Constitutive activity of the M_1–M_4 subtypes of muscarinic receptors in transfected CHO cells and of muscarinic receptors in the heart cells revealed by negative antagonists

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Abstract **We investigated whether muscarinic receptors of the M_1–M_4 receptor subtypes are constitutively active. We have found that the synthesis of cyclic AMP was enhanced by the muscarinic antagonists atropine and *N*-methylscopolamine (NMS) in Chinese hamster ovary (CHO) cells stably transfected with human m2 and m4 muscarinic receptor genes and in rat cardiomyocytes expressing the M_2 receptor subtype, and that the production of inositol phosphates was inhibited by atropine and NMS in CHO cells stably transfected with human m1 and m3 and with rat m1 muscarinic receptor genes. The muscarinic antagonists quinuclidinyl benzilate and AF-DX 116 had no effect in some cases and acted like atropine and NMS in others. We conclude that the M_1–M_4 subtypes of muscarinic receptors are constitutively active in the CHO cell lines expressing them and in cardiomyocytes and that atropine and NMS act as negative antagonists on these receptor subtypes by stabilizing them in the inactive conformation.**

Key words: Muscarinic receptor; Receptor, muscarinic; Constitutive receptor activity; Atropine; Methylscopolamine; CHO cell; Cardiomyocyte

1. Introduction

It has been discovered recently that some of the receptors which are coupled to G proteins display constitutive (spontaneous) activity in the absence of agonists [1,2]. Most probably, such receptors oscillate between the inactive and the active conformation [3,4] or one of the active conformations if these conformations are multiple [5,6]. In the active conformation, they are able to activate the corresponding G protein. The binding of agonists shifts the equilibrium between the inactive and active receptors towards the active state. On the other hand, the binding of antagonists may have two different effects: some antagonists do not distinguish between the inactive and active conformations and do not shift the equilibrium between them (neutral antagonists), while other antagonists bind preferentially to the inactive receptors and shift the equilibrium in their favour (negative antagonists) [7,8].

By decreasing the proportion of receptors which are in the spontaneously active state the negative antagonists influence the activity of G proteins and their effector molecules (enzymes and ion channels) in a direction which is opposite to the action of corresponding agonists. Accordingly, the use of negative antagonists permitted the demonstration of the constitutive activity of δ opioid receptors [7], α_2-adrenoceptors [9], β_2-adrenoceptors [10–12], 5-hydroxytryptamine$_{2C}$ receptors [13] and bradykinin receptors [14].

The present report describes the effects of four muscarinic antagonists on two processes which are controlled by muscarinic receptors, namely the synthesis of cyclic AMP (normally inhibited by the M_2 and M_4 subtypes of muscarinic receptors) and the hydrolysis of phosphoinositides (normally activated by the M_1, M_3 and M_5 subtypes of muscarinic receptors – review [15]). No reports were available on the action of muscarinic antagonists applied alone on these processes by the time our experiments were started. Recently, however, Burstein et al. [16] described an inhibitory effect of atropine and pirenzepine on the hydrolysis of phosphoinositides in cells equipped with M_3 muscarinic receptors and excess G_q protein.

2. Materials and methods

2.1. Materials

[^{3}H]adenosine (25 Ci/mmol), cyclic [^{14}C]AMP (283 Ci/mol), [^{3}H]inositol (83 Ci/mmol) and [^{14}C]inositol-1-phosphate (55 Ci/mol) were from Amersham Int. (Little Chalfont, UK). Dulbecco's modified Eagle's medium (Hybri-Max DME) and alumina WN/3 were from Sigma (St. Louis, MO), culture medium E-199 was from USOL (Prague, Czechia), the mixture of proteases (P-TC proteases for cell culture) was from TK Media Co. (Bratislava, Slovakia), and cytosine arabinoside was from Mack (Illertissen, Germany). Atropine sulphate and *N*-methylscopolamine (NMS) were from Sigma (St. Louis, MO), *R*(−)-quinuclidinyl benzilate (QNB) was from RBI (Natick, MA), and AF-DX 116 (Hammer et al., 1986) was kindly provided by Dr. H. Ladinsky.

