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Human muscarinic receptors expressed in A9L and CHO cells: activation by full and partial agonists

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1 A comparative study of receptor activation by ten full and partial muscarinic agonists was undertaken on the five subtypes of human muscarinic receptors expressed at similar receptor densities in Chinese hamster ovary (CHO-K1) cells. In addition, m_1, m_2 and m_3 receptors were expressed in mouse fibroblast A9L cells in order to compare the influences of cell type on agonist activation of these receptors.

2 Receptor-effector coupling efficiencies were greater in CHO than A9L cells and agonists displayed greater potencies and similar or greater intrinsic activities at $CHOm_1$ and $CHOm_3$ than $A9Lm_1$ and $A9Lm_3$ receptors. Although m_2 receptor density was 6 fold higher in A9L than CHO cells, carbachol elicited significantly greater inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation in $CHOm_2$ cells. These data suggest that not only receptor density but receptor-effector coupling and/or coupling efficiencies play significant roles in agonist-induced responses.

3 In CHO cells, receptor-effector coupling efficiencies were $m_3 = m_1 > m_5$. Although $CHOm_5$ receptors were the least efficiently coupled, some partial agonists displayed higher intrinsic efficacies at m_5 than m_3 receptors suggesting that, in CHO cells, m_5 and m_3 receptors may activate different G proteins and/or effectors to stimulate inositol monophosphate (IP1) formation.

4 McN-A-343 was a functionally selective m_4 agonist. It had little or no agonist activity at m_3 receptors expressed in either A9L or CHO cells. The slopes of McN-A-343 concentration-response curves in $CHOm_2$ cells were significantly lower than the slopes obtained with this compound in $CHOm_4$ cells suggesting that the mode of activation by McN-A-343 differed between the two muscarinic receptors negatively coupled to adenylyl cyclase.

5 Cloned receptors provide valuable tools for the study of agonist-receptor interaction and agonist-receptor activation but caution should be applied in assuming that the results are valid for all cell types or for tissue-expressed receptors.

Keywords: Recombinant muscarinic receptors; agonist relative efficacy; receptor-effector coupling efficiencies

Introduction

A great deal of effort has been expended in trying to find agonists that selectively activate subtypes of muscarinic receptors. Until recently, only tissues or cultured cells expressing endogenous receptors were available for comparing drug activities. Confounding factors included the necessity of using different tissues or cells as model systems for different receptor subtypes. Thus, differences in species, tissue preparations or cell culture conditions as well as the possible presence of multiple subtypes of receptors and/or effectors could obscure the interpretation of the results. More recently, there is evidence that a given subtype of receptor may activate different responses in different tissues, e.g. in olfactory bulb and striatum, putative m_4 receptors respectively activate (Olianas & Onali, 1991) or inhibit (McKinney *et al.*, 1991; Dokas & Ting, 1993) adenosine 3':5'-cyclic monophosphate (cyclic AMP) formation. In addition, in rat heart, where >92% of muscarinic receptors are of one type (Li *et al.*, 1991), there are multiple responses to muscarinic receptor activation, suggesting that in the same tissue one receptor may activate more than one type of G protein and/or effector (Kenakin & Boselli, 1990). Thus, in tissues, comparison of a series of agonists is confounded not only by the possible occurrence of multiple receptor subtypes but also by differences in receptor densities, the repertoire and relative levels of G proteins and/or isoforms of effectors available for activation by the liganded receptor. The question arises as to whether agonists can truly differentiate among receptor subtypes. This is of more than academic interest since selective muscarinic agonists may be therapeutically useful for the treatment of memory deficits in patients with Alzheimer's disease (see Caulfield, 1993).

It is now known that the amino acid sequences of the five subtypes of muscarinic receptors are highly similar especially in the transmembrane regions that form the ligand binding pocket (reviewed by Hulme *et al.*, 1990). This also suggests that agonists may not differentiate among muscarinic receptor subtypes but that receptor reserve or postreceptor-activation events determine the apparent selectivity observed.

Currently, there are cell lines with few or no endogenous muscarinic receptors that have been transfected with genes for one of the muscarinic receptor subtypes. These cells provide a means to control for differences in receptor densities and repertoires of G proteins and effectors. Chinese hamster ovary (CHO-K1) cells or mouse fibroblast (A9L) cells, transfected with human muscarinic receptor genes, were used in this study. The inhibition constants (K_i or IC_{50} values obtained from radioligand binding studies) of a series of agonists were determined for the five subtypes of receptors in CHO cells and for m_1 and m_3 receptors expressed in A9L cells. This same series of muscarinic agonists was used to compare receptor activation in the seven systems. Receptor-effector coupling efficiencies as well as agonist functional dissociation constants (K_A) and relative efficacies were determined for $CHOm_1$, $CHOm_3$ and $CHOm_5$ receptors. Some of the data have been presented in abstract form (Richards *et al.*, 1993).

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Methods

Cell culture

A9L mouse fibroblasts or Chinese hamster ovary cells, transfected with human genes encoding one of the five subtypes of muscarinic receptors, were purchased from Research Genetics, Inc. (Bethesda, MD, U.S.A.). Wild-type A9L (ATCC CCL-1.4) and CHO-K1 (ATCC CCL-61) cells were purchased from American Type Culture Collection (Rockville, MD, U.S.A.). All materials used in cell cultures were obtained from Gibco (Cergy Pontoise, France). Upon arrival, the cell lines were put into culture and the first passages were aliquoted and stored as stock cultures. Passage numbers indicate the number of times the cells were passaged in our institute. A9L cells were maintained in Dulbecco's Modified Eagle Medium (DMEM, Gibco 041-1965), 10% foetal calf serum (FCS), penicillin G (10 u ml^{-1}), streptomycin (10 μg ml^{-1}), 4 mM L-glutamine and 1 mM sodium pyruvate. CHO cells were maintained in DMEM-Nutrient Mixture F-12 (1:1, Gibco 041-1331), 10% FCS, penicillin G (10 u ml^{-1}) and streptomycin (10 μg ml^{-1}), 1% non-essential amino acids, 2 mM L-glutamine, 0.5 mM sodium pyruvate. Both cell lines were grown in humidified incubators at 37°C, 5% CO_2. After 20–22 passages (cyclic AMP assay) or about 30 passages (IP1 formation), the cells were discarded and cells of lower passage number were put into culture. Cell membranes for the binding assays were prepared from confluent cells detached by scraping, washed twice in magnesium- and calcium-free phosphate buffered saline and stored frozen at −80°C.

Binding

Frozen membranes were thawed and homogenized in 10 ml ice-cold 50 mM sodium potassium phosphate buffer (pH 7.4) using a Polytron (setting 6 for 15 s). The homogenate was centrifuged at 40,000 *g* for 10 min at 4°C. The resulting pellet was resuspended in homogenizing buffer to obtain 0.05–0.5 mg protein ml^{-1} (protein content determined by the method of Lowry *et al.*, 1951). The incubation mixtures (0.5 ml final volume) contained buffer, homogenate, test compound and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) at 0.3 nM final concentration. Non-specific binding was defined by use of 2 μM atropine. In saturation experiments, the radioligand concentration varied between 0.025 and 1 nM. After incubation at room temperature (1 h for m_1-m_4, 1.5 h for m_5), incubation was terminated by rapid filtration through Whatman GF/B glass fibre filters presoaked in water. The filters were rinsed with saline (three times 3 ml using a Brandel cell harvester or for 10 s using a Skatron 96 well cell harvester) and the radioactivity determined by liquid (Beckman 6000 LS) or solid (1205 Betaplate, Wallac) scintillation spectrometry.

