



# Inhibition of GTPase activity of G<sub>i</sub> proteins and decreased agonist affinity at M<sub>2</sub> muscarinic acetylcholine receptors by spermine and methoctramine

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**1** The effects of spermine and methoctramine, a selective M<sub>2</sub> muscarinic receptor antagonist, were studied on the high-affinity GTPase activity of G proteins, and on ligand binding to M<sub>2</sub> muscarinic receptors in pig heart sarcolemma.

**2** The spontaneous GTP hydrolysis by pig heart sarcolemma and its stimulation by mastoparan or carbachol were prevented by pertussis toxin and inhibited by methoctramine (IC<sub>50</sub>s: 21, 13 and 0.005 μM, respectively), and spermine (IC<sub>50</sub>s: 967, 278 and 11 μM). Spermine and methoctramine also inhibited spontaneous GTP hydrolysis by rat peritoneal mast cell membranes which do not respond to carbachol.

**3** The neutral muscarinic antagonists, AF-DX 116 and atropine, did not modify the inhibitory effect of high concentrations of methoctramine, indicating that this effect was not related to the antagonist binding site of muscarinic receptors. We suggest that methoctramine behaves as a receptor antagonist at nanomolar concentrations and interacts with G proteins at micromolar concentrations.

**4** Spermine did not modify the binding of the tritiated muscarinic antagonist [<sup>3</sup>H]-NMS, but decreased the binding of the agonist [<sup>3</sup>H]-Oxo-M. Spermine elicited a rightward shift of the carbachol/[<sup>3</sup>H]-NMS binding isotherm with a decrease in the proportion of sites with high-affinity for carbachol, suggesting that polyamines uncouple G<sub>i</sub> proteins from receptors.

**5** The inhibition of GTPase activity by polyamines, preventing the re-association of α and βγ subunits of G<sub>i</sub> proteins, might sustain the regulatory effect of G<sub>i</sub> subunits on downstream effectors. The level of intracellular polyamines might be important for the control of the transduction of extracellular signals through G<sub>i</sub> protein-coupled receptors.

**Keywords:** G protein; GTPase activity; mastoparan; methoctramine; muscarinic M<sub>2</sub> acetylcholine receptor; spermine; rat mast cells; pig sarcolemma; polyamines

**Abbreviations:** GPant-2 or [pGlu<sup>5</sup>,D-Trp<sup>7,9,10</sup>] SP<sub>5–11</sub>, pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH<sub>2</sub>; GppNHp, guanylylimido-diphosphate; NMS, N-methylscopolamine; Oxo-M, oxotremorine-M

## Introduction

The three polyamines, spermine, spermidine and putrescine are found in every eukaryotic cell up to millimolar concentrations (Seiler, 1991). Their first recognized physiological function has been their requirement for proliferative processes (Fozard *et al.*, 1980). In biochemical studies, polyamines have been shown to modulate many enzymatic systems, but little physiological correlation has been demonstrated (Seiler, 1991; Seiler *et al.*, 1996). The polycationic property of polyamines led to the assumption that they could affect physiological systems by binding to anionic sites of membranes and nucleic acids (Schuber, 1989) and by modulating ion channels (Scott *et al.*, 1993). The most widely studied effects of polyamines with physiological relevance has been the modulation (inhibition or stimulation) of NMDA receptors and of inwardly rectifying potassium channels (K<sub>IR</sub>) (Johnson, 1996, for review).

Only limited experimental evidence has been obtained to suggest a role for intracellular polyamines in the control of the transduction of extracellular signals through membrane receptors. Natural polyamines have been shown to decrease the basal and PGE<sub>1</sub>-stimulated adenylyl cyclase activity of

cultured cardiac cells (Clo *et al.*, 1988; Pignatti *et al.*, 1990). The inhibition of basal adenylyl cyclase activity by polyamines has also been observed in erythrocyte membranes (Khan *et al.*, 1990). Polyamines also induced phospholipase C activation in rat peritoneal mast cells, leading to histamine secretion (Bueb *et al.*, 1991; 1992). The effects of polyamines on cardiomyocytes and mast cells were completely prevented by pretreating cells with pertussis toxin, which is known to ADP-ribosylate G<sub>i</sub> and G<sub>o</sub> proteins. These observations suggested that the modulation of the activity of adenylyl cyclase or phospholipase C by polyamines was related to the interaction of polyamines with receptors or G proteins, rather than with enzymatic effectors.

Interestingly, receptor-independent effects have been reported for the synthetic polyamine, methoctramine. This polymethylene tetramine is a highly potent and selective antagonist at M<sub>2</sub> muscarinic acetylcholine receptors (Melchiorre *et al.*, 1987). Lee *et al.* (1989) showed that methoctramine, at concentrations over 5 μM, is a powerful activator of phosphoinositide hydrolysis in dissociated rat cerebral cortex. Muscarinic receptor antagonists were unable to block methoctramine-stimulated phosphoinositide hydrolysis but the underlying mechanism was not determined. Methoctramine also induced concentration-dependent

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(0.003–1 mM) histamine release and phosphoinositide breakdown from rat peritoneal mast cells which do not express muscarinic receptors (Chahdi *et al.*, 1998a). The effects of methoctramine on rat cerebral cortex and peritoneal mast cells were prevented by pertussis toxin-pretreatment indicating the possible involvement of G proteins. These observations suggested that the receptor-independent effects of methoctramine might be related to its polyamine structure and might involve the regulation of G protein activity.

The aim of the present paper was to study the effect of spermine and methoctramine on pertussis toxin-sensitive G proteins. Pig atrial sarcolemma offered the opportunity to study the spontaneous GTPase activity of this class of G protein constitutively coupled with muscarinic M<sub>2</sub> receptors in the absence of agonist (Hilf & Jakobs, 1992; Daeffler *et al.*, 1999). This model also allows us to activate the G protein cycle with muscarinic agonists or with mastoparan, a peptide interacting with G proteins and mimicking agonist-liganded receptors (Higashijima *et al.*, 1988). Alternatively, rat peritoneal mast cells were also used as these cells do not express muscarinic receptors, but contain G<sub>i</sub> proteins involved in their secretory response to peptides and polyamines (Bueb *et al.*, 1990; Aridor *et al.*, 1993).

## Methods

### Preparation of membranes

Pig atrial sarcolemma were prepared according to the method of Peterson & Schimerlik (1984) as described previously (Daeffler *et al.*, 1999). Membranes from bovine and rat brain cortex, were prepared similarly. Peritoneal rat mast cells were collected and purified as previously described (Chahdi *et al.*, 1998c). Cells from 20 rats were pooled ( $17\text{--}20 \times 10^6$ ; purity >95%), disrupted by sonication and centrifuged at 4°C for 8 min at  $1000 \times g$ . The supernatant was centrifuged for 40 min at  $40,000 \times g$  and the final membrane pellet was suspended in triethanolamine hydrochloride 50 mM-NaOH pH 7.4, including (in mM): ATP 1, EDTA 0.1, dithiothreitol 1, NaCl 150 and MgCl<sub>2</sub> 1.