2.2. Cells and cell cultures

Untransfected wild-type Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Rockville, MD). CHO cell lines stably transfected with genes for the human M_1, M_2, M_3 or M_4 subtypes of muscarinic receptors (subsequently denoted as hm1–hm4 genes and hM_1–hM_4 subtypes) [17] were kindly provided by Drs. T. I. Bonner and M. R. Brann. The gene for the rat M_1 muscarinic receptor subtype (subsequently denoted as rm1 gene and rM_1 subtype) was kindly donated by Dr. C. Fraser and subcloned into the pCMV-3 mammalian expression vector supplied by Dr. D. W. Russell. CHO cells were co-transfected with pCMV-3 and pMSV$_{neo}$ and treated as described [18]. Clonal cell lines stably transfected with the rm1 gene were screened to select cells which express high or low receptor number. The high-expression and the low-expression cell lines used contained 41.5 ± 5.2 fmol and 6.6 ± 2.7 fmol of [^{3}H]NMS binding sites (determined as described [20]) per 1 million cells, respectively. Cell cultures were treated as described [18–20]. Cells were harvested using mild treatment with a mixture of proteases 4–5 days after subculturing. The binding properties of muscarinic receptors on the CHO cell lines expressing the hm1–hm4 genes and the rm1 gene were as determined by Jakubík et al. [20] and Zhu et al. [18], respectively.

Cardiomyocytes were obtained from minced hearts of 4-day-old rats

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by trypsin treatment and trituration. Fibroblasts were removed by preplating and 24 h exposure to cytosine arabinoside. Cells were grown in E-199 medium with 10% fetal calf serum. After 7 days in culture, they were harvested using mild treatment with a mixture of proteases, washed by repeated centrifugation and used for experiments.

2.3. Assay of [^{3}H]cyclic AMP formation

The method was modified from that described [19]. Cells were suspended in Krebs–Henseleit buffer, preincubated for 1 h at 37 °C with [^{3}H]adenine (10 μCi/ml), washed twice by centrifugation and resuspended in Krebs–Henseleit buffer containing 1 mM isobutylmethylxanthine. They were equilibrated for 15 min at 37 °C, after which they were incubated for 10 min with the investigated muscarinic ligand, with or without 20 μM forskolin. The incubation was in a volume of 0.8 ml per tube, with 300,000–400,000 cells per tube. It was stopped with 2.5 M HCl and the content of the tube was applied on a column filled with 1.5 g alumina [21,22]. The column was washed with 2 ml of 100 mM ammonium acetate (pH 7.0) and the retained cyclic [^{3}H]AMP was eluted with 4 ml of 100 mM ammonium acetate and quantified by liquid scintillation spectrometry. Cyclic [^{14}C]AMP was used as standard to check the recovery. The synthesis of cyclic [^{3}H]AMP was measured as the difference between the content of cyclic [^{3}H]AMP in the samples at the end and in the beginning of the 10 min incubation period.

2.4. Assay of the formation of [^{3}H]inositol phosphates

The assay was as reported [19] but diisoproplyfluorophosphate was not used. Inositol mono-, bis- and trisphosphate were collected together as described [23] because their separation was unlikely to provide relevant additional information.

2.5. Data analysis

Experiments were performed three times, with incubations in triplicates. The maximum effect (E_{max}) which a muscarinic ligand had on the measured parameter (the synthesis of cyclic [^{3}H]AMP or of [^{3}H]inositol phosphates) and the values of EC_{50} (concentration of the ligand producing one half of the maximum effect) and nH (Hill slope factor) were computed by fitting the following equation [24,25] to the data:

$$E = 100 + E_{max} \times \frac{[L]^{nH}}{[L]^{nH} + (EC_{50})^{nH}} \quad \textbf{(Eqn. 1)}$$

where E = measured parameter (expressed as % of its control value) in the presence of muscarinic ligand L at the concentration [L], and E_{max} is the maximum effect expressed as % change of control value. Marqvardt's least square method with proportional weighting was used for the fitting. Statistical significance of differences from control values was evaluated with Student's t-test and differences at $P < 0.05$ were regarded as significant.