Inositol monophosphate (IP1) formation

Cells at >90% confluency were gently rinsed twice with physiological buffer (see below) and then incubated 2–5 min with 5 mM EDTA in 10 mM Na_2HPO_4, 0.25 M sucrose (pH = 7.4). The solution was gently decanted, the cells detached with a rubber 'cell scraper' into buffer and precipitated by low speed centrifugation. The cells were resuspended in a known volume of buffer and aliquots taken immediately to determine the percentage of cells excluding trypan blue. The volume of the cell suspension was then adjusted so as to obtain about 2×10^5 trypan blue-excluding cells per assay tube. The cells were added to tubes containing buffer with 5 mM LiCl and 0.1 μM [^{3}H]-*myo*-inositol and incubated for 30 min in a shaking bath at 37°C before the addition of different concentrations of standard or test agonists (final incubation volume = 300 μl). Twenty-five minutes after adding agonists, the reaction was stopped with 940 μl chloroform:methanol (1:2, v:v) and [^{3}H]-inositol monophosphates were quantified by column chromatography following the procedure of Brown *et al.* (1984), as modified by Richards (1990). Each treatment, i.e. blank (no cells), basal or a given concentration of an agonist, was determined in triplicate. Results are expressed as a percentage of the maximum response above basal obtained with a standard agonist (oxotremorine-M or carbachol) included in each experiment.

Measurement of cyclic AMP levels

We observed that cells near or at confluency tended to have higher basal cyclic AMP levels but the response to forskolin was unchanged, thus giving rise to a smaller stimulation. To avoid confluency, cells were seeded at a low density 20–22 h before the experiment began. The cells were detached, resuspended and counted as described above and the volume adjusted to obtain about 2×10^5 trypan blue-excluding cells per assay tube. Each tube contained physiological buffer and 0.1 mM 3-isobutyl-1-methyl-xanthine (IBMX). After an initial incubation of 10 min in a shaking bath at 37°C, agonists were added, followed 10 min later by forskolin (final concentration = 1 or 3 μM). The final volume was 250 μl. The reactions were stopped 10 min after the addition of forskolin with 250 μl ice-cold 10% perchloric acid (PCA). The protein was precipitated by centrifugation and quantified by the Lowry procedure. An aliquot of each supernatant was extracted with Freon:trioctylamine (1:1, v:v) to remove the PCA and the cyclic AMP content determined using radioimmunoassay (RIA) kits.

Each treatment was assayed in duplicate samples of cells and duplicate samples of each assay tube were subjected to radioimmunoassay (RIA). Blank values (no cells), which varied from 0 to an equivalent of around 200 fmol cyclic AMP, were determined in each experiment. Blank values were subtracted from sample values which were then converted to pmol [mg^{-1} protein (% trypan-blue-excluding cells)]. The percentage inhibition relative to the standard agonist (carbachol or oxotremorine-M included in every experiment) was calculated for each agonist.

Receptor alkylation

The active aziridinium ion of propylbenzilylcholine mustard was obtained by the procedure of Hu & El-Fakahany (1990). Receptor alkylation proceeded for 15 min ($CHOm_4$) or 20 min ($CHOm_1$, $CHOm_3$, $CHOm_5$) at 37°C in a shaking water bath. Excess propylbenzilylcholine mustard was removed by centrifugation and the cells were resuspended in physiological buffer to obtain the desired number of trypan blue-excluding cells per assay tube as described above.

Drugs and chemicals

Radioimmunoassay kits and [^{3}H]-*myo*-inositol (18–20 Ci $mmol^{-1}$) were purchased from DuPont de Nemours S.A., Les Ulis, France and Amersham France, Les Ulis, France. Propylbenzilylcholine mustard and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS, 83 Ci mol^{-1}) were purchased from DuPont de Nemours S.A., Les Ulis, France. RS 86 (2-ethyl-8-methyl-2,8-diazaspiro-[4,5]-decan-1,3-dion hydrobromide) was a gift from Sandoz (Basel, Switzerland). Oxotremorine-M (N,N,N,-trimethyl-4-)2-oxo-1-pyrrolidinyl)-2-butyn-1-ammonium iodide) and McN-A-343 (4-hydroxy-2-butynyl)-1-trimethylammonium-*m*-chlorocarbanilate chloride) were purchased from Research Biochemicals Incorporated (Natick, MA, U.S.A.). The following products were purchased from Sigma Chimie, La Verpillière, France: arecoline hydrobromide, carbamyl-β-methyl choline chloride (bethanechol), carbamylcholine chloride (carbachol), (±)-acetyl-β-methylcholine chloride (methacholine), (±)-muscarine chloride, oxotremorine sesquifumarate, pilocarpine hydrochloride, bovine albumin fraction V, IBMX, N-[2-hydroxyethyl]piperazine-N′-

[2-ethanesulphonic acid] (HEPES), ethylenediaminetetraacetic disodium salt (EDTA), and Freon (1,1,2-trichlorotrifluoroethane). Trioctylamine was from Aldrich-Chimie, Strasbourg, France. Physiological buffer was prepared with analytical grade chemicals and contained (in mM): NaCl 118, KCl 5.0, $CaCl_2$ 1.3, KH_2PO_4 1.0, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10 and HEPES 10, pH 7.4

Calculations

(1) Binding assay Displacement curves were analysed by InPlot GraphPad (GraphPad Software, Inc., San Diego California, U.S.A.) to obtain Hill slopes and inhibition values (IC_{50}). In cases where the slope ≥ 0.8, K_i values were calculated using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). The dissociation constants (K_D) for [^{3}H]-NMS were obtained from saturation experiments performed under the same conditions as the agonist binding assays.

(2) Functional assays A standard logistic equation was fitted to the data obtained from the functional assays (i.e. stimulation of IP1 formation or inhibition of forskolin-stimulated cyclic AMP accumulation relative to the standard agonist) using a curve-fitting programme (GraFit, Erithacus Software Limited, London, UK, or SigmaPlot, Jandel Scientific, Erkrath, Germany) to obtain estimates of the parameters EC_{50}, slope and maximum response.