### GTPase activity measurements

GTPase activity was determined by a method adapted from Hilf & Jakobs (1989) in 50 mM triethanolamine hydrochloride-NaOH, pH 7.4, including (in mM): NaCl 150, ATP 1, EDTA 0.1, dithiothreitol 1 and MgCl<sub>2</sub> 1. Samples of membranes (10 µg of protein) were first preincubated for 30 min at 25°C with drugs. The reaction was started by addition of 20 µl [ $\gamma$ -<sup>32</sup>P]-GTP (30 Ci mmol<sup>-1</sup>) to the samples, so as to reach a final concentration of 0.1 µM in a final volume of 100 µl. Alternatively this concentration was varied from 0.03 to 0.8 µM to determine  $K_M$  and  $V_{max}$ . GTP hydrolysis was stopped after 15 min of incubation at 25°C, by the addition of 0.7 ml of an ice-cold 5% (w v<sup>-1</sup>) charcoal suspension in 50 mM KH<sub>2</sub>PO<sub>4</sub>-HCl, pH 7.4. The mixture was centrifuged at  $800 \times g$  for 20 min at 4°C. A fraction of the supernatant (0.4 ml) was put into counting vials containing 3.6 ml scintillation solution. The high affinity GTPase activity was calculated by subtracting the <sup>32</sup>P<sub>i</sub> released in the presence of 50 µM unlabelled GTP, from total <sup>32</sup>P<sub>i</sub> accumulation (Cassel & Selinger, 1976; Hilf & Jakobs, 1989). Spontaneous high-affinity GTPase activity was determined as the activity measured in the absence of ligands.

### Pertussis toxin-catalyzed ADP-ribosylation of the sarcolemma membranes

Pertussis toxin was preactivated for 30 min at 37°C in 100 µl of 50 mM Tris-HCl, pH 7.5 including 5 mM dithiothreitol. Pig atrial sarcolemma (0.8 mg) was resuspended after centrifugation at 4°C for 20 min at  $44,000 \times g$ , in 900 µl of ADP-ribosylation buffer 50 mM Tris-HC, pH 7.5, including (in mM): thymidine 10, EDTA 1, MgCl<sub>2</sub> 5, ATP 0.4, GTP 0.4 and NAD<sup>+</sup> 0.5. Preactivated pertussis toxin solution was then added to each 900 µl membrane suspension, to reach a final concentration of 10, 20 or 40 µg ml<sup>-1</sup>, and incubated for 2 h at 30°C. The reaction was stopped by addition of 1 ml ice-cold 50 mM triethanolamine pH 7.4 including (in mM): NaCl 150, ATP 1, EDTA 0.1, dithiothreitol 1 and MgCl<sub>2</sub> 1. The membrane suspension was centrifuged at 4°C for 20 min at  $44,000 \times g$ . The pellets were washed twice to remove toxin, and resuspended with a glass-teflon tissue homogenizer in the same buffer and used immediately for GTPase activity determination.

### SDS-PAGE and immunoblotting

Electrophoresis was performed using a minigel system (Mini-PROTEAN II; Bio-Rad, Ivry sur Seine, France) in discontinuous gel system described by Laemmli (1970) with minor modifications. Briefly, stacking and resolving gels contained 4 and 12% acrylamide, respectively. Membrane samples (10 µg protein) were solubilized by boiling for 5 min in Laemmli's sample buffer containing 350 mM dithiothreitol. Gels were run at 200 V until the dye front reached the bottom of the resolving gel (about 2 h). Electrophoretic transfer of resolved proteins to nitrocellulose membranes (Hybond ECL, Amersham, U.K.) was carried out for 2 h at 100 V using a mini Trans-Blot transfert cell (Bio-Rad). Before exposure to antibodies, nitrocellulose membranes were saturated by incubation overnight in a blocking solution containing 100 mM NaCl, casein 0.1% (w v<sup>-1</sup>). Nitrocellulose membranes were then incubated for 1 h with primary rabbit polyclonal (G<sub>13</sub>α) or mouse monoclonal (G<sub>11</sub>α, G<sub>12</sub>α and G<sub>0</sub>α) antibodies at a final dilution of 1/2000, 1/2000, 1/3000 and 1/3000, respectively. The nitrocellulose membrane was finally, after incubation with secondary antibodies for 1 h, incubated for 2 min in ECL reagents (Amersham, U.K.). The bound antibodies were visualized by autoradiography for 2 min with Kodak X-OMAT films.

### Radioligand binding studies

Competition experiments, were carried out with 2 nM [<sup>3</sup>H]-oxotremorine-M ([<sup>3</sup>H]-Oxo-M, 86.6 Ci mmol<sup>-1</sup>) and with 0.2 nM [<sup>3</sup>H]-N-methylscopolamine ([<sup>3</sup>H]-NMS, 80.4 Ci mmol<sup>-1</sup>). All binding studies were carried out at 25°C in 10 mM Tris-HCl, pH 7.4 including 150 mM NaCl and 1 mM MgCl<sub>2</sub>. Incubation mixture (0.5 ml) contained 20 and 40 µg of protein, for [<sup>3</sup>H]-NMS and [<sup>3</sup>H]-Oxo-M binding, respectively. Samples were incubated for 1 h to reach the equilibrium. Non-specific binding was determined in the presence of 1 µM atropine (4–5% of total binding) as described previously (Daeffler *et al.*, 1999). Saturation experiments with the radiolabelled ligands demonstrated saturability in each condition used and Scatchard analysis of the saturation isotherms was used to calculate the density of binding sites ( $B_{max}$ ),  $0.41 \pm 0.10$  ([<sup>3</sup>H]-Oxo-M),  $1.45 \pm 0.39$  ([<sup>3</sup>H]-NMS) and  $1.48 \pm 0.35$  pmol. (mg of protein)<sup>-1</sup> ([<sup>3</sup>H]-NMS + GppNHp) and dissociation constants ( $K_D$ ),  $1.20 \pm 0.37$ ,  $0.24 \pm 0.08$  and  $0.16 \pm 0.06$  nM, respectively (means  $\pm$  s.e.mean,  $n = 3$ ).

