# EFFECTS OF CHOLINERGIC AGENTS ON RAT LIVER PLASMA MEMBRANE GUANYLATE CYCLASE

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## 1. Introduction

Guanylate cyclase (EC 4.6.1.2) catalyzes the formation of guanosine 3′,5′-monophosphate (cyclic GMP), which is postulated to act as an intracellular messenger on the action of various biologically-active compounds, including cholinergic agonists [1–3]. Acetylcholine is able to increase the intracellular concentrations of hepatic cyclic GMP [4], which has been implicated in the parasympathetic control on the liver glycogen metabolism [5,6]. Although tissue levels of cyclic GMP are increased several-fold under cholinergic stimulation [1,4], an appreciable activation of guanylate cyclase by acetylcholine and other muscarinic agents has never been demonstrated [7–10]. Here, we show significant activation by muscarinic agonists on the guanylate cyclase activity in isolated plasma membranes from rat liver. Moreover, this stimulation was inhibited by atropine, a muscarinic blocker. Preliminary results were reported in abstract from [11].

## 2. Materials and methods

### 2.1. *Chemicals*

Cyclic GMP, GTP, GDP, GMP, creatine phosphate, creatine phosphokinase from rabbit muscle, type I, Lubrol PX, acetylcholine chloride, carbamoylcholine and atropine sulphate were purchased from Sigma Co. Sucrose was from BDH Chemicals. Aquasol was from New England Nuclear. Cyclic GMP RIA was from Amersham. Bovine serum albumin (fraction V) was from Armour. All other reagents used were of analytical grade.

### 2.2. *Preparation of rat liver plasma membranes*

The original procedure is detailed in [12]. Rat liver was perfused in situ through the portal vein with 0.154 M NaCl followed by buffer I (0.25 M sucrose–5 mM Tris–HCl (pH 8.0)) cleaned and minced. The minced tissue (40 g) was suspended in 1 vol. buffer I/g wet tissue and homogenized (Potter-Elvehjem, Arthur Thomas type C) and centrifuged at 750 × *g* for 10 min. The supernatant was saved and the sediment extracted twice as above. All supernatants were pooled and centrifuged at 31 000 × *g* for 15 min giving a crude mitochondrial fraction as sediment. The post-mitochondrial supernatant was spun at 105 000 × *g* for 90 min and the sediment was the 'microsomal fraction'. To purify the plasma membrane fraction, a discontinuous sucrose gradient was used. The microsomal fraction was dispersed by homogenization (5 strokes) in 1.28 M sucrose (1 ml/g wet tissue) and 7.5 ml of the latter suspension was placed on the bottom of SW 25.1 tube and overlayered with 15 ml 0.82 M sucrose and 7.5 ml 0.25 M sucrose. This gradient was centrifuged at 53 000 × *g* for 12 h, the plasma membranes fraction collected at the 0.25/0.82 M sucrose interface, diluted with 5 mM Tris–HCl (pH 8.0) and centrifuged at 150 000 × *g* for 30 min. The sediment was suspended in small volume of buffer I. This plasma membrane fraction was used immediately or kept at −70°C. The entire procedure was carried out at 4°C and all sucrose solutions were buffered with 5 mM Tris–HCl (pH 8.0).

### 2.3. *Guanylate cyclase assay*

Unless otherwise indicated in the figure legends, the reaction mixtures (140 μl total vol.) contained