(3) Dissociation constants, relative efficacy and receptor occupancy The dissociation constants (K_A) of oxotremorine-M for $CHOm_1$, $CHOm_3$ and $CHOm_5$ receptors were determined by the partial receptor occlusion method (Furchgott & Bursztyn, 1967) using propylbenzilylcholine mustard as the alkylating agent. Relative efficacies and dissociation constants of partial agonists were estimated from the slopes and intercepts of plots of $[A]\,[P]^{-1}$ vs [A] for equieffective concentrations of standard [A] and partial agonist [P] (Kenakin, 1987). The averaged values of the standard agonist, oxotremorine-M, and individual experimental results of partial agonists were used. For weak partial agonists, the response elicited by a 1 mM concentration was determined. A plot of log relative efficacy against % maximal response (Kenakin, 1987) for partial agonists in the series provided a rough estimation, by extrapolation (NB), of the relative efficacies of these weak partial agonists at $CHOm_3$ and $CHOm_5$ receptors.

To compare receptor-effector coupling efficiencies among the different receptor subtypes expressed in CHO cells, or of the same receptor subtype expressed in the two cell lines, the ratios of dissociation or inhibition constant (K_A or K_i) to EC_{50} (Kenakin, 1987) were calculated using oxotremorine-M as agonist.

(4) Statistical analysis The data obtained from curve fitting procedures and the relative efficacies were analysed for statistical differences using single factor ANOVA and Dunnett's *t* test. The parameters obtained with oxotremorine-M served as control. EC_{50} values were converted to the negative log values for statistical analysis, the latter being more likely to be normally distributed (Fleming *et al.*, 1972). Student's *t* test was used to compare differences obtained with an agonist at a given receptor expressed in two cell lines. Differences were statistically significant when $P<0.05$.

Results

Binding

The receptor densities and K_D values of [^{3}H]-NMS at the different muscarinic receptor subtypes expressed in A9L or CHO cells are indicated in Table 1. The K_A values of [^{3}H]-NMS were similar to the range of values reported by Buckley *et al.* (1989) in CHO cells and using somewhat different assay conditions. Analysis of competition curves to displace specifically bound [^{3}H]-NMS by agonists was used to calculate IC_{50} values and slopes for these agents (Table 2). For agonists with slopes equal to or greater than 0.8, the IC_{50} was converted to K_i.

The IC_{50}/K_i values obtained with seven muscarinic agonists were similar in A9L and CHO cells expressing m_1 receptors (differences <5 fold) except for oxotremorine (ratio of K_i at $CHOm_1/A9Lm_1 = 6$). Differences of 4 fold or less were observed with m_3 receptors in the two cell lines. In CHO cells, all agonists but McN-A-343 and RS 86 displayed the lowest IC_{50} value for m_2 receptors, higher values were observed at m_4, m_5 and m_3 receptors, and the highest IC_{50} values were for m_1 receptors. With three exceptions, all agonists displayed IC_{50} values that were more than 30 fold lower at m_2 than at m_1 receptors. Pilocarpine, RS 86 and the so-called 'M_1-selective' agonist, McN-A-343, did not distinguish (differences of 3 fold or less) among the five subtypes of muscarinic receptors.

The Hill slopes from the data obtained with these agonists were lowest for m_2 receptors, followed by m_4 receptors, both coupled predominantly to G proteins that inhibit adenylyl cyclase activity. The interactions of the agonists with the three receptor subtypes coupled to phospholipase C(s) gave rise to slopes of 0.80 or greater except carbachol at $A9Lm_1$, $A9Lm_3$ and $CHOm_3$ receptors.

IP1 formation induced by full and partial agonists at m_1, m_3 and m_5 receptors

The current methods used to quantify receptor-activated phosphatidylinositol hydrolysis have been reviewed by Nahorski and coworkers (Nahorski & Challiss, 1991; Wojcikiewicz *et al.*, 1993) and the advantages and disadvantages of each method considered. The continuous labelling method used in this study could give rise to erroneous conclusions if the two cell lines incorporate the label into different inositol pools with different sensitivities to agonists such that the agonist-induced responses are due to differences in specific radioactivity rather than or in addition to receptor-activation. We observed differences in the amount of labelling and in the kinetics of IP1 formation following activation of m_1 receptors expressed in CHO and A9L cells (Richards & van Giersbergen, 1995) but the extent of phospholipid hydrolysis obtained at two different incubation times with 1 mM carbachol were similar for A9Lm1 (times basal) (30 min: 10.6 ± 3.5; 60 min: 16.3 ± 4.6, each $n = 4$), A9Lm3 (12.0 and 10.3 in 2 experiments at 30 min; 60 min: 15.8 ± 5.8, each $n = 3$), CHOm1 (30 min: 11.8 ± 3.1; 60 min:

Table 1 Dissociation constants (K_D in pM) for [^{3}H]-N-methylscopolamine and receptor densities (B_{max} = fmol binding sites mg^{-1} protein) of the different receptor subtypes expressed in A9L or CHO cells

	A9L m_1	*CHO* m_1	*A9L* m_2	*CHO* m_2	*A9L* m_3	*CHO* m_3	*CHO* m_4	*CHO* m_5
K_D	87 ± 9	214 ± 26	207 ± 26	226 ± 9	175 ± 53	112 ± 18	81 ± 11	426 ± 15
B_{max}	369 ± 73	824 ± 59	2625 ± 449	410 ± 42	611 ± 42	651 ± 177	531 ± 29	414 ± 97

$n = 3$

Table 2 Affinity constants (K_i, μM) of agonists determined by the inhibition of the specific binding of [^{3}H]-N-methylscopolamine to human muscarinic receptors expressed in A9L or CHO cells

