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Dual effects of muscarinic M_2 acetylcholine receptors on the synthesis of cyclic AMP in CHO cells: dependence on time, receptor density and receptor agonists

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1 Muscarinic M_2 receptors normally inhibit the production of cyclic AMP *via* G_i proteins, but a stimulatory component occurs in their effect at high agonist concentrations, believed to be based on the activation of G_s proteins. We investigated the conditions which determine the occurrence and extent of the stimulatory component in CHO cells stably expressing muscarinic M_2 receptors.

2 Biphasic concentration-response curves (decline followed by return towards control values) were obtained after 10 min incubation with carbachol, oxotremorine-M, acetylcholine, arecoline and arecaidine propargyl ester, but the upward phase was missing with oxotremorine, methylfurmethide, furmethide and pentylthio-TZTP. Shortening the incubation favoured the occurrence of the stimulatory component. Carbachol (1 mM) and oxotremorine-M (1 mM) brought about net stimulation (above 100% of control) of cyclic AMP synthesis during 2 min incubations. The stimulatory components disappeared after the density of receptors had been lowered with oxyphenonium mustard.

3 All agonists stimulated the synthesis of cyclic AMP in cells pretreated with pertussis toxin.

4 Most differences between agonists regarding the stimulatory component of their effect on cyclic AMP synthesis could be explained by differences in their efficacy and the induced receptor internalization.

5 We propose that the G_s-mediated stimulatory component of the effect of muscarinic M_2 receptors on cyclic AMP synthesis only occurs if the density of activated receptors is high enough to saturate the G_i proteins and proportionate to the receptors' low affinity for the G_s proteins. It tends to be abolished by receptor internalization.

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Abbreviations: CHO-M_2 cells, Chinese hamster ovary cells stably expressing the human gene for muscarinic receptors of the M_2 subtype; $EC_{50\text{-}I}$, EC_{50} for inhibitory effect; $EC_{50\text{-}S}$, EC_{50} for stimulatory effect; [^{3}H]-NMS, *[3H-methyl]*-N-methylscopolamine

Introduction

Muscarinic M_2 acetylcholine receptors are usually coupled with the G_i proteins and have an inhibitory effect on the activity of adenylyl cyclase (Caulfield, 1993). However, we have found previously that the muscarinic agonist, carbachol, had both inhibitory and stimulatory effects on the synthesis of cyclic AMP in Chinese hamster ovary (CHO) cells stably transfected with muscarinic M_2 or M_4 receptors (Jakubík *et al*., 1996). Although carbachol inhibited the synthesis of cyclic AMP at low concentrations, its effect diminished at high concentrations (yielding an upward inflection of the concentration-response curve), and became stimulatory after the cells had been pretreated with pertussis toxin. Similar dual effects of agonists acting on receptors known to couple preferentially with the G_i proteins have been noted in other studies of muscarinic receptors (Jones *et al*., 1991; Migeon & Nathanson, 1994; Vogel *et al*., 1995), α_2-adrenoceptors (Fraser *et al*., 1989; Eason *et al*., 1992; Pepperl & Regan, 1993; Eason & Liggett, 1995; Nasman *et al*., 1997; Sautel & Milligan, 1998) and somatostatin sst_5 receptors (Carruthers *et al*., 1999). Apparently, both muscarinic receptors (Dittman *et al*., 1994) and α_2-adrenoceptors (Eason *et al*., 1994) can stimulate adenylyl cyclase *via* the G_s protein. Conditions which determine the occurrence of the stimulation of cyclic AMP synthesis by muscarinic M_2 and M_4 receptors have not been fully investigated.

We describe here experiments in which we monitored the way in which the synthesis of cyclic AMP in CHO cells stably expressing muscarinic M_2 receptors is affected by several muscarinic agonists and also the effect of the duration of incubation, the density of receptors in cell membranes and the functioning of the G_i proteins on agonist responses. Our observations can be accommodated within a scheme presuming that the M_2 muscarinic receptors can simultaneously activate both the G_i and the G_s proteins

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when the density of the activated receptors is sufficiently high to be commensurate with their low affinity for the G_s proteins. An abstract of preliminary data has been published (Michal *et al*., 1999a).

Methods

Cells

Experiments were performed on CHO cells stably transfected with the human gene for muscarinic M_2 receptor subtype (CHO-M_2 cells; Buckley *et al*., 1989; Jakubík *et al*., 1995). They were grown in DMEM with 10% foetal calf serum and 0.005% geneticin, harvested after 5 days in culture and treated as described (Jakubík *et al*., 1997; Michal *et al*., 1999b). They were washed twice through centrifugation in the incubation medium consisting of (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.3, $NaHCO_3$ 25 and glucose 1.2 and saturated with 95% O_2/5% CO_2. In some experiments, cells were exposed to pertussis toxin (0.1 μg ml^{-1}) or cholera toxin (1 μg ml^{-1}) during the last 24 h in culture (before harvesting). Foetal calf serum was omitted from the culture medium in some of the experiments with cholera toxin (during the period of exposure to cholera toxin), but no difference was apparent between the effects of the toxin in the presence or the absence of foetal calf serum.

Measurement of the synthesis of cyclic AMP

CHO-M_2 cells were loaded with [^{3}H]-adenine during 60 min preincubation at 37°C (1×10^7 cells in 1 ml of the incubation medium containing 10 μCi [^{3}H]-adenine), sedimented and washed twice by resuspension and recentrifugation. Cells were then suspended in the incubation medium containing 1 mM isobutylmethylxanthine, and after 15 min, distributed into individual incubation tubes (1 million cells per tube) containing forskolin (25 μM) ± the tested muscarinic agonist. Final incubation volume was 0.8 ml and the incubation was at 37°C and lasted for 0.5–20 min. The reaction was stopped by the addition of 0.05 ml of 2.5 M HCl and of tracer amount (1500 d.p.m.) of cyclic [^{14}C]-AMP. Cyclic AMP was isolated by single-step chromatography (Johnson *et al*., 1994; Jakubík *et al*., 1996) on acid alumina. The sample was poured on dry alumina (1.5 g in 0.8×10 cm column), the tube was washed with 1 ml water and elution was performed with 7 ml water, followed by 2 ml 0.2 M ammonium acetate and an additional 3 ml 0.2 M ammonium acetate. Cyclic [^{3}H]-AMP and cyclic [^{14}C]-AMP were found in the last 3 ml of the eluate and their radioactivities were measured by liquid scintillation spectrometry. The amount of cyclic [^{3}H]-AMP found in the eluate was corrected for losses occurring during chromatography, as indicated by the recovery of cyclic [^{14}C]-AMP (80–90%). To determine the amount of cyclic [^{3}H]-AMP that had been synthesized during a certain incubation period, the amount of cyclic [^{3}H]-AMP present in the cell suspension before it was mixed with forskolin (± the agonist) (i.e., the content of cyclic [^{3}H]-AMP at time zero) was subtracted from the amount of cyclic [^{3}H]-AMP discovered at the end of the incubation.

Determination of the density of muscarinic binding sites

The density of muscarinic binding sites was determined according to the binding of [^{3}H]-NMS either at a single high concentration of the radioligand (2 nM, i.e. about 10 times the K_D), or by performing saturation binding experiments with [^{3}H]-NMS. Suspended cells (3×10^5 per tube) or membranes (corresponding to 10^6 cells per tube) were incubated for 1 h at 37°C with [^{3}H]-NMS at six different concentrations (25–800 pM), and the bound radioactivity was trapped by rapid filtration in a Brandel cell harvester on Whatman GF/B glass fibre filters presoaked in 0.3% polyethylenimine. Atropine (5 μM) was used to measure non-specific binding of [^{3}H]-NMS. All incubations were performed in triplicate.

