

# Constitutive Inhibitory Action of Muscarinic Receptors on Adenylyl Cyclase in Cardiac Membranes and Its Stereospecific Suppression by Hyoscyamine

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## Summary

Muscarinic acetylcholine receptors in the heart have been shown to display agonist-independent spontaneous (constitutive) activity which causes changes in the opening of cardiac ion channels and in the activity of G proteins. We investigated whether an inhibition of the constitutive activity of muscarinic receptors induced by the binding of antagonist brings about a change in the synthesis of cyclic AMP in rat cardiac membranes, and whether the action of the antagonist is stereospecific. Atropine and S(-)-hyoscyamine were indeed found to enhance the forskolin-stimulated synthesis of cyclic AMP in rat cardiac (both atrial and ventricular) membranes by up to 24%. The effect was stereospecific and the potency of R-(+)-hyoscyamine was 30 fold lower than that of the S(-) enantiomer, confirming that the action of hyoscyamine is receptor-mediated. The effect did not depend on the presence of endogenous acetylcholine in the system used. The results strongly suggest that the adenylyl cyclase in the heart is exposed to continuous mild inhibition by constitutively active muscarinic receptors in the membranes of cardiomyocytes.

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## Key words

Muscarinic receptors • Adenylyl cyclase • Constitutive activity of receptors • Hyoscyamine • Atropine • Heart • Inverse agonist action

## Introduction

Cardiac muscarinic receptors were probably the first receptors on which the phenomenon of constitutive (agonist-independent) activity was noted. Giles and Noble (1976) detected that atropine (applied in the absence of a muscarinic agonist) increased the slow inward  $\text{Na}^+/\text{Ca}^{2+}$  current in cells of frog heart atria (i.e. an effect opposite to that of acetylcholine). Similarly, Soejima and Noma (1984) discovered that atropine and

scopolamine depressed the acetylcholine-sensitive  $\text{K}^+$  current in rabbit atrial cells in the absence of muscarinic agonists. Thorough analysis of the effects of muscarinic antagonists on ion channels in cardiocyte membranes left little doubt that the antagonists affect the opening of cardiac  $\text{K}^+$  and  $\text{Ca}^{2+}$  channels by eliminating the spontaneous activity of muscarinic receptors (Hanf *et al.* 1993). The inhibitory effect of muscarinic antagonists on the agonist-independent activity of muscarinic receptors has also been demonstrated by their inhibitory effect on

the binding of GTP $\gamma$ S (guanosine 5'-O-[ $\gamma$ -thio]triphosphate) to cardiac membranes (Hilf and Jakobs 1992). These findings are in accord with the concept that the G protein-coupled receptors in general display agonist-independent transitions between inactive and active conformations and that the antagonists inhibit the agonist-independent constitutive activity by favoring the inactive conformations (Costa *et al.* 1992, Lefkowitz *et al.* 1993, Scheer and Cotecchia 1997, Burstein *et al.* 1997).

Spontaneous activity of muscarinic receptors may have profound effects on the function of corresponding cells, and is likely to have pharmacological implications. Our previous work on genetically engineered CHO (Chinese hamster ovary) cell lines has shown that the constitutive activity of muscarinic receptors tonically activates the formation of inositol phosphates (M<sub>1</sub> and M<sub>3</sub> receptor subtypes) and tonically inhibits the synthesis of cyclic AMP (M<sub>2</sub> and M<sub>4</sub> receptor subtypes) (Jakubík *et al.* 1995), in accordance with other data in the literature (Migeon and Nathanson 1994; Vogel *et al.* 1995; Burstein *et al.* 1995, 1996; Liu *et al.* 1996).

In the present work, we describe experiments in which we measured the synthesis of cyclic AMP in membranes prepared from the hearts of adult rats, and its changes induced by muscarinic antagonists atropine (the racemate) and the corresponding enantiomers, S-(–)-hyoscyamine and R-(+)-hyoscyamine. Comparisons between the two enantiomers of hyoscyamine were stimulated by observations suggesting that the pharmacologically less active (R)-(+)-isomer is more potent than the S-(–)-isomer in its action on constitutively active presynaptic receptors in the nerve terminals (Ghelardini *et al.* 1997). To our knowledge, this is the first attempt to investigate enantiomer selectivity of this inhibition directly by a negative antagonist (inverse agonist) of the constitutive activity of a G protein-coupled receptor.

