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Comparative pharmacology of recombinant human M_3 and M_5 muscarinic receptors expressed in CHO-K1 cells

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1 Affinity estimates were obtained for several muscarinic antagonists against carbachol-stimulated [^{3}H]-inositol phosphates accumulation in Chinese hamster ovary (CHO-K1) cells stably expressing either human muscarinic M_3 or M_5 receptor subtypes. The rationale for these studies was to generate a functional antagonist affinity profile for the M_5 receptor subtype and compare this with that of the M_3 receptor, in order to identify compounds which discriminate between these two subtypes.

2 The rank order of antagonist apparent affinities (pK_B) at the muscarinic M_5 receptor was atropine (8.7) $\geqslant$ tolterodine (8.6) = 4-diphenylacetoxy-N-methylpiperidine (4-DAMP, 8.6) > darifenacin (7.7) $\geqslant$ zamifenacin (7.6) > oxybutynin (6.6) = para-fluorohexahydrosiladifenidol (p-F-HHSiD, 6.6) > pirenzepine (6.4) $\geqslant$ methoctramine (6.3) = himbacine (6.3) > AQ-RA 741 (6.1).

3 Antagonist apparent affinities for both receptor subtypes compare well with published binding affinity estimates. No antagonist displayed greater selectivity for the muscarinic M_5 subtype over the M_3 subtype, but himbacine, AQ-RA 741, p-F-HHSiD, darifenacin and oxybutynin displayed between 9- and 60 fold greater selectivity for the muscarinic M_3 over the M_5 subtype.

4 This study highlights the similarity in pharmacological profiles of M_3 and M_5 receptor subtypes and identifies five antagonists that may represent useful tools for discriminating between these two subtypes. Collectively, these data show that in the absence of a high affinity M_5 selective antagonist, affinity data for a large range of antagonists is critical to define operationally the M_5 receptor subtype.

Keywords: Muscarinic M_3 and M_5 receptors; inositol phosphates accumulation; CHO-K1 cells; muscarinic antagonists

Abbreviations: A, agonist; $[A]_{50}$, concentration of agonist causing 50% effect; B, antagonist; $[B]_{50}$, concentration of antagonist causing 50% effect; CHO-K1, Chinese hamster ovary cells; 4-DAMP, 4-diphenylacetoxy-N-methylpiperine; DPM, disintegrations per minute; EC_{50}, concentration producing half maximal effect; EDTA, ethylenediaminetetraacetic acid; E_m, maximal effect; InsPs, inositol phosphates; nH, Hill slope; PBS, phosphate buffered saline; pEC_{50}, potency; p-F-HHSiD, para-fluorohexahydrosiladifenidol; pK_B, antagonist apparent affinity estimate; r, correlation coefficient; SSQ, sum of squares of differences ($\sum(x-y)^2$)

Introduction

The existence of five subtypes of the muscarinic receptor is now established and identification of pharmacological correlates for all but the fifth gene product has been achieved. However, despite the lack of an endogenous correlate for this subtype it has been given the upper case nomenclature (M_5 rather than m5) based on data generated in cultured cell lines (see Caulfield & Birdsall, 1998 for review). The inability to identify clearly an endogenous population of M_5 receptors and to ascribe a function to this gene product stems from its restricted anatomical expression and the lack of selective antagonists for this subtype. The muscarinic M_5 receptor is 532 amino acids long and, like the M_3 receptor possesses a large third intracellular loop (Reever *et al*., 1997). The muscarinic M_5 subtype also exhibits an antagonist affinity profile similar to that of the M_3 subtype, which has complicated the identification of a functional correlate. Moreover, it is possible that an endogenous M_5 population has been observed but incorrectly classified as an M_3 receptor. Several groups (Buckley *et al*., 1989; Dörje *et al*., 1991; Bolden *et al*., 1992; Lazareno & Birdsall, 1993) have reported antagonist affinity profiles at the M_5 receptor subtype derived from binding studies using membrane homogenates of cells expressing the recombinant cloned receptor, including our own (Eglen *et al*., 1997). However, comparable data have not been reported for functional responses mediated *via* M_5 receptors.

The aim of the present studies was to establish antagonist affinity estimates for a range of muscarinic receptor antagonists against carbachol-stimulated [^{3}H]-inositol phosphates accumulation in CHO-K1 cells stably expressing either human recombinant muscarinic M_3 or M_5 receptors. Having established these functional antagonist affinity estimates these values were compared with published binding affinity estimates for these compounds to determine concordance with the literature, but were also compared with each other, in order to identify compounds which discriminate between these two receptor subtypes. In this way compounds would be identified that could represent useful tools in discriminating between these two receptor subtypes.

Preliminary accounts of this work have been presented to the British Pharmacology Society (Eglen *et al*., 1997; Watson *et al*., 1998).