Determination of the affinity of agonists for their binding sites

This was performed by evaluating the ability of agonists to compete with [^{3}H]-NMS for binding to intact CHO-M_2 cells, or to membranes prepared from these cells as described previously (Michal *et al*., 1999b). The procedure of the binding assay was similar to that used for the determination of the density of muscarinic binding sites. Suspended cells (3×10^5 per tube) or membranes (corresponding to 10^6 cells per tube) were incubated for 2 h or 1 h respectively, at 37°C in the presence of 360 pM [^{3}H]-NMS and of various concentrations of the agonists. Bound radioactivity was separated by rapid filtration on Whatman GF/B glass fibre filters and measured by scintillation counting.

Reduction of the density of muscarinic binding sites by their covalent modification

Oxyphenonium mustard was kept in a stock solution in methanol (1.6×10^{-3} M). In order to become activated, it was diluted in the incubation medium to a concentration of 4×10^{-8} M and kept at room temperature for 60 min. Suspended CHO-M_2 cells were then exposed to different concentrations of the activated mustard for 15 min at 37°C after which the mustard was inactivated with sodium thiosulphate (1 mM final concentration). Sodium thiosulphate was added also to control cells. Suitable concentrations of the mustard were found in preliminary experiments and actual receptor densities were determined in each experiment using the single point (2 nM [^{3}H]-NMS) method.

Loss of muscarinic binding sites during exposure to agonists

CHO-M_2 cells were seeded in 3.8 cm^2 plastic wells (10^6 cells per well) and grown in DMEM with 10% foetal calf serum and 0.005% geneticin at 37°C. After 2 days in culture, agonists were added to fresh medium and cells were incubated for 2–30 min in their presence. They were then washed three times with ice-cold saline, and incubated on ice for 4 h in a medium containing 1.9 nM [^{3}H]-NMS, and (mM) NaCl 112, KCl 3, $CaCl_2$ 3, $MgSO_4$ 2, NaH_2PO_4 1, Na-HEPES 25 (pH 7.4), and glucose 6. Then the medium was

aspirated, the cultures were washed three times with ice-cold saline, the cells were released and solubilized with 0.3 ml of 1% Triton X-100 and 0.2 ml of 0.2% trypsin, and the radioactivity that had been bound to the cells was measured by liquid scintillation spectrometry.

Data analysis

B_{max}, K_D, IC_{50} and EC_{50} values were evaluated by non-linear regression with the use of the GraphPad (San Diego, CA, U.S.A.) Prism programme. The equation proposed by Cheng & Prusoff (1973) was used to compute K_i from IC_{50} values. The following equation was fitted to data demonstrating the biphasic effects of muscarinic agonists on the synthesis of cyclic AMP:

$$Y = 100 \times (1 - \text{Depression}) \times (1 + \text{Stimulation}), \quad (1)$$

where Y = observed rate of synthesis in the presence of a given concentration of the agonist, expressed as per cent of synthesis in its absence, and:

$$\text{Depression} = \frac{\frac{100-MDA}{100} \times 10^X}{10^X + 10^{\log IC_{50}}} \quad (2)$$

whereas

$$\text{Stimulation} = \frac{\frac{MSA-MDA}{MDA} \times 10^X}{10^X + 10^{\log IC_{50}}} \quad (3)$$

where X = log of the concentration of the agonist (M), and MDA and MSA are the rates of synthesis at maximum depression and maximum stimulation, respectively (expressed as per cent of control synthesis in the absence of the agonist).

Reagents

[^{3}H]-NMS ([*^{3}H-methyl*]-N-methylscopolamine, 79 Ci mmol^{-1}), [8-^{3}H]-adenine (23 Ci mmol^{-1}) and cyclic [adenine-U-^{14}C]-AMP (240 Ci mol^{-1}) were from Amersham plc (Little Chalfont, U.K.). Oxyphenonium mustard (methylchlorethyl-aminohydroxyethyl ester of α-phenylcyclohexylglycolic acid) was kindly provided by Dr S. Shelkovnikov (St. Petersburg), methylfurmethide and furmethide by Dr J. Bajgar (Hradec Králové), and pentylthio-TZTP (a thio-analogue of xanomeline) by Dr P. Sauerberg (Copenhagen). Acetylcholine, carbachol, oxotremorine, arecoline, arecaidine propargyl ester, forskolin, isobutylmethylxanthine, acid alumina (type WA-1), Dulbecco's modified Eagle's medium (DMEM), and toxins of Bordetella pertussis (pertussis toxin) and of Vibrio cholerae (cholera toxin) were from Sigma-Aldrich s.r.o. (Prague), and oxotremorine-M was from RBI (Natick, MA, U.S.A.).

Results

Inhibition of [^{3}H]-NMS binding to CHO-M_2 cells and membranes by muscarinic agonists

The density of [^{3}H]-NMS binding sites, as revealed from saturation binding experiments on suspended cells, corresponded to $(692 \pm 33) \times 10^8$ sites per million cells, with the mean K_D value for [^{3}H]-NMS binding equal to 170 ± 10 pM. On CHO-M_2 membranes, the number of [^{3}H]-NMS binding sites recovered from 1 million cells was $(76 \pm 8.3) \times 10^8$ with a mean K_D value of 191 ± 12 pM. The affinities for the binding of the agonists carbachol, oxotremorine-M, oxotremorine and metylfurmethide were determined in competition binding experiments in suspended cells and isolated membranes. Curves describing the inhibition of [^{3}H]-NMS binding by the tested agonists were steep and corresponded to the one-site model on whole cells, but those obtained using isolated membranes were better described by the two-site model. Binding parameters computed by non-linear regression are summarized in Table 1. [^{3}H]-NMS binding was completely suppressed by carbachol, oxotremorine, oxotremorine-M and methylfurmethide at 1 mM concentrations both in whole cells and membranes.

Effects of agonists on cyclic AMP synthesis during incubations lasting 10 min

Initial experiments designed to characterize the effect of increasing concentrations of agonists on the synthesis of cyclic AMP were performed using incubations lasting 10 min. All agonists tested in these experiments (carbachol, oxotremorine-M, oxotremorine, methylfurmethide, acetylcholine, arecoline, arecaidine propargyl ester, furmethide and pentylthio-TZTP) brought about an inhibition of the synthesis of cyclic AMP in control CHO-M_2 cells (Figures 1 and 3) and stimulated the synthesis in cells that had been pretreated with PTX (Figures 2 and 3). While the inhibitory effects of increasing concentrations of oxotremorine, methylfurmethide, furmethide and pentylthio-TZTP were unimodal after 10 min incubations, those of the other agonists were bimodal and the degree of inhibition diminished when they were present at high concentrations.

Table 1 Summary of data on the inhibition of [^{3}H]-NMS binding to whole CHO-M_2 cells and to CHO-M_2 cell membranes by muscarinic agonists

	Whole cells		*Membranes*				
	pK_i	n_H	n_H	$pK_{i\text{-}high}$	$pK_{i\text{-}low}$	$K_{i\text{-}low}/K_{i\text{-}high}$	f_{high}
Carbachol	5.37 ± 0.017	0.94 ± 0.027	0.46 ± 0.04	7.90 ± 0.14	5.42 ± 0.05	302	0.24 ± 0.02
Oxotremorine-M	5.93 ± 0.033	0.92 ± 0.061	0.46 ± 0.03	8.18 ± 0.13	5.94 ± 0.06	174	0.33 ± 0.02
Oxotremorine	5.78 ± 0.028	0.93 ± 0.053	0.56 ± 0.03	8.34 ± 0.12	6.45 ± 0.05	78	0.31 ± 0.03
Methylfurmethide	5.23 ± 0.014	0.94 ± 0.024	0.59 ± 0.04	7.66 ± 0.13	5.62 ± 0.04	110	0.22 ± 0.02

Data are means ± s.e.mean of three experiments performed with incubations in triplicates. In experiments on isolated membranes, the fit was significantly better on the assumption that the agonists bound to two populations of binding sites. The computed proportion of sites with a high affinity for the agonists has been denoted as f_{high}.