Agonist	$A9Lm_1$			$CHOm_1$		$CHOm_2$		$A9Lm_3$		$CHOm_3$		$CHOm_4$		$CHOm_5$	
	IC_{50}	K_i	Slope	IC_{50}	K_i	Slope	IC_{50}	Slope	IC_{50}	K_i	Slope	IC_{50}	Slope	IC_{50}	K_i	Slope
Oxo-M	23 ±5	7 ±1	0.83 ±0.03	34 ±9	13 ±3	0.80 ±0.04	0.8 ±0.4	0.53 ±0.05	38 ±8	11 ±2	0.85 ±0.03	54 ±22	25 ±3	0.86 ±0.05	7.2 ±1.4	0.67 ±0.03	1.7 ±0.3	0.9 ±0.04	1.06 ±0.04
Arecoline	34 ±6	10 ±2	0.83 ±0.04	75 ±10	31 ±3	0.93 ±0.03	2.3 ±0.3	0.58 ±0.02	31 ±1	9.3 ±0.3	0.88 ±0.03	36 ±5	12 ±1	0.93 ±0.03	44 ±2	0.76 ±0.01	34 ±5	20 ±3	0.86 ±0.05
Bethanechol	657 ±71	197 ±23	0.85 ±0.06	1837 ±294	771 ±15	0.91 ±0.03	25 ±4	0.59 ±0.04	278 ±25	84 ±7	0.92 ±0.03	631 ±8	214 ±6	0.95 ±0.02	317 ±60	0.93 ±0.16	393 ±51	232 ±36	0.92 ±0.04
Carbachol	500 ±70		0.76 ±0.07	465 ±123	320 ±75	0.82 ±0.07	4.5 ±1.4	0.51 ±0.04	173 ±30		0.76 ±0.01	417 ±19		0.76 ±0.00	49 ±9	0.76 ±0.04	196 ±53	125 ±22	0.83 ±0.06
McN-A-343	21 ±3	6 ±1	0.85 ±0.06	61 ±21	17 ±1	0.88 ±0.03	37 ±7	0.85 ±0.07	16 ±1	5 ±1	0.93 ±0.02	29 ±5	10 ±2	0.98 ±0.04	27 ±3	0.77 ±0.03	21 ±3	13 ±2	0.98 ±0.02
Methacholine	NT			766 ±97	316 ±35	0.88 ±0.02	2.6 ±0.5	0.56 ±0.01	NT			134 ±11	46 ±2	0.92 ±0.03	52 ±14	0.78 ±0.08	62 ±8	36 ±5	0.85 ±0.06
Oxotremorine	1.8 ±0.2	0.55 ±0.07	0.92 ±0.03	7.6 ±0.2	3.2 ±0.04	0.85 ±0.10	0.19 ±0.05	0.61 ±0.04	3.5 ±0.4	1.0 ±0.1	0.97 ±0.07	2.7 ±0.3	0.9 ±0.2	0.89 ±0.08	3.4 ±0.7	0.78 ±0.06	1.4 ±0.2	0.8 ±0.1	0.90 ±0.02
Pilocarpine	13 ±0.6	3.9 ±0.2	0.97 ±0.06	26 ±3	11 ±1	0.92 ±0.04	5.7 ±1.1	0.79 ±0.06	5.6 ±0.7	1.7 ±0.2	0.87 ±0.04	21 ±2	7.1 ±0.4	0.90 ±0.04	20 ±1	0.77 ±0.04	9 ±1	5.1 ±0.9	0.91 ±0.02
RS 86	11 ±3	3.3 ±0.1	0.90 ±0.03	38 ±13	16 ±6	0.93 ±0.03	16 ±4	0.79 ±0.06	7.1 ±1.3	2.1 ±0.4	0.94 ±0.05	11 ±1	3.8 ±0.6	0.87 ±0.03	27 ±4	0.85 ±0.08	13 ±2	7.5 ±1.2	0.96 ±0.05

Values are mean ± s.e.mean; $n = 3$.
Oxo-M = oxotremorine-M.
NT = not tested.

17.7 ± 4.2, each $n = 3$) and CHOm5 (30 min: 9.8 ± 2.6; 60 min: 25.6 ± 4.2, each $n = 9$) whereas significantly smaller stimulations were observed in CHOm3 cells (30 min: 3.6 ± 0.6; 60 min: 4.7 ± 0.5, each $n = 3$). Although the inositol pools were not at isotopic equilibrium, the time course and levels of IP1 accumulation induced by carbachol that we observed in $CHOm_1$ cells using the continuous labelling method were similar to those observed in $CHOm_1$ cells labelled for 48 h with [^{3}H]-*myo*-inositol (Atack *et al.*, 1993; degree of isotopic labelling not indicated). Moreover, results we obtained with the continuous labelling method were largely similar (see Discussion) to those obtained using a pulse-labelling procedure (Wang & El-Fakahany, 1993) or results obtained with agonist-stimulated GTPase activity (Lazareno *et al.*, 1993).

Oxotremorine-M, carbachol and muscarine were full agonists at m_1, m_3 and m_5 receptors expressed in CHO cells (Table 3). Arecoline and oxotremorine were full or strong partial (> 50% maximal response) agonists. Pilocarpine and RS 86 were partial agonists at the three receptor subtypes. Bethanechol was the least potent agonist, displaying similar EC_{50} values at the three receptors where it was a full or strong partial agonist. McN-A-343 was a potent full agonist at $CHOm_1$ receptors, 18 fold less potent and a partial agonist at $CHOm_5$ receptors and almost inactive at $CHOm_3$ receptors.

Comparison of m_1 receptors expressed in A9L and CHO cells

CHO cells expressed 2.2 times more m_1 receptors than did A9L cells and all agonists except RS 86 were more potent in $CHOm_1$ cells (Table 3). Differences in agonist potency in the two cell lines fell into two groups: differences of less than 3 fold (bethanechol, carbachol, McN-A-343, pilocarpine, RS 86) and differences of 6 fold or greater (oxotremorine-M, arecoline, methacholine, muscarine, oxotremorine). As might be expected from a slightly higher receptor density in CHO cells, McN-A-343 and RS 86 displayed greater intrinsic activity in CHO than A9L cells. Oxotremorine and pilocarpine appeared to be strong partial agonists. In six out of seven experiments with $A9Lm_1$ cells, RS 86 was a partial agonist (32% ± 7% of maximum response) but in one experiment it was a full agonist (no change in potency). It thus appears likely that RS 86 is a partial agonist of similar potency in both cell lines. Bethanechol was again the least potent but a full agonist in the two systems.

Comparisons of m_3 receptors expressed in A9L and CHO cells

Although receptor densities for m_3 receptors expressed in CHO and A9L cells were similar, all agonists (except McN-A-343) were significantly more potent at stimulating m_3 receptors in CHO cells (Table 3); differences in agonist potencies at m_3 receptors in the two cell lines were 8 fold or greater except for bethanechol. The agonists displayed a similar order of intrinsic activities at m_3 receptors in the two cell lines except oxotremorine which was a partial agonist in $A9Lm_3$ and a full agonist in $CHOm_3$ cells ($P = 0.0011$). McN-A-343 was inactive or a weak partial agonist at m_3 receptors expressed in either cell line. A striking difference was observed with RS 86 and pilocarpine which were partial agonists, as potent as carbachol at m_3 receptors expressed in CHO cells but only weakly active at m_3 receptors expressed in A9L cells.

Stimulation of cyclic AMP formation with forskolin

A9L and CHO cells (wildtype) were incubated for 10 min in the absence or presence of increasing concentrations (0.1 to 30 μM) of forskolin and the levels of cyclic AMP were determined. Basal activity was significantly lower in the A9L

Table 3 Agonist-induced inositol monophosphate (IP1) accumulation in A9L or CHO cells expressing human muscarinic receptors

