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Inverse agonist activity of pirenzepine at M_2 muscarinic acetylcholine receptors

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1 The intrinsic properties of muscarinic ligands were studied through their binding properties and their abilities to modulate the GTPase activity of G proteins coupled to muscarinic M_2 receptors in pig atrial sarcolemma.

2 Competition binding experiments were performed with [^{3}H]-oxotremorine-M to assess the affinity of receptors coupled to G proteins (R*), with [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) to estimate the affinities of coupled and uncoupled receptors (R*+R) and with [^{3}H]-NMS in the presence of GppNHp to assess the affinity of uncoupled receptors (R).

3 The ranking of K_i values for the agonist carbachol was R*<<R*+R<<R (0.95, 124 and 1017 nM). K_i values for atropine and AF-DX 116 were similar for the three binding conditions (0.34, 0.42, 0.41 and 19, 22, 32 nM). The ranking of K_i values for pirenzepine was R*>R*+R>R (174, 155, 115 nM), suggesting inverse agonism.

4 The V_{max} of the basal high affinity GTPase activity of pig atrial sarcolemma was increased by mastoparan and decreased by GPAnt-2 indicating the relevance of this activity to G proteins coupled to receptors (R*). The K_M value (0.26–0.33 μM) was not modified by mastoparan or GPAnt-2.

5 Carbachol increased the V_{max} of GTP hydrolysis (EC_{50} 8.1±0.3 μM), whereas atropine and AF-DX 116, up to 1 mM, did not modify it. Pirenzepine decreased the V_{max} of GTP hydrolysis (EC_{50} 77.5±10.3 μM). This effect was enhanced when KCl was substituted for NaCl (EC_{50} 11.0±0.8 μM) and was antagonized by atropine and AF-DX 116 (IC_{50} 0.91±0.71 and 197±85 nM).

6 Pirenzepine is proposed as an inverse agonist and atropine and AF-DX 116 as neutral antagonists at the muscarinic M_2 receptor.

Keywords: Inverse agonism; muscarinic M_2 receptors; G protein; pirenzepine; mastoparan; GPAnt-2; GppNHp

Abbreviations: [^{3}H]-NMS, [^{3}H]-N-methylscopolamine; [^{3}H]-Oxo-M, [^{3}H]-oxotremorine-M; GppNHp, guanylylimidodiphosphate; AF-DX 116, 11-[[2-[(diethyl-amino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one; GPAnt-2, [P-Glu^5,D-Trp^{7,9,10}]-substance P_{5-11}, pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2

Introduction

The concept that the activation of a receptor by an agonist involves a conformational change in the receptor has been recently applied to G protein-coupled receptors (Leff, 1995, for review). This model invokes the existence at equilibrium between two receptor states, a resting one (R) and an active one (R*). Agonists have preferential affinity for R*, inverse agonists (also called negative antagonists) for R, while neutral antagonists have equal affinities for both. G protein-coupled receptors exist in a constitutively active state R* able to initiate a biochemical response in the absence of agonist and to reveal inverse agonism (Milligan *et al*., 1995; Tucek, 1997; Kenakin, 1997; for review and discussion).

Related features of G protein-coupled receptor ligands were already demonstrated from first binding experiments with β-adrenoceptor ligands (Maguire *et al*., 1976; Lefkowitz *et al*., 1976). [^{3}H] antagonist/agonist competition curves tend to be shallow, suggesting an apparent heterogeneity of the receptor population. In the presence of guanyl nucleotides, [^{3}H] antagonist/agonist curves shifted to lower affinities. A similar behaviour was first reported for muscarinic acetylcholine receptor ligands (Birdsall *et al*., 1978; Hulme *et al*., 1978). However, the binding properties of both muscarinic agonists and antagonists, determined by [^{3}H] antagonist/ligand curves, were modulated by guanyl nucleotides (Berrie *et al*., 1979; Rosenberger *et al*., 1980; Burgisser *et al*., 1982; Hosey, 1982; Hulme *et al*., 1981; Martin *et al*., 1984). These observations suggested two distinct forms of muscarinic receptors, R_1 with high affinity for antagonists and low affinity for agonists, and R_2 with opposite affinities (Burgisser *et al*., 1982). The interconversion of R_2 to R_1 in the presence of guanyl nucleotides suggested that R_1 and R_2 could represent the R and R* states of G protein-coupled receptors described above. The fact that muscarinic antagonists have been considered to have a higher affinity for the R state (Burgisser *et al*., 1982), suggest that these ligands behave as inverse agonists. This interpretation is in line with recent results showing that the binding of GTP to cardiac membranes is increased by agonists and decreased by all the antagonists used, whereas such ligands should not modify the affinity of G proteins for GTP (Hilf & Jakobs, 1992).