## Methods

### *Source of reagents*

Creatine phosphokinase (type I from rabbit muscle), adenosine deaminase (type VIII from calf intestinal mucosa), acetylcholinesterase (type V-S from electric eel), ATP, GTP, creatine phosphate, alamethicin,

forskolin, atropine sulphate, S-(–)-hyoscyamine and HEPES (N-2-hydroxyethylpiperazine-N-4-butanedisulfonic acid) were from Sigma (St. Louis, MO). R-(+)-hyoscyamine was prepared according to Gualtieri *et al.* (1991).

### *Preparation of membranes*

Male Wistar-type rats of approx. 200 g body weight were sacrificed by cervical dislocation and decapitation. Heart atria and ventricles were isolated, cleaned from connective tissue and homogenized with an Ultra-Turrax homogenizer (Jankel and Kunkel, Staufen, Germany; 2 x 20 s at 20 500 rpm) in 250 mmol/l sucrose containing 20 mmol/l Na-HEPES (pH 7.4) and 2.5 mmol/l Na-EDTA. The homogenate was centrifuged twice for 5 min at 400 x g, and the combined supernatants were centrifuged for 30 min at 47 000 x g. The sediment was resuspended in 250 mmol/l sucrose with 20 mmol/l Na-HEPES (pH 7.4). Experiments were performed with freshly prepared membranes.

### *Determination of adenylyl cyclase activity*

The activity of adenylyl cyclase was measured according to the production of cyclic AMP, with fluorescent detection of derivatized cyclic AMP (modified from De Petrillo *et al.* 1990). Membranes were incubated for 5-10 min at 30 °C in 80  $\mu$ l of a medium consisting of 62.5 mmol/l sucrose, 30 mmol/l Na-HEPES (pH 7.4), 0.5 mmol/l Na-EGTA, 2.5 mmol/l MgCl<sub>2</sub>, 1 mmol/l 3-isobutyl-1-methylxanthine, 3.75 mmol/l creatine phosphate, 0.5 mmol/l Na-ATP, 0.1 mmol/l Na-GTP, 10  $\mu$ mol/l forskolin, 3.3 unit/ml creatine phosphokinase, and 0.125 unit/ml adenosine deaminase. Dithiothreitol (1 mmol/l) was also present in the incubation medium in initial experiments but no difference was found between the data obtained in its presence or absence. The concentration of membranes was 0.2-0.8 mg protein/ml. The incubation was terminated by warming the tubes to 95 °C for 5 min, the tubes were centrifuged and the supernatants used for determinations of cyclic AMP. Excess ATP in them was removed by precipitation with 25  $\mu$ l of 0.25 mol/l ZnSO<sub>4</sub> and 25  $\mu$ l of 0.25 mol/l Na<sub>2</sub>CO<sub>3</sub>, followed by centrifugation. Cyclic AMP was derivatized to fluorescent 1,N<sup>6</sup>-etheno derivative by mixing 100  $\mu$ l of the supernatant with 10  $\mu$ l of 1 mol/l Na-acetate (pH 7.4) and 10  $\mu$ l of 25 % chloroacetaldehyde, with subsequent 15 min heating at 95 °C.

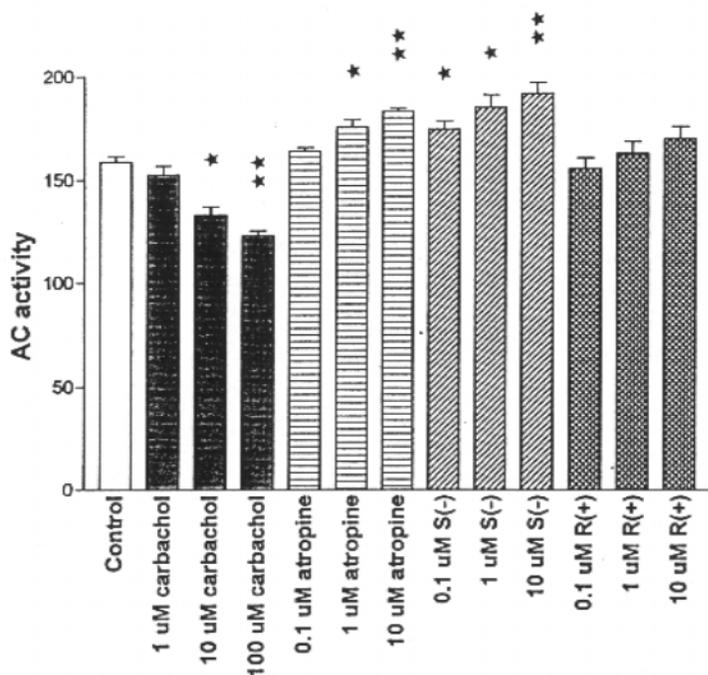
The mixture was separated by HPLC on a silica C18 column (5  $\mu\text{m}$ , 3x150 mm), with the mobile phase consisting of 50 mmol/l ammonium acetate and 10 % acetonitrile. Fluorescence was recorded at 230 or 290 nm excitation and 415 nm emission wavelengths.