	$A9Lm_1$		$CHOm_1$		$A9Lm_3$		$CHOm_3$		$CHOm_5$	
Agonist	EC_{50}	R_{max}	EC_{50}	R_{max}	EC_{50}	R_{max}	EC_{50}	R_{max}	EC_{50}	R_{max}
Oxotrem.-M	0.64	112	0.10	99	3.7	107	0.22	108	0.39	107
(n = 9,14,5,9,8)	±0.12	±7	±0.03	±3	±1.1	±4	±0.07	±7	±0.06	±4
Arecoline	14[abe]	102	1.06[ab]	85	7.2	59*	0.96[acd]	77*	6.5[a]	79*
(n = 8,8,3,6,3)	±5	±12	±0.16	±6	±1.4	±8	±0.12	±8	±4	±6
Bethanechol	72[+]	98	35[+]	98	77[+]	71*	14.5[+]	72*	32[abce]	91
(n = 6,10,8,4,3)	±12	±6	±7	±7	±19	±4	±2.9	±4	±17	±3
Carbachol	5.3[a]	96	1.83[abc]	98	9.4[b]	101	0.90[ac]	100	1.31	100
(n = 17,18,10,13,9)	±0.9	±6	±0.26	±2	±2.1	±2	±0.23	±1	±0.35	±1
McN-A-343	5.0[a]	51*	1.74[ab]	90	(x)	2*	(x)	9*	33[abce]	49*
(n = 5,9,3,6,5)	±1.8	±10	±0.24	±7		±2		±3	±16	±9
Methacholine	8.2[a]	115	0.83[ab]	89	6.0	102	0.33	83	nt	
(n = 8,6,4,5,–)	±2.9	±11	±0.22	±6	±2.5	±12	±0.07	±9		
Muscarine	5.2[a]	127	0.55[a]	93	8.4[b]	92	0.29	94	0.76	108
(n = 7,8,5,6,3)	±2.2	±9	±0.12	±4	±2.3	±6	±0.08	±8	±0.20	±2
Oxotremorine	1.3	91	0.13	78*	2.8	56*	0.33	88	0.55	74*
(n = 4,11,7,4,3)	±0.3	±11	±0.03	±7	±1.2	±5	±0.05	±4	±0.22	±8
Pilocarpine	5.6[a]	86	4.1[abcd]	76*	(x)	11*	2.19[abcd]	43*	(x)	23*
(n = 6,9,6,4,3)	±1.1	±6	±1.7	±5		±7	±0.36	±9		±5
RS-86	4.4[a]	45*	5.8[abcdf]	78	(x)	13*	1.06[a]	50*	(x)	10*
(n = 7,9,6,3,4)	±1.5	±14	±2.8	±9		±4	±0.18	±11		±5

EC_{50} = μM; [a–f] or [+]indicate statistical differences among EC_{50} values: [a]from oxo-M, [b]from oxotremorine, [c]from muscarine,[d]from methacholine, [e]from carbachol, [f] from arecoline, [+]from all other agonists. x = response obtained with 1 mM agonist. R_{max} = % of maximum response (above basal) obtained with a standard agonist; * = R_{max} values significantly different from oxotremorine-M.

(15.6 ± 3.8 pmol cyclic AMP mg^{-1} protein) than in CHO cells (47.0 ± 7.5 pmol cyclic AMP mg^{-1} protein; n = 4 and 5, respectively, P = 0.01). At 30 μM forskolin, the stimulation was significantly greater in CHO than in A9L cells: 27.4 ± 3.1 (n = 5) and 5.9 ± 0.9 (n = 4, P < 0.01) times basal, respectively.

Comparison of m_2 receptors expressed in A9L and CHO cells

Carbachol inhibited cyclic AMP formation at m_2 receptors expressed in these cell lines with similar potencies (P > 0.05): 114 ± 28 nM at $A9Lm_2$ (n = 7), 41 ± 13 nM at $CHOm_2$ (n = 5). However, the intrinsic activity was significantly greater in CHO than A9L cells despite the 6 fold greater receptor density in the latter cell line. The maximum inhibition elicited by carbachol in $A9Lm_2$ cells was 51.3 ± 5.1% (n = 7) compared to 89.1 ± 1.2% (n = 5) in $CHOm_2$ (P < 0.01).

Effect of preincubation with carbachol on forskolin-stimulated cyclic AMP formation

$CHOm_4$ cells (as were $A9Lm_2$ and $CHOm_2$ cells) were preincubated 10 min with the agonists to allow ligand-receptor equilibration before the addition of forskolin. In order to determine if preincubation could desensitize the m_4 receptors. $CHOm_4$ cells were exposed to carbachol for 0 to 30 min before the addition of forskolin. The inhibition of forskolin-stimulated cyclic AMP formation by 0.3 μM carbachol was constant for up to 20 min: inhibition was 75 ± 4% at time 0, 79 ± 1% at 10 min and 78 ± 2% at 20 min incubation (n = 3 at each time). A significantly smaller inhibition by carbachol was observed after 30 min preincubation (51 ± 7%, n = 3; P < 0.05).

Inhibition in $CHOm_4$ and $CHOm_2$ cells of forskolin-stimulated cyclic AMP formation by full and partial agonists (Table 4)

Activation of $CHOm_2$ or $CHOm_4$ receptors by oxotremorine-M inhibited forskolin-stimulated cyclic AMP formation to a similar extent: 85% ± 3% (n = 6) at m_2, 79% ± 1% (n = 16; P = 0.062) at m_4 receptors. In $CHOm_4$ cells, none of the maximum responses differed significantly from that of oxotremorine-M. However, the maximum response elicited by bethanechol was significantly less than the responses elicited by carbachol and oxotremorine, suggesting that bethanechol may be a strong partial agonist at these receptors.

Table 4 Agonist-induced inhibition of forskolin-stimulated cyclic AMP formation in CHO cells transfected with human genes for m_2 or m_4 muscarinic receptors

	$CHOm_2$		$CHOm_4$	
Agonist	EC_{50} (nM)	R_{max} (%)	EC_{50} (nM)	R_{max} (%)
Oxotrem.-M	23	106	1.2	105
(n = 6,18)	±6	±6	±0.3	±2
Arecoline			21[a]	101
(n = –,6)			±7	±3
Bethanechol			787[x]	88[bc]
(n = –,5)			±218	±6
Carbachol	41	102	20[ab]	107
(n = 5,19)	±13	±4	±4	±4
McN-A-343	1659[ac]	72[ac]	50[ab]	109
(n = 4,6)	±632	±5	±12	±5
Methacholine			26[ab]	105
(n = –,6)			±6	±4
Muscarine			154[a]	97
(n = –,7)			±5	±4
Oxotremorine			7[a]	114
(n = –,7)			±4	±7
Pilocarpine			5804[x]	110
(n = –,6)			±1041	±7
RS-86			151[x]	107
(n = –,6)			±39	±5

Values are means ± s.e.means for n experiments.
[a] = significantly different from oxotremorine-M; [b] = significantly different from oxotremorine; [c] = significantly different from carbachol; [x] = significantly different from all other agonists.

Three agonists were also used to inhibit forskolin-stimulated cyclic AMP formation via m_2 receptors, in CHO cells, expressed at a similar density as m_4 receptors. Carbachol, a full agonist, displayed similar potencies (P > 0.05) at the two receptors. Both oxotremorine-M and McN-A-343 were significantly (P < 0.01) more potent at m_4 than m_2 receptors. McN-A-373 displayed greater intrinsic activity at

m_4 than m_2 receptors ($P<0.05$). The slopes of the concentration-response curves for McN-A-343 at m_2 receptors (0.51 ± 0.03, $n = 4$) were significantly less ($P = 0.0493$) than those obtained at m_4 receptors (0.98 ± 0.15, $n = 8$).

Experiments with propylbenzilylcholine mustard

Incubation of $CHOm_4$ cells with 30 nM propylbenzilylcholine mustard shifted the concentration-response curve of oxotremorine-M to the right 15.1 ± 3.1 fold ($n = 3$) with no change in the maximum response. Higher concentrations of propylbenzilylcholine mustard, up to 10 μM, further shifted the concentration-response curves of oxotremorine-M but had no effect on the maximum response.