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50 mM Tris–HCl (pH 7.6); 3 mM $MnCl_2$ or $MgCl_2$; 1–2 mM GTP, a GTP regenerating system (5 mM creatine phosphate and 1 IU phosphocreatine kinase in 0.1% of defatted bovine serum albumin); 20–70 μg membrane protein; 0.9% NaCl and atropine sulphate or deionized water. Incubations were initiated by the addition of cholinergic agents or deionized water in control experiments (basal activity) and continued for 10 min at 37°C. Reactions were terminated by immersion in liquid nitrogen for 15 s and boiling at 90°C for 3 min. To determine non-enzymic cGMP generation, blank experiments containing the different additions were done in the presence of pre-boiled enzyme. Cyclic GMP formed was determined by radioimmunoassay in 30 μl of the supernatant of the reaction mixture obtained after centrifugation at 12 000 × *g* × 5 min at 4°C. The radioactivity was determined by liquid scintillation spectrometry, using Aquasol as scintillation fluid. The amount of GTP remaining after the incubation was found to be >80% of the initial one, as checked by high-pressure liquid chromatography, under the experimental conditions described for a Varian LCS 1000 [13]. Protein was estimated by the method in [14], as modified in [15], using bovine serum albumin as standard.

### 3. Results and discussion

It would be worthwhile to investigate whether cholinergic control of liver glycogen metabolism is brought about by mechanisms which may include a cholinergic receptor–guanylate cyclase system at the hepatic level. Assuming that the latter system is operative in some tissues, it would be important to demonstrate experimentally that cholinergic receptors coupled to guanylate cyclase are present in liver plasma membranes, and that cholinergic agonists influence this enzyme activity. Here we show that guanylate cyclase is present in a liver plasma membrane fraction derived from sinusoidal front hepatocyte [12] and acetylcholine and carbamoylcholine are able to increase this guanylate cyclase activity. Liver plasma membrane guanylate cyclase was stimulated by both acetylcholine and carbamoylcholine in the presence of $MnCl_2$ (fig.1) or $MgCl_2$ (fig.2) as divalent cofactors. These data demonstrate that cholinergic stimulation of guanylate cyclase is independent of the divalent cation used in the assay mixture. The maximal stimulation achieved with acetylcholine was at $10^{-7}$ M and carbamoylcholine at $10^{-9}$ M; atropine, a muscarinic blocker, at $10^{-6}$ M was able to decrease this acetylcholine- or carbamoylcholine-induced stimulation, suggesting that stimulation was through a muscarinic receptor. To demonstrate the reproducibility of the acetylcholine stimulation of membrane-bound guanylate cyclase, experiments were done

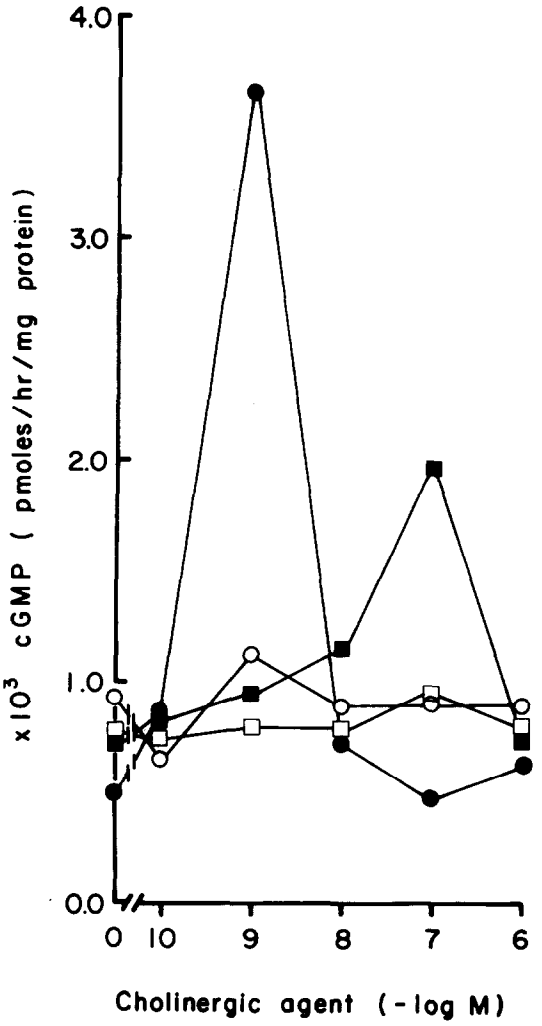


Fig.1. Effects of carbamoylcholine (•——•) and acetylcholine (▪——▪) on guanylate cyclase from rat liver plasma membrane. The effect of atropine ($10^{-6}$ M), in the presence of carbamoylcholine (o——o) and acetylcholine (□——□). Enzymic activity was assayed in presence of 3 mM $MnCl_2$ as divalent cofactor and 1 mM GTP. Each point represents the mean of 2 individual expt performed in duplicate. These experiments were done with different membrane preparations. Details are in section 2.

