basal, 2 100 μM, 2 10 μM, 4 1 μM and 5 0 .1 μM GppNHp. The figure represents one of the five similarly conducted experiments.

Adenylyl cyclase activity

A substantial reduction of adenylyl cyclase activity was observed in membranes isolated from tissues pre-incubated with 1 μM methacholine for 30 minutes (Fig. 2). The reductions were 62.5%, 78% and 60% in the basal, GTP plus isoprenaline and

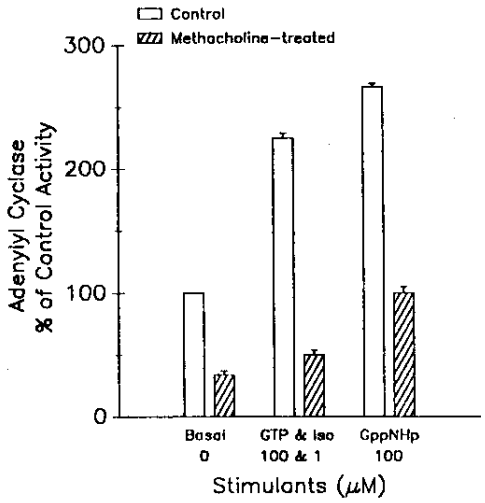

Fig. 2: **The adenylyl cyclase activity in membrances prepared from guinea-pig trachealis pre-treated with methacholine; Effect of GTP (100 μM) and isoprenaline (1 μM) and GppNHp (100 μM):** Prior to making the membrances, the tracheal smooth muscle was incubated at 37°C for 30 minutes without or with 1 μM methacholine in Krebs hensleit buffer bubbled with O_2 + CO_2 (95% + 5%). Subsequently, the adenylyl cyclase activity was measured. Each value is an average (± S.E.M) of three to four observations. Iso: isoprenaline

GppNHp-stimulated respectively. The inhibitory effect was reversed in the membranes exposed to atropine prior to methacholine treatment (Fig. 3). Clearly, adenylyl cyclase activity, basal or stimulated by either G protein or receptor, is susceptible to inhibition with methacholine. The measurement of adenylyl cyclase activity in the membranes pre-incubated with the G_i-specific antibody, SG1 is elicited in Fig. 4. An enhancement of the GppNHp-stimulated and basal activity is evident from this figure. Clearly, the methacholine-caused inhibition of GTP-induced stimulation of adenylyl cyclase was prevented in the membranes exposed to the antibody (Fig. 5).

We have investigated the functional role of G_i-coupled muscarinic receptor in the tracheal smooth muscle of the guinea pig. Despite being the product of separate genes, the subtypes of muscarinic receptor show a remarkable sequence homology with regard to their transmembrane stretches. However, the third cytoplasmic loop is considerably diverse in different subtypes and determines the type of G protein for coupling and the effector enzyme in turn. The studies carried out on individual muscarinic acetylcholine receptor subtypes, expressed from the cloned DNAs in *Xenopous* oocytes and mammalian cells indicate that each subtype exhibits a selectivity, albeit not exclusive in coupling with the effector systems [24,25]. Therefore, selective interaction of receptor with G proteins plays an important role in

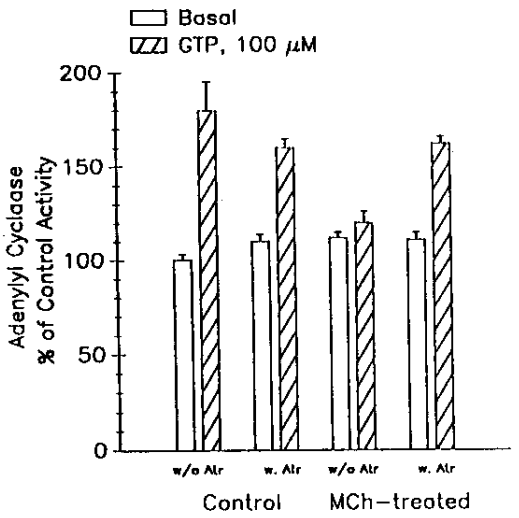

Fig. 3: **The adenylyl cyclase activity in guinea-pig trachealis membranes: Effect of atropine on methacholine-mediated inhibition of GTP-stimulated activity.** The membranes were prepared from tracheal smooth muscle pre-incubated at 37°C for 30 minutes without or with 1 μM methacholine, in the absence or presence of 1 mM atropine in Krebs Hensleit buffer bubbled with O_2 + CO_2 (95% + 5%). Subsequently, the adenylyl cyclase activity was measured. Each value is an average (± S.E.M) of three observations. w/o: without, w: with, Atr: atropine. Mch: methacholine.