In $CHOm_1$ and $CHOm_3$ cells, incubation with 10 μM propylbenzilylcholine mustard decreased the maximum responses and increased the EC_{50} values obtained with oxotremorine-M (Figure 1a and b). The shift in the EC_{50} values were similar for $CHOm_1$ and $CHOm_3$: 77 ± 15 fold for m_1 ($n = 5$) and 85 ± 25 fold for m_3 ($n = 6$). Similar decreases in the maximum stimulation of IP1 to $46 \pm 10\%$ for m_1 ($n = 5$) and $42 \pm 7\%$ for m_3 ($n = 6$) were observed. From these data, the dissociation constants (K_A) for oxotremorine-M in $CHOm_1$ and $CHOm_3$ cells were found to be in good agreement (differences $\leqslant 2$) with the corresponding K_i values (Tables 2 and 5).

In $CHOm_5$ cells, incubation with 1 μM propylbenzilylcholine mustard shifted the concentration-response curve of oxotremorine-M to the right 27 ± 13 fold ($n = 4$) and decreased the maximal response to $58 \pm 6\%$ of the response to oxotremorine-M in the absence of propylbenzilylcholine mustard (Figure 1c). From these data, the K_A was found to be 4.8 ± 0.7 μM ($n = 4$), somewhat greater than the K_i of 0.90 ± 0.04 μM ($n = 3$).

The dissociation constants of some partial agonists (Table 5) differed by 5 fold or less from their corresponding K_i values (Table 2). The exception was oxotremorine at $CHOm_1$ receptors (9 fold difference).

Relative efficacies, receptor-effector coupling efficiencies

Receptor-effector coupling efficiencies were similar for m_3 receptors (K_A/EC_{50} for oxotremorine-M = 60) and m_1 receptors ($K_A/EC_{50} = 58$) whereas m_5 receptors were less efficiently coupled ($K_A/EC_{50} = 12$). Comparing ratios of K_i/EC_{50} for oxotremorine-M at m_1 and m_3 receptors expressed in CHO and A9L cells suggest that receptor-effector coupling is more efficient in CHO cells. In A9L cells, m_1 receptors appeared to be coupled somewhat more efficiently than m_3 receptors.

The relative efficacies (compared to oxotremorine-M) of partial agonists at m_1, m_3 and m_5 receptors expressed in CHO cells are presented in Table 5. Relative efficacies of McN-A-343 at $CHOm_3$ and pilocarpine and RS 86 at $CHOm_5$ receptors are estimated by extrapolation from plots of log relative efficacy versus % maximal response of more active partial agonists. Linear regression of the points plotted from data obtained with $CHOm_3$ had a correlation coefficient of 0.97; standard errors of the slope and intercept were 10% and 14% of the corresponding values. Linear regression of the three points obtained with $CHOm_5$ receptors had a correlation coefficient of 1, the standard errors of the slope and intercept were less than 2% of their corresponding variables. Pilocarpine displayed greater intrinsic acitivity at m_1 than at m_3 or m_5 receptors. RS 86 was more efficacious at m_1 and m_3 than m_5 receptors. Although $CHOm_5$ receptors were less efficiently coupled than $CHOm_1$ or $CHOm_3$ receptors, arecoline and oxotremorine were more efficacious at m_5 than m_3 (and, for oxotremorine, m_1) receptors.

The apparent inability of propylbenzilylcholine mustard to block $CHOm_4$ receptors sufficiently prevented an estimation of the K_A value for oxotremorine-M and calculating an inhibition constant, K_i, is of dubious value due to the low Hill slope. However, substituting K_i for K_A would allow a very rough estimate of the efficacy of oxotremorine-M. We have found that GTP analogues such as GppNHp steepen and shift the binding concentration-response curves of oxotremorine-M to the right so using the K_i value would give a *minimal* figure for the receptor reserve for this agonist. In our transfected CHO cells, oxotremorine-M-activated m_4 receptors are coupled with a high degree of efficiency as indicated by an apparent receptor reserve >900.

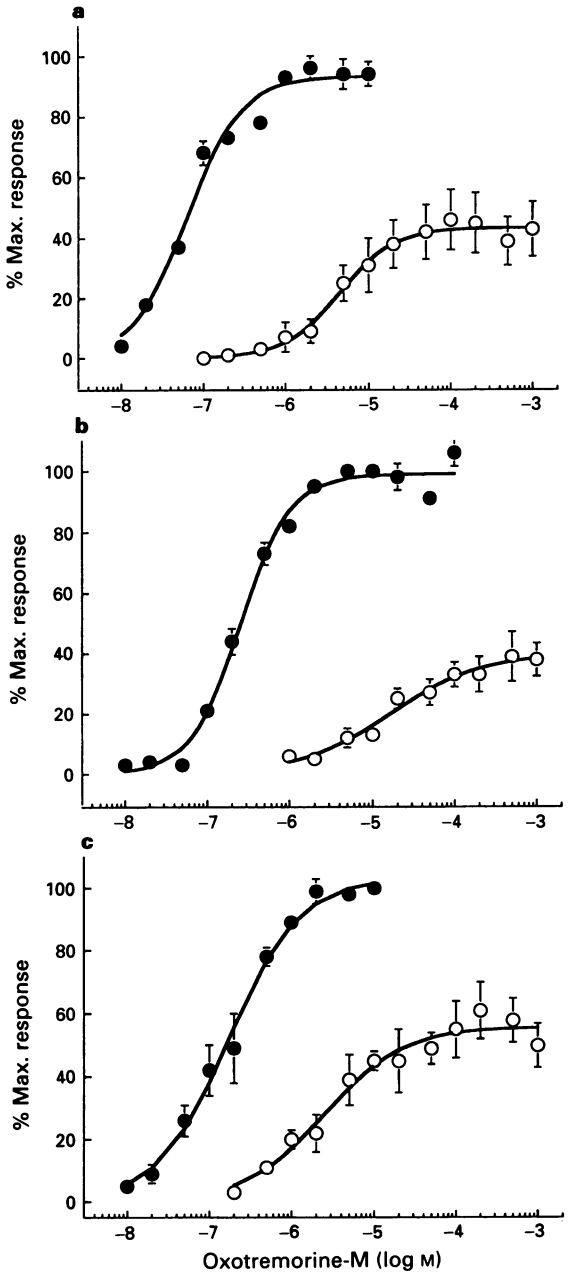

Figure 1 Concentration-response curves of oxotremorine-M in the absence (●) or presence (○) of propylbenzilylcholine mustard (20 min incubation). (a) $CHOm_1$ cells ($n = 5$), 10 μM propylbenzilylcholine mustard; (b) $CHOm_3$ cells ($n = 5$), 10 μM propylbenzilylcholine mustard; (c) $CHOm_5$ cells ($n = 4$), 1 μM propylbenzilylcholine mustard.