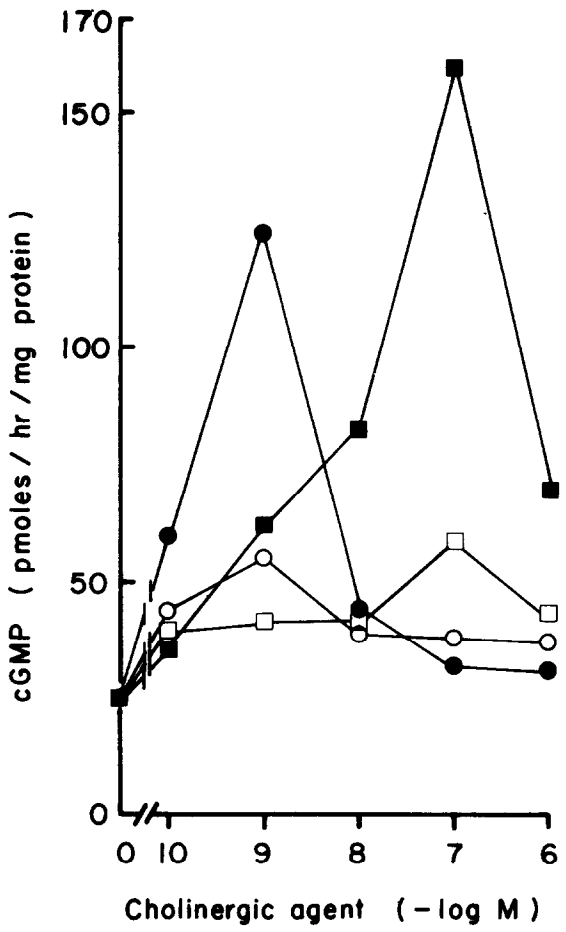


Fig.2. Effects of carbamoylcholine (●——●) and acetylcholine (■——■) on guanylate cyclase from rat liver plasma membranes. The effect of atropine ($10^{-6}$ M) in the presence of carbamoylcholine (○——○) and acetylcholine (□——□). Enzymic activity was assayed in presence of 3 mM $MgCl_2$ as divalent cofactor and of 1 mM GTP. Each point represents the mean of 2 individual expt performed in duplicate. Each set of experiments was done with different membrane preparations. Details are in section 2.

using several membrane preparations under different experimental conditions as shown in fig.3.

A GTP-regenerating system (creatine phosphate plus creatine phosphokinase) was required for maximal activity of guanylate cyclase bound to membranes. An explanation for this requirement is the existence of a very active GTP hydrolyzing enzyme in rat liver plasma membranes fractions [16]. Moreover, the basal $Mn^{2+}$-dependent guanylate cyclase was higher than the $Mg^{2+}$-dependent one, as reported in [10].