The relative affinities of muscarinic ligands in displacing radiolabelled antagonists and agonists, have also been proposed to test their efficacy (Freedman *et al*., 1988; 1993; Sharif *et al*., 1995; Van Gelderen *et al*., 1996). Agonists exhibited high positive ratios of K_i for displacing [^{3}H] antagonists versus K_i for displacing [^{3}H] agonists. These ratios reflected the ability of agonists to stimulate coupled effectors, measuring phosphoinositide turnover (Freedman *et al*., 1988) or GTPase activity of coupled G proteins (Van Gelderen *et al*.,

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1996). Results obtained with muscarinic antagonists were less clear, varying from slightly positive (Freedman *et al*., 1988; 1993; Van Gelderen *et al*., 1996) to negative ratios (Sharif *et al*., 1995), i.e. leading to putative neutral antagonist or inverse agonist properties.

Thus, previous studies have not clearly distinguished between inverse agonist and antagonist properties of muscarinic ligands. In the present study, we reexamined such putative properties using three binding conditions to determine the affinity of ligands for R and R* states of M_2 receptors and measuring the induced modulation of the GTPase activity of associated G proteins. Data were obtained from porcine atrial sarcolemma, which previously has been shown to exhibit constitutively active muscarinic acetylcholine M_2 receptors (Hilf & Jakobs, 1992), i.e. a significant amount of R* in the absence of agonist.

Methods

Preparation of pig atrial sarcolemma membranes

Pig atrial sarcolemma were prepared according to Peterson & Schimerlik (1984). Briefly, fresh pig atria were kept on ice from the local slaughterhouse and cut into small pieces excluding adipose tissue. The tissue was homogenized with a polytron tissue homogenizer in Tris-HCl 10 mM, pH 7.4 including EDTA 1 mM, sodium azide (NaN_3) 0.02% w v^{-1} and phenylmethylsulphonylfluoride (PMSF) 0.1 mM. The homogenate was then centrifuged at $250 \times g$ for 15 min at 4°C and the pellet discarded. The supernatant was filtered through four layers of cheesecloth and then centrifuged at $44{,}000 \times g$ for 1 h at 4°C, in a Beckman JA 20 rotor. The resulting pellet was resuspended in the buffer using a glass teflon tissue homogenizer (10 ml per 100 g starting tissue). This suspension was then centrifuged at $44{,}000 \times g$ for 1 h at 4°C through a discontinuous sucrose gradient consisting of 10 ml 28%, and 10 ml 13% (w $v)^{-1}$ sucrose solutions in the above buffer. The layer between the two sucrose solutions was removed, diluted 3 fold in the above buffer and recentrifuged at $44{,}000 \times g$ for 1 h at 4°C. The final pellet was resuspended in the buffer and stored at −80°C.

Binding studies

Competition experiments, were carried out with [^{3}H]-oxotremorine-M ([^{3}H]-Oxo-M 2 nM, 86.6 Ci $mmol^{-1}$) and with [^{3}H]-N-methylscopolamine ([^{3}H]-NMS 0.2 nM, 84 Ci $mmol^{-1}$) in the absence or presence of 100 μM GppNHp, (Van Gelderen *et al*., 1996). All binding studies were carried out at 25°C in Tris-HCl 10 mM, pH 7.4 including NaCl 150 mM and $MgCl_2$ 1 mM. The incubation mixture (0.5 ml) contained 20 and 40 μg of protein, for [^{3}H]-NMS and [^{3}H]-Oxo-M binding experiments, respectively. Samples were incubated for 1 h to reach equilibrium. Non-specific binding was determined in the presence of 1 μM atropine (4–5% of total binding). Filtration was carried out using Whatman GF/C glass fibre filters presoaked in 0.3% polyethylenimine to reduce non-specific binding to the filters as previously described (Gies *et al*., 1989). Filters were washed twice with 5 ml of ice-cold washing buffer (Tris-HCl 25 mM, pH 7.4), dried and put into 10 ml counting vials containing 6 ml of scintillation solution. Under the different conditions used, the steady-state for specific binding and the linear relationship between specific radiolabelled ligand binding and the amount of membrane added were verified. Saturation experiments with the radiolabelled ligands demonstrated saturability under each condition used and Scatchard analysis of the saturation isotherms was used to calculate the density of binding sites (B_{max}), 0.41 ± 0.10 ([^{3}H]-Oxo-M), 1.45 ± 0.39 ([^{3}H]-NMS) and 1.48 ± 0.35 pmol (mg of protein)$^{-1}$ ([^{3}H]-NMS + GppNHp) and dissociation constants (K_D), 1.20 ± 0.37, 0.24 ± 0.08 and 0.16 ± 0.06 nM, respectively (means ± s.e.mean, $n = 3$). Protein concentration was determined by the method of Bradford (1976) with bovine γ-globulin as standard.