3. Results and discussion

3.1. Effects of muscarinic antagonists on the synthesis of cyclic [^{3}H]AMP in CHO cells expressing the hM_2 or hM_4 subtype of muscarinic receptors and in rat cardiomyocytes

Changes in the basal and the forskolin-stimulated rates of cyclic [^{3}H]AMP accumulation were investigated. As shown in

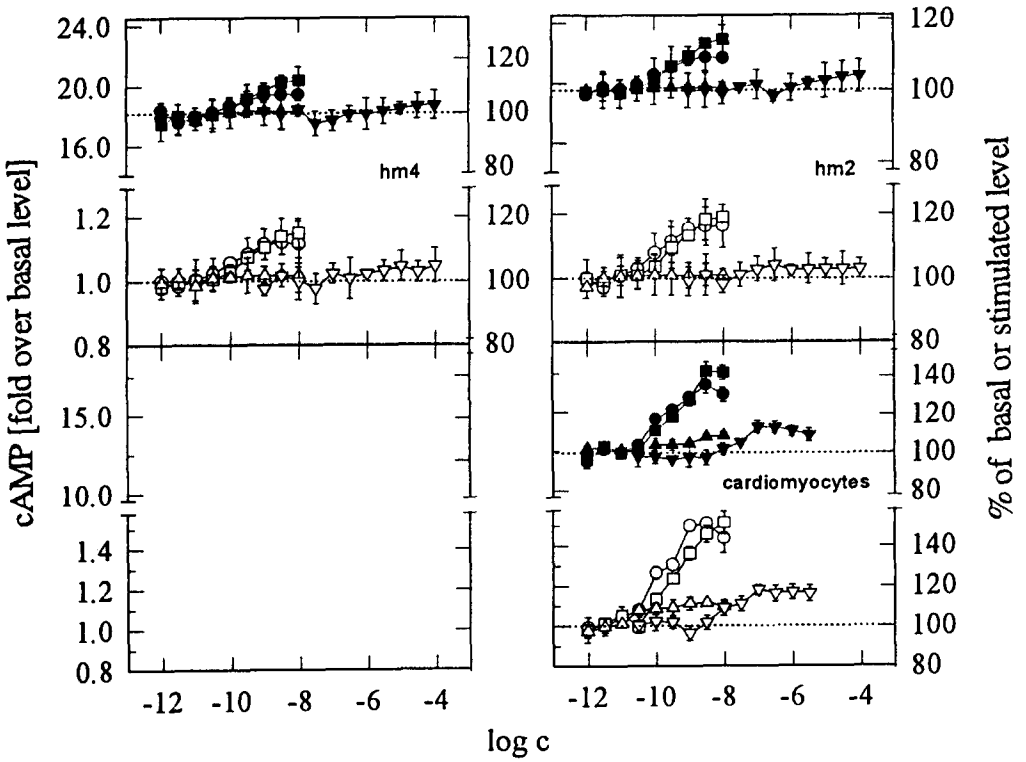

Fig. 1. Effect of muscarinic antagonists on the synthesis of cyclic [^{3}H]AMP in CHO cells expressing the human m4 (upper left) or m2 (upper right) muscarinic receptor gene or in rat cardiomyocytes (lower right), either in the presence of 20 μM forskolin (full symbols) or in its absence (open symbols). Abscissa: $\log_{10}$ of antagonist concentration (M). Left ordinate: cyclic [^{3}H]AMP accumulation during 10 min incubation, expressed as a multiple of the basal accumulation rate observed in the absence of forskolin and antagonists, which was in the range of 85–107 fmol cyclic [^{3}H]AMP per tube. Right ordinate: cyclic [^{3}H]AMP accumulation, expressed as % of the basal unstimulated accumulation (for experiments without forskolin) or as % of the basal stimulated accumulation (in the presence of forskolin but without any antagonist). NMS, ○ ●; atropine, □ ■; QNB, △, ▲; AF-DX 116, ▽, ▼. Data are weighted means ± S.E.M. of three experiments with incubations in triplicates. All increases in cyclic [^{3}H]AMP accumulation observed in all three types of cells in the presence of atropine and NMS were statistically significant ($P < 0.05$) at 10^{-9} M and higher concentrations but in some cases even lower antagonist concentrations caused a significant change. In experiments with cardiomyocytes, the effects of QNB were significant at $10^{-9.5}$ M and higher concentrations and the effects of AF-DX 116 were significant at $10^{-7.5}$ M and higher concentrations.