Discussion

A series of muscarinic agonists were used to study agonist-receptor interaction and agonist-receptor activation of muscarinic receptor subtypes expressed in mouse fibroblasts (A9L) and Chinese hamster ovary (CHO-K1) cells. The

Table 5 Dissociation constants (K_A) and relative efficacies of partial agonists compared to the standard agonist oxotremorine-M

	$CHOm_1$		$CHOm_3$		$CHOm_5$	
	K_A (μM)	*Rel. Eff.*	K_A (μM)	*Rel. Eff.*	K_A (μM)	*Rel. Eff.*
Oxo-M (5,5,4)	5.82 ± 1.03	1.0	13.20 ± 1.96	1.0	4.80 ± 0.67	1.0
Arecoline (–,5,3)			7.38 ± 1.34	0.058[a] ± 0.020	15.79 ± 4.41	0.198 ± 0.057
Bethanechol (–,4,–)			44.79 ± 5.87	0.028 ± 0.002		
McN-A-343 (–,–,4)				0.0012*	18.53 ± 7.47	0.025[x] ± 0.005
Oxotremorine (9,4,3)	0.35 ± 0.07	0.023[a] ± 0.003	2.10 ± 0.47	0.058[a] ± 0.015	2.31 ± 0.50	0.139 ± 0.029
Pilocarpine (8,4,–)	4.32 ± 1.16	0.022 ± 0.002	4.19 ± 0.79	0.007[b] ± 0.002		0.0041*
RS 86 (8,3,–)	4.40 ± 1.27	0.021 ± 0.003	1.58 ± 0.18	0.010 ± 0.003		0.0017*

Values are means ± s.e.mean, *n* number of experiments is indicated in parentheses. Statistical analysis across receptor subtypes for a given agonist. a = significantly different from m_5; b = significantly different from m_1. Statistical analysis among agonists for a given receptor subtype: [x] = significantly different from arecoline. *relative efficacy values obtained by extrapolation.

parameters examined included agonist inhibition values determined in a binding assay, a comparison of intrinsic activities and potencies of a series of agonists activating a given receptor in the two expression systems, and receptor-effector coupling efficiencies and relative agonist efficacies at homologous receptors expressed in the same cell line at similar receptor densities. We considered differences of 5 fold or less as unlikely to be physiologically relevant even if these differences were statistically significant. This was based, in part, on the observations that atropine, a putatively non-selective antagonist, displayed five fold differences among muscarinic receptor subtypes in both functional (Richards, 1990) and binding studies (Nivelbrant & Sparf, 1986) and that estimates of muscarinic antagonist affinity values at a given receptor subtype may vary by three fold or more (Mitchelson, 1988; Caulfield, 1993).

The conditions used to determine agonist inhibition values were those generally used with antagonists, i.e. non-physiological buffer and membrane preparations devoid of cytosolic factors that influence agonist binding. As far as we are aware, no other study has systematically compared inhibition values for a large series of agonists at all subtypes of cloned muscarinic receptors expressed in the same cell line or of a given receptor expressed in different types of cells. A significant difference in IC_{50} values was observed with carbachol using membranes prepared from recombinant (Results) or endogenous m_3 and m_4 receptors (Baumgold & White, 1989, membranes prepared in physiological buffer) whereas no difference was observed in intact CHO cells (Schwarz *et al.*, 1993). Carbachol did not distinguish between $CHOm_1$ and $CHOm_3$ receptors in membrane preparations (Results) or intact cells (Hu & El-Fakahany, 1990; Schwarz *et al.*, 1993). However, Hu & El-Fakahany (1990) found that the K_i of McN-A-343 was 23 fold greater in intact $CHOm_1$ than $CHOm_3$ cells, in contrast to Schwarz *et al.* (1993), also using intact cells, or in membrane preparations (Results), where no differences were found. Six out of nine agonists clearly distinguished between $CHOm_1$ and $CHOm_2$ receptors with differences in IC_{50} values of 30 fold or greater (Table 2) whereas in intact cells differences among (fewer) agonists were six fold or less (Schwarz *et al.*, 1993). Slopes and affinities of agonists binding to muscarinic receptors that inhibit adenylyl cyclase are more readily modulated by changes in the ionic strength of the buffer and the presence or absence of GTP than are muscarinic receptors linked to phosphatidylinositol turnover (references cited in Richards, 1991). Although both $CHOm_2$ and m_4 receptors inhibited adenylyl cyclase, the slopes determined in the binding assay were consistently lower at m_2 (except that of McN-A-343) than at m_4 receptors suggesting differences in agonist-receptor-G protein interactions but which produce the same end response.

Ligand-receptor equilibrium dissociation constants (K_i) at $CHOm_1$, $CHOm_3$ and $CHOm_5$ receptors were compared with 'functional' dissociation constants (K_A) obtained using the null method (Furchgott & Bursztyn, 1967; Kenakin, 1987). In most cases K_i and K_A values differed by 5 fold or less. The exception was a 9 fold difference for oxotremorine at $CHOm_1$ receptors. It is not known if this difference is due to chance, i.e. arose from scatter in the data, or reflect a particular receptor-interaction mode for this agonist sensitive to the differences in the two assay conditions. The utility of binding constants and functional dissociation constants as indicators of agonist-receptor interactions has been criticized by Colquhoun (1987). Specifically, in both radioligand binding assays and receptor alkylation studies, the constants obtained will depend not only on the affinity but also the efficacy of the agonist, especially for strong agonists. The inability to model quantitatively the various steps between agonist-receptor binding and the subsequent biological response and thus obtain precise information concerning agonist affinity and efficacy means that these terms are not strictly quantitative and their use to compare a series of agonists are indicative of relative rather than absolute differences.

The relative inefficacy of propylbenzilylcholine mustard to inactivate muscarinic receptors in CHO cells was unexpected. Despite high muscarinic receptor densities in rat striatum and rat cerebral cortex (2–3 pmol mg^{-1} protein, Wall *et al.*, 1991; Yasuda *et al.*, 1993), 10 nM propylbenzilylcholine mustard (McKinney *et al.*, 1989) or 10 nM benzilylcholine mustard (Keen & Nahorski, 1988) was sufficient to decrease the maximum responses to carbachol in these tissues. In contrast, cloned m_1 and m_3 receptors were more resistant to propylbenzilylcholine mustard, requiring 200 nM of the alkylating agent to decrease the maximum response to carbachol in CHO cells expressing low receptor densities ($B_{max} < 300$ fmol binding sites mg^{-1} protein; Hu & El-Fakahany, 1990) and 10 μM propylbenzilylcholine mustard in cells expressing moderate (this paper) or high receptor densities ($B_{max} = 2900$ fmol mg^{-1} protein; Schwarz *et al.*, 1993). This suggests that cloned muscarinic receptors, at least in CHO cells, are much more efficiently linked to effectors than are the corresponding muscarinic receptors in rat brain.