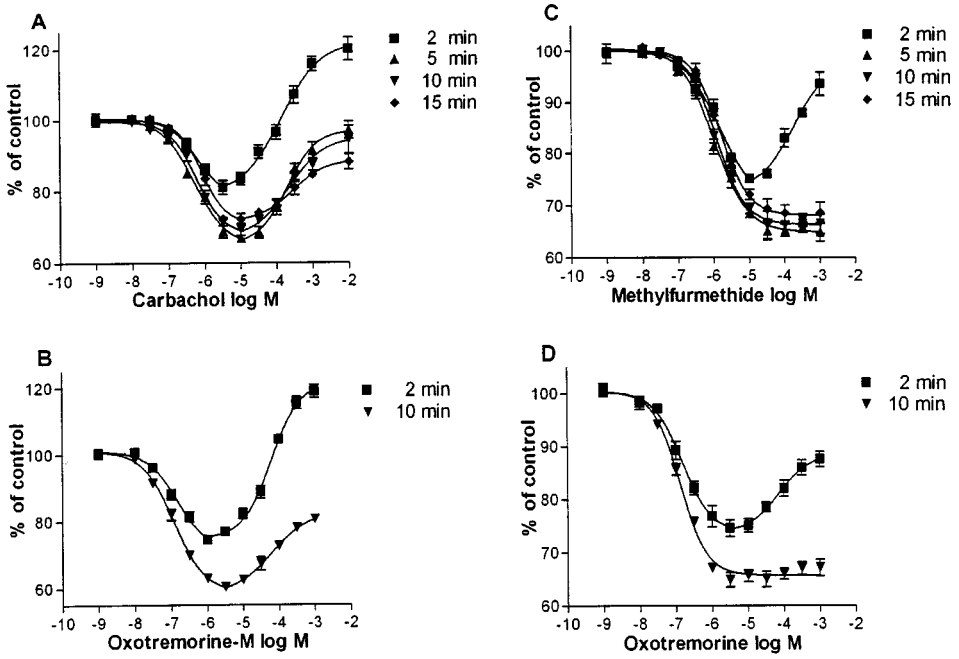

Figure 1 Effects of carbachol (A), oxotremorine-M (B), methylfurmethide (C) and oxotremorine (D) on forskolin-stimulated synthesis of cyclic AMP in CHO-M_2 cells during incubations lasting 2–15 min. Abscissa: log of the concentration (M) of the agonist. Ordinate: cyclic AMP synthesis in the presence of the agonist, expressed as per cent of the synthesis in its absence. Each point represents the mean (±s.e.mean) of three experiments, with incubations performed in triplicates.

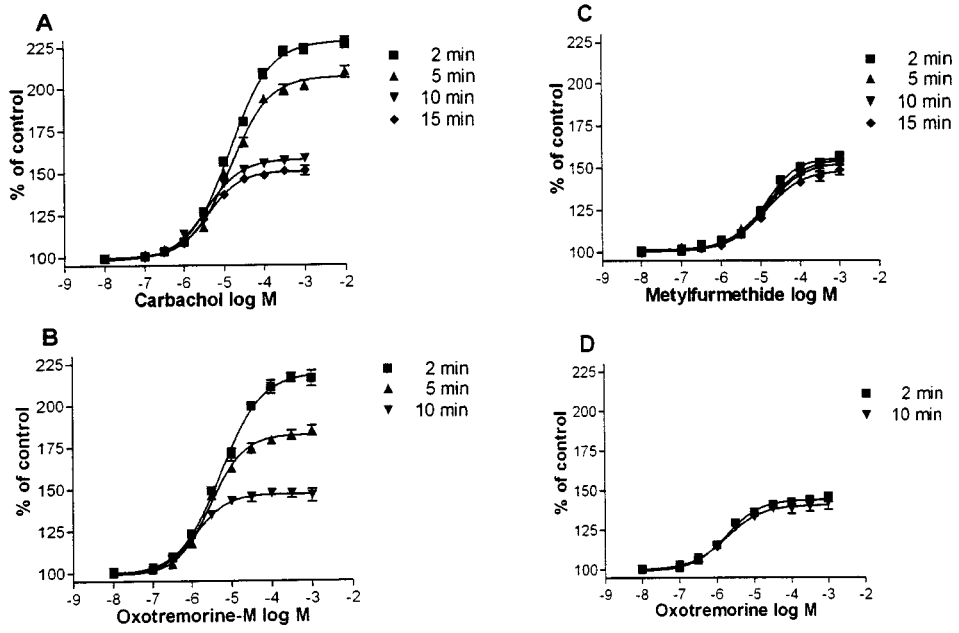

Figure 2 Effects of carbachol (A), oxotremorine-M (B), methylfurmethide (C) and oxotremorine (D) on forskolin-stimulated synthesis of cyclic AMP in CHO-M_2 cells that had been pretreated with pertussis toxin (0.1 μg ml^{-1} for 24 h) during incubations lasting 2–15 min. Abscissa: log of the concentration (M) of the agonist. Ordinate: cyclic AMP synthesis in the presence of the agonist, expressed as per cent of the synthesis in its absence. Each point represents the mean (±s.e.mean) of three experiments, with incubations performed in triplicates.