## Data analysis

Experimental data for the saturation and the inhibition binding studies were analysed using the non-linear regression analysis described by Munson & Rodbard (1980) (LIGAND program, Elsevier-Biosoft, Cambridge, U.K.) to yield equilibrium dissociation constants and receptor densities. The precision of the fit to a one- or two-site model was determined with an *F* test ( $P < 0.01$ ), by comparing the residual sum of squares for fitting data to a one- or two-site model. Data were weighted with the reciprocal of the variance.  $K_i$  values were calculated from  $IC_{50}$ s according to Cheng & Prusoff (1973) for competitive interactions, taking into account the concentration and the  $K_D$  of tritiated ligands. Values for binding experiments are expressed as geometric means  $\pm$  s.e.mean to decrease the errors associated with estimation of the means from logarithmic curves (Fleming *et al.*, 1972).

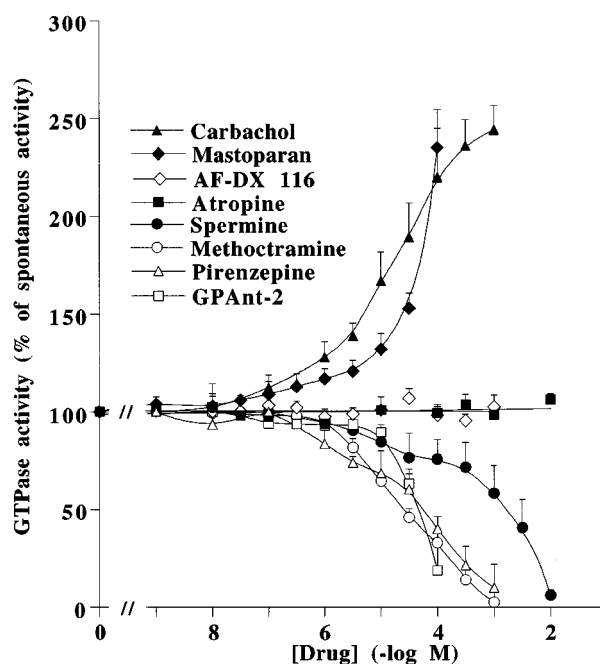
## Materials

Atropine, carbachol, spermine, Tween 20 and guanylylimidodiphosphate (GppNHp) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Methoctramine and pirenzepine were obtained from RBI (Natick, U.S.A.). AF-DX 116 (11-[[2-[(diethyl-amino)-methyl]-1-piperidinyl] acetyl]-5,11-dihydro-6H-pyrido[2,3-b] [1,4] benzodiazepin-6-one) was a gift from Boehringer-Ingelheim (Ingelheim, Germany). The substance-P related peptide GPant-2 also referred to as [P-Glu<sup>5</sup>,D-Trp<sup>7,9,10</sup>]-substance P<sub>5-11</sub>, (pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH<sub>2</sub>) was purchased from Bachem (Paris, France) and mastoparan from Neosystem (Strasbourg, France). [<sup>3</sup>H]N-methylscopolamine ([<sup>3</sup>H]-NMS, 80.4 Ci mmol<sup>-1</sup>), [<sup>3</sup>H]-oxotremorine-M ([<sup>3</sup>H]-Oxo-M, 86.6 Ci mmol<sup>-1</sup>), and [ $\gamma$ -<sup>32</sup>P]-GTP (30 Ci mmol<sup>-1</sup>) were purchased from New England Nuclear (Boston, MA, U.S.A.). Glass fibre filters (GF/C) were obtained from Whatmann (Clifton, U.S.A.). Casein was purchased from Tropix (Bedford, MA, U.S.A.). Acrylamide, bis-acrylamide, Laemmli's sample buffer and horseradish peroxidase-conjugated goat anti-rabbit IgG antiserum were from Bio-Rad (Ivry sur Seine, France). Horseradish peroxidase-conjugated anti-mouse IgG antiserum was from Jackson ImmunoResearch (West Grove, U.S.A.). Nitrocellulose (Hybond ECL) membranes and ECL detection reagents were obtained from Amersham (U.K.). Anti-G<sub>123</sub> subunit, rabbit polyclonal antibody was purchased from Upstate (Lake Placid, U.S.A.). Anti-G<sub>121</sub>, -G<sub>122</sub>, -G<sub>02</sub> mouse monoclonal antibodies were obtained from Chemicon (Temecula, U.S.A.). All drugs were prepared in the different buffers described above, with the exception of AF-DX 116 and GPant-2 which were dissolved in 0.1 N HCl and 80% CH<sub>3</sub>COOH, respectively, and diluted with buffers described above.

## Results

### Inhibition of high-affinity GTPase activity by spermine and methoctramine

Figure 1 shows that the muscarinic receptor agonist, carbachol, and the G protein ligand, mastoparan, increased the spontaneous GTP hydrolysis by pig atrial sarcolemma at concentrations corresponding to their affinity for their respective targets. Carbachol and mastoparan did not modify  $K_M$  for GTP, but increased  $V_{max}$  (Table 1). The spontaneous and stimulated GTP hydrolysis were prevented by pertussis toxin pretreatment (Table 2). The neutral muscarinic



**Figure 1** Modulation of high affinity GTPase activity of pig atrial sarcolemma by various drugs. Spontaneous high-affinity GTPase activity (100%) was  $925 \pm 155$  fmol P<sub>i</sub> min<sup>-1</sup> pmole of receptor<sup>-1</sup>. Values are geometric means  $\pm$  s.e.mean of three independent experiments.

**Table 1** Michaelis-Menten constants,  $K_M$  and  $V_{max}$ , for high affinity GTPase activity of pig atrial sarcolemma, in the presence of different drugs

	$K_M$ ( $\mu$ M)	$V_{max}$ (pmol P <sub>i</sub> min <sup>-1</sup> mg protein <sup>-1</sup> )
Control	$0.31 \pm 0.06$	$3.63 \pm 0.24$
Carbachol, 0.1 mM	$0.28 \pm 0.04$	$5.48 \pm 0.18$
Mastoparan, 0.06 mM	$0.28 \pm 0.04$	$9.50 \pm 0.46$
GPant-2, 0.1 mM	$0.27 \pm 0.05$	$2.37 \pm 0.24$
Methoctramine, 0.1 mM	$0.50 \pm 0.03$	$0.60 \pm 0.23$
Spermine, 1 mM	$0.48 \pm 0.07$	$1.20 \pm 0.34$
GppNHp, 0.03 mM	$0.96 \pm 0.08$	$3.23 \pm 0.32$
Pirenzepine 0.1 mM	$0.33 \pm 0.06$	$2.02 \pm 0.63$

