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Carbamylcholine inhibits β-adrenergic receptor-coupled G_s protein function proximal to adenylate cyclase

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The specific mechanism by which the inhibitory guanine nucleotide binding protein (G_i) mediates the inhibition of adenylate cyclase activity is still unclear. The subunit dissociation model, based on studies in purified or reconstituted systems, suggests that the $\beta\gamma$ subunit, which is dissociated with activation of G_i, inhibits the function of the stimulatory guanine nucleotide binding protein (G_s) by reducing the concentration of the free α_s subunit. In the present study, G_s protein function is determined by measuring cholera toxin-blockable, isoproterenol-induced increases in guanosine triphosphate (GTP) binding capacity to rat cardiac ventricle membrane preparations. Carbamylcholine totally inhibited this β-adrenergic receptor-coupled G_s protein function. Pretreatment of the cardiac ventricle membrane preparation with pertussis toxin prevented this muscarinic agonist effect. These results confirm the possibility of an inhibitory agonist-receptor coupled effect through G_i on G_s protein function proximal to the catalytic unit of adenylate cyclase in an intact membrane preparation.

G protein; Adenylate cyclase; β-Adrenergic receptor; Muscarinic receptor

1. INTRODUCTION

Adenylate cyclase activity is dually regulated by membrane receptors that activate specific guanine nucleotide binding proteins. Stimulatory receptors (e.g. β-adrenergic receptors) activate a specific adenylate cyclase-stimulatory G protein (G_s) while inhibitory receptors (e.g. muscarinic recptors) activate an inhibitory guanine nucleotide binding protein (G_i) (for review, see [1]).

The mechanism of adenylate cyclase activation by G_s is well understood. Specifically, hormones or neurotransmitters interact with their specific receptors to stimulate the binding of guanosine triphosphate (GTP) to the α subunit of G_s, which is concomitantly followed by subunit dissociation. Thus formed, the GTP-bound α_s directly activates adenylate cyclase [1,2].

The specific mechanism by which G_i mediates the inhibition of adenylate cyclase activity is still unclear and controversial. The subunit dissociation model [1,3,4] proposes that the $\beta\gamma$ subunit, which is dissociated with activation of G_i, indirectly inhibits adenylate cyclase by reducing the concentration of the free α_s subunit. The resulting association of $\beta\gamma$ and α_s forms an inactive trimer of G_s. Two additional models propose direct inhibition of adenylate cyclase by either the activated α_i [5–7] or $\beta\gamma$ subunits [8]. All the above models are based on experiments carried out mostly on purified or reconstituted components of the receptor-G protein-adenylate cyclase complexes.

An important characteristic of G proteins is their increased guanine nucleotide binding following agonist stimulation which in turn leads to their activation. Although basal binding of guanine nucleotides to certain crude membrane preparations is not exclusive to G proteins [9], there is no dispute that agonist-induced increases in guanine nucleotide binding are an exclusive characteristic of G proteins and may serve to assess receptor-coupled G protein function proximal to adenylate cyclase [10,11].

In the present study, G_s protein function was determined by measuring cholera toxin-blockable, β-adrenergic agonist-induced increases in [^{3}H]GTP binding to membrane preparations from rat cardiac ventricles to test the possibility of an inhibitory agonist-receptor coupled effect through G_i on G_s protein function proximal to adenylate cyclase.

2. MATERIALS AND METHODS

2.1. *Cardiac ventricle membrane preparation*

Male Sprague-Dawley rats (200–250 g) were decapitated, and the cardiac ventricles were rapidly removed and homogenized in buffer A (25 mM Tris, pH 7.4, 1 mM dithiothreitol (DTT)) containing 1 mM ethyleneglycol-bis-(β-aminoethylether)-N (EGTA). Cardiac ventricle homogenate was then passed through double-layer cheesecloth. Membranes were obtained by centrifuging the preparations twice for 10 min at 10000 × *g* and 4°C.

2.2. *[^{3}H]GTP binding to rat cardiac ventricle membranes*

200 μl of membranes (0.5–1.5 mg protein) suspended in 25 mM Tris (pH 7.4), 1 mM adenosine triphosphate (ATP), 2.2 mM Mg^{2+},

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1 mM EGTA, and 1 mM DTT, were pipetted into plastic microfuge tubes containing varying concentrations of [^{3}H]GTP (0.04–0.6 μM). The incubation was carried out at room temperature for 5 min (equilibrium conditions) and the reaction was stopped by adding 5 vols of ice-cold buffer A, followed by centrifugation at 10000 × g for 2 min. The pellets were washed rapidly 3 times with ice-cold buffer A by repeating centrifugation and were then resuspended in 200 μl of the same buffer. Bound radioactivity was measured by liquid scintillation spectrometry by adding a 150 μl aliquot dissolved in scintillation liquid. All assays were carried out in triplicate, together with triplicate control samples containing 100 μM unlabeled Gpp(NH)p to determine nonspecific binding. Either isoproterenol or carbamylcholine was added at a final concentration of 50 μM.