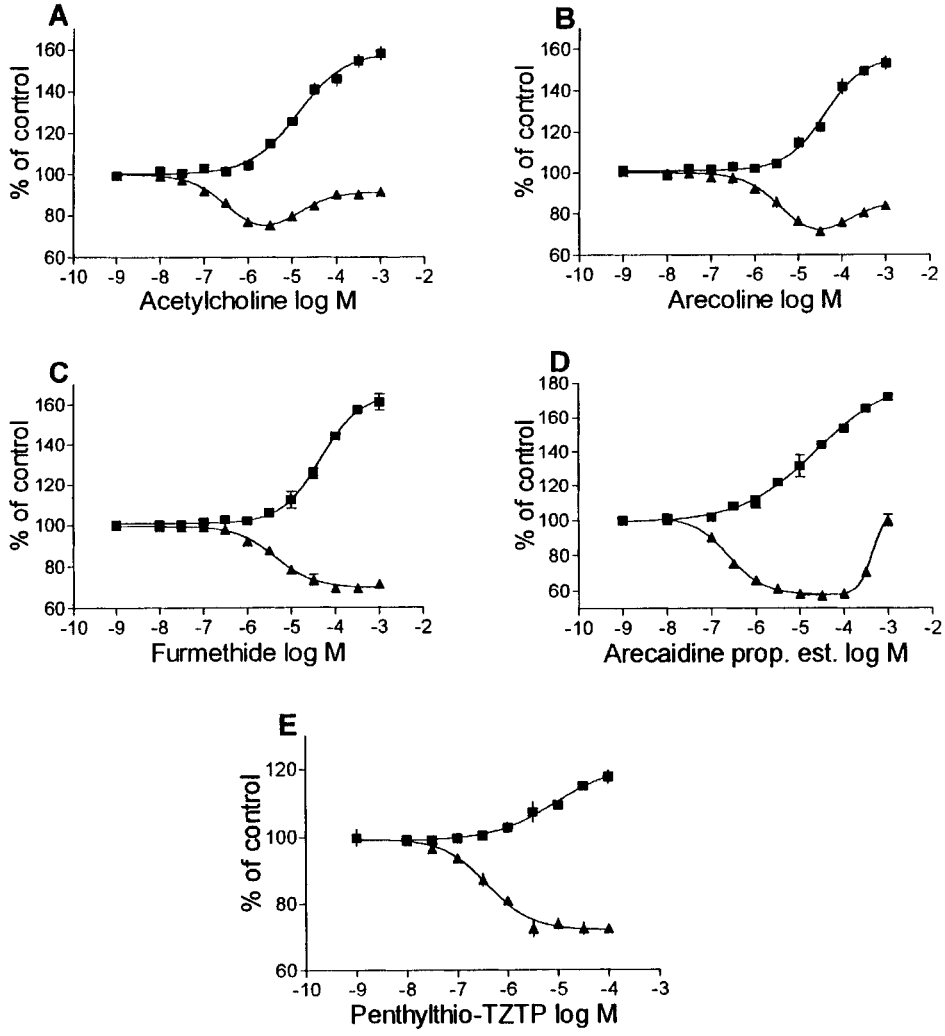

Figure 3 Effects of acetylcholine (A), arecoline (B), furmethide (C), arecaidine propargyl ester (D) and pentylthio-TZTP (E) on forskolin-stimulated synthesis of cyclic AMP in CHO-M_2 cells during incubations lasting 10 min. The cells either had not been treated with pertussis toxin (triangles) or had been preincuated for 24 h with 0.1 μg ml^{-1} pertussis toxin (squares). Abscissa: log of the concentration (M) of the agonist. Ordinate: cyclic AMP synthesis in the presence of the agonist, expressed as per cent of the synthesis in its absence. Each point represents the mean ($\pm$s.e.mean) of two experiments, with incubations performed in triplicates.

Effects of agonists on cyclic AMP synthesis during incubations lasting 2, 5 and 15 min

Four agonists have been chosen for closer examination: carbachol, oxotremorine-M, oxotremorine and methylfurmethide (Figures 1 and 2). Compared to data obtained after 10 min incubations with these agonists, the results obtained with incubations lasting 2 min differed in several respects: (a) at high concentrations (0.3 and 1.0 mM), carbachol and oxotremorine-M caused a true stimulation (increase above 100% of control) of cyclic AMP synthesis in cells not treated with pertussis toxin (Figure 1A,B), (b) the 'bimodality' of the inhibitory action (less inhibition at high agonist concentrations) was also seen with methylfurmethide and oxotremorine (Figure 1C,D), (c) carbachol and oxotremorine-M had much stronger stimulatory effects on the synthesis of cyclic AMP in pertussis toxin-treated cells (increased by 125%; Figure 2A,B) than oxotremorine and methylfurmethide (increased by 50%; Figure 2C,D).

Concentration-response curves for carbachol and methylfurmethide obtained on control cells after incubations lasting 5 or 15 min were closely similar to those obtained after 10-min incubations (Figure 1). On cells pretreated with pertussis toxin, the stimulation of cyclic AMP synthesis by high concentrations of carbachol and oxotremorine-M diminished progressively between incubations lasting 2 and 10 min, whereas the effects of methylfurmethide and oxotremorine were not significantly affected by changes in the duration of incubation within the same time range (Figure 2). Values of maximum observed inhibition and stimulation of cyclic AMP synthesis and computed values of pEC_{50} for the inhibition and the stimulation of cyclic AMP

Table 2 Effects of muscarinic agonists on the synthesis of cyclic AMP in control CHO-M_2 cells and in CHO-M_2 cells treated with pertussin toxin (PTX)

		Control cells				*PTX-treated cells*	
Agonist	*Duration of incubation (min)*	$pEC_{50\text{-}I}$	$pEC_{50\text{-}S}$	$EC_{50\text{-}S}/EC_{50\text{-}I}$	*Max. obs. inibition (%)*	$pEC_{50\text{-}S}$	*Max. obs stimulation (%)*
Carbachol	2	6.29±0.127	3.90±0.082	245	22±1	4.84±0.036	128±2
	5	6.25±0.051	3.76±0.071	309	35±2	4.84±0.054	107±2
	10	6.22±0.060	3.75±0.097	295	32±2	5.44±0.041	58±1
	15	6.02±0.087	3.89±0.177	135	29±1	5.40±0.024	51±1
Oxotremorine-M	2	6.84±0.095	4.26±0.074	380	29±4	5.27±0.041	119±3
	10	6.87±0.031	4.30±0.076	372	41±1	5.89±0.020	47±1
Oxotremorine	2	6.76±0.040	4.24±0.097	331	26±2	5.74±0.045	44±1
	10	6.89±0.050	–	–	34±1	5.74±0.093	41±1
Methylfurmethide	2	5.86±0.075	3.94±0.137	83	30±4	4.90±0.043	56±2
	5	5.99±0.047	–	–	36±1	4.88±0.071	55±2
	10	5.93±0.035	–	–	34±1	4.85±0.042	53±2
	15	5.81±0.027	–	–	32±1	4.90±0.064	49±2
Acetylcholine	10	6.45±0.067	4.94±0.106	32	27±2	4.87±0.068	59±5
Arecoline	10	5.38±0.074	3.94±0.178	28	33±3	4.34±0.072	56±5
Furmethide	10	5.38±0.056	–	–	30±2	4.32±0.032	65±3
Arecaidine propargyl ester	10	6.53±0.051	+	+	42±4	4.61±0.108	83±8
Pentylthio-TZTP	10	6.41±0.076	–	–	27±3	5.05±0.173	21±5

Data for carbachol, oxotremorine-M, oxotremorine, methylfurmethide and furmethide are means±s.e.mean of three experiments with incubations performed in triplicates. Data for acetylcholine, arecoline, arecaidine propargyl ester and pentylthio-TZTP are means±s.e.mean of two experiments with incubations performed in triplicates. $pEC_{50\text{-}I}$ and $pEC_{50\text{-}S}$ values relate to the inhibitory (descending) and stimulatory (ascending) phases of concentration-response curves, respectively. Values of maximum inhibition and stimulation indicated in the table are those that were directly observed. On control cells, maximum stimulation corresponds to the difference between the synthesis at maximum inhibition and at its maximum elevation at high agonist concentrations (calculated in per cent of control synthesis). –, denotes the absence of the stimulatory phase; +, indicates the stimulation was present but could not be evaluated because of atypical concentration-response relationship.

Table 3 Reductions of [^{3}H]-NMS binding sites induced by preincubations with oxyphenonium mustard

Mustard concentration (nM)	K_D *(pM, by saturation binding)*	B_{max} *(%, by saturation binding)*	B_{max} *(%, by single-point determination)*	*'Nominal' receptor density (%)*
0	170±10	100	100	100
0.5	164±9	48±3	52±4	50
1.0	177±7	32±2	28±3	30
10	161±7	9±2	11±3	10

CHO-M_2 cells were preincubated for 15 min with various concentrations of activated oxyphenonium mustard (see Methods) and the density of [^{3}H]-NMS binding sites was then determined in saturation binding experiments (yielding also the K_D value for [^{3}H]-NMS binding) or by meausring the binding of [^{3}H]-NMS at a single concentration of 2 nM. Data are means±s.e.mean of two experiments performed with incubation in triplicates. B_{max} determined by saturation binding in control cells corresponds to $692\times10^8\pm33\times10^8$ binding sites per 10^6 cells.

formation have been listed in Table 2. Also included in Table 2 are the values of $EC_{50\text{-}S}/EC_{50\text{-}I}$ ratios, which have been derived from the bimodal concentration-response curves and which indicate how many times higher agonist concentration was required using control cells to elicit the stimulation, as compared to the inhibition of cyclic AMP synthesis.