Methods

Tissue culture

Chinese hamster ovary cells (CHO-K1) stably expressing human recombinant muscarinic M_3 and M_5 receptor protein

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were cultured to confluence in tissue culture flasks (T-162). Culture flasks containing Ham's F-12 nutrient medium supplemented with 10% foetal bovine serum, geneticin (150 μg ml^{-1}) and penicillin/streptomycin (30 μg ml^{-1}, 30 μg ml^{-1}) at 37°C in 7% CO_2. Cells were used at between passages 8 and 20 for both systems.

Inositol phosphates accumulation

The method used was a modification of the procedure reported by Brown and colleagues (1984). Briefly, transfected cells were grown to 80% confluence and washed twice in phosphate buffered saline (PBS) prior to incubating overnight in 15 ml inositol-free Ham's F-12 containing 10% dialyzed foetal bovine serum and 25 μCi [^{3}H]-*myo*-inositol. Following incorporation, the medium was aspirated and the cells washed with PBS to remove unincorporated [^{3}H]-*myo*-inositol. PBS containing ethylene diamine tetraacetic acid (EDTA, 30 μM for 5–10 min at 37°C) was used to dissociate cells from the flask. The cell suspension was centrifuged for 5 min at 500 × g at 37°C and washed three times in PBS. The cells were resuspended in inositol-free Ham's to 10^6 to 3×10^6 cells ml^{-1} M_3 and M_5, respectively. Reactions were performed in triplicate in tubes containing 300 μl final reaction volume. Cell suspension (240 μl) was added to 30 μl antagonist or vehicle for pre-equilibration at 37°C for 20 min. The reaction was initiated with the addition of 30 μl agonist or vehicle, containing LiCl (final concentration 10 mM). Tubes were then gently mixed and placed in a 37°C bath for 5 or 10 min for M_5 and M_3 cells, respectively. Reactions were terminated by the addition of 50 μl ice-cold perchloric acid (20%). Tubes were allowed to sit in an ice-water bath for 20 min and samples were then neutralized with 160 μl KOH (1 M) and vortex-mixed. Samples were then diluted with the addition of 2 ml Tris-HCl (50 mM, pH 7.5) and decanted onto disposable columns containing 1 ml Dowex AG 1 × 8, chloride form (1 : 1, w v^{-1}) slurry which had been washed with 5 ml distilled H_2O. Columns were then washed with 20 ml distilled H_2O and the eluate discarded. [^{3}H]-Inositol phosphates (InsPs) were eluted with 3 ml HCl (1 M) into scintillation vials containing 15 ml Ready-Safe liquid scintillation cocktail.

Data analysis

Accumulated [^{3}H]-InsPs were measured by liquid scintillation spectroscopy using a Packard 1900TR (using standard [^{3}H]-window settings) and expressed as disintegration per minute (DPM). DPM values were imported into Microsoft Excel for determination of mean and standard deviation values for each triplicate. Iterative, nonlinear curve-fitting methods using Kaleidagraph software were used to fit data to the general logistic functions: Effect = basal + E_m. $A^{nH}/(A^{nH} + [A]_{50}{}^{nH})$ for

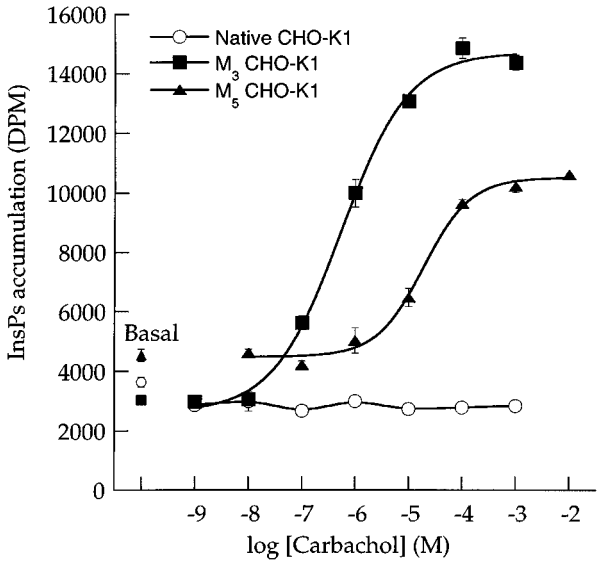

Figure 1 Carbachol-stimulated [^{3}H]-InsPs accumulation in native CHO-K1 cells and those expressing human recombinant muscarinic M_3 and M_5 receptors. Data shown are from single experiments performed in triplicate. Within-experiment means and standard deviations are shown.