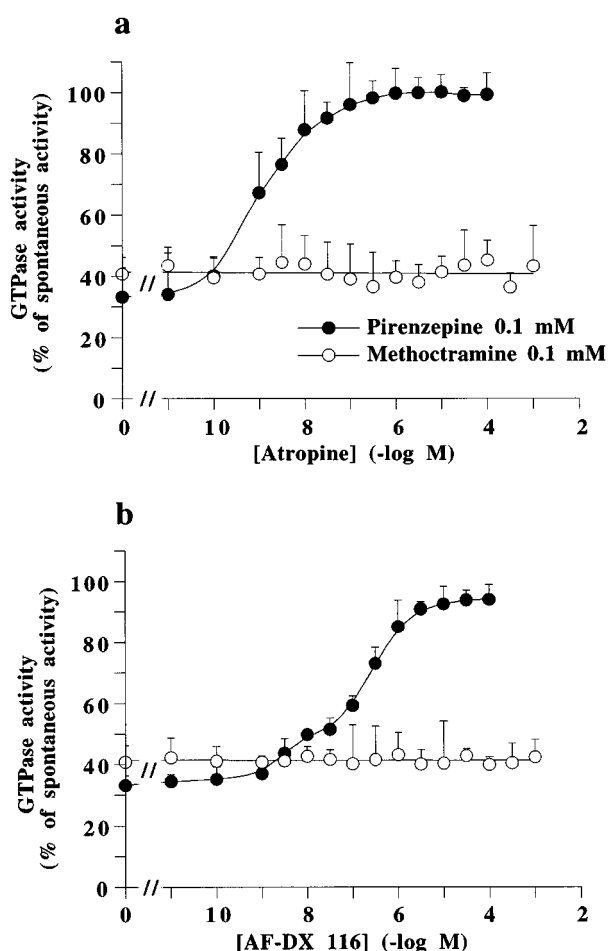
$V_{max}$  and  $K_M$  for GTP hydrolysis were obtained by non-linear regression analysis of Michaelis-Menten plots by the UltraFit program (Aladdin Systems, Inc. and Raymond Lau). Values are arithmetic means  $\pm$  s.e.mean from three independent experiments.

antagonists, AF-DX 116 and atropine did not modify the spontaneous GTPase activity (Figure 1). Unexpectedly, the M<sub>2</sub> selective antagonist, methoctramine, inhibited the spontaneous GTP hydrolysis (Figure 1), with an increase in  $K_M$  for GTP and a decrease in  $V_{max}$  (Table 1). The active concentrations of methoctramine ranged from micro to millimolar levels. Millimolar concentrations of the natural polyamine, spermine, also inhibited the spontaneous GTP hydrolysis by pig atrial sarcolemma. GPant-2, a substance P analogue which prevents the muscarinic receptor-G protein interaction (Mukai *et al.*, 1992), also inhibited GTP hydrolysis. This effect was related to a decreased  $V_{max}$ , with no modification in  $K_M$  for GTP (Table 1). The non-hydrolysable analogue of GTP, GppNHp, increased the  $K_M$  value without modifying  $V_{max}$ . The inverse agonist of muscarinic M<sub>2</sub> receptors, pirenzepine (Daeffler *et al.*, 1999), also decreased  $V_{max}$  for GTP, but did not modify  $K_M$

**Table 2** Modulation by pertussis toxin-pretreatment of the high-affinity GTPase activity of pig atrial sarcolemma

Compound	- Pertussis toxin	GTPase activity (% of control)		
		10 $\mu\text{g ml}^{-1}$	+ Pertussis toxin 20 $\mu\text{g } \mu\text{l}^{-1}$	40 $\mu\text{g ml}^{-1}$
Control	100	76.9 $\pm$ 2.8	68.3 $\pm$ 1.0	61.2 $\pm$ 2.1
Carbachol, 0.1 mM	169.5 $\pm$ 10.5	101.2 $\pm$ 10.6	87.9 $\pm$ 1.4	70.9 $\pm$ 13.0
Mastoparan, 0.07 mM	30.2.7 $\pm$ 22.5	215.2 $\pm$ 6.5	161.6 $\pm$ 28.8	99.3 $\pm$ 17.0

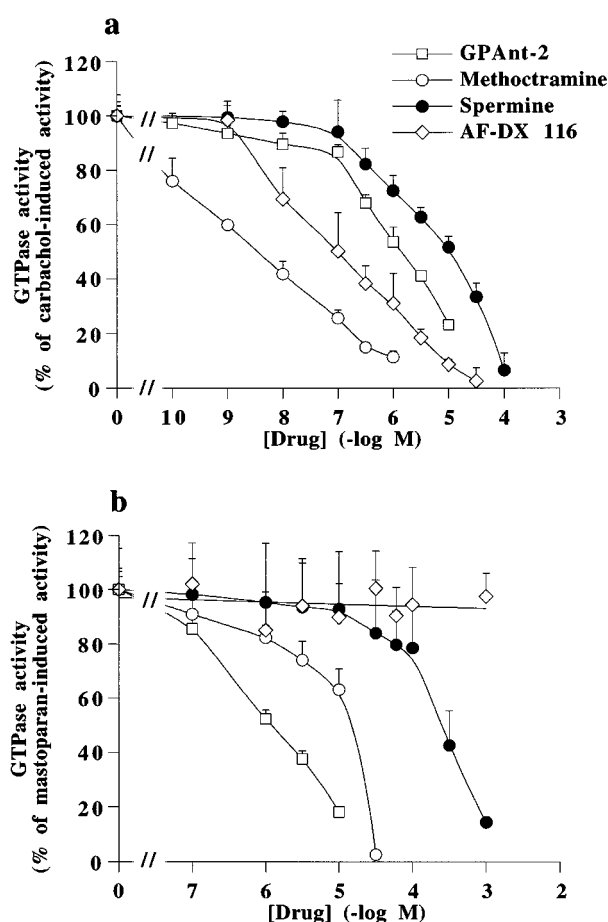
Spontaneous high-affinity GTPase activity (100%) was  $1046 \pm 166$  fmol  $\text{P}_i \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$ . Values are geometric means  $\pm$  s.e. mean from three independent experiments.



**Figure 2** Concentration-dependent effect of atropine and AF-DX 116 on the high affinity GTPase activity of pig atrial sarcolemma observed in the presence of 0.1 mM methoctramine or pirenzepine. Spontaneous high affinity GTPase activity (100%) was  $596 \pm 77$  fmol  $\text{P}_i \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$ . In the presence of methoctramine or pirenzepine, the GTPase activity was  $243 \pm 37$  or  $196 \pm 25$  fmol  $\text{P}_i \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$ , respectively. Results are geometric means  $\pm$  s.e. mean from four experiments.

(Table 1). In contrast to pirenzepine, the inhibitory effect of methoctramine on the spontaneous GTP hydrolysis was not inhibited by the neutral antagonists, AF-DX 116 and atropine (Figure 2).