Time course of agonist effects on cyclic AMP synthesis

Differences between the concentration-response curves obtained after incubations of varying duration stimulated us to examine the time course of agonist action on cyclic AMP synthesis in more detail. As shown in Figure 4A, carbachol, oxotremorine, oxotremorine-M and methylfurmethide all inhibited the synthesis of cyclic AMP at all time intervals between 0.5 and 20 min when applied at 10 μM concentrations. When applied at 1 mM concentrations, however, carbachol and oxotremorine-M (but not oxotremorine and methylfurmethide) brought about stimulation of cyclic AMP synthesis during incubations lasting 0.5–3 min and inhibition during incubations lasting more than 5 min (Figure 4B). In cells that had been pretreated with pertussis toxin, the stimulation of cyclic AMP synthesis by 1 mM carbachol and 1 mM oxotremorine-M (but not that by methylfurmethide or oxotremorine) was

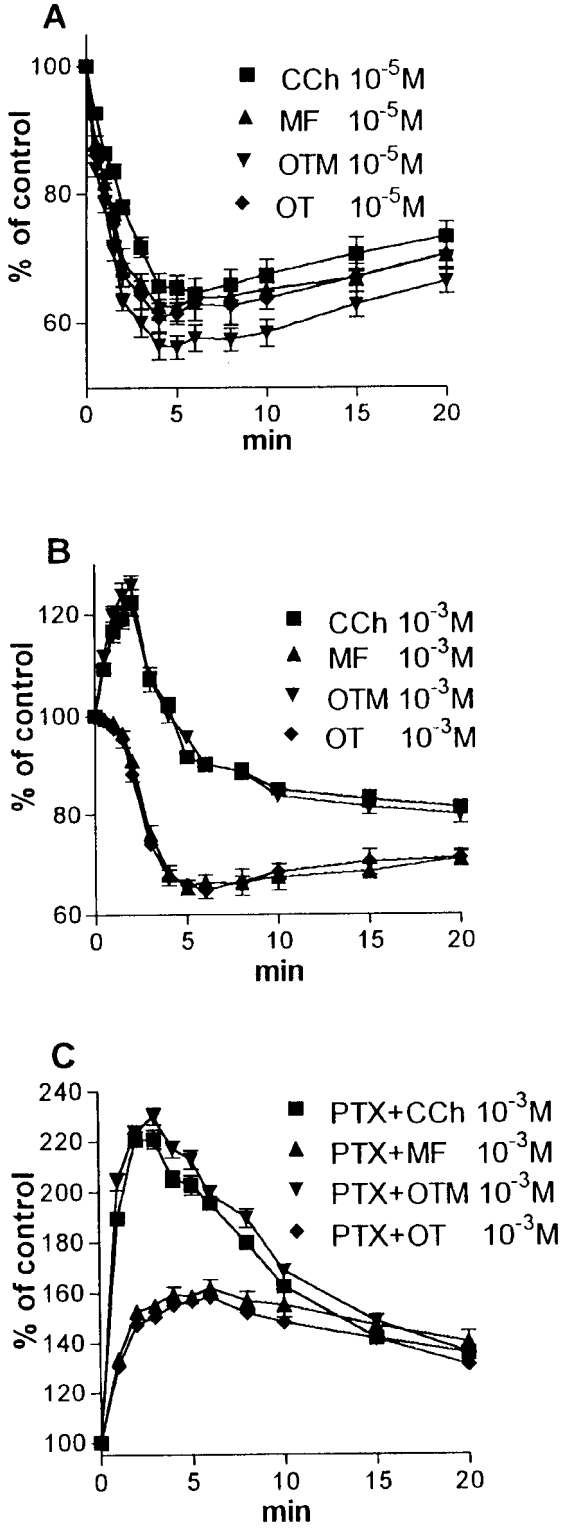

Figure 4 Time course of the effects of muscarinic agonists on forskolin-stimulated synthesis of cyclic AMP in control CHO-M_2 cells (A, B) and in CHO-M_2 cells pretreated with pertussis toxin (C; 0.1 μg ml^{-1} for 24 h). Four different agonists were applied at a concentration of 10^{-5} M (A) or 10^{-3} M (B, C) and the content of cyclic AMP in the cells was determined at time intervals of 0.5–20 min after the addition of the agonist. Abscissa: time after the addition of the agonist. Ordinate: cyclic AMP synthesis in the presence of the agonist, expressed as per cent of the synthesis in its absence. Data are means ± s.e.mean of six incubations, and similar results have been obtained in additional experiments.

much greater during short than during long incubations (Figure 4C).

Changes in the effects of carbachol and methylfurmethide induced by irreversible blockade of a proportion of muscarinic receptors by oxyphenonium mustard

Preincubations of CHO-M_2 cells with oxyphenonium mustard diminished the number of [^{3}H]-NMS binding sites without altering the K_D for [^{3}H]-NMS binding (Table 3). The conditions of inactivation were set so as to reduce receptor density close to 50, 30 and 10% of control ('nominal values' in Table 3). It is apparent that there was little difference between data on receptor densities obtained by the 'saturation binding' or 'single point' procedure.

Cells with reduced receptor densities have been applied to investigate changes in the effects of carbachol and methylfurmethide on cyclic AMP synthesis. With both carbachol and methylfurmethide, diminishing receptor density by 50% shifted the concentration-response curves to the right, without diminishing the maximum inhibition of cyclic AMP synthesis (Figure 5). When the density of muscarinic receptors was diminished by more than 50%, the maximum inhibitory effects (E_{max}) of both agonists became less. The stimulatory effects of carbachol on control cells (as revealed by the upward phase of the concentration-response curves at high agonist concentrations) was diminished in experiments with 2 min incubation (Figure 5A) and completely lost in experiments with 10 min incubation (Figure 5B) if the density of receptors had been lowered. The stimulatory phase of the methylfurmethide concentration-response curve observed in experiments with 2-min incubations also disappeared if the density of receptors had been lowered. The effect of reductions in the density of muscarinic receptors on the concentration-response curves for oxotremorine-M and oxotremorine were similar to those observed with carbachol and methylfurmethide, respectively (Figure 6).

In cells pretreated with pertussis toxin, the loss of receptors caused by oxyphenonium mustard diminished the maximum stimulation by both carbachol (Figure 7A,B) and methylfurmethide (Figure 7C,D).

Effect of carbachol on cells pretreated with cholera toxin

The synthesis of cyclic AMP was more than six times enhanced in cells that had been pretreated with cholera toxin. While the inhibitory action of carbachol was preserved in these cells, the second (stimulatory) phase of its concentration-response curves disappeared (Figure 8).