#### Determination of protein

The method of Peterson (1977) was employed.

#### Analysis of data

Statistical significance of differences was evaluated by unpaired two-tailed Student's t-test or, for multiple groups, by one-way ANOVA, and  $p < 0.05$  was considered significant. GraphPad Prism for Windows program (GraphPad Software Inc., San Diego, CA) was used to fit the concentration-response curves and compute the  $\text{EC}_{50}$  values.



**Fig. 1.** Changes in the forskolin-stimulated synthesis of cyclic AMP ( $\text{pmol}\cdot\text{min}^{-1}\cdot(\text{mg protein})^{-1}$ ) by rat ventricular membranes in the presence of carbachol, atropine, S(-)-hyoscyamine and R(+)-hyoscyamine at the concentrations indicated in the Figure. The concentration of forskolin was 10  $\mu\text{mol/l}$ . Data are means  $\pm$  S.E.M. of 3-6 measurements. Statistical significance of differences from control samples: \* $p < 0.05$ , \*\* $p < 0.01$ .

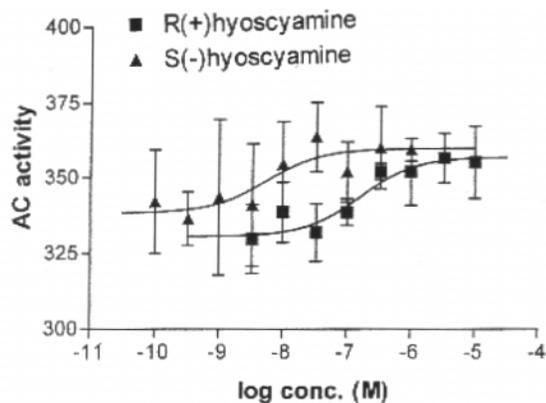
## Results

Under the conditions described in Methods section, basal adenylyl cyclase activity was close to 20 pmol/min per mg protein in the membranes of cardiac ventricles, and 10  $\mu\text{mol/l}$  forskolin caused an 8-12 fold stimulation. The forskolin-stimulated activity was inhibited by 23 % in the presence of 100  $\mu\text{mol/l}$  carbachol (Fig. 1). On the other hand, it was enhanced in the presence of atropine or S(-)-hyoscyamine (20 % enhancement by 10  $\mu\text{mol/l}$  S(-)-hyoscyamine in Fig. 1). Although the enhancement of cyclic AMP synthesis by R(+)-hyoscyamine was not significant in the experiments shown in Fig. 1, a low-potency positive effect of R(+)-hyoscyamine was observed in several other sets of experiments, one of which is shown in Fig. 2. Here, the computed  $\text{EC}_{50}$  value for R(+)-hyoscyamine was 30 fold higher than that for the S(-) enantiomer (190 nmol/l vs. 5.7 nmol/l). On the average, the  $\text{EC}_{50}$  value for the R(+)

enantiomer was  $29.9 \pm 1.2$  times (mean  $\pm$  S.E.M. of 5 experiments) higher than that for the S(-) enantiomer. The experiments just described were performed on ventricular membranes, but the effects of muscarinic ligands on the synthesis of cyclic AMP were the same on membranes from the ventricles and the atria (compare Figs. 1 and 3).