We also studied the effect of these various drugs on the rates of GTP hydrolysis stimulated by carbachol or mastoparan. AF-DX 116 (Figure 3b) and atropine (not shown) did not modify mastoparan-induced GTP hydrolysis. AF-DX 116 and methoctramine inhibited the carbachol-induced effect, at concentrations in the range of their known



**Figure 3** Concentration-dependent effect of methoctramine, spermine, GPant-2 and AF-DX 116 on 0.1 mM carbachol- (a) or 0.06 mM mastoparan-stimulated (b) high affinity GTPase activity of pig atrial sarcolemma. Spontaneous GTPase activity was  $722 \pm 84$  fmol  $\text{P}_i \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$ . Carbachol- and mastoparan-induced activities (100%) reached  $1432 \pm 109$  and  $1408 \pm 109$  fmol  $\text{P}_i \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$ , respectively. Results are geometric means  $\pm$  s.e. mean from three independent experiments.

antagonist activity at muscarinic receptors (Figure 3a, Table 3). Methoctramine also inhibited mastoparan-induced GTP hydrolysis, but this effect was observed only at millimolar concentrations (Figure 3b), i.e. similar to that required for the inhibition of spontaneous GTP hydrolysis (Table 3). Spermine inhibited the carbachol-induced GTPase activity in the micromolar range of concentrations, but higher concentrations of spermine were required to inhibit the spontaneous or mastoparan-induced GTPase activity. GPant-2 similarly inhibited both spontaneous and induced activities (Figure 3, Table 3).

### Tissue selectivity of the effect of spermine and methoctramine

Figure 4 shows that carbachol, unlike mastoparan, did not modify the spontaneous rate of GTP hydrolysis in membranes from rat peritoneal mast cells. Spermine and methoctramine inhibited spontaneous GTP hydrolysis by mast cell membranes with  $IC_{50}$ s of  $141.3 \pm 9.2$  and  $23.1 \pm 4.8 \mu\text{M}$ , respectively, i.e. similar to those observed for pig atrial sarcolemma (Table 3). Comparative experiments were also performed with rat and bovine brain membranes (Table 4). Spermine (10 mM) inhibited totally the spontaneous GTP hydrolysis in rat mast cell membranes, about 94% in pig atrial membranes, 44% in rat brain, and only 20% in bovine brain membranes. Similar results were observed in the presence of 1 mM methoctramine (Table 4). The various pertussis toxin-sensitive G proteins present in these membrane preparations were characterized by immunoblotting detection of their alpha subunits in membrane samples containing  $10 \mu\text{g}$  of protein per lane (Figure 5).  $G_{i1\alpha}$  was not revealed in rat mast cell membranes, but was observed in pig atria, rat and bovine brain membranes.  $G_{i2\alpha}$  was mainly revealed in pig atria with traces in the other tissues.  $G_{i3\alpha}$  was observed in all membrane preparations.  $G_{o\alpha}$  was

revealed mainly in rat and bovine brain. A lower amount of  $G_{o\alpha}$  was observed in pig atria, but  $G_{o\alpha}$  was not detected in rat mast cell membranes.

### Displacement of tritiated muscarinic agonist and antagonist binding by spermine and methoctramine

Figure 6 shows binding curves for increasing concentrations of methoctramine and spermine performed with the agonist [ $^3\text{H}$ ]-Oxo-M and with the antagonist [ $^3\text{H}$ ]-NMS. Methoctramine fully displaced the specific binding of both radioligands. However, the apparent affinity of methoctramine for [ $^3\text{H}$ ]-NMS binding sites was higher than its affinity for [ $^3\text{H}$ ]-Oxo-M sites ( $K_i$ ,  $0.97 \pm 0.4$  and  $2.0 \pm 0.3 \text{ nM}$ ) with Hill coefficients ( $n_H$ ) of  $0.97 \pm 0.03$  and  $1.01 \pm 0.04$ , respectively. Spermine, up to 1 mM, did not modify [ $^3\text{H}$ ]-NMS binding, but partly decreased, from  $0.1 \mu\text{M}$ , the specific binding of [ $^3\text{H}$ ]-Oxo-M. Controls were performed with mastoparan and GPAnt-2 (Figure 7). Both G protein ligands also decreased [ $^3\text{H}$ ]-Oxo-M specific binding without affecting [ $^3\text{H}$ ]-NMS binding. Figure 8 shows that spermine induced a concentration-dependent rightward shift of the carbachol/[ $^3\text{H}$ ]-NMS specific binding isotherm. The Hill coefficient for carbachol displacing [ $^3\text{H}$ ]-

**Table 3** Inhibition of high affinity GTPase activity by various compounds in pig sarcolemma

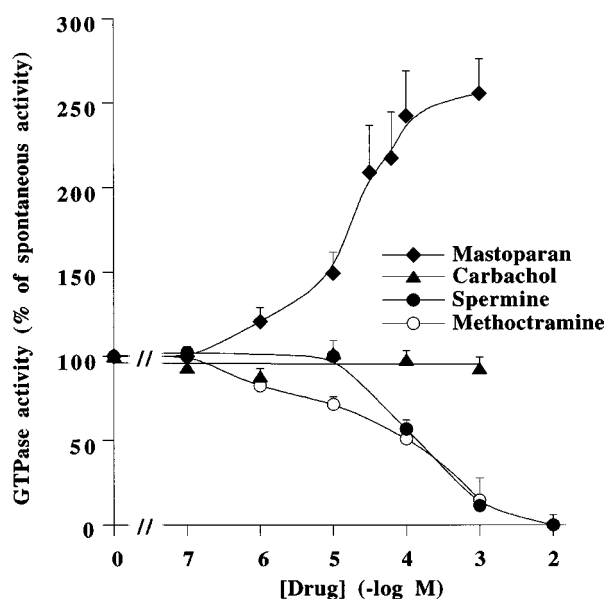
Compound	Inhibition of GTPase activity ( $IC_{50}$ , $\mu\text{M}$ )		
	Spontaneous activity	Mastoparan-induced activity	Carbachol-induced activity
AF-DX 116	(no effect)	(no effect)	$0.08 \pm 0.02$
Methoctramine	$21 \pm 5$	$13 \pm 3$	$0.005 \pm 0.001$
Spermine	$967 \pm 191$	$278 \pm 165$	$11.0 \pm 1.5$
GPAnt-2	$31 \pm 6$	$1.1 \pm 0.1$	$1.5 \pm 0.4$

$IC_{50}$  were calculated from results shown on Figures 1 and 3. Values are geometric means  $\pm$  s.e.mean from three independent experiments.