GTPase activity measurements

GTPase activity was determined by a method adapted from Hilf & Jakobs (1989) in triethanolamine-NaOH 50 mM, pH 7.4 including NaCl 150 mM, ATP 1 mM, EDTA 0.1 mM, dithiothreitol (DTT) 1 mM and $MgCl_2$ 1 mM. Alternatively, KCl was substituted for NaCl. Samples of sarcolemma (10 μg of protein) were first preincubated for 30 min at 25°C with drugs. The reaction was started by addition of 20 μl [γ-^{32}P]-GTP (30 Ci $mmol^{-1}$) 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 0.05–0.7 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 $v)^{-1}$ charcoal suspension in KH_2PO_4-HCl 50 mM, 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 $^{32}P_i$ released in the presence of 50 μM unlabelled GTP, from total $^{32}P_i$ accumulation (Hilf & Jakobs, 1989; Cassel & Seliuger, 1976). Basal levels of pig atrial membrane high-affinity GTPase activity, i.e. activity in the absence of ligands, are given in legends and were taken as 100% of the activity. To facilitate comparison between the different membrane preparations, we used the B_{max} from saturation binding experiments with [^{3}H]-NMS for each preparation, to calculate the basal GTPase specific activity (pmol of P_i released.min^{-1} (pmol of [^{3}H]-NMS binding sites)$^{-1}$), considering the density of muscarinic binding sites as an index of sarcolemma purification.

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 & Prussoff (1973) for competitive interactions, taking into account the concentration and the K_D of tritiated ligands. V_{max} and K_M for GTP hydrolysis were obtained by non-linear regression analysis of the Michaelis-Menten plots by the UltraFit program (Aladdin Systems, Inc. and Raymond Lau). All values for binding experiments and GTPase activity monitoring are expressed as geometric means ± s.e.mean to decrease the errors associated with estimation of the means from logarithmic curves (Fleming *et al*., 1972).

Materials

Carbachol, atropine and guanylylimidodiphosphate (GppNHp) were purchased from Sigma Chemical (St. Louis,

U.S.A.). Pirenzepine was obtained from RBI (Natick, U.S.A.). 11-[[2-[(diethyl-amino)-methyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepin-6-one (AF-DX 116) was a gift from Boehringer-Ingelheim (Ingelheim, Germany). The substance-P related peptide GPAnt-2, [P-Glu^5,D-Trp^{7,9,10}]-substance P_{5-11}, (pGlu-Gln-D-Trp-Phe-D-Trp-D-Trp-Met-NH_2) was purchased from Bachem (Paris, France) and mastoparan from Neosystem (Strasbourg, France). [^{3}H]N-methylscopolamine ([^{3}H]-NMS, 84 Ci mmol^{-1}), [^{3}H]-oxotremorine-M ([^{3}H]-Oxo-M, 86.6 Ci mmol^{-1}), and [γ-^{32}P]-GTP (30 Ci mmol^{-1}) were purchased from New England Nuclear (Boston, MA, U.S.A.). Glass fibre filters (GF/C) were obtained from Whatmann (Clifton, 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 HCl 0.1 N and 80% CH_3COOH, respectively, and diluted with buffers described above.

Results

Figure 1 shows competition binding curves for increasing concentrations of muscarinic ligands performed with the agonist [^{3}H]-Oxo-M and the antagonist [^{3}H]-NMS in the absence or presence of 100 μM GppNHp, a non-hydrolysable analogue of GTP. Affinities (K_i) and slope factors (Hill coefficients, n_H) were first calculated from a one-site receptor-ligand interaction model (Table 1). The affinity of carbachol for [^{3}H]-Oxo-M binding sites was 130 fold higher than its affinity for [^{3}H]-NMS binding sites in the absence of GppNHp. However, the Hill coefficient for carbachol displacing [^{3}H]-NMS binding was significantly different to unity (Table 1) and was best fitted by a two-site binding model (Table 2), GppNHp induced a large rightward shift of the [^{3}H]-NMS displacement curve for carbachol with an increase in slope (Figure 1) and a correlative increase in low affinity binding sites from 65–91% (Table 2).

The affinity of AF-DX 116 for [^{3}H]-Oxo-M binding sites was not significantly different from its affinity for [^{3}H]-NMS binding sites (Figure 1, Table 1). GppNHp induced a slight decrease in affinity of AF-DX 116 for [^{3}H]-NMS sites. The affinity of atropine for [^{3}H]-Oxo-M binding sites was slightly higher than its affinity for [^{3}H]-NMS sites in the absence or presence of GppNHp. The affinity of pirenzepine for [^{3}H]-Oxo-M binding sites was slightly lower than its affinity for [^{3}H]-NMS binding sites (Table 1). This difference was amplified in the presence of GppNHp which shifted the [^{3}H]-NMS binding isotherm to the left (Figure 1). Moreover, the [^{3}H]-NMS displacement curve for pirenzepine in the presence of GppNHp was best fitted to a two-site binding model (Table 2) with a