The possibility was considered that the enhancement of cyclic AMP synthesis induced by muscarinic antagonists might be due to the presence of endogenous acetylcholine in the preparations of the membranes used. To exclude such a possibility, experiments were performed on atrial membranes (since the concentration of acetylcholine is higher in the atria than in the ventricles) in which purified acetylcholinesterase had been added to the membrane preparations in order to ensure the hydrolysis of any acetylcholine with which they might be contaminated. As shown in Fig. 3, the addition of acetylcholinesterase brought about no

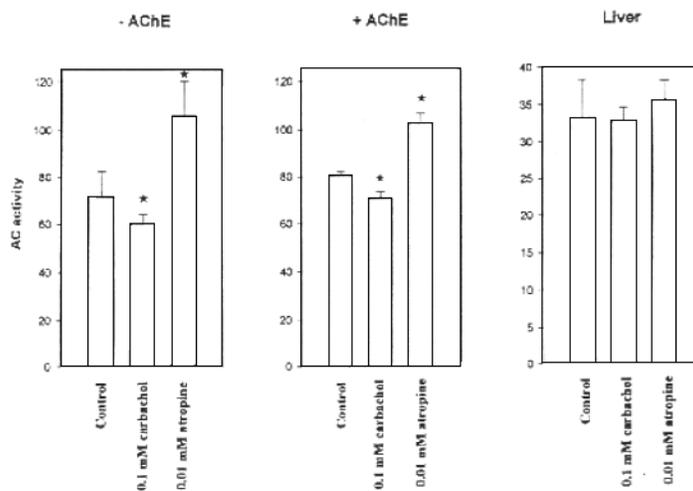
change in the potentiating effect of atropine on cyclic AMP synthesis.



**Fig. 2.** Concentration - response curves for the effects of S(-)-hyoscyamine and R(+)-hyoscyamine on the activity of adenylyl cyclase (stimulated by 10  $\mu\text{mol/l}$  forskolin) in rat ventricular membranes. Abscissa: log of the concentration of hyoscyamine (mol/l). Ordinate: cyclic AMP synthesis ( $\text{pmol}\cdot\text{min}^{-1}\cdot(\text{mg protein})^{-1}$ ). Data are means  $\pm$  S.E.M. of 3-6 measurements.

**Fig. 3.** Comparison of the effects of carbachol (100  $\mu\text{mol/l}$ ) and atropine (10  $\mu\text{mol/l}$ ) on the activity of adenylyl cyclase in rat atrial membranes incubated in the absence of added acetylcholinesterase (left) or in the presence of added acetylcholinesterase (middle), and on the activity of adenylyl cyclase in liver membranes (right). Membranes were incubated in the presence of the standard medium containing 10  $\mu\text{mol/l}$  forskolin. In the middle graph, acetylcholinesterase (Sigma type V-S; 5 I. U. per ml) had been added to the medium. Ordinate:

cyclic AMP synthesized ( $\text{pmol}\cdot\text{min}^{-1}\cdot(\text{mg protein})^{-1}$ ). Data are means  $\pm$  S.E.M. of 3-5 measurements. Statistical significance of differences from control samples: \* $p < 0.05$ .



## Discussion

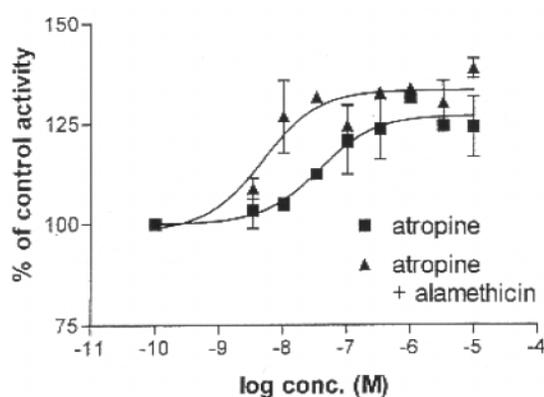
The most important finding of the present work is the observation that the synthesis of cyclic AMP in cardiac ventricular membranes becomes enhanced in the presence of atropine or S(-)-hyoscyamine, and that the effect of hyoscyamine is stereospecific (enantioselective).

It was also possible that carbachol or atropine influenced the activity of adenylyl cyclase directly, without an involvement of muscarinic receptors. However, it was found in experiments on liver membranes (known to lack muscarinic receptors) that carbachol and atropine had no effect on the synthesis of cyclic AMP in this preparation (Fig. 3).

The concentrations of atropine and S(-)-hyoscyamine required for changes in the synthesis of cyclic AMP to occur were high compared to data in the literature on the affinities of these antagonists for muscarinic receptors. One of the reasons might consist in that muscarinic receptors were not easily accessible to their ligands under the conditions used, perhaps because of the vesicularization of membrane fragments. The concentration-response curve for atropine was indeed shifted to the left if the incubation was performed in the presence of alamethicine, a peptide known to increase the permeability of cardiac membranes (Fig. 4).