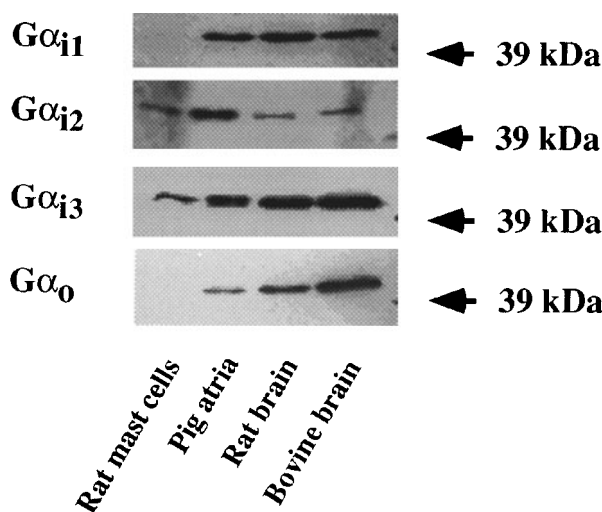
**Table 4** Inhibitory activities of spermine and methoctramine on high affinity GTPase activity of various membranes

	GTPase activity (% of control)			
	Spermine		Methoctramine	
	1 mM	10 mM	0.1 mM	1 mM
Pig atria	$58.4 \pm 14.2$	$6.2 \pm 2.7$	$33.0 \pm 5.3$	$2.5 \pm 5.1$
Rat mast cells	$11.5 \pm 4.8$	$0.04 \pm 6.05$	$51.1 \pm 8.1$	$14.7 \pm 13.2$
Rat brain	$81.4 \pm 2.7$	$46.4 \pm 4.2$	$72.0 \pm 4.4$	$32.8 \pm 7.7$
Bovine brain	$81.7 \pm 4.9$	$80.2 \pm 7.5$	$90.7 \pm 6.5$	$76.4 \pm 12.5$

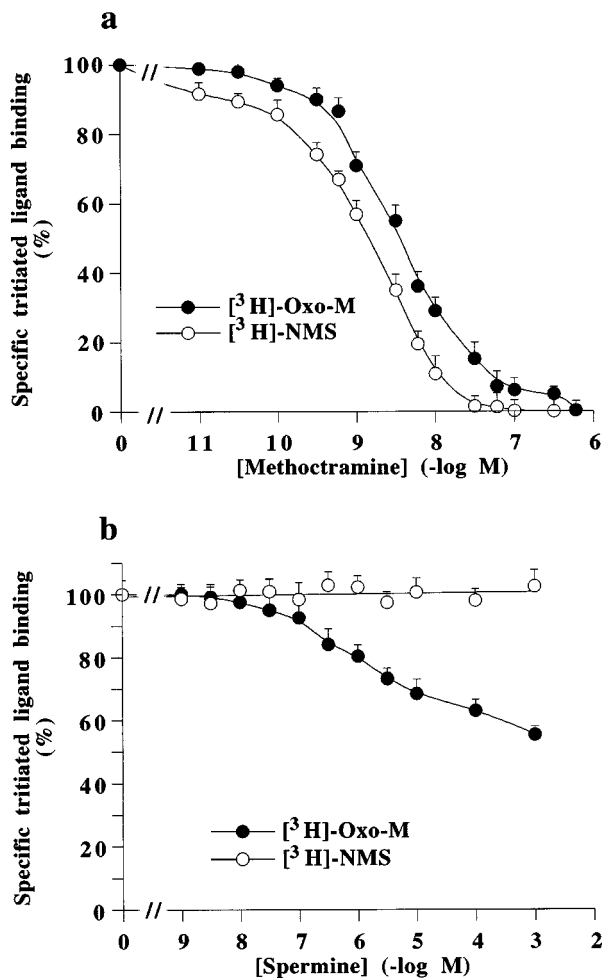
Control values of GTP hydrolysis in the absence of drugs (100%) were  $665 \pm 88$  for pig atria,  $425 \pm 32$  for rat mast cells,  $953 \pm 78$  for rat brain and  $1195 \pm 146 \text{ pmol P}_i \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$  for bovine brain, respectively. Values are arithmetic means  $\pm$  s.e.mean from three independent experiments. Full concentration-effect curves for pig atria and rat mast cell membranes are shown on Figures 1 and 4.



**Figure 4** Modulation of high affinity GTPase activity of rat peritoneal mast cells by various drugs. Spontaneous GTPase activity (100%) was  $425 \pm 32 \text{ fmol P}_i \cdot \text{min}^{-1} \cdot \text{mg}$  of protein $^{-1}$ . Values are geometric means  $\pm$  s.e.mean from three independent experiments.



**Figure 5** Immunoblot analysis of the expression of  $G_{i2}$  and  $G_{o\alpha}$  proteins in various membrane preparations. Results shown are representative of three independent experiments.



**Figure 6** Inhibition of specific binding of [ $^3\text{H}$ ]-Oxo-M and [ $^3\text{H}$ ]-NMS on pig atrial sarcolemma by methoctramine (a) or spermine (b). The binding of [ $^3\text{H}$ ]-NMS was performed in the presence of  $100\ \mu\text{M}$  GppNHp. Total specific binding in the absence of drugs (100%) correspond to  $720 \pm 70$  [ $^3\text{H}$ ]-Oxo-M and  $1627 \pm 131$  [ $^3\text{H}$ ]-NMS) d.p.m. per assay. Values are geometric means  $\pm$  s.e.mean from three independent experiments.

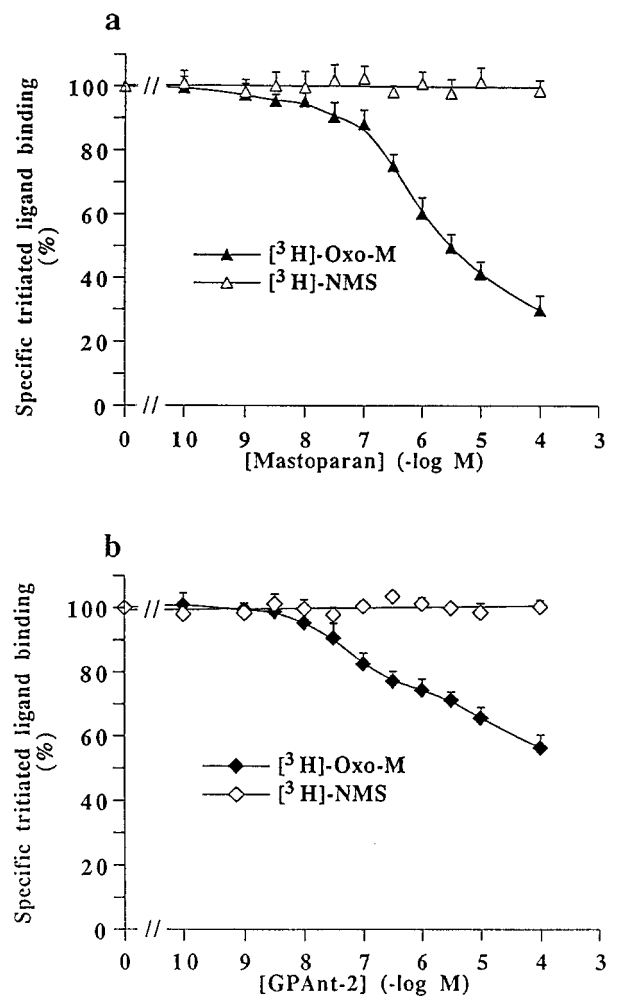
NMS binding was significantly different from unity and was best fitted by a two-site binding model in each case (Table 5). The rightward shift induced by spermine was accompanied by a decrease in the proportion of high affinity binding sites ( $R_h$ ) (Table 5).