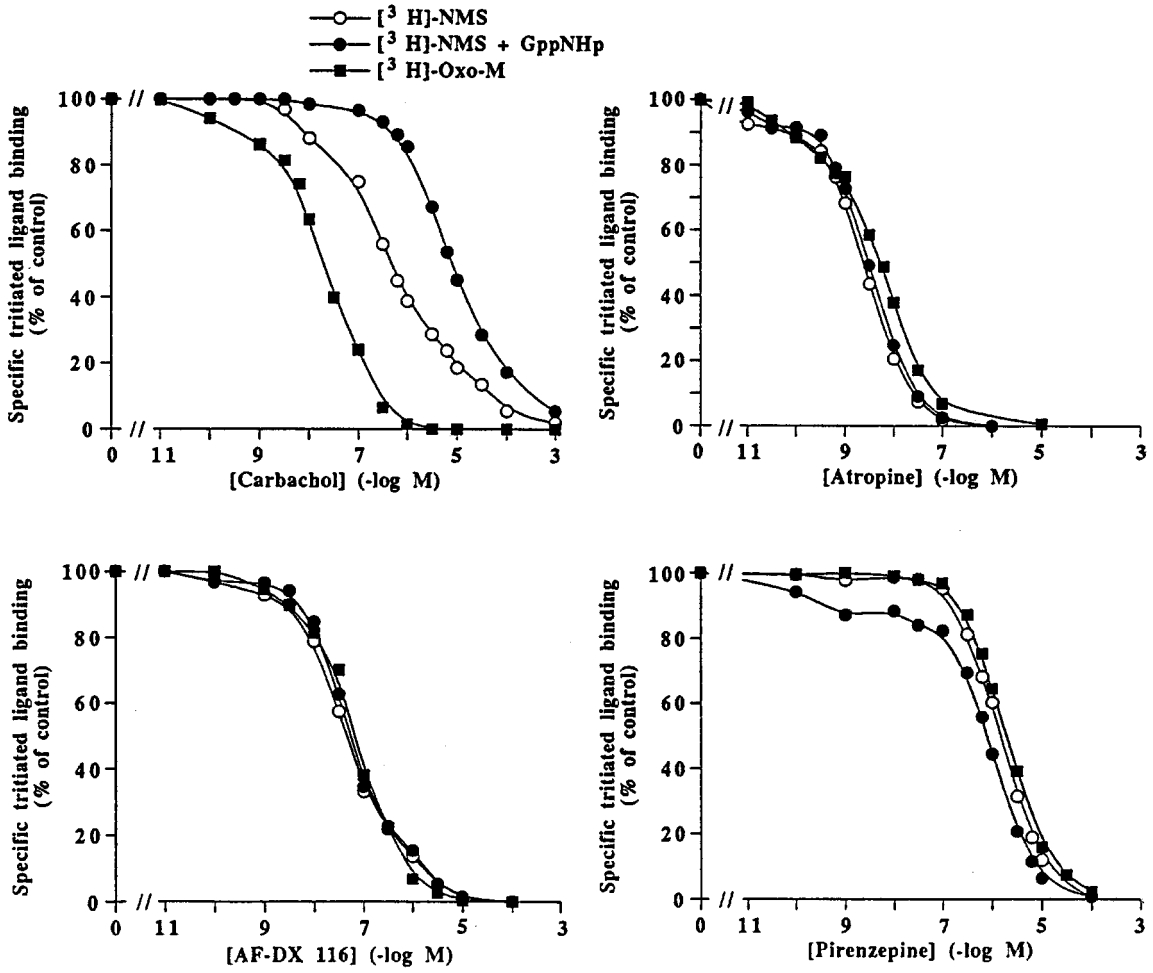

Figure 1 Inhibition of specific binding of [^{3}H]-oxotremorine-M ([^{3}H]-Oxo-M) and of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) with or without GppNHp to pig atrial sarcolemma by carbachol, atropine, AF-DX 116 and pirenzepine. Total specific binding in the absence of competitors (100%) corresponded to 596±179 ([^{3}H]-Oxo-M), 2068±168 ([^{3}H]-NMS) and 2367±185 d.p.m. per assay ([^{3}H]-NMS+GppNHp). Values shown are means from three independent experiments. Results are analysed in Table 1 and Table 2.

Table 1 One-site analysis of the inhibition of [^{3}H]-Oxo-M and [^{3}H]-NMS binding in pig atrial sarcolemma shown on Figure 1

Ligand	*[^{3}H]-Oxo-M*		*[^{3}H]-NMS*		*[^{3}H]-NMS + GppNHp*		*[^{3}H]-NMS/ [^{3}H]-Oxo-M*	*[^{3}H]-NMS+ GppNHp/ [^{3}H]-NMS*	*[^{3}H]-NMS+ GppNHp/ [^{3}H]-Oxo-M*
	K_i (nM)	n_H	K_i (nM)	n_H	K_i (nM)	n_H	K_i ratio	K_i ratio	K_i ratio
Carbachol	0.95±0.38	0.91	124±28*	0.52*	1017±222*	0.79*	130	8.20	1070
AF-DX 116	18.9±5.3	1.01	22.0±4.3	0.99	32.0±6.1	0.92	1.16	1.45	1.69
Atropine	0.34±0.10	0.97	0.42±0.06	1.02	0.41±0.12	0.98	1.23	0.97	1.20
Pirenzepine	174±19	0.96	155±11	0.93	115±7*	0.98*	0.89	0.74	0.66

(*) binding curves fitting to a two-site model to determine high and low affinity binding constants (Table 2). Values are geometric means±s.e.mean from three independent experiments.