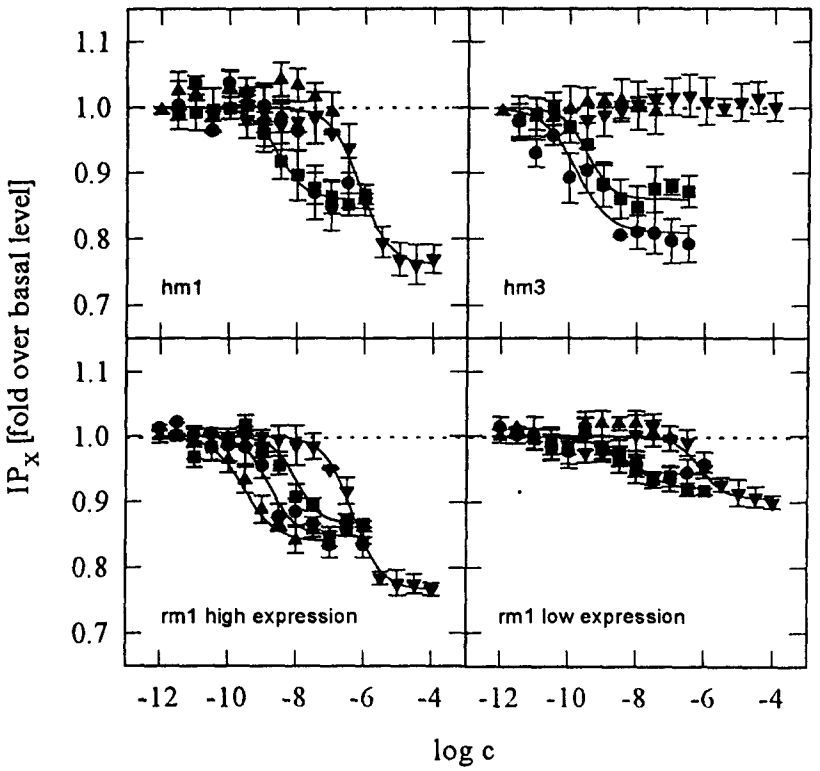

Fig. 2. Effect of muscarinic antagonists on the production of [^{3}H]inositol phosphates in CHO cells expressing the human m1 (upper left) or m3 (upper right) muscarinic receptor gene or expressing the rat m1 gene with a high density (lower left) or a low density (lower right) of muscarinic receptors. Abscissa: $\log_{10}$ of antagonist concentration (M). Ordinate: accumulation of [^{3}H]inositol phosphates (IP_x = sum of inositol mono-, bis- and trisphosphate) during 60 min incubation in the presence of an antagonist, expressed as a multiple of the accumulation in control samples without the antagonist; the latter value was in the range of 24–28 fmol [^{3}H]inositol phosphates per tube. NMS, ●; atropine, ■; QNB, ▲; AF-DX 116, ▼. Data are weighted means ± S.E.M. of three experiments with incubations in triplicates. All effects induced by atropine with all types of cells were statistically significant ($P < 0.05$) at concentrations of $10^{-8.5}$ M and higher and those induced by NMS were significant at concentrations of 10^{-8} M and higher. In the cells with the high expression of the rm1 gene, significant changes were also observed at QNB concentrations of 10^{-10} M and higher and at AF-DX 116 concentrations of 10^{-7} M and higher. In the cells with the low expression of the rm1 gene, AF-DX 116 produced significant changes at 10^{-6} M and higher concentrations.