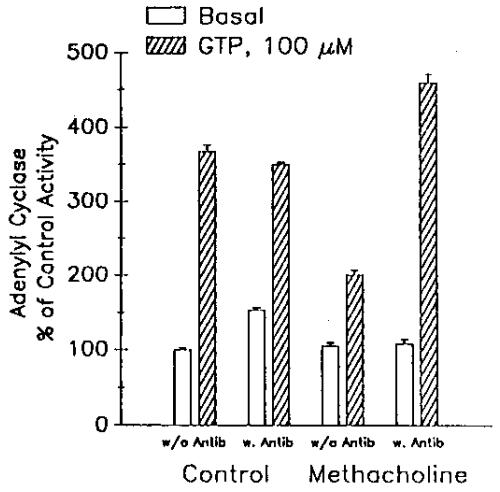

Fig. 4: **The adenylyl cyclase activity in guinea-pig trachealis membranes: Effect of SGI, the Gi-specific antibody on methacholine-mediated inhibition of the GTP-stimulated activity.** The tracheal smooth muscle was pre-incubated at 37°C for 30 minutes without or with 1 μM methacholine, in the absence or presence of 1 μM atropine in Krebs Hensleit buffer bubbled with O_2 + CO_2 (95% + 5%). The membranes were then prepared and incubated at 37°C for 45 minutes in the absence or presence of the antibody in a final dilution of 200. Subsequently, the adenylyl cyclase activity was measured. Each value is an average (± S.E.M) of three to four observations. w/o: with, Antib: antibody

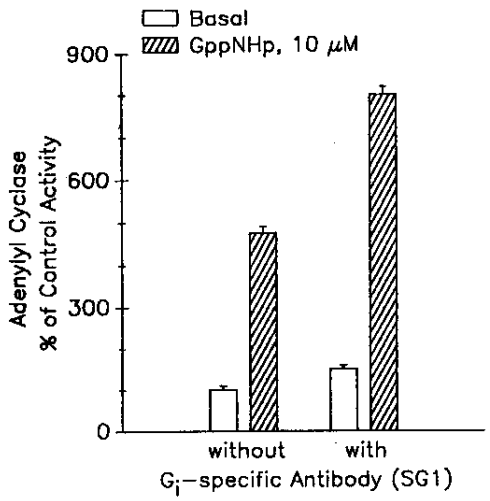

Fig. 5. **Effect of Gi-specific antibody, SGI, on GppNHp-stimulated adenylyl cyclase activity in guinea-pig trachealis membranes.** The freshly prepared membranes were incubated at 37°C for 45 minutes in the absence or presence of the antibody in a final dilution of 200. Subsequently, the adenylyl cyclase activity was measured. Each value is an average (± S.E.M) of three to four observations

determining the specificity of the cellular effects of muscarinic agonists [24,25]. The coupling of M_1 and M_2 receptors with various effector enzymes through pertussis toxin-insensitive and sensitive G proteins respectively has been proposed [25,26].

The modulation of pertussis toxin-sensitive G protein with muscarinic receptor was demonstrated by the reduction in pertussis toxin-catalyzed ADP-ribosylation in methacholine-treated tissue (Fig. 1). The blockade of adenylyl cyclase activity in methacholine-treated tissue and its inhibition with atropine (Fig. 2& 3) clearly indicated that a subtype of muscarininc receptor is involved in the regulation of this pathway. However, atropine does not discriminate between the known muscarinic receptor subtypes. The G_i specific antibody, SG1, that identifies the C-terminal decapeptide of G_i1 and G_i2 indiscriminately, was used to study the uncoupling of muscarininc receptor from adenylyl cyclase. A marked increase of GppNH—stimulated adenylyl cyclase activity in the membranes pre-treated with the antibody (Fig. 5), supports the functional role of G_i in tracheal smooth muscle. Due to binding of SG1, G_i, was unable to interact with the receptor and thereby the message of methacholine-activated muscarinic receptor was not conveyed to the effector enzyme. As a result, the activitation of adenylyl cyclase stimulated with the guanine nucleotides, GTP and GppNHp was left uncontrolled. This clearly suggests that in the guinea-pig trachealis, a subtype of the muscarinic receptor is operative that modulates adenylyl cyclase activity through G_i, (G_i1, or G_i 2). The reversal of methacholine-mediated inhibition of adenylyl cyclase with SG1 further evidenced the functional role of G_i-coupled muscarinic receptor in the guinea-pig trachealis.