Table 2 Two-site analysis of the inhibition of [^{3}H]-NMS binding in the absence* or presence† of GppNHp in pig atrial sarcolemma shown on Figure 1

Ligand	K_{ih} (nM)	% R_h	K_{il} (nM)	% R_l
Carbachol*	1.1±0.2	34.4±1.3	990±54	65.6±1.2
Carbachol†	4.3±0.9	8.8±2.7	1166±95	91.2±2.7
Pirenzepine†	0.4±0.1	17.1±1.9	101.7±9.6	82.5±1.9

Values are geometric means±s.e.mean from three independent experiments.

high-affinity binding component representing 17% of the total [^{3}H]-NMS binding sites.

Preliminary experiments were performed to characterize the GTPase activity of pig atrial sarcolemma. The activity corresponding to a high affinity for GTP was 25±1% (mean±s.e.mean, $n=15$) of the total GTPase activity of the sarcolemmal preparation. Figure 2a, Figure 3 and Table 3 show that the basal high affinity GTPase activity could be increased, with no K_M modification, by mastoparan, a peptide interacting with G proteins, the mastoparan-G protein interaction mimicking the receptor-G protein interaction (Higashijima *et al*., 1988; Mousli *et al*., 1990). In contrast, the basal activity was inhibited, with no modification of the K_M for GTP, by GPAnt-2, a peptide inhibiting the receptor-G protein interaction (Mukai *et al*., 1992). The maximal effects of mastoparan and GPAnt-2 were not reached at the highest concentrations used, preventing the determination of EC_{50} or IC_{50} values, but effects were observed at concentrations previously shown to be active (Higashijima *et al*., 1988; Mukai *et al*., 1992). The non-hydrolysable analogue of GTP, GppNHp, competing for the substrate, increased the K_M with no modification of V_{max}, (Table 3). A pretreatment of membranes with pertussis toxin led to a 38.9±2.1% inhibition ($n=3$) basal high affinity GTPase activity (0.8 mg of membrane protein pretreated with 40 μg of pertussis toxin in 1 ml for 2 h at 30°C, data not shown).

The effect of the receptor ligands used in binding experiments was studied on the GTPase activity. Figure 2b shows that carbachol induced a concentration-dependent increase of GTP hydrolysis (EC_{50} 8.1±0.3 μM). Atropine and AF-DX 116 did not modify the activity, but pirenzepine decreased it (EC_{50} 77.5±10.3 μM). These modifications corresponded to increases or decreases of the V_{max} with no alteration of the K_M for GTP (Table 3). The potency of pirenzepine to inhibit GTP hydrolysis was increased when KCl was substituted for NaCl in the assay medium (Figure 4, EC_{50} 11.0±0.8 μM). KCl also led to a large increase in basal GTPase activity (Figure 4). The inhibitory effect of pirenzepine on GTP hydrolysis was concentration-dependently antagonized by atropine and AF-DX 116 (Figure 5, IC_{50} 0.91±0.71 and 197±85 nM, respectively). Atropine, AF-DX 116 and