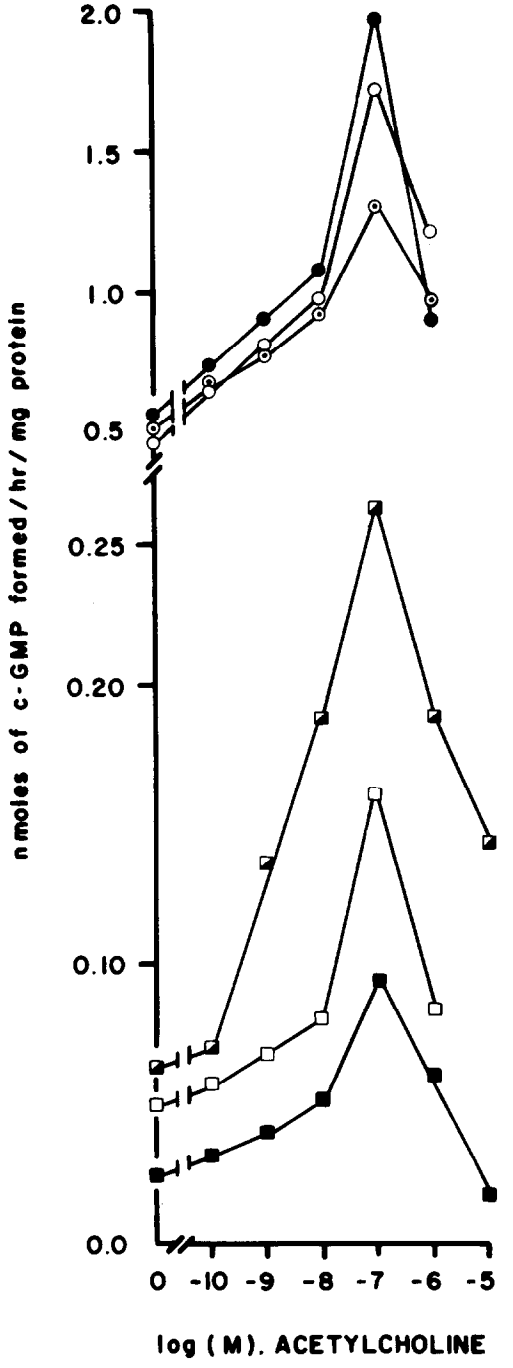


Fig.3. Effects of acetylcholine on guanylate cyclase from rat liver plasma membranes. The experimental conditions are: Acetylcholine + regenerating system with 1 mM GTP and 3 mM $MnCl_2$ (○——○, ⊙——⊙, ●——●). Acetylcholine + regenerating system with 3 mM $MgCl_2$ in the presence of 2 mM GTP (▣——▣) or 1 mM GTP (□——□) acetylcholine – regenerating system in the presence of 3 mM $MgCl_2$ and 2 mM GTP (■——■).

Table 1
Effect of Lubrol PX on basal and acetylcholine-stimulated guanylate cyclase activity

| Acetylcholine (M) | −Lubrol PX | +Lubrol PX |
|---|---|---|
| 0 | 25 ± 6 | 844 ± 118 |
| $10^{-10}$ | 36 ± 9 | 703 ± 106 |
| $10^{-9}$ | 63 ± 17 | 553 ± 134 |
| $10^{-8}$ | 83 ± 6 | 689 ± 191 |
| $10^{-7}$ | 160 ± 21 | 508 ± 178 |
| $10^{-6}$ | 70 ± 14 | 650 ± 180 |

Results are expressed as pmol cyclic GMP formed . $h^{-1}$ . mg $protein^{-1}$. The enzymic activity was assayed in presence of 3 mM $MgCl_2$ as divalent cofactor and 1 mM GTP. Data represent the mean ± SD of 4 expt done in duplicate

These findings agree with the effect of acetylcholine increasing the intracellular concentrations of hepatic cyclic GMP [4], possibly mediated by stimulation of a muscarinic–guanylate cyclase complex associated to rat liver plasma membranes.

The stimulation of the guanylate cyclase by acetylcholine was completely lost when Lubrol PX was added in the presence of either $Mg^{2+}$ (table 1) or $Mn^{2+}$ (not shown). The latter effect may be due to the capacity of the detergent molecules to disrupt some interactions between the cholinergic receptor (muscarinic) and the catalytic unit of the membrane-bound guanylate cyclase. Moreover, table 1 shows that Lubrol PX activated this guanylate cyclase activity 30–34-fold, as described for cultured Balb 3T3 fibroblasts [17].

These observations serve as a starting point for studies designed to clarify the relationship between the rat liver muscarinic receptor and the catalytic subunit of the guanylate cyclase, which ultimately may provide new insight into the molecular mechanisms of action of the cholinergic agents.

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