## Discussion

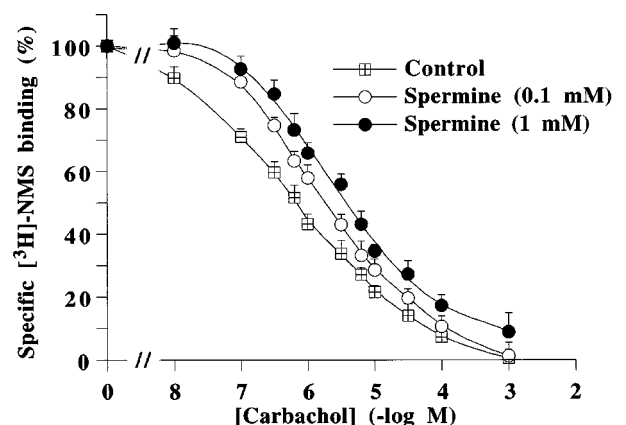
The function of heterotrimeric G proteins is regulated cyclically by exchange of GDP to GTP on the  $\alpha$  subunit, dissociation of  $\alpha$  and  $\beta\gamma$  subunits leading to the activation of effectors and hydrolysis of GTP to GDP allowing the reassociation of  $\alpha$  and  $\beta\gamma$  subunits (Gilman, 1987).

### *The effect of polyamines on $G_i$ and $G_o$ proteins*

The present results show that spermine inhibits the high-affinity and pertussis toxin-sensitive GTPase activity of membrane preparations from pig atria and rat mast cells (Figures 1 and 4). These properties indicate that the GTP hydrolysis measured in these preparations is relevant, at least partly, of  $G_i$  and/or  $G_o$  proteins, considering the property of pertussis toxin to uncouple receptors from G proteins through



**Figure 7** Inhibition of specific binding of [ $^3\text{H}$ ]-oxotremorine-M and [ $^3\text{H}$ ]-N-methylscopolamine on pig atrial sarcolemma by mastoparan (a) or GPant-2 (b). Total specific bindings in the absence of drugs (100%) corresponded to  $716 \pm 66$  [ $^3\text{H}$ ]-Oxo-M and  $1690 \pm 176$  [ $^3\text{H}$ ]-NMS) d.p.m. per assay. Values are geometric means  $\pm$  s.e.mean of three independent experiments.



**Figure 8** Inhibition of specific [ $^3\text{H}$ ]-N-methylscopolamine binding to pig atrial sarcolemma by carbachol in the absence (control) or presence of 1 or 10 mM spermine. Values are geometric means  $\pm$  s.e.mean of three experiments performed in triplicate.

ADP-ribosylation of a cysteine residue of the C terminus of  $G_i$  and  $G_o$  (Gilman, 1987, for review).  $G_o$  was not detected in mast cell membranes which contained  $G_{i3}$  and  $G_{i2}$ , confirming

**Table 5** Two-site analysis of the inhibition of [<sup>3</sup>H]-NMS binding by carbachol in the presence of spermine in pig atria sarcolemma shown in Figure 8

	$nH$	$K_iH$ (nM)	$\%R_H$	$K_iL$ (nM)	$\%R_L$
Control	0.52	$3.5 \pm 0.6$	$42.5 \pm 2.4$	$1029 \pm 185$	$57.5 \pm 2.4$
Spermine 0.1 mM	0.68	$2.7 \pm 0.6$	$31.6 \pm 1.3$	$986 \pm 86$	$68.4 \pm 1.3$
Spermine 1 mM	0.78	$2.8 \pm 0.4$	$19.3 \pm 2.2$	$1008 \pm 102$	$80.7 \pm 2.2$

Values are geometric means  $\pm$  s.e.mean from three independent experiments.

previous observations (Aridor *et al.*, 1993). In pig atria sarcolemma, G<sub>o</sub> was detected besides G<sub>i1</sub>, G<sub>i2</sub> and G<sub>i3</sub>. This indicates that the GTP hydrolysis measured was entirely (mast cells) or predominantly (atria) relevant of G<sub>i</sub> proteins. Thus, the inhibitory effect of spermine for GTP hydrolysis by G proteins can be ascribed to the G<sub>i</sub> subfamily.

We observed previously that natural polyamines increase GTP hydrolysis by G proteins purified from bovine cortex containing mostly G<sub>o</sub> (Bueb *et al.*, 1991; 1992). In line with this observation, we show here (Table 4) that concentrations of spermine which fully inhibited GTP hydrolysis in mast cells or atria membranes, decreased only partially the hydrolysis in brain membranes characterized by presence of G<sub>o</sub> (Figure 5). The maximal inhibitory effect of polyamines in membranes might be limited by their G<sub>o</sub> content. Different effects of drugs on G protein subfamilies has been previously observed (Chahdi *et al.*, 1998b, for review). For instance, melittin activates G<sub>i</sub> and G<sub>o</sub> proteins (Higashijima *et al.*, 1990), but inhibits G<sub>s</sub> (Fukushima *et al.*, 1998).

#### *Alterations of effector activity, a common consequence of the activation of the G protein cycle and of the inhibition of GTPase activity*

We show here that mastoparan stimulates the GTPase activity of G<sub>i</sub> proteins, whereas spermine and methoctramine inhibit this activity. Both types of drug activate phospholipase C promoting exocytosis in mast cells (Mousli *et al.*, 1990a; Bueb *et al.*, 1992; Chahdi *et al.*, 1998a). These properties of spermine and methoctramine are shared with non-hydrolyzable analogues of GTP which also activate phospholipase C and trigger secretion when introduced into mast cells (Cockcroft & Gomperts, 1985; Howell *et al.*, 1987). Thus, two groups of mast cell 'triggers' activating phospholipase C can be distinguished. Activators of the G<sub>i</sub> protein cycle include mastoparan and putatively other cationic peptides (Mousli *et al.*, 1990b). Inhibitors of GTPase activity comprise non-hydrolyzable analogues of GTP, natural polyamines and methoctramine.