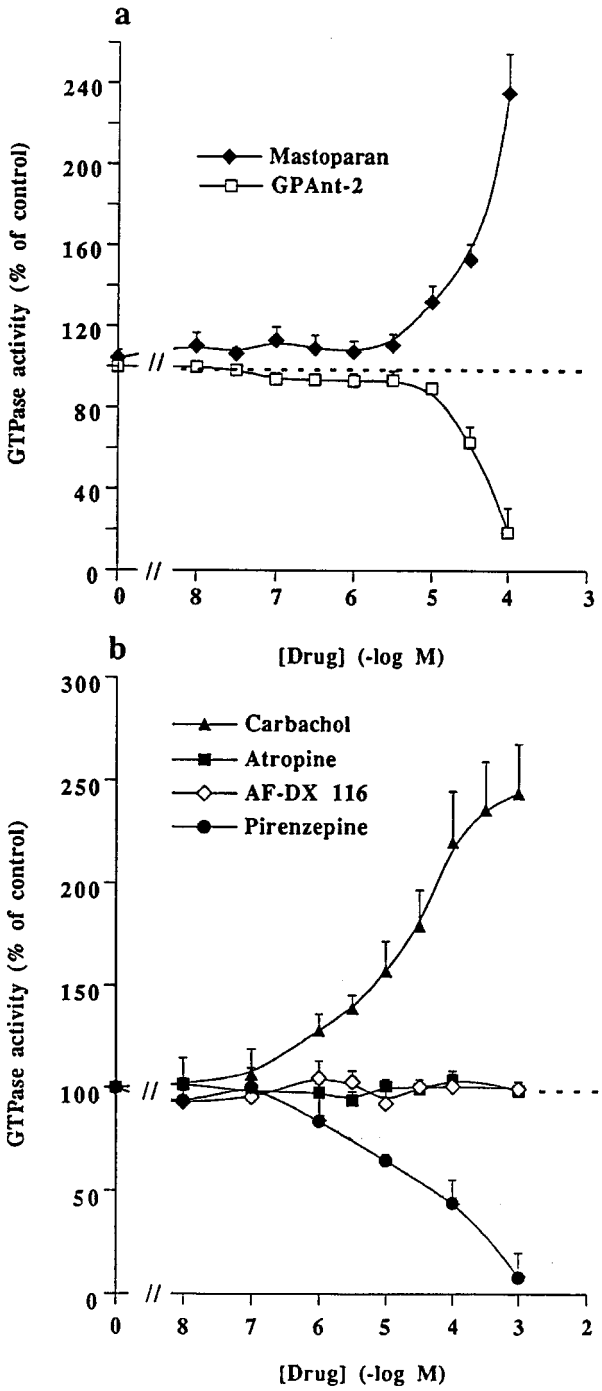

Figure 2 Modulation of the high-affinity GTPase activity of pig atrial sarcolemma membranes by (a) mastoparan and [P-Glu^5,D-Trp^{7,9,10}]-substance P_{5-11} (GPAnt-2) or (b) carbachol, atropine, AF-DX 116 and pirenzepine. Control basal GTPase activity (100%) was 941±122 (a) and 833±181 (b) fmol P_i min^{-1}. (pmol of receptor)$^{-1}$. Values are means±s.e.mean from five independent experiments.

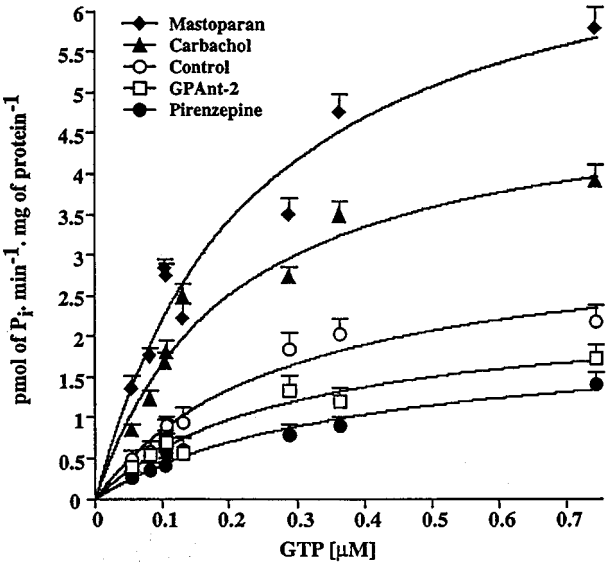

Figure 3 Michaelis-Menten representation of the effect of 0.1 mM carbachol, 0.1 mM pirenzepine, 0.06 mM mastoparan and 0.1 mM GPAnt-2 on the high-affinity GTPase activity of pig atrial sarcolemma, for various concentrations of substrate, [γ-^{32}P]-GTP. Values are means$\pm$s.e.mean from three independent experiments. Results are analysed on Table 3.

Table 3 Michaelis-Menten constants, V_{max} and K_M, for GTPase activity of pig atrial sarcolemma, in the presence of different drugs

	V_{max} (pmol P_i.min^{-1}.mg of protein^{-1})	K_M (μM)
Control	3.23$\pm$0.32	0.26$\pm$0.04
Mastoparan (0.06 mM)	7.47$\pm$0.41	0.28$\pm$0.03
Carbachol (0.1 mM)	5.03$\pm$0.42	0.29$\pm$0.02
Pirenzepine (0.1 mM)	2.02$\pm$0.63	0.33$\pm$0.06
GPAnt-2 (0.1 mM)	2.34$\pm$0.65	0.26$\pm$0.03
GppNHp (0.03 mM)	3.61$\pm$0.27	0.45$\pm$0.06

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

pirenzepine potently antagonized the GTPase stimulatory effect of 0.1 mM carbachol (EC_{50} 0.6$\pm$0.3, 0.7$\pm$0.2 and 6.0$\pm$0.3 μM respectively, $n=3$, data not shown).

Discussion

We show here that pirenzepine, previously considered as a selective muscarinic M_1 antagonist (Hammer *et al.*, 1980), fulfils the criteria for exhibiting inverse agonist activity at M_2 receptors, based on its affinity for the different states of the receptor and on the signal transduced to G proteins. The different states of the M_2 receptors of porcine atrial sarcolemma were studied by competition binding experiments performed with [^{3}H]-Oxo-M to test the affinity of receptors coupled to G proteins (R*), with [^{3}H]-NMS to approach the affinity of coupled and uncoupled receptors (R* + R), and with [^{3}H]-NMS in the presence of GppNHp to test the affinity of uncoupled receptors (R).

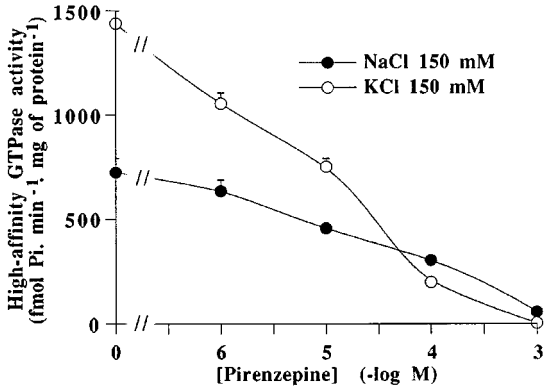

Figure 4 Effect of pirenzepine on the high-affinity GTPase activity of pig atrial sarcolemma in the presence of 150 mM NaCl or KCl. The basal activities, in the absence of pirenzepine, were 726$\pm$69 (NaCl) and 1440$\pm$59 fmol P_i min^{-1}.(pmol of receptor)$^{-1}$ (KCl). Each value is the mean$\pm$s.e.mean from three experiments.