Agonist activation of human m_4 receptors in CHO cells was compared with the results of McKinney *et al.* (1991), who used a similar series of agonists at endogenous m_4 receptors in N1E-115 cells and putative m_4 receptors in rat striatum. The m_4 receptor density in rat striatum is around

1 pmol mg^{-1} protein (Yasuda *et al.*, 1993; see Table 1 for density of $CHOm_4$ receptors). All agonists were more potent in inhibiting cyclic AMP formation in $CHOm_4$ cells than in rat striatum. The ratios of EC_{50} values at $CHOm_4$ versus striatal receptors (from McKinney *et al.*, 1991) were 118 for oxotremorine-M, 26–43 for six other agonists and 2 for pilocarpine. In the three systems, identical rank orders of potencies were observed for seven of the eight agonists: oxotremorine-M ≥ oxotremorine > muscarine > carbachol > arecoline > McN-A-343 > bethanechol, providing additional evidence that the muscarinic receptors inhibiting adenylyl cyclase in rat striatum are of the m_4 subtype. The exception was pilocarpine which was less potent than bethanechol in $CHOm_4$ cells, more potent than bethanechol at striatal receptors and more potent than muscarine in N1E-115 cells.

Expressing a given receptor in two cell lines permitted comparison of the influences of different cell systems on receptor activation. We observed differences in the kinetics of IP1 formation in A9L and CHO cells transfected with m_1 (Richards & van Giersbergen, 1995) and m_3 receptors (unpublished observations). We also observed differences in cyclic AMP formation in these two cell lines (Results) suggesting differences in the levels and/or complement of G proteins or adenylyl cyclase(s) in these cells. Differences in receptor-effector coupling and/or coupling efficiency are suggested by the observation that the inhibition of forskolin-stimulated cyclic AMP formation by carbachol was significantly greater at m_2 receptors expressed in CHO cells despite the higher density of m_2 receptors in A9L cells.

Eight of the ten agonists displayed greater intrinsic activities and (except for RS 86) greater potencies in $CHOm_1$ than $A9Lm_1$ cells, consistent with the somewhat greater receptor density and apparently more efficient receptor-effector coupling observed in $CHOm_1$ cells. However, comparison of agonist potencies in the two cell lines suggested that the 10 agonists could be divided into two groups (see Results). The reasons for this are unknown. However, data obtained from point mutations of m_1 receptors (Hibert *et al.*, 1995) and from comparison of agonist potency ratios (Richards & van Giersbergen, 1995) suggest that agonist occupancy and activation of m_1 receptors differ among agonists and that m_1 receptors may complex with different isoform(s) of couplers or effectors depending on agonist structure.

We also compared activation of m_3 receptors expressed in either A9L or CHO cells. Although receptor densities were similar, the greater coupling efficiency in CHO cells was reflected in greater agonist potencies and intrinsic activities in these cells. The exception was bethanechol which had the same intrinsic activity in the two cell lines. Ratios of K_i/EC_{50} close to unity for bethanechol and arecoline in the apparently less efficiently coupled A9L cells suggests that receptor occupancy and response are approximately linearly related. This is compared to ratios of 14 and 12, respectively, in the more efficiently coupled CHO cells. Thus for cloned receptors, as for tissue receptors (Kenakin, 1987), the functions relating receptor occupancy and response may not be linear even for drugs that occupy 100% of the receptors to produce their maximal response.

We compared relative efficacies of partial agonists at m_1, m_3 and m_5 receptors expressed in CHO cells at similar receptor densities. Some agonists were more efficacious at activating the less efficiently coupled $CHOm_5$ receptors. For example, agonists producing about 75% of the maximal response in $CHOm_5$ cells were 14–20% as efficient at oxotremorine-M whereas in $CHOm_1$ and $CHOm_3$ cells, agonists producing about the same maximal response were only 2–6% as efficient as oxotremorine-M. Thus, in CHO cells, m_5 receptors may activate different G proteins and/or different phospholipases C(s) to stimulate IP1 formation.

Lazareno *et al.* (1993) determined agonist potencies and intrinsic activities of a series of agonists stimulating GTPase activity in human m_1, m_2, m_3 and m_4 receptors expressed in CHO cells at high density (1–7 pmol binding sites mg^{-1} protein). Wang & El-Fakahany (1993) also studied agonist activation (second messenger formation) using rat m_1 and m_3 and human m_2, m_4 and m_5 muscarinic receptors expressed in CHO cells at relatively low densities (B_{max} = 165–545 fmol mg^{-1} protein). Comparing data obtained in three independent laboratories using these cloned muscarinic receptors highlighted the many similarities despite differences in receptor densities and techniques. For example, both groups that determined agonist potencies (Lazareno *et al.*, 1993; present study) found that full agonists were at least 10 fold more potent at m_2 and m_4 than m_1 and m_3 receptors. Pilocarpine and McN-A-343 were weak partial agonists at m_5 receptors (Wang & El-Fakahany, 1993; present study). All three groups found McN-A-343 to display functional selectivity for m_4 receptors, to be a partial agonist at m_2 receptors and a weak partial agonist or inactive at m_3 receptors. The low slope of the concentration-response curves we observed with McN-A-343 suggests that m_2 receptor activation by this agonist may differ from the mode of activation observed at m_4 receptors. Non-classical interaction of McN-A-343 with muscarinic binding sites in rat myocardial membranes was observed by Birdsall *et al.* (1983).

There were also some notable differences among the results from these groups. Although the m_4 receptor densities were quite similar in our study and that of Wang & El-Fakahany (1993), we found the intrinsic activities of bethanechol and McN-A-343 to be less or equal, respectively, to that of carbachol whereas Wang & El-Fakahany (1993) found the intrinsic activities of both compounds to be significantly greater than that of carbachol. In addition, Wang & El-Fakahany (1993) reported that, in CHO cells, the order of receptor-effector coupling efficiencies was $m_5 > m_3 > m_1$ whereas we found that m_1 and m_3 receptors were equally and much more efficiently coupled to IP1 formation than were m_5 receptors.

Other contradictory results observed with muscarinic receptors expressed in CHO cells include a report by Schwarz *et al.* (1993) that PI turnover is more sensitive to muscarinic agonists than the cyclic AMP assay, clearly different from the data of Lazareno *et al.* (1993), Wang & El-Fakahany (1993) and the present results. Although the cloned muscarinic receptors used in three of these studies are from the same source, there are differences in receptor gene expression, receptor-effector coupling efficiencies and agonist activation of these receptors. These differences might arise because the insertion of genes into the cellular genome could influence not only receptor expression but perhaps the expression of other proteins that contribute to the cellular response subsequent to receptor activation. In our cultures, one of the transfected lines of CHO cells differ in morphology and growth curves from the other transfected CHO cells (J.-P. Ledig, personal communication). In addition, although the CHO cell line was found to have a relatively stable karyotype (Worton *et al.*, 1977), genetic 'drift' has been observed (Worton, 1978) and could be a factor in changes in gene expression or cell responses that occur with passage number or culture conditions (personal observations; for an example with another cell line, see Varrault *et al.*, 1992), variables that are not often reported in the literature. Cloned receptors are useful tools for the study of potential receptor-coupler-effector activation models in tissues but until the factors determining receptor-activation and response are better understood, it seems premature to extrapolate agonist profiles from cloned to tissue-expressed receptors.

In conclusion, agonist-induced responses depend not only on receptor density but also on a particular mode of receptor-effector coupling and the efficiency of this process.

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