Atropine and S(-)-hyoscyamine are highly specific competitive muscarinic antagonists and their effect on the synthesis of cyclic AMP might be achieved in one of two ways: (i) they might act by eliminating the binding of the endogenous agonist (i.e. acetylcholine), or (ii) they might act by preventing the constitutive activity of the receptors (i.e., by stabilizing their inactive conformations). It is

highly unlikely that there was enough acetylcholine present in the membrane preparations used which might cause receptor activation, since the membranes contain cholinesterases and had been prepared in the absence of cholinesterase inhibitors and washed during preparation. Moreover, the addition of exogenous acetylcholinesterase did not change the effect of muscarinic antagonists. Consequently, it is likely that the antagonists acted by stabilizing the inactive receptor conformations, and that the activation of the synthesis of cyclic AMP was a consequence of the inhibition of the constitutive activity of the receptors. As far as we know, this is the first direct demonstration that the blockade of the constitutive activity of muscarinic receptors by their antagonists is stereospecific (selective with regard to the enantiomer applied).



**Fig. 4.** Effect of increasing concentrations of atropine on the synthesis of cyclic AMP (stimulated by 10  $\mu\text{mol/l}$  forskolin) in the absence and presence of alamethicin. Ventricular membranes had been pretreated with 0.1 mg/ml alamethicin for 15 min at room temperature. The treatment with alamethicin diminished the activity of adenylyl cyclase by about 40 % and the activities in the presence of atropine were therefore expressed as percentage of corresponding control activities in the absence of atropine. Ordinate: log of the concentration of atropine (mol/l). Data are means ( $\pm$  S.E.M.) of 3-5 measurements.

Conceivably, the antagonists might also directly affect adenylyl cyclase catalytic activity. However, they had no effect on adenylyl cyclase activity in liver cell membranes, and the likely explanation is that this was due to the fact that hepatocytes lack muscarinic receptors. A receptor-mediated mechanism is strongly supported by

the observed stereospecificity of the action of hyoscyamine enantiomers.

The concentrations of atropine and S(-)-hyoscyamine at which the changes of cyclic AMP synthesis occurred were higher than the effective concentrations usually reported in experiments with radioligand binding or in isolated tissues. The reason of this could not be established with certainty, but two factors may have played a role: (a) Inverse membrane vesiculation (bringing about receptor sequestration on the inner surface of the vesicles) probably occurred during the homogenization and incubation conditions used. With regard to cardiac adenylyl cyclase, this phenomenon has been described by Sethi *et al.* (1993). In accordance with this assumption, the concentration – response curve for the effect of atropine on the synthesis of cyclic AMP was shifted to the left by alamethicin (Fig. 4), a peptide known to increase the permeability of lipid membranes for small molecules (Jones *et al.* 1980). However, alamethicin was not used in other experiments, because it diminished the amount of cyclic AMP synthesized. (b) In order to block constitutive activity, antagonists have to associate with receptors in active conformations. Such receptors are expected to display a higher-than-average affinity for agonists but a lower-than-average affinity for antagonists (Samama *et al.* 1994, Burstein *et al.* 1997).

It is generally accepted that the affinity of muscarinic receptors for S(-)-hyoscyamine is higher than that for the R-(+) enantiomer. In functional experiments on guinea-pig ileum, Barlow *et al.* (1973) found a 300 fold difference in affinities, whereas Ghelardini *et al.* (1997) discovered a 50 fold difference in the rat atrium. On genetically engineered CHO cells, the affinity of the  $M_2$  muscarinic receptors for S(-)-hyoscyamine was 36 times higher than that for the R-(+) enantiomer (Ghelardini *et al.* 1997). At the same time, however, Ghelardini *et al.* (1997) observed phenomena which led them to suggest that R-(+)-hyoscyamine blocks the constitutive activity of neuronal muscarinic receptors with a higher potency than the S(-) isomer. The present observations indicate that this does not happen on muscarinic receptors in the cardiac muscle.

In conclusion, our data support the notion that adenylyl cyclase in the surface membranes of cardiocytes is under continuous inhibitory influence of spontaneously (constitutively) active muscarinic receptors, and reveal stereospecificity in the blockade by hyoscyamine of this constitutive inhibition.

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