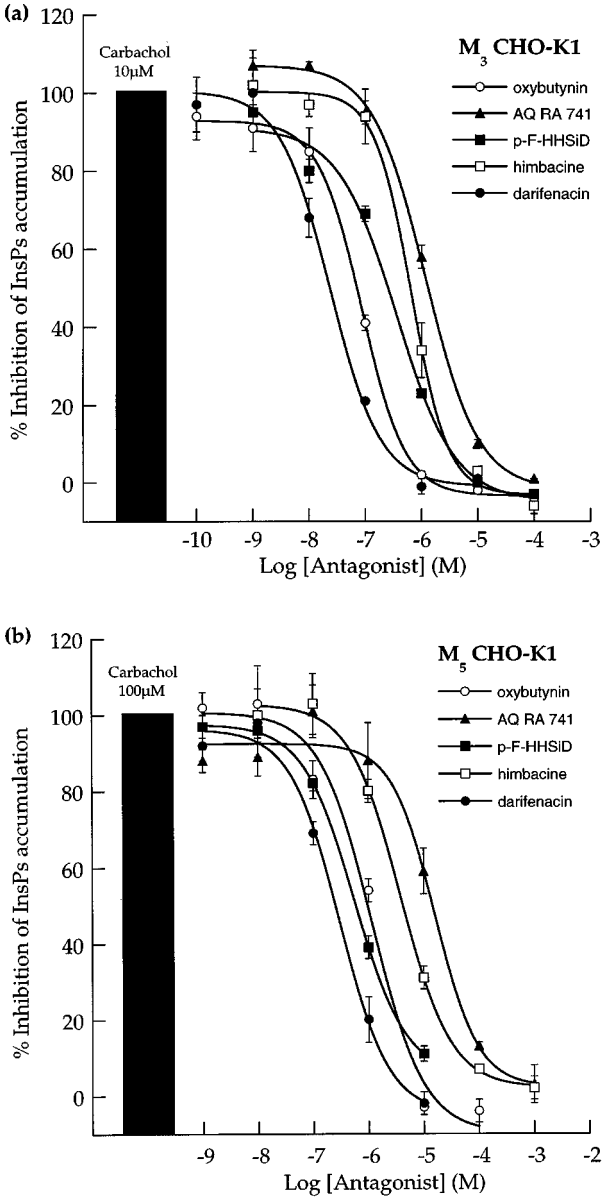

Figure 2 Inhibition of carbachol-stimulated [^{3}H]-InsPs accumulation in CHO-K1 cells expressing human recombinant muscarinic M_3 (a) and M_5 (b) receptors. Responses were obtained to a single concentration of carbachol alone and after equilibration with increasing concentrations of antagonist (20 min antagonist equilibration, 10 and 5 min agonist stimulation for M_3 and M_5, respectively). Data are from representative experiments performed in triplicate. Within-experiment means and standard deviations are shown.

agonist stimulation curves and, effect = basal + E_m − (E_m. B^{nH}/(B^{nH} + $[B]_{50}{}^{nH}$)) for antagonist inhibition curves, in order to establish agonist EC_{50} ($[A]_{50}$) antagonist EC_{50} ($[B]_{50}$), maxima (E_m) and Hill slopes (nH) for each curve. EC_{50} being the concentration of agonist or antagonist producing a half maximal effect. Apparent affinity values of antagonists (pK_B) were calculated using the Leff & Dougall (1993) modification of the Cheng-Prusoff equation (Cheng & Prusoff, 1973) such that $K_B = [B]_{50}/((2 + ([A]/[A]_{50})^{nH})^{1/nH} - 1)$. All values quoted are the mean ± s.e.mean of 3–4 individual experiments each performed in triplicate, unless otherwise stated.

Materials

Ham's F-12 nutrient medium, phosphate buffered saline, geneticin (G418), foetal bovine serum (qualified and dialyzed), penicillin/streptomycin and versene (EDTA) were obtained from Gibco (Gaithersburg, MD, U.S.A.). *Myo*-[2-^{3}H] inositol (10–20 Ci mmol^{-1}) was obtained from Amersham (Arlington Heights, IL, U.S.A.). Disposable plastic columns (CC-09-M) were obtained from E&K Scientific Products (Saratoga, CA, U.S.A.). Carbachol was obtained from Research Biochemicals International (Natick, MA, U.S.A.). Bulk chemicals and reagents were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). All other compounds were synthesized by Medicinal Chemistry, Neurobiology Unit, Roche Bioscience (Palo Alto, CA, U.S.A.). Chinese hamster ovary cells (CHO-K1) expressing human muscarinic M_3 and M_5 receptor proteins were kindly prepared by Mr Steve Delgado in the laboratory of Dr David Chang, Center for Biological Research, Roche Bioscience (Palo Alto). Kaleidagraph Software was purchased from Synergy Software (Reading, PA, U.S.A.). Ready-Safe liquid scintillation cocktail was purchased from Baxter Scientific (McGraw Park, IL, U.S.A.). Himbacine was kindly provided by Professor Taylor (University of New South Wales, Australia). AQ-RA 741 was a generous gift from Boehringer Ingelheim Pharmaceuticals Inc., U.S.A. Darifenacin and zamifenacin were generously provided by Pfizer Central Research (Kent, England). All drugs were prepared using distilled water, with the exception of darifenacin and zamifenacin, these were prepared in DMSO (10% in 10^{-2} M stock solution). All dilutions were prepared using distilled water.