Fig. 1, 20 μM forskolin itself increased the rate of cyclic [^{3}H]AMP synthesis more than 18-fold in the hM_2 and the hM_4 transfected CHO cells and more than 12-fold in cardiomyocytes. Its effect in the wild-type CHO cells was similar (not shown). Both the basal and the forskolin-stimulated rates of cyclic [^{3}H]AMP accumulation were augmented by the muscarinic receptor antagonists atropine and NMS in all three cell types examined, whereas QNB and AF-DX 116 had no effect on CHO cells and a positive effect on cardiomyocytes. The computed maximum effects induced by NMS and atropine were 33–54% in the cardiomyocytes and 8–20% in the transfected CHO cells (Table 1). The concentrations of antagonists producing half-maximal stimulation of cyclic [^{3}H]AMP accumulation were in the range of 347–489 pM for atropine and 110–126 pM for NMS. None of the four muscarinic antagonists tested had any effect on the accumulation of cyclic [^{3}H]AMP in the wild-type CHO cells when applied at the concentrations used in Fig. 1 (not shown).

The possibility that atropine and NMS produce their effects by preventing the action of endogenous acetylcholine (originating from some unknown source) appeared unlikely because the experiments were performed in the absence of cholinesterase inhibitors and the enhancement of cyclic [^{3}H]AMP accumulation by atropine and NMS was also observed at a time when the potent muscarinic antagonist QNB had no effect. In two experiments (performed with incubations in triplicates) which we made on cardiomyocytes, the addition of either acetylcholinesterase (10 units/ml) or eserine sulphate (50 μM) to the incubation medium had no effect on the rate of cyclic [^{3}H]AMP accumulation in the absence of NMS and did not change the stimulation of cyclic [^{3}H]AMP accumulation induced by NMS.

3.2. *Effect of muscarinic antagonists on the production of [^{3}H]inositol phosphates in CHO cells expressing the hM_1, rM_1 or hM_3 subtypes of muscarinic receptors*

The formation of [^{3}H]inositol phosphates was diminished by atropine both in CHO cells expressing the hM_1 and hM_3 receptor subtypes and in CHO cells expressing the rM_1 receptor subtype at high or low density (Fig. 2, Table 2). The effects of NMS were similar to those of atropine, but data obtained with NMS on cells expressing the hM1 receptor subtype did not fit Equation 1 and relevant parameters of NMS action were therefore not computed in Table 2. QNB was without effect on cells expressing the hM_1 and hM_3 receptor subtypes and on cells with low expression of the rM_1 subtype, but it had an inhibitory effect on cells with a high expression of the rM_1 subtype. In the presence of AF-DX 116, the accumulation of inositol phos-

Table 1
Quantitative parameters of the action of muscarinic antagonists on the synthesis of cyclic [^{3}H]AMP in CHO cells expressing the human m2 or m4 muscarinic receptor gene and in rat cardiomyocytes