The predominant existence of M_2 receptor over M_3 on smooth muscle preparations, detected by Northern blot has been reported [27]. In the circular smooth muscle from rabbit caecum, the inhibition of adenylyl cyclase, mediated by M_2 receptor via pertussis toxin-sensitive G protein has been demonstrated [28]. The presence of M_4 sites through antagonist binding and Northern blot studies has been reported in the rabbit lung [29]. Both M_2 and M_4 are coupled to the inhibition of adenylyl cyclase (reviewed by Peralta *et al.*, 1988., Hulme *et al.*, 1990., Richards, 1991) [5-7].

Thus, the conclusion can be drawn that in guinea-pig trachealis, probably M_2 and/or M_4 type muscarinic receptor is responsible for exerting the inhibitory control over adenylyl cyclase through the intermediacy of G_i. However, the type of M_2 mAChR could be different from the M_2 of parasympathetic nerve, most probably $M_{2\beta}$ as has been mentioned in the introduction to this article. It is also possible that the decline in cAMP mediated by $M_{2\beta}$ and/or M_4 actually contributes towards the contractile response of this tissue.

Experimental

All chemicals and toxin were from Sigma Chemical company (UK). The nucleotides guanylylimidodiphosphate (GppNHp), guanosine 5`-triphosphate (GTP) and adenosine 5`-triphosphate (ATP) were obtained from Boehringer Mannheim (Germany). [^{3}H] cyclic AMP and [α-^{32}P]-ATP were

purchased from Amersham International plc (UK). [^{32}P]-NAD (nicotinamide adenine dinucleotide) was from Dupont (USA). SGI was a kind gift from Dr. G. Milligan (University of Glasgow). Horse-radish peroxidase linked antibody was a gift from the Scottis antibody production unit (Carluke, Scotland).

Dissection of Tracheal Smooth Muscle

Guinea-pig was stunned by a blow on the head, trachea was immediately dissected out and rinsed in Krebs-Hensleit buffer. The smooth muscle strip was dissected clean of the connective tissue under a microscope.

Incubation of Tracheal Smooth Muscle Strip

The smooth muscle strips were incubated in two sets, each containing 10 ml of Krebs-Hensleit buffer, pH 7.4 constantly bubbled with carbogen (O_2:CO_2, 95:5%) for 10 minutes at 37°C. Then methacholine (1 μM) was added to one of the vial and a further incubation of 30 minutes was allowed. Treatment with atropine (1 μM) involved a 10 minutes incubation prior to the addition of methacholine.

Preparation of Tracheal Smooth Muscle Membranes

The smooth muscle strips were homogenized in 2 ml of 10 mM Tris-HCl buffer (pH 7.4) containing 1 mM ethylenediaminetetra acetic acid, 250 mM sucrose, 0.1 mM phenylmethyl sulphonyl fluoride and 2 mM benzamidine. The homogenate was centrifuged at 50,000 g for 20 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 0.2 ml of the buffer (0.4 mg ml^{-1} protein).

Pertussis Toxin Catalysed ADP-Ribosylation of G_i

Pertussis toxin-catalyzed ADP-ribosylation was performed according to Pyne *et al.*, (1989) [21]. The reaction mixture contained membranes (10 μg protein(, 80 μM potassium phosphate (pH 7.5) 30 μM thymidine, 20 μM arginine, 1 μM $MgCl_2$. After adding 2 μCi [^{32}P]-NAD^+ and pre-activated 0.6 μg/assay of pertussis toxin, the reaction mixture was incubated at 37°C for 30 minutes. The incubation was terminated by the addition of trichloroacetic acid (final concentration 6% w/v) and deoxycholate (0.01% w/v). The precipitated proteins were pelleted by centrifugation at 15,000 g for 10 min. at 4°C and resuspended in 10 μl of 1 M Tris/HCl and 20 μl of Laemmli buffer. The samples were boiled for 5 minutes and subjected to sodium dodecyl-polyacrylamide gel (SDS-PAGE) (10% acrylamide) electrophoresis as described by Laemmli (1970) [22].

After electrophoresis, the bands of proteins separated on the gels were transferred to nitrocellulose sheets which were then auto-radiographed.