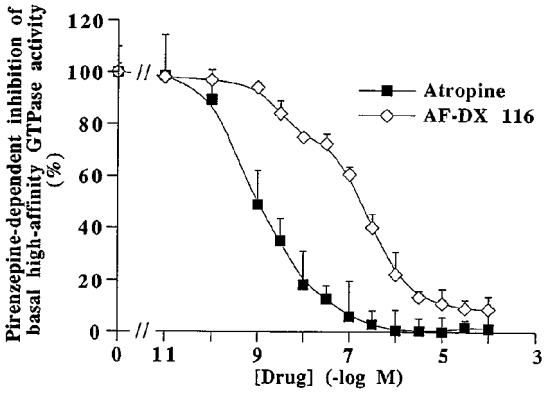

Figure 5 Concentration-dependent effect of atropine and AF-DX 116 on the inhibitory effect of 0.1 mM pirenzepine on the high-affinity GTPase activity of pig atrial sarcolemma. The GTPase activity in the presence of pirenzepine (100%) was 460$\pm$75 fmole P_i min^{-1}. (pmol of receptor)$^{-1}$. This activity in the absence of pirenzepine was 689$\pm$57 fmole P_i min^{-1}. (pmol of receptor)$^{-1}$. Results are means$\pm$s.e.mean from four independent experiments.

The validity of this approach was attested by the behaviour of carbachol which, in agreement with its full agonist activity, showed a very large preference for R* compared to R* + R and to R (K_i for R* < < R* + R < < R) (Figure 1 and Table 1). The [^{3}H]-Oxo-M displacement curve was best fitted to a one-site model, presumably corresponding to R*. The [^{3}H]-NMS curve was best fitted to a two-site model, allowing calculation of a high affinity component for carbachol binding similar to that determined with [^{3}H]-Oxo-M (Table 1 and Table 2). Also, the analysis of the [^{3}H]-NMS displacement curve for carbachol shows that the relative percentage of R* to R was about 35/65 assuming that the high and low affinities corresponded to R* and R, respectively (Table 2). The presence of GppNHp increased the percentage of low affinity binding sites from 65–91% (Table 2). This suggests that the uncoupling of receptors to G protein might not be fully achieved. However, it has to be kept in mind that the R/R* model is an over-simplification of the different states of a receptor (for discussion see Tucek, 1997; Kenakin, 1997).

Assuming a one-site receptor-ligand interaction process (Table 1), the affinity of pirenzepine in displacing [^{3}H]-NMS was slightly higher than its affinity in displacing [^{3}H]-Oxo-M. Moreover, the presence of GppNHp increased the affinity of

pirenzepine, suggesting inverse agonism (K_i for $R > R + R^* > R^*$). Curiously, the presence of GppNHp led to an apparent heterogeneity of the displacement curve for pirenzepine (Figure 1) with a super high affinity for 17% of the binding sites using a two-site interaction model (Table 2). We have presently no explanation for this observation which warrants further thorough study.

Results obtained with atropine and AF-DX 116 show some limits of interpretation of binding data in the characterization of the intrinsic activity of receptor ligands. A small inverse agonist, activity might have been proposed considering the binding curves (Figure 1), but this was invalidated by calculating K_i which suggested a slight partial agonist activity (Table 1). These observations show that the ratios of [^{3}H]-antagonist versus [^{3}H]-agonist affinity binding constants is useful in the prediction of agonist properties, as proposed earlier (Freedman *et al*., 1988; 1993; Sharif *et al*., 1995; Van Gelderen *et al*., 1996), but is not sufficient to clearly discriminate between neutral antagonists and inverse agonists which lead to only slightly different binding isotherms.

Measuring GTPase activity of pig atrial sarcolemma allowed us to confirm the inverse agonist activity of pirenzepine. The basal high affinity GTPase activity measured in this membrane preparation was decreased by the G protein antagonist GPAnt-2 (Figure 2a), a peptide inhibiting receptor-G protein interaction (Mukai *et al*., 1992) and by pertussis toxin (see Results). The ADP-ribosylation induced by pertussis toxin, prevents the G protein-receptor interaction, but does not modify the activity of purified G proteins (Gilman, 1987). Thus, results obtained with GPAnt-2 and pertussis toxin indicate that the basal GTPase activity measured in pig atrial sarcolemma is dependent on the receptor-G protein complex corresponding to the R* state. The lack of effect of atropine and AF-DX 116 on the basal GTPase activity confirms that this activity is not related to the presence of acetylcholine in the membrane preparation as discussed by Hilf & Jakobs (1989).