Loss of [^{3}H]-NMS binding sites during incubations with agonists

Changes in the number of cell surface [^{3}H]-NMS binding sites occurring during 2–30 min incubations with four muscarinic agonists, each at 1 mM concentration, are shown in Figure 9A. It is apparent that the number of cell surface [^{3}H]-NMS binding sites is substantially diminished during incubations with carbachol, oxotremorine-M and oxotremorine, but not with methylfurmethide. The concentration dependence of the effects of the agonists on the density of

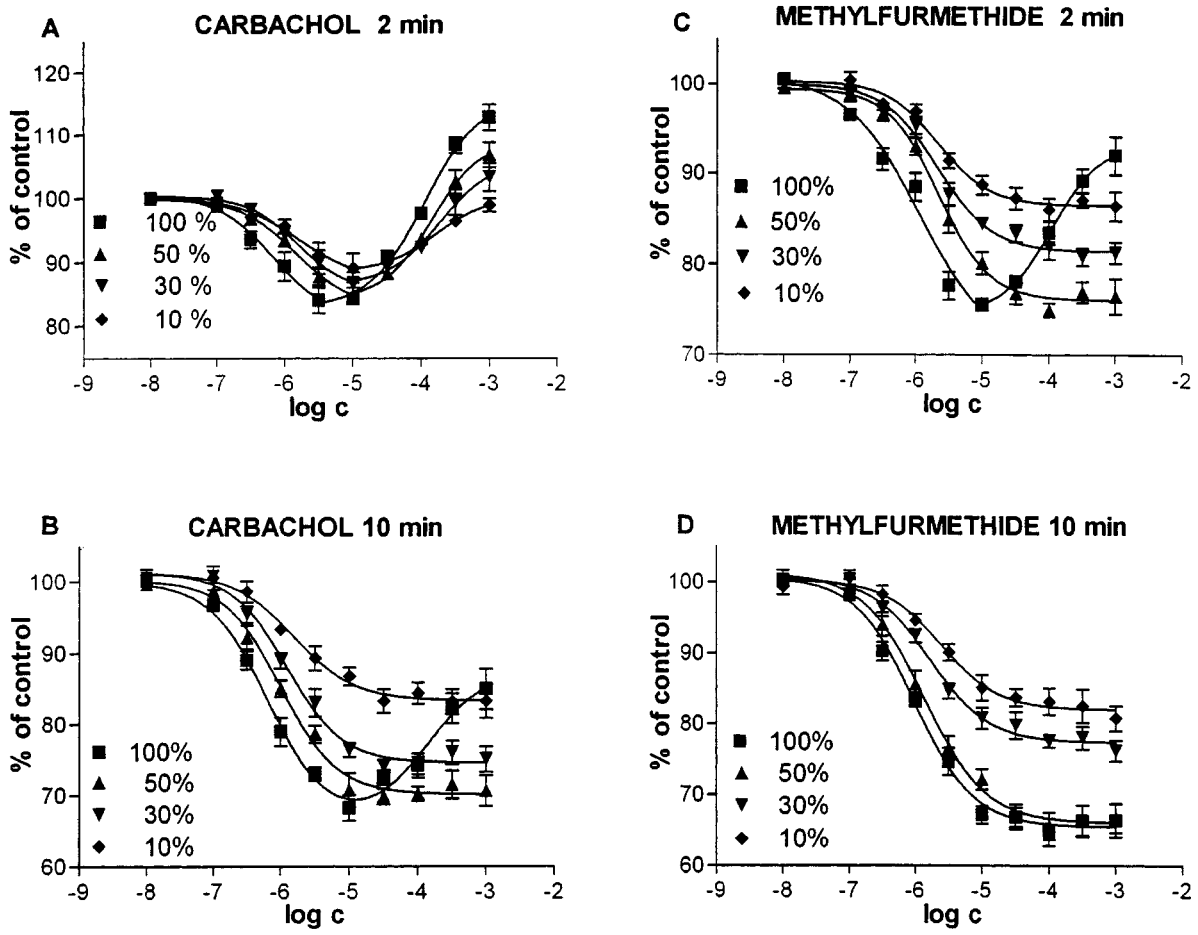

Figure 5 Variations in the effects of carbachol (A, B) and methylfurmethide (C, D) on forskolin-stimulated synthesis of cyclic AMP in CHO-M_2 cells, depending on the density of muscarinic receptors in cell surface membranes, with incubations lasting 2 min (A, C) or 10 min (B, D). Cells had been exposed to the covalent ligand oxyphenonium mustard so as to diminish the density of muscarinic receptors in their membranes close to 50, 30 or 10% of control (see 'nominal' receptor densities in Table 3). Abscissa: log of the concentration (M) of the agonist. Ordinate: cyclic AMP synthesis in the presence of the agonist, expressed as per cent of the synthesis in identically treated cells in the absence of the agonist. Data are means ± s.e.mean of two experiments with incubations performed in duplicates, and similar results have been obtained in additional experiments.

cell surface [^{3}H]-NMS binding sites has been tested during incubations lasting 5 min (Figure 9B); pEC_{50} values computed from data in Figure 9B were 3.92 for carbachol, 5.05 for oxotremorine-M, 3.89 for oxotremorine, and 2.91 for methylfurmethide.

Discussion

Our data confirm that muscarinic M_2 receptors are able both to inhibit and to stimulate the synthesis of cyclic AMP. The stimulation is greater in cells pretreated with pertussis toxin, which indicates that it does not depend on the G_i proteins, be it their α or $\beta+\gamma$ subunits. Earlier investigations of the adenylyl cyclase-stimulating effects of muscarinic M_2 and M_4 receptors and α_2 adrenoceptors (Dittman *et al.*, 1994; Eason *et al.*, 1994; Jakubík *et al.*, 1996) led to the conclusion that these effects are mediated by the G_s proteins. It seems unlikely that changes in the intracellular concentration of Ca^{2+} ions played a role in the phenomena we observed since the effects of muscarinic stimulation on $[Ca^{2+}]_i$ are negligible in the CHO-M_2 cells utilized in the present work (Doležal *et al.*, 1997). In addition, stimulation of adenylyl cyclase activity by muscarinic M_2 receptors could also be observed in experiments on isolated membranes (Vogel *et al.*, 1995). Muscarinic receptors of the M_1 subtype have been also found to be able to communicate with the G_s proteins instead of their usual partner, which is G_q (Burford & Nahorski, 1996).

The following observations seem of particular interest:

(i) The occurrence and extent of the stimulatory component in the action of muscarinic agonists diminished with the duration of incubation.

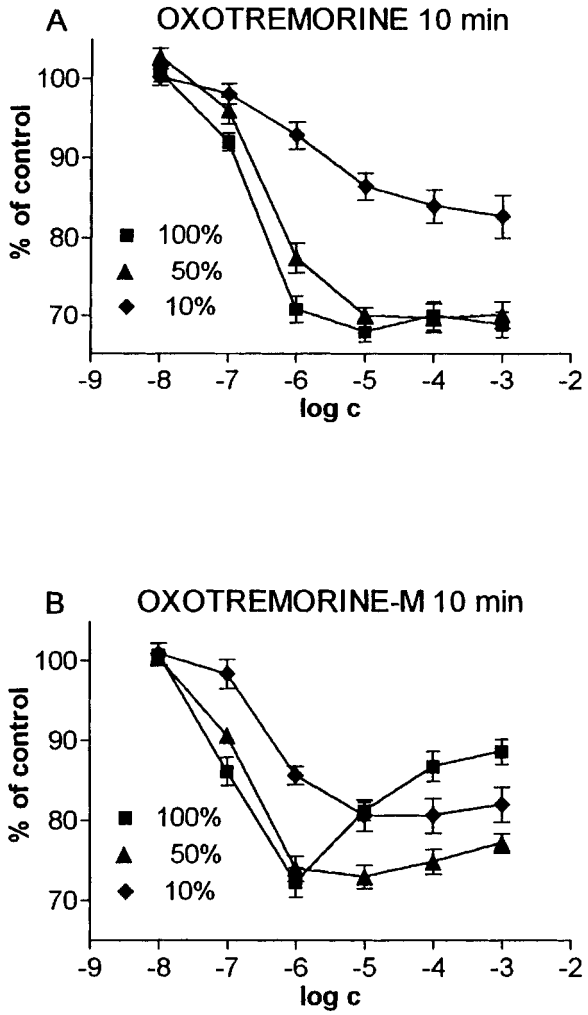

Figure 6 Effects of oxotremorine (A) and oxotremorine-M (B) on the synthesis of cyclic AMP during 10 min incubations of control CHO-M_2 cells (squares) and of cells in which the number of cell surface M_2 receptors had been reduced with oxyphenonium mustard close to 50% (triangles) or 10% (diamonds) (see 'nominal densities' in Table 3). Abscissa: log of the concentration (M) of the agonist. Ordinate: cyclic AMP synthesis in the presence of the agonist, expressed as per cent of the synthesis in identically treated cells in the absence of the agonist. Data have been obtained in a single experiment and are means ± s.e.mean of three incubations.