2.3. *Adenosine diphosphate (ADP) ribosylation*

Membranes were suspended in 1 ml buffer containing 25 mM Tris, pH 7.4, 10 mM nicotinamide adenine dinucleotide (NAD), 1 mM ATP, 1 mM EGTA, 100 μM GTP, 2 mM DTT, 5 mM $MgCl_2$, 10 mM thymidine, 20 mM creatine phosphate, and 40 $\mu g \cdot ml^{-1}$ creatine phosphokinase. ADP ribosylation was carried out for 15 min at 30°C by adding cholera toxin (50 $\mu g \cdot ml^{-1}$) preactivated for 10 min at 37°C with 20 mM DTT, or pertussis toxin (25 $\mu g \cdot ml^{-1}$) preactivated for 10 min at 30°C with 20 mM Tris. The reaction was stopped by adding 25 ml ice-cold 25 mM Tris, pH 7.4, and 5 mM $MgCl_2$ immediately followed by centrifugation at 10000 × g for 10 min. [^{3}H]GTP binding was then carried out as described.

3. RESULTS AND DISCUSSION

Membrane G_s protein function was determined by measuring β-adrenergic agonist modulation of GTP binding characteristics. In membranes prepared from rat cardiac ventricles, isoproterenol induced over a 20% increase in [^{3}H]GTP binding capacity, which was blocked by propranolol (10 μM), with no significant effect on the affinity of GTP (fig.1, table 1). This β-adrenergic agonist-induced increase in [^{3}H]GTP binding specifically characterizes G_s protein function as it is totally abolished by cholera toxin-catalyzed ADP ribosylation and is unaffected by pertussis toxin (table 1). The β-adrenergic agonist effect of increasing GTP binding capacity may be caused by stimulation of the dissociation of $G_s \cdot$guanosine diphosphate (GDP), which must precede the GTP binding, and by direct stimulation of GTP binding [1,12].

In contrast to isoproterenol, carbamylcholine did not induce any detectable increases in GTP binding capacity (table 1). These differences in the ability of the β-adrenergic and the muscarinic agonists to alter GTP binding characteristics in cardiac membranes, may be explained by the findings of Gilman et al. [3–5] who reported that at low concentrations of Mg^{2+}, the tendency of G_i to dissociate appears to be greater than that of G_s. This characteristic of G_i is an important feature in the subunit dissociation model of action of this protein as an inhibitory regulator of adenylate cyclase activity [3–5]. In a previous study, we showed that pertussis toxin-blockable, carbamylcholine-induced increases in GTP binding capacity are mediated by the pharmacologically defined M_1 muscarinic receptors, as M_1-selective antagonists had a 100-fold greater ability than M_2-selective antagonists to block carbamylcholine effects in rat cerebral cortex [11]. Moreover, the carbamylcholine effect was exclusively detected in tissues such as the cerebral cortex and hippocampus, which are predominantly populated by M_1 receptors, whereas cerebellum and cardiac ventricles, which are mostly populated by M_2 receptors, failed to show carbamylcholine effects on GTP binding to G proteins [11]. The underlying biochemical mechanisms of these differences in the carbamylcholine effect are as yet unknown and may stem from different coupling mechanisms of the various muscarinic receptor subtypes. As subtypes of muscarinic receptors differ in their primary transducing effect (i.e. induction of phosphatidylinositol (PI) turnover vs adenylate cyclase inhibition) [13–15], different members of the G protein

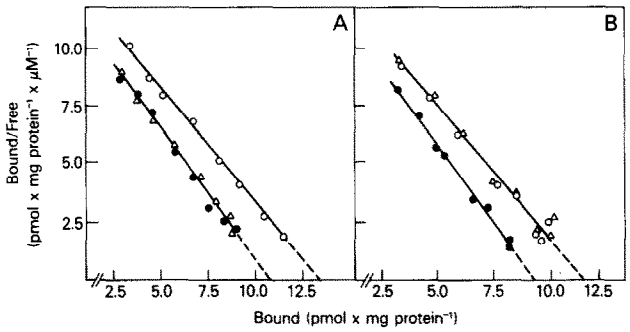


Fig.1. Pertussis toxin-blockable muscarinic agonist inhibition of β-adrenergic agonist-induced increases in GTP binding capacity. Representative Scatchard plots of basal (●) and isoproterenol-induced [^{3}H]GTP binding in the absence (○) or presence (□) of carbamylcholine to membranes prepared from rat cardiac ventricles. (A) Untreated membranes exposed to ribosylation conditions in the absence of pertussis toxin. (B) Membranes pretreated with pertussis toxin. Both isoproterenol and carbamylcholine concentrations were 50 μM.