The fact that pirenzepine decreased the basal high affinity GTPase activity, whereas atropine and AF-DX 116 did not (Figure 2b), but prevented its inhibition by pirenzepine (Figure 5), demonstrates the inverse agonist activity of pirenzepine through its interaction with the antagonist binding site of the M_2 receptor. The effect of pirenzepine was observed at rather high concentrations when compared to the affinity of pirenzepine for muscarinic M_2 receptors. Due to its lipophilicity, pirenzepine might interact non-selectively with membranes, disturbing the coupling of M_2 receptors to G proteins. However, the inhibitory effect of pirenzepine is blocked in a concentration-dependent manner by atropine at a range of concentrations similar to that required to displace the tritiated ligand in binding studies. This demonstrates that pirenzepine is acting at the antagonist binding site of muscarinic M_2 receptors to inhibit the basal GTPase activity of pig atrial sarcolemma. In contrast, the inhibitory effect of GPAnt-2 is related to its competition with the receptor for interacting with G proteins (Mukai *et al*., 1992). Pirenzepine and GPAnt-2 decreased the V_{max} of the enzyme reaction without altering the affinity for GTP (similar K_M values, Table 3). In contrast, the non-hydrolysable analogue of GTP, GppNHp, competing for the substrate, increased the K_M of the reaction with no modification of V_{max}. Whereas pirenzepine was selective for the inhibition of basal GTPase activity, atropine, AF-DX 116, as well as pirenzepine, potently antagonized the GTPase stimulatory effect of carbachol. The EC_{50} value for atropine (0.6 μM) was rather high considering the affinity determined by binding experiments, but this has also been observed by Hilf & Jakobs (1989) on the same atrial preparation.

The efficiency of antagonists on GTPase activity has been reported by Costa & Hertz (1989) for δ opioid receptors in membranes of neuroblastoma-glioma hybrid NG108-15 cells. Basal GTPase activity was inhibited by only some Leu-enkephalin analogues (inverse agonists) but not by drugs such as MR 2266 (neutral antagonists), although both types of ligands inhibited agonist-stimulated GTPase activity (Costa & Hertz, 1989; Costa *et al*., 1992). Another similarity between the present results and those obtained on δ opioid receptors is the enhancement of the inhibitory effect of the inverse agonist when NaCl is replaced with KCl. It is noteworthy that under physiological conditions G proteins are exposed to the intracellular milieu, where the concentration of K^+ is high and that of Na^+ is low. However, whereas the basal GTPase activity and the inhibitory effect of inverse agonists are increased in the presence of KCl, the stimulatory effect of agonists is decreased (Hilf & Jakobs, 1989; Costa & Hertz, 1989 and present results). These ionic dependencies remain unexplained (see Hilf & Jakobs, 1989 and Costa & Hertz, 1989, for extensive discussion).

The GTPase stimulation of G proteins by the agonist-liganded receptor is preceded by exchange of G-protein-bound GDP by GTP (Gilman, 1987, for review). Hilf & Jakobs (1992) reported, when using the same atrial membrane preparation as in the present study, that muscarinic antagonists, including atropine, AF-DX 116 and pirenzepine, not only competitively inhibited the agonist-induced stimulation of GTPγS binding to G proteins, but also reduced binding in the absence of agonist. No evidence for heterogeneity with regards to negative intrinsic activity of antagonists was obtained. The same experimental approach applied to 5-HT_{1A} receptors was able to distinguish neutral antagonism (no modification of GTPγS binding) and inverse agonism (inhibition of basal binding) (Newman-Tancredi *et al*., 1997). Thus, the discrepancies of results obtained using the same tissue with GTPγS binding (Hilf & Jakobs, 1992) and GTPase activity (present results) cannot be presently explained.

Previous studies have already proposed inverse agonist properties for muscarinic ligands. Atropine was observed to decrease GTPγS binding (Akam *et al*., 1996) and to increased the production of cyclic AMP (Jakubik *et al*., 1995) in Chinese hamster ovary (CHO) cells expressing human M_2 receptors. In contrast, AF-DX 116 did not modify cyclic AMP production in CHO-M_2 cells (Jakubik *et al*., 1995), suggesting neutral antagonist activity, whereas atropine and NMS were proposed as inverse agonists. In these transfected cells, the coupling of M_2 receptor with G proteins might be modified due to the over-expression of the receptor. Such an over-expression has been reported to increase the proportion of constitutively active receptors (Milligan *et al*., 1995). Also, a direct interaction of drugs with adenylyl cyclase, for instance, has not been excluded. Moreover, in the different studies suggesting inverse agonism properties for muscarinic ligands, no evidence has been presented for interaction of these ligands with neutral antagonists. The fact that atropine and AF-DX 116 antagonize the inhibitory effect of pirenzepine on GTP hydrolysis (Figure 5) is the strongest evidence in the present study to propose inverse agonism activity for pirenzepine.

In conclusion, pirenzepine behaves as an inverse agonist (or negative antagonist) at acetylcholine muscarinic M_2 receptors, whereas atropine and AF-DX 116 are characterized as neutral antagonists. We propose the atropine-sensitive inhibition of basal high affinity GTPase activity of membranes in the presence of KCl as a potent assay to discriminate inverse

agonists among muscarinic antagonists. Studies should be extended to include other muscarinic receptor subtypes to approach the selectivity of muscarinic inverse agonists and to consider their potential therapeutic interest.

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