Results

Carbachol-stimulated [^{3}H]-InsPs accumulation

Preliminary studies investigating the time-course of carbachol-stimulated [^{3}H]-InsPs accumulation indicated that a 10 min and 5 min agonist exposure time was within the linear portion of the [^{3}H]-InsPs accumulation curve in the CHO-K1 M_3 and M_5 receptor systems, respectively (data not shown). These,

Table 1 Antagonist affinity estimates derived from InsPs studies compared with Tris-Krebs binding data at muscarinic M_3 receptors

Antagonist	*pK_i	pK_B	*s.e.mean*	*Hill slope*	*s.e.mean*	n
Atropine	9.5	8.6	0.1	1.1	0.1	4
Pirenzepine	6.8	6.4	0.1	1.0	0.1	4
Methoctramine	6.1	6.8	0.1	1.1	0.3	3
AQ-RA 741	7.2	7.1	0.1	0.9	0.1	3
Himbacine	6.9	7.2	0.1	1.1	0.1	3
4-DAMP	9.3	8.8	0.1	1.1	0.1	4
p-F-HHSiD	7.5	7.6	0.1	1.0	0.2	3
Darifenacin	8.9	8.9	0.1	0.9	0.1	3
Zamifenacin	7.9	7.9	0.1	1.1	0.1	3
Tolterodine	8.5	8.5	0.3	1.6	0.4	4
Oxybutynin	8.7	8.4	0.2	1.1	0.1	4

*Binding affinity estimates are taken from Eglen *et al*. (1997) with the exception of AQ-RA 741 which is taken from Dörje *et al*. (1991), and oxybutynin which is unpublished data provided by D.N. Loury and D.W. Bonhaus where $n = 6$ (Roche Bioscience, Palo Alto). n = number of individual experiments each performed in triplicate.

Table 2 Antagonist affinity estimates derived from InsPs studies compared with Tris-Krebs binding data at muscarinic M_5 receptors

Antagonist	*pK_i	pK_B	*s.e.mean*	*Hill slope*	*s.e.mean*	n
Atropine	9.1	8.7	0.1	1.0	0.2	3
Pirenzepine	6.9	6.4	0.1	1.0	0.2	4
Methoctramine	6.4	6.3	0.2	1.0	0.1	3
AQ-RA 741	6.1	6.1	0.2	0.8	0.2	4
Himbacine	6.1	6.3	0.2	0.8	0.2	4
4-DAMP	8.9	8.6	0.2	1.2	0.3	4
p-F-HHSiD	6.7	6.6	0.1	1.4	0.4	3
Darifenacin	8.1	7.7	0.1	0.9	0.2	3
Zamifenacin	7.4	7.6	0.1	1.2	0.1	3
Tolterodine	8.6	8.6	0.2	0.9	0.1	3
Oxybutynin	7.6	6.6	0.1	0.9	0.1	3

*Binding affinity estimates are taken from Eglen *et al*. (1997) with the exception of AQ-RA 741 which is taken from Dörje *et al*. (1991), and oxybutynin which is unpublished data provided by D.N. Loury and D.W. Bonhaus where $n = 6$ (Roche Bioscience, Palo Alto). n = number of individual experiments each performed in triplicate.

therefore, provided appropriate conditions for evaluating antagonist affinity estimates.

Carbachol produced a 6.5 ± 0.4 fold stimulation of inositol phosphates accumulation with a potency (pEC_{50}) of 5.9 ± 0.1 in cells expressing muscarinic M_3 receptors (mean $\pm$ s.e.mean of 11 experiments performed in triplicate). In cells expressing muscarinic M_5 receptor, carbachol produced a 2.9 ± 0.2 fold stimulation of inositol phosphates accumulation, with a potency (pEC_{50}) of 4.8 ± 0.1 (mean $\pm$ s.e.mean of 13 experiments performed in triplicate). No carbachol-stimulated accumulation of inositol phosphates was detected in untransfected CHO-K1 cells (Figure 1). The CHO-K1 cells expressed the M_3 receptor protein at a density of 6075 fmol mg^{-1} protein, which was 10 fold higher than the density of M_5 receptor expression (600 fmol mg^{-1} protein).