Both the C- and the N-termini of G<sub>z</sub> proteins have been proposed to contribute to the binding site of cationic peptides (Mousli *et al.*, 1990a; Higashijima & Ross, 1991; Conklin & Bourne, 1993; Chahdi *et al.*, 1998b). Concerning inhibitors, non-hydrolysable analogues of GTP such as GppNHP, are considered to compete for GTP binding as confirmed here by the increase of  $K_M$  with no modification of  $V_{max}$  (Table 1). In contrast, spermine and methoctramine modified both  $V_{max}$  and  $K_M$  for GTP, suggesting some allosteric process leading to the inhibition of GTP hydrolysis. Further studies are required to locate their putative binding sites. Nevertheless, the present results show that the opposite modulation of the GTPase activity of G<sub>i</sub> proteins can correlate with the activation of phospholipase C. Similarly, the activation of adenylyl cyclase or cyclic GMP-phosphodiesterase can be achieved both through the receptor-dependent activation of the functional

cycle of G<sub>s</sub> and G<sub>t</sub>, respectively, or through the inhibition of GTPase activity by cholera toxin which ADP-ribosylates G<sub>s</sub> and G<sub>t</sub> in the vicinity of their GTP binding site, promoting the dissociation of  $\alpha$  and  $\beta\gamma$  subunits (Cassel & Selinger, 1997; Abood *et al.*, 1982; Kahn & Gilman, 1984). We propose that the inhibition of GTPase activity of G<sub>i</sub> by spermine and methoctramine, at high concentrations, prevent the reassociation of  $\alpha$  and  $\beta\gamma$  subunits, maintaining the regulation of effectors by G<sub>i</sub> subunits. This proposal fits with the activation of phospholipase C observed in brain cortical cells and in mast cells (Lee *et al.*, 1989; Bueb *et al.*, 1992; Chahdi *et al.*, 1998a) and with the inhibition of adenylyl activity observed in cardiomyocytes and erythrocytes (Melchiorre *et al.*, 1987; Clo *et al.*, 1988; Pignatti *et al.*, 1990; Khan *et al.*, 1990), these two types of regulation involving  $\beta\gamma$  and  $\alpha$  subunits, respectively (Clapham & Neer, 1997, for review).

#### *The decreased affinity of muscarinic agonists, a consequence of the polyamine-G protein interaction*

Binding experiments performed with  $\beta$ -adrenoceptor and muscarinic receptor ligands revealed that guanylyl nucleotides shifted the [<sup>3</sup>H] antagonist/agonists binding curves to lower affinities, with a decreased proportion of high-affinity binding sites for agonists (Maguire *et al.*, 1976; Lefkowitz *et al.*, 1976; Birdsall *et al.*, 1978; Hulme *et al.*, 1978). We observe here a guanylyl nucleotide-like effect for spermine (Figure 8, Table 5). The spermine-induced decrease in agonist affinity was observed directly in [<sup>3</sup>H]-Oxo-M binding assays (Figure 6). These results strongly suggest that spermine, by decreasing GTPase activity as observed with GppNHP, might uncouple G<sub>i</sub> proteins from M<sub>2</sub> muscarinic receptors, increasing the proportion of uncoupled receptors with low affinity for agonists (referred to as R; see for review Leff, 1995) and decreasing the proportion of M<sub>2</sub> receptors spontaneously coupled to G<sub>i</sub> with high affinity for agonists (referred to as R\*).

The inhibitory effect of spermine on spontaneous and mastoparan-induced GTP hydrolysis was observed at millimolar concentrations (Figure 1, Table 3). The potency of spermine decreased to the micromolar concentration range when observed on carbachol-induced GTP hydrolysis (Figure 3, Table 3). These results agree with the proposal that spontaneous GTP hydrolysis is related to G proteins coupled to R\* in the absence of agonist, and with the property of the mastoparan-G protein interaction to mimic the receptor-G protein interaction (Higashijima *et al.*, 1988). The high potency of spermine to decrease the agonist-dependent GTPase activity might arise from the spermine-induced decrease of receptor affinity for agonists which abolishes the stimulation of the G protein cycle by the agonist-liganded receptor. Such a feedback effect could not arise for mastoparan-dependent and for spontaneous, R\*-dependent, activations which are both agonist-independent, leading to a lower potency of spermine to decrease corresponding GTPase activities.

### The dual effect of methoctramine

Nanomolar concentrations of methoctramine decreased the carbachol-dependent GTP hydrolysis (Figure 3, Table 3), and displaced [<sup>3</sup>H]-NMS binding (Figure 6), suggesting that the decrease of carbachol-dependent GTP hydrolysis was related to the antagonist activity of methoctramine at M<sub>2</sub> receptors. The observation of an apparent higher affinity of methoctramine for [<sup>3</sup>H]-antagonist (NMS) sites compared to the [<sup>3</sup>H]-agonist (Oxo-M) sites, might suggest inverse agonist activity for methoctramine, as shown before for pirenzepine (Daeffler *et al.*, 1999). However, the inhibitory effect of methoctramine on GTPase activity was not prevented by neutral muscarinic antagonists, (Figure 2), excluding this hypothesis.

Micromolar concentrations of methoctramine were required to inhibit spontaneous and mastoparan-induced GTP hydrolysis, both in pig atria and mast cell membranes, the latter lacking muscarinic receptors (Table 4). Receptor-independent, but pertussis toxin-sensitive activation of phosphoinositide hydrolysis by micromolar concentrations of methoctramine have been reported in dissociated rat cerebral cortex and peritoneal mast cells (Lee *et al.*, 1989; Chahdi *et al.*, 1998a). These observations can be explained

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by the above proposal that high concentrations of methoctramine prevent the reassociation of  $\alpha$  and  $\beta\gamma$  subunits of G<sub>i</sub> proteins allowing the activation of phospholipase C. It is conceivable that the interaction of methoctramine with G proteins decreases the affinity of the receptor for agonists, as proposed for spermine, lowering the apparent affinity for [<sup>3</sup>H]-Oxo-M binding sites compared to [<sup>3</sup>H]-NMS sites (Figure 6). Due to the potent antagonist activity of low concentrations of methoctramine at M<sub>2</sub> receptors, its putative potency to induce, at high concentrations, a decrease of receptor affinity for agonists was not demonstrable through the shift of carbachol / [<sup>3</sup>H]-NMS curves as was observed for spermine (Figure 8).

In conclusion, spermine and methoctramine inhibit the GTPase activity of G<sub>i</sub> proteins, preventing the reassociation of  $\alpha$  and  $\beta\gamma$  subunits, maintaining the activation of effectors and decreasing the affinity of receptors for agonist. The effects of spermine were observed at concentrations within physiological limits for mammalian tissues (Seiler, 1991). The level of intracellular polyamines might be important for the regulation of the interaction of receptors and their related G proteins and for the modulation of downstream transduction events.



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