Adenylyl Cyclase Assay

Adenylyl cyclase activity was assayed according to Salomon *et al* (1974) [23]. The reaction cocktail in 12.5 mM Tris-HCl (pH 7.8) contained 20-30 μg membrane proteins and other reactants in final concentrations (mM): ATP 0.1, cyclic AMP 0.4, creatine phosphate 2.5, creatine kinase 1.5 units, KCl 7.5 and sucrose 30. The [α-^{32}P]-ATP was added (1 μCi) as the substrate and the reaction was carried out at 37°C for 30 minutes. The reaction was terminated by adding 40 mM ATP and boiling for 2 minutes. The contents were chromatographed first through a Dowex and then on an alumina column. The isolated [^{32}P]-cAMP was measured by liquid scintillation counting.

References

1. F. Murad, Y.M. Chi, T.W. Rall, and E.W. Sutherland, *J. Biol. Chem.* 237, 1233 (1962).
2. K.H. Jacobs, K. Aktories, and G. Schultz, *Naunyn Schmiedeberg's Arch. Pharmacol.* 310: 113 (1979)
3. J.W., Fleming, R.A. Strawbridge, and A.M.J. Watanabe, *Mol. Cell. Cardiol.* **19**: 47 (1987)
4. T. Evans, M.M. Smith, L.T. Tanner, and T.K. Harden, *Mol. Pharmacol.* **26**, 395 (1984)
5. E.G. Peralta. A. Ashkenazi, J.W. Winslow, J. Ramachandran, and D.J. Capon, *Nature* **334**, 434 (1988)
6. E.C. Hulme, N.J.M. Birdsall, and N.J. Buckley, *Annu. Rev. Pharmacol. Toxicol.* **30**, 633 (1990)
7. M.H. Richards, *Biochem. Pharmacol.* **42**, 1645 (1991).
8. H. Kilbinger, R. Schneider, H., Siefken, D. Wolf, and G. Agostino, *Br. J. Pharmacol.* **103**, 1757 (1991)

9. M. Eltze, and M. Galvan, *Pulm. Pharmacol.* 7, 109 (1994)
10. S. Matsumoto, T. Nagayama, T. Kanno, M. Yamasaki, and T.J. Shimizu, *Auton, Nerv. Syst.* 53, 126 (1995)
11. C. Kaiser, and J. Rzeszotarsky, Chapter 14 in Receptor Pharmacology and Function, ed. by M. Williams, R.A. Glennon and P.B.W.M. Timmerman, Marcel Dekker Inc. New York (1989)
12. R. Hammer, E. Giraldo, G.B. Schiavi, E. Monferini, and H. Ladinsky, *Life Sci.* 38, 1653 (1986)
13. P.J. Silver, and J.T. Stull, *J. Biol. Chem.*, 257, 6145 (1982).
14. M.J. Berridge, and R.F. Irvine, *Nature*, 312, 315 (1981)
15. Y. Nishizuke, *Nature*, 308, 693 (1984)
16. E.R. Chilvers, J. Challis, P.J. Barnes, and S.R. Nahorski, *Eur. J. Pharmacol.*, 614, 587 (1989)
17. S. Katsuki, and F. Murad, *Mol. Pharmacol.* 13, 330 (1977)
18. J.J. Pyne, M.W. Grady, D. Shehnaz, P.A. Stevens. S. Pyne. and I.W. Rodger, *Br. J. Pharmacol.*, 107, 881 (1992).
19. A.M. Spirgel, A Shenker, and L.S. Weinstein, *Endocr. Rev.*, 13, 536 (1992).
20. M.C. Olianas, and P. Onali, *Mol. Pharmacol.*, 49, 22 (1996).
21. N.J. Pyne, G.J. Murphy, G. Milligan, and M.D. Houslay, *FEBS Lett.*, 243, 77 (1989).
22. U.K. Laemmli, *Nature*, 227, 680 (1970).
23. Y. Salomon, C. Londos, and M. Rodbell, *Anal. Biochem.*, 56, 502 (1974).
24. D.F. Matesic, D.R. Manning, and G.R. Luthin, *Mol. Pharmacol.*, 40, 347 (1991).
25. J. Lai, S.L. Waite, J.W. Bloom, H.I. Yamamura, and W.R. Roeske, *J. Pharmacol. Exp. Ther.*, 258, 938 (1991).
26. P.C. Sternweis, and A.V. Smrcka, *Trends Biochem. Sci.*, 17, 502 (1992).
27. A. Maeda, T. Kubo, M. Mishina, and S. Numa, *FEBS Lett.*, 239, 339 (1988).
28. P. Cuq, R. Magous, and J-P. Bali, *Mol. Cell. Biochem.*, 140, 65 (1994).
29. S. Lazareno, N.J. Buckley, and F.F. Roberts, *Mol. Pharmacol.*, 38, 805 (1992).