Given the relatively low potency of carbachol in cells expressing M_5 receptors, several muscarinic agonists were evaluated in order to establish whether a more potent agonist could be identified. However, there was no difference in either the potency or the magnitude of maximal [^{3}H]-InsPs accumulation induced by (+)-cis-dioxolane ($pEC_{50} = 5.2$), carbachol ($pEC_{50} = 4.7$), or oxotremorine M ($pEC_{50} = 4.6$). In contrast, pilocarpine produced no detectable [^{3}H]-InsPs accumulation over the concentration range studied (1 nM – 10 mM).

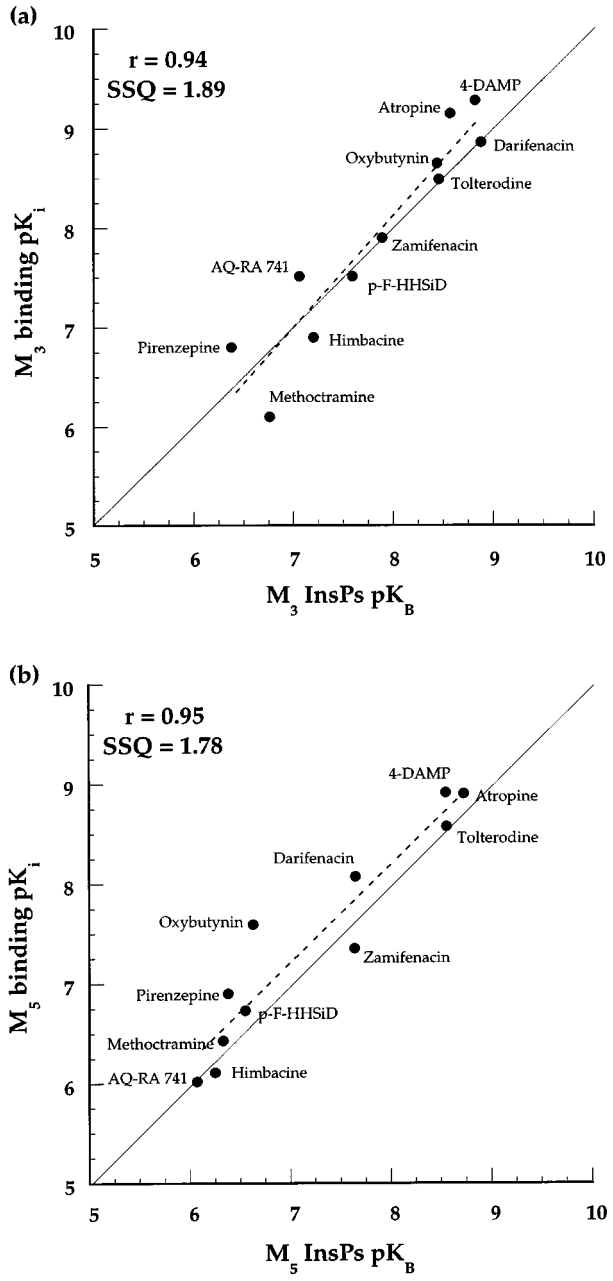

Figure 3 Correlation plots showing the relationship of binding affinity data (pK_i) generated at human recombinant muscarinic M_3 (a) and M_5 receptors (b) expressed in CHO-K1 cells with functional affinity data (pK_B) generated in the present study (see Tables 1 and 2 for source of these data). The solid line represents the line of identity, SSQ = sum of squares of differences between affinity estimates ($\Sigma(x-y)^2$), while the dotted line represents the correlation, where r = correlation coefficient.