Table 1

Inhibition of isoproterenol-induced increases in GTP binding by carbamylcholine: effects of cholera toxin- and pertussis toxin-catalyzed ADP ribosylation

Agonist	Percent increase in [^{3}H]GTP binding capacity		
	Membrane treatment		
	None	Cholera toxin	Pertussis toxin
Basal	10.2 ± 0.53	10.7 ± 0.6	10.3 ± 0.8
Isoproterenol	12.9 ± 0.77*	10.4 ± 0.58	13.2 ± 0.7*
Carbamylcholine	10.3 ± 0.36	10.2 ± 0.47	10.3 ± 0.8
Isoproterenol + carbamylcholine	10.6 ± 0.6	10.3 ± 0.8	12.5 ± 0.68*

Rat cardiac ventricle membranes were preactivated with either cholera toxin or pertussis toxin to induce ADP ribosylation of G_s and non-G_s proteins, respectively. Untreated membranes were exposed to ribosylation conditions in the absence of toxins. The effects of isoproterenol (50 μM), carbamylcholine (50 μM), or isoproterenol + carbamylcholine on [^{3}H]GTP specific binding were then examined. * $P < 0.01$ for [^{3}H]GTP maximal binding capacity, as compared with basal binding capacity

family (i.e. G_i, G_o, G_p) may be involved in the coupling of the various muscarinic subtypes to their second messenger signaling systems (for review, see [16]). Moreover, Florio and Sternweis [17] showed that the mechanism of guanine nucleotide exchange induced by hormones and neurotransmitters is more complex than previously postulated. Working on the mechanisms of muscarinic receptor action on G_o in reconstituted phospholipid vesicles, these authors found that muscarinic receptors stimulate guanine nucleotide exchange on G_o protein both by increasing the rates of dissociation of nucleotides and by altering their relative affinities such that binding of GTP becomes highly favored over GDP.

The fact that carbamylcholine does not induce increases in GTP binding capacity to pertussis toxin-sensitive G proteins in cardiac ventricles (table 1) [11] is advantageous for exploring the possibility of a muscarinic receptor-coupled effect through G_i on G_s protein function proximal to adenylate cyclase.

Fig.1A shows that carbamylcholine attenuates isoproterenol-induced increases in GTP binding capacity to G_s. This inhibitory effect of carbamylcholine was totally abolished by atropine (10 μM). This carbamylcholine effect reflects inhibitory influences exerted by a pertussis toxin-sensitive G protein on G_s as the inhibition by carbamylcholine of β-adrenergic-coupled G_s function was totally abolished by pretreatment of the cardiac membranes with pertussis toxin (table 1, fig.1B).

Fleming et al. [18] have shown that muscarinic agonists inhibit GTP-activated but not Gpp(NH)p-activated adenylate cyclase activity, suggesting a role for GTP hydrolysis in the mechanism of inhibition of adenylate cyclase. Further studies by this group [19] indicate that muscarinic receptor stimulation of high affinity GTPase activity, which is dependent on functional pertussis toxin substrate(s), is closely linked to the mechanism of muscarinic inhibition of adenylate cyclase activity. This muscarinic agonist-stimulated GTPase activity may explain our failure to detect carbamylcholine-induced increases in GTP binding to cardiac membranes, as well as our failure to detect isoproterenol-induced increases in GTP binding in the presence of carbamylcholine. Indeed, preliminary findings from this laboratory indicate that carbamylcholine is able to increase the binding capacity of [^{3}H]Gpp(NH)p to cardiac membranes and that isoproterenol-induced increases in [^{3}H]Gpp(NH)p binding capacity are not inhibited by carbamylcholine.

Thus, we show an inhibition of G_s protein function by a pertussis toxin-sensitive G protein (presumably G_i) proximal to adenylate cyclase. We speculate that the $\beta\gamma$ subunit complex dissociated upon activation of G_i by the cholinergic agonist is responsible for the inactivation of GTP bound α_s protein, probably by favoring hydrolysis of the bound GTP. Using an intact membrane preparation, our present results provide supportive evidence for the subunit dissociation model of inhibition of adenylate cyclase by G_i, which was exclusively based on findings in purified and reconstituted systems.

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