(ii) The four agonists which were more closely investigated (carbachol, oxotremorine-M, oxotremorine and methylfurmethide) differed in their ability to produce the stimulatory phase of the concentration-response curves, although they were all full agonists if evaluated by their ability to inhibit the synthesis of cyclic AMP.

(iii) Inactivation of a proportion of receptors by oxyphenonium mustard brought about a decrease or disappearance of the stimulatory component of cyclic AMP response.

(iv) The EC_{50} values for inducing the relative stimulation of the synthesis of cyclic AMP in cells not treated with pertussis toxin were substantially higher that the EC_{50} values for inducing the inhibition. Based on experiments with either 2 or 10 min incubation times, the difference was 224–251 fold for carbachol, 245–371 fold for oxotremorine-M, 347 fold for oxotremorine, but only 78 fold for methylfurmethide, 31 fold for acetylcholine and 23 fold for arecoline.

(v) All tested agonists stimulated the synthesis of cyclic AMP in cells pretreated with pertussis toxin. In the case of carbachol, oxotremorine-M, oxotremorine and methylfurmethide, the EC_{50} values for stimulation were at least 8 fold lower on pertussis toxin-treated cells as compared to control cells, but the difference was smaller in the case of arecoline and absent in the case of acetylcholine. The treatment with pertussis toxin is likely to increase the pool of activated receptors which are available for interaction with the G_s proteins by eliminating the possibility of their interaction with the G_i proteins.

Although the data we have do not directly reveal the mechanistic basis of the dual effects of M_2 muscarinic receptors on the synthesis of cyclic AMP, they all fit a single concept which may be described as follows. The activated M_2 receptor has a much higher affinity for the G_i than the G_s proteins (Table 2). The stimulatory effect of the M_2 receptor activation only occurs if the density of the M_2 receptors is sufficiently high (Figure 5) so that (a) the available G_i proteins become saturated with activated receptors and (b) the concentration of the activated M_2 receptors is commensurate with their low affinity for the G_s proteins. The occurrence of the stimulatory effect is facilitated by an inactivation of the G_i proteins with pertussis toxin (Figure 2). At high agonist concentrations and high receptor densities, both the G_i and G_s proteins become activated simultaneously. The stimulatory effect may diminish, outweigh or even surpass the inhibitory effect (Figures 1 and 4). The stimulatory effect is more affected by receptor desensitization, however, and diminishes or disappears faster than the inhibitory effect during prolonged receptor activation (Figure 4). It seems natural to anticipate that the stimulatory component will be more likely to occur in cells with a high ratio of G_s/G_i concentrations. The exact stoichiometric relations between the receptors, the G_i and G_s proteins and the effector enzyme (adenylyl cyclase) are difficult to characterize at present, however, because of uncertainties with regard to the compartmentalization of the components of signal transduction pathways in cells (Neubig, 1994; Strange, 1999; Milligan, 2000), the possibility of receptor-G protein precoupling and also in view of the catalytic nature of receptor and G protein actions.

Although all agonists displayed some stimulatory effects, differences between them were conspicuous. Comparing the four agonists which have been more closely tested, the stimulatory effects were much easier to obtain with carbachol and oxotremorine-M than with oxotremorine and methylfurmethide. Judging by the ability to inhibit the synthesis of cyclic AMP, carbachol, oxotremorine-M, oxotremorine and methylfurmethide were all full agonists, producing 34–41% maximum inhibition of cyclic AMP synthesis during 10 min incubations (Figure 1, Table 2). The maximum inhibitory effects of carbachol and methylfurmethide were not dimin-

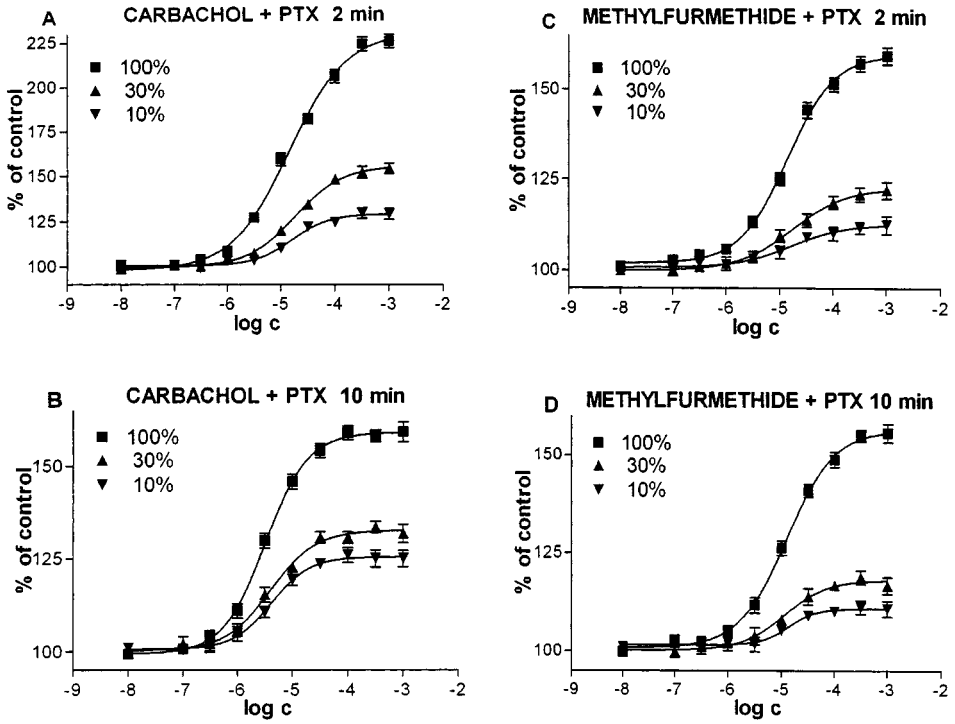

Figure 7 Variations in the effects of carbachol (A, B) and methylfurmethide (C, D) on forskolin-stimulated synthesis of cyclic AMP in CHO-M_2 cells that had been pretreated with pertussis toxin (0.1 μg ml^{-1} for 24 h), depending on the density of muscarinic receptors in cell surface membranes. Incubations lasted 2 min (A, C) or 10 min (B, D). Cells had been exposed to the covalent ligand oxyphenonium mustard so as to diminish the density of muscarinic receptors in their membranes close to 50, 30 or 10% of control (see 'nominal' receptor densities in Table 3). Abscissa: log of the concentration (M) of the agonist. Ordinate: the synthesis of cyclic AMP in the presence of the agonist, expressed as per cent of the synthesis in identically treated cells in the absence of the agonist. Data are means±s.e.mean of two experiments with incubations performed in duplicates.