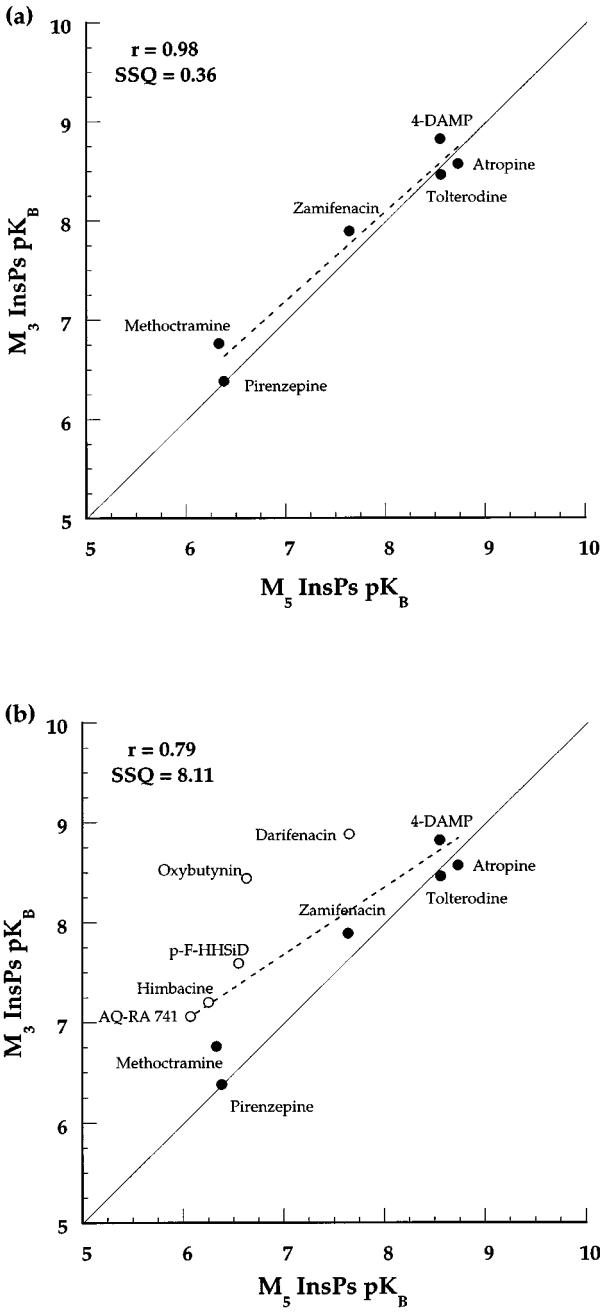

Figure 4 Correlation plots showing the relationship of functional affinity data (pK_B) of the human recombinant M_3 with the M_5 muscarinic receptor (see Tables 1 and 2 for source of these data). The solid line represents the line of identity, SSQ = sum of squares of differences between affinity estimates ($\Sigma(x-y)^2$), while the dotted line represents the correlation, where r = correlation coefficient.

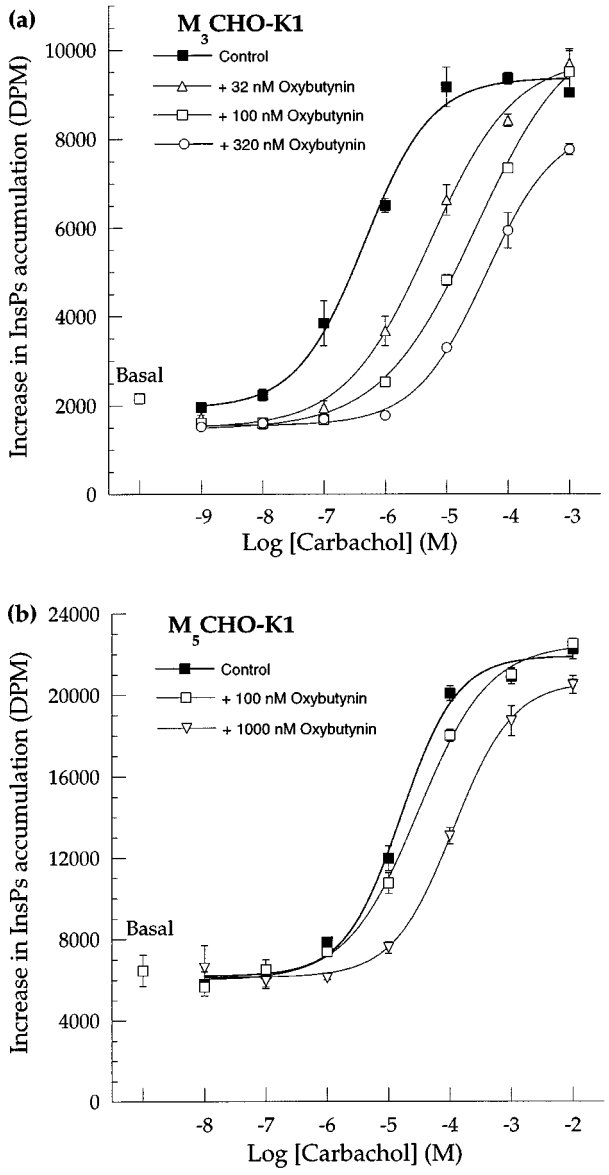

Figure 5 Inhibition of carbachol-stimulated [^{3}H]-InsPs accumulation in CHO-K1 cells expressing human recombinant muscarinic M_3 (a) and M_5 (b) receptors. Carbachol concentration-effect curves were performed in the absence and presence of oxybutynin as indicated. Data are from representative experiments performed in triplicate. Within-experiment means and standard deviations are shown.