Ligand	Forskolin	CHO cells, hm2 gene			CHO cells, hm4 gene			Rat cardiomyocytes		
		pEC_{50}	E_{max} (% change)	*nH*	pEC_{50}	E_{max} (% change)	*nH*	pEC_{50}	E_{max} (% change)	*nH*
NMS	0	9.94 ± 0.49	+16.4 ± 0.8	1.03 ± 0.01	9.95 ± 0.42	+12.1 ± 0.1	1.02 ± 0.06	9.96 ± 0.19	+49.1 ± 2.4	1.08 ± 0.00
	20 μM	9.90 ± 0.30	+11.5 ± 0.5	0.98 ± 0.05	9.92 ± 0.14	+8.1 ± 0.3	1.03 ± 0.07	9.92 ± 0.23	+33.3 ± 0.8	1.13 ± 0.00
Atropine	0	9.41 ± 0.26	+19.7 ± 0.3	0.95 ± 0.00	9.46 ± 0.36	+15.0 ± 0.4	1.01 ± 0.02	9.36 ± 0.27	+54.3 ± 0.9	0.89 ± 0.04
	20 μM	9.37 ± 0.03	+18.0 ± 1.1	0.95 ± 0.06	9.40 ± 0.42	+13.5 ± 0.2	0.97 ± 0.06	9.31 ± 0.21	+47.7 ± 1.0	0.85 ± 0.01
R(−)-QNB	0		no effect			no effect		10.4 ± 0.14	+11.0 ± 0.4	1.17 ± 0.02
	20 μM		no effect			no effect		9.95 ± 0.11	+8.7 ± 0.3	0.81 ± 0.02
AF-DX 116	0		no effect			no effect		7.88 ± 0.31	+17.0 ± 0.3	1.18 ± 0.05
	20 μM		no effect			no effect		7.58 ± 0.23	+13.0 ± 0.2	1.18 ± 0.02

Values in the Table have been computed from the results of experiments shown in Fig. 1; they are weighted means ± S.E.M. of three experiments with incubations in triplicates. $pEC_{50} = -\log_{10}$ of the concentration of antagonist producing one half of the maximum effect; E_{max} = maximum effect expressed as % of control value, with stimulation denoted by + and inhibition by − signs; *nH* = Hill slope factor.

phates was inhibited in cells expressing the hM_1 or rM_1 receptor subtypes. Conspicuously, the effects of atropine, NMS and AF-DX 116 were higher on the cells expressing the rM_1 receptor subtype at the high density than on those expressing it at the low density, and the effect of QNB was only apparent on the cells expressing the rM_1 receptor subtype at the high density (Table 2).

None of the four antagonists tested at the concentrations shown in Fig. 2 had any effect on the accumulation of [^{3}H]inositol phosphates in untransfected (wild-type) CHO cells (not shown).

3.3. Effects of carbachol

Functional responses to the stable muscarinic agonist carbachol were tested in parallel experiments. At 1 μM concentration, carbachol inhibited the rates of the basal and the forskolin-stimulated cyclic [^{3}H]AMP accumulation in CHO cells expressing the hm2 and hm4 genes by more than 43% and in cardiomyocytes by more than 23%. The rate of the accumulation of [^{3}H]inositol phosphates was enhanced 5.6-fold and 9.4-fold in CHO cells expressing the hm1 and hm3 genes, respectively, and 4.7-fold and 5.2-fold in CHO cells expressing the rm1 gene at the high and the low receptor density, respectively.

3.4. Inferences

By producing an increase in the synthesis of cyclic [^{3}H]AMP in CHO cells expressing the M_2 and M_4 muscarinic receptor subtypes and in rat cardiocytes and by diminishing the production of [^{3}H]inositol phosphates in CHO cells expressing the M_1 and M_3 muscarinic receptor subtypes, the muscarinic antagonists atropine and NMS had exactly opposite effects than the muscarinic agonist carbachol on these cells. It appears justified to assume that the effects of atropine and NMS were mediated by muscarinic receptors because they were not present in wild-type CHO cells untransfected with the receptor gene and the EC_{50} values were close to those expected for the binding of atropine and NMS to muscarinic receptors [17,20].