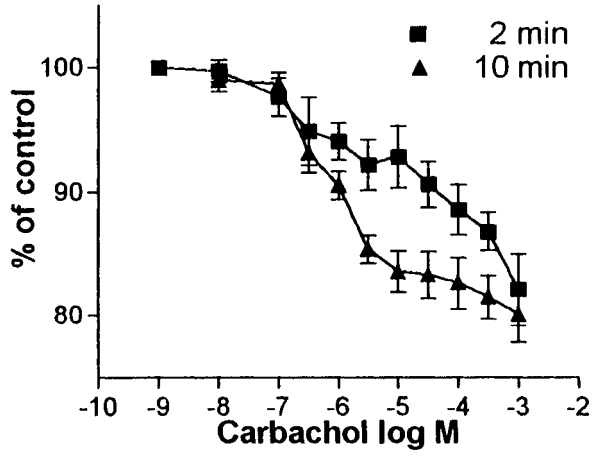

Figure 8 Effects of carbachol on forskolin-stimulated synthesis of cyclic AMP in control CHO-M_2 cells and in cells that had been pretreated with cholera toxin (1 μg ml^{-1} for 24 h). Abscissa: log of the concentration of carbachol (M). Ordinate: cyclic AMP synthesis in the presence of carbachol, expressed as per cent of the synthesis in its absence. The synthesis of cyclic AMP was more than 7 fold higher in cells pretreated with cholera toxin. Data are means±s.e.mean of three experiments with incubations performed in triplicates.

ished after an irreversible blockade of 50% of receptors, but both became smaller when the density of receptors was diminished by 70% (Figure 5). This indicates that, with regard to the inhibition of cyclic AMP synthesis, both carbachol and methylfurmethide acted with a 'receptor reserve' of >50%. Yet it is questionable whether the degree of the maximum inhibition alone is an appropriate indicator of agonist efficacy in a system in which inhibition may be accompanied and at least partly overshadowed by stimulation. A comparison of the ratios of $K_{i\text{-low}}/K_{i\text{-high}}$ values for agonists obtained in competition binding experiments on isolated membranes and listed in Table 1 suggests that the efficacies of carbachol and oxotremorine-M are higher than those of oxotremorine and methylfurmethide. Within the scheme outlined above, higher agonist efficacy is bound to facilitate the occurrence of the stimulatory effect.

Another factor likely to be responsible for differences in the observed agonist effects were differences in agonist-induced receptor internalization. Under the conditions of our experiments, the loss of receptors from the cell surface was fastest and most profound in the presence of oxotremorine, and slowest and least profound in the presence of methylfurmethide (Figure 9). Rapid internalization of receptors probably diminished the stimulatory component of the effect of oxotremorine.

A third factor which might cause differences between agonists would be the induction of agonist-specific receptor conformations, with different affinities for the G_i protein on the one hand and the G_s protein on the other. Evidence pointing to the existence of agonist-specific conformations has recently been presented with regard to muscarinic M_1 receptors (Gurwitz *et al.*, 1994), α_1- and β_2-adrenoceptors (Perez *et al.*, 1996; Krumins & Barber, 1997), and

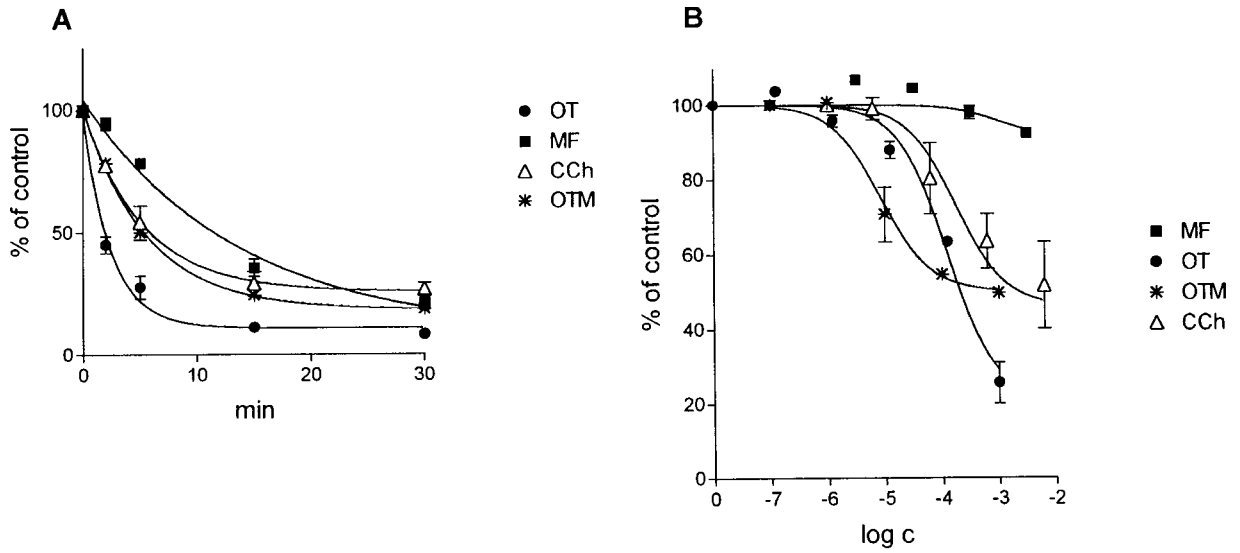

Figure 9 Loss of [^{3}H]-NMS binding sites on the surface of cells exposed to agonists. (A) Cells were incubated for 2–30 min at 37°C in the presence of methylfurmethide (MF), carbachol (CCh), oxotremorine-M (OTM) or oxotremorine (OT) at 1 mM concentrations, washed and further incubated for 4 h at 4°C with 2 nM [^{3}H]-NMS. (B) Cells were incubated for 5 min at 37°C in the presence of methylfurmethide, carbachol, oxotremorine M or oxotremorine at the concentrations indicated on the abscissa (log M). Then they were washed and further incubated for 4 h at 4°C with 2 nM [^{3}H]-NMS. Abscissa: [^{3}H]-NMS binding after the exposure to agonists, expressed as per cent of the binding to identically treated cells that had not been exposed to agonists. Data are means ± s.e.mean of three experiments (in A) or two experiments (in B), with incubations performed in triplicates.

cannabinoid receptors (Bonhaus *et al*., 1998). It would be expected that differences in the affinities of agonist-receptor complexes *vis-a-vis* G_s or G_i will be reflected by differences in the ratios of $EC_{50\text{-}S}/EC_{50\text{-}I}$. Data in Table 2 do not indicate any significant differences between carbachol, oxotremorine-M and oxotremorine but suggest that, in the case of acetylcholine, arecoline and methylfurmethide the gap between the concentrations required for the stimulation and the inhibition of the synthesis of cyclic AMP is smaller than in the case of carbachol, oxotremorine-M and oxotremorine. Thorough investigation of this aspect will be made on simpler models.

We have recently described evidence suggesting the presence of two tandemly arranged binding subsites on muscarinic receptors (Jakubík *et al*., 2000). It seems possible to exclude the possibility, however, that the stimulation of cyclic AMP synthesis by high concentrations of agonists as observed in the present work was a consequence of the binding of two agonist molecules to the same receptor. If that had been so, the stimulatory effect should persist after treatment with oxyphenonium mustard performed to reduce the density of functional receptors.

It will be important to find out if intense stimulation of muscarinic M_2 receptors proceeding under physiological or pathological conditions *in vivo* may also produce simultaneous inhibition and stimulation of the synthesis of cyclic AMP. It seems worth considering whether some of the unusual effects of muscarinic agonists on the heart (positive inotoropy – Imai & Ohta, 1982; Eglen *et al*., 1988; Kenakin & Boselli, 1990; Webb & Pappano, 1995) can be explained by an M_2 receptor-mediated enhancement of cyclic AMP production.

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