Antagonist-induced inhibition of carbachol-stimulated [^{3}H]-InsPs accumulation

Concentrations of 10 μM and 100 μM carbachol were selected as approximately EC_{90}s against which inhibition curves to antagonists were generated in the CHO-K1 M_3 and M_5 receptor systems, respectively. All antagonists produced concentration-dependent inhibition of carbachol-stimulated [^{3}H]-InsPs accumulation (Figure 2) from which IC_{50} values and slopes were determined for estimation of affinities (pK_B; see Leff & Dougall, 1993). Antagonists had no effect on basal [^{3}H]-InsPs levels (data not shown). Tables 1 and 2 show the antagonist affinity estimates established in the present study at the M_3 and M_5 subtypes, respectively. These data were compared with those obtained previously from radioligand binding studies in similar recombinant cells.

Figure 3 shows a significant correlation between the affinity estimates previously reported from binding studies and the estimates established in the present study. (M_3: $\sum(x-y)^2=1.89$, $r=0.94$, pK_i vs pK_B and M_5: $\sum(x-y)^2=1.78$, $r=0.95$, pK_i vs pK_B). Figure 4 shows the correlation between the antagonist affinity estimates generated in the present study at the M_3 and M_5 receptors. This demonstrates the similarity in the antagonist affinity profile of these two subtypes but also highlights those antagonists (b), which are able to discriminate between these subtypes.

Oxybutynin-induced shifts in carbachol concentration-effect curves

In cells expressing the M_3 subtype oxybutynin produced concentration-dependent rightward shifts in the concentration-effect curves to carbachol (Figure 5a) yielding sufficient data for a Schild plot, with a slope not significantly different from unity (1.0 ± 0.1) and an antagonist affinity of 8.4 ± 0.2 ($n=3$). In cells expressing the M_5 subtype oxybutynin also produced a rightward shift in the concentration-effect curve to carbachol, but because of the low potency of carbachol, insufficient data could be generated for Schild analysis (Figure 5b). Nevertheless, the apparent affinity estimate generated from the shift by a single concentration of oxybutynin was 6.4 ± 0.2 ($n=3$). These values for oxybutynin at the M_3 and M_5 receptors are in good agreement with the estimates established using antagonist inhibition curves (pK_B; see Leff & Dougall, 1993) in Tables 1 and 2, respectively.

Discussion

The present study has compared functional affinity estimates of several muscarinic antagonists at muscarinic M_3 and M_5 receptors expressed in CHO-K1 cells. These data demonstrate a good correlation between the functional affinity estimates obtained herein and binding affinity estimates previously established in these cells (Buckley *et al*., 1989; Dörje *et al*., 1991; Bolden *et al*., 1992; Lazareno & Birdsall, 1993; Eglen *et al*., 1997). These data also highlight the similarity in the pharmacology of these two receptor subtypes (Choppin *et al*., 1998). Reever *et al*. (1997) previously reported that AQ-RA 741 and AF-DX 384, are able to discriminate between M_3 and M_5 receptor subtypes. In the present study, we have confirmed these findings with AQ-RA 741 and in addition we have identified additional compounds, himbacine, p-F-HHSiD, darifenacin, and oxybutynin, that are also able to discriminate between muscarinic M_3 and M_5 receptors. However, all these compounds display higher affinity for the M_3 subtype and as yet no compound has been identified which can discriminate between M_3 and M_5 receptors by displaying a higher affinity at the M_5 receptor subtype.

Agonist studies

In the present study, an initial determination of the functional antagonist affinity estimates was carried out in the CHO-K1 M_3 cells by performing shifts of the carbachol-stimulated [^{3}H]-InsPs accumulation using three different concentrations of antagonists. This enabled Schild analysis to be performed and a pA_2 value to be determined for each antagonist (Arunlakshana & Schild, 1959). However, when the response to carbachol was evaluated in the CHO-K1 M_5 receptor system the potency was 10 fold lower, (4.8 ± 0.1 versus 5.9 ± 0.1) such that shifts to only a single antagonist concentration could be

achieved. The reasons for the difference in carbachol potency may reflect differences in the density of receptor protein, which was 10 fold greater in the CHO-K1 M_3 system as compared to the M_5 system. The potencies of other muscarinic agonists were evaluated in the CHO-K1 M_5 system in an effort to identify a more potent agonist, which might enable Schild analysis to be performed in the CHO-K1 M_5 cell system. However, there was little difference in the potency or the magnitude of maximal [^{3}H]-InsPs accumulation induced by (+)-cis-dioxolane, carbachol or oxotremorine M, and pilocarpine was unable to stimulate [^{3}H]-InsPs accumulation. Therefore, since it was not technically feasible to generate curve shifts with multiple concentrations of antagonist in the CHO-K1 M_5 system, an alternative approach was used. A concentration of carbachol was selected which caused approximately 90% of the maximum [^{3}H]-InsPs accumulation (100 μM) and antagonist-inhibition curves were performed which enabled an affinity estimate to be generated based on the Leff & Dougall (1993) derivation of the Cheng-Prusoff equation. The experiments in the CHO-K1 M_3 receptor system were then repeated using the same approach, in order to facilitate comparison between these two cell systems. There was, however, good agreement between antagonist affinities established in the CHO-K1 M_3 receptor systems using Schild analysis and those established using the method of Leff & Dougall (1993). Given the difference between binding and functional affinity estimates for oxybutynin in these studies at the CHO-K1 M_5 receptor system, both the Schild and Leff-Dougall approaches were used with oxybutynin to corroborate the lower than expected affinity estimate obtained.