On the other hand, the effects of QNB and AF-DX 116 varied between cell types and receptor subtypes. Differences in the effects of different receptor antagonists on receptor activity have been noted in the work with δ opioid receptors [7] and in subsequent studies on other types of G protein coupled recep-

Table 2
Quantitative parameters of the action of muscarinic antagonists on the synthesis of [^{3}H]inositol phosphates in CHO cells expressing the human m1 or m3 gene or the rat m1 gene at two different levels of expression

Ligand	pEC_{50}	E_{max} (% change)	*nH*	pEC_{50}	E_{max} (% change)	*nH*
	CHO cells, hm1 gene			**CHO cells, hm3 gene**		
NMS		lack of fit		9.82 ± 0.56	−19 ± 1	0.88 ± 0.01
Atropine	8.58 ± 0.17	−14 ± 2	1.07 ± 0.02	9.49 ± 0.17	−14 ± 0	1.20 ± 0.03
R(−)-QNB		no effect			no effect	
AF-DX 116	6.16 ± 0.09	−24 ± 2	0.99 ± 0.01		no effect	
	CHO cells, rm1 gene, high expression			**CHO cells, rm1 gene, low expression**		
NMS	8.77 ± 0.19	−15 ± 2	1.11 ± 0.00	8.69 ± 0.09	−6 ± 1	1.11 ± 0.03
Atropine	8.50 ± 0.22	−13 ± 1	1.13 ± 0.02	8.21 ± 0.14	−8 ± 1	0.78 ± 0.02
R(−)-QNB	9.53 ± 0.16	−16 ± 1	0.92 ± 0.03		no effect	
AF-DX 116	6.33 ± 0.11	−23 ± 2	1.01 ± 0.02	6.14 ± 0.08	−9 ± 1	1.19 ± 0.02

Values in the Table have been computed from the results of experiments shown in Fig. 2; they are weighted means ±S.E.M. of three experiments with incubations in triplicates. $pEC_{50} = -\log_{10}$ of the concentration of antagonist producing one half of the maximum effect; E_{max} = maximum effect expressed as% of control value, with stimulation denoted by + and inhibition by − signs; *nH* = Hill slope factor.

tors [10,11,13,14]. It has been predicted that the same antagonist may have different effects on the same receptor subtype depending on the magnitude of the allosteric constant describing the equilibrium between the active and inactive receptors in a given cell population and on the stoichiometric ratio of receptors to G proteins [26]. The difference between the stoichiometric ratios of rM_1 receptors and G proteins in cells with the high and the low receptor expression was probably responsible for the observed differences in the magnitude of the effects of atropine, NMS, AF-DX 116 and QNB on the accumulation of inositol phosphates in these two kinds of cells.

Two main conclusions may be drawn from the present data. (a) Muscarinic receptors of the M_1–M_4 subtypes expressed in genetically engineered CHO cells, and also native muscarinic receptors of the M_2 subtype expressed in cardiomyocytes in culture are constitutively active and their activity can be revealed by the muscarinic antagonists atropine and NMS and, less systematically, QNB and AF-DX 116. Constitutive activity of the receptors maintains slight inhibition of the synthesis of cyclic AMP by the M_2 and M_4 receptor subtypes and slight stimulation of phosphoinositide hydrolysis by the M_1 and M_3 receptor subtypes in the absence of a receptor agonist. (b) Muscarinic antagonists differ in their effect on the constitutive activity of muscarinic receptors. While atropine and NMS behaved as negative antagonists on all cell types and receptor subtypes applied in the present study, QNB and AF-DX 116 behaved as neutral antagonists in some and as negative antagonists in other models.

The degree of the spontaneous activity of muscarinic receptors is likely to depend on many factors and its extent and significance in the various types of cells of multicellular organisms in vivo are difficult to predict and will have to be determined by direct measurements. Apparently, the constitutive activity of muscarinic receptors has to be taken into account in explanations of metabolic and functional events in cells and muscarinic antagonists have to be evaluated not only with regard to their potency and subtype selectivity, but also with regard to their influence on the equilibrium between the receptors in the active and inactive conformations.

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