Antagonist studies

The antagonist apparent affinity estimates obtained in the present study compare well with binding affinity estimates, with two notable exceptions. Atropine appeared to be approximately 10 fold less potent at M_3 receptors and oxybutynin was approximately 10 fold less potent at the M_5 receptor, compared to the binding affinity estimates for these two antagonists. The low affinity of atropine might be explained by endogenous atropine esterase activity associated with the CHO-K1 system, however, a series of experiments performed in the presence of methyl butyrate (100 μM to inhibit atropine esterase activity), failed to improve the affinity estimate of atropine (data not shown). Given that the binding affinity estimates were obtained in membrane preparations at 20°C and the functional affinity estimates obtained in whole cells at 37°C it is perhaps not surprising to observe some differences. However, the correlation between functional and binding affinity estimates was in general very good as reflected by the correlation plots.

It is unclear why oxybutynin displayed lower affinity for muscarinic M_5 receptors in the present study than that predicted by binding studies. However, the increased M_3 over M_5 receptor selectivity resulting from these data may relate to the >10 fold disparity between the muscarinic M_3 and M_5 receptor densities in the CHO-K1 cell clones. Interpretation of these data would be strengthened if antagonist affinities were established at these receptor subtypes when expressed at similar densities, however, these studies were not undertaken. Therefore, while the implication from the current finding, using InsPs derived affinity estimates, is an improved discrimination of oxybutynin, between muscarinic M_3 and M_5 receptors, the utility of oxybutynin as a discriminator between M_3 and M_5 receptors should be confirmed in cell systems where receptor expression levels are comparable. This could be achieved using alternative clonal cell lines with similar expression levels or receptor alkylation techniques could be undertaken to reduce the number of M_3 receptors to a level comparable to the M_5 CHO-K1 system.

The present study highlights the need for a range of antagonists to be used in the characterization of muscarinic receptor subtypes in order that an unambiguous characterization may be achieved. Characterization of a new receptor subtype is often performed using a very simplistic approach. The non-selective antagonist atropine is included, to determine the muscarinic nature of the receptor, and then a single antagonist selective for M_1, M_2 and M_3 receptor subtypes (e.g.: pirenzepine – M_1 selective, methoctramine – M_2 selective and 4-DAMP – M_3 selective antagonist) are included. As is evident from Figure 4a such a characterization would not adequately discriminate between M_3 and M_5 receptors. Figure 4b identifies the compounds that would improve the characterization and enable discrimination between these two subtypes. Reevers *et al*. (1997), have previously reported that AQ-RA 741 shows some 10 fold lower affinity for M_5 receptors than for the M_3 subtype, an observation which is confirmed by the present study. In addition, however, the present study identifies himbacine as having lower affinity for the M_5 subtype than for the M_3 subtype. Darifenacin and p-F-HHSiD, both M_3 selective antagonists, provide advantages over the other M_3 antagonists, 4-DAMP and zamifenacin, in that they have lower affinity at the M_5 receptor subtype. Additionally, oxybutynin with lower affinity at the M_5 subtype compared to the M_3, provides additional separation. However, no compound was identified in the present study as having higher affinity for the M_5 subtype over the M_3 subtype and clearly such a compound would be a most useful tool.

Implications for receptor characterization

These observations raise questions with regard to responses reportedly mediated by muscarinic M_3 receptors, particularly when the characterization was based on a limited number of antagonists of limited selectivity. In particular ocular tissues, such as iris and ciliary smooth muscle appear to be candidates for a peripheral M_5 receptor mediated response. Thus, immunoprecipitation studies in human iris-ciliary body identified the presence of M_5 as well as M_3 receptors (Gil *et al*., 1997). Bognar *et al*. (1989; 1992) performed an extensive characterization of the muscarinic receptor mediating contractions of the rabbit iris sphincter. These authors reported the presence of a muscarinic receptor with an M_3-like pharmacology, but which displayed low affinity for p-F-HHSiD and secoverine, suggesting the possibility of an M_5 subtype. However, recent studies in rabbit iris sphincter muscle from our group (Choppin *et al*., 1998) have not confirmed the low affinity of p-F-HHSiD and secoverine in this preparation. Consequently it was concluded that these data are more consistent with M_3 receptors mediating contraction of this tissue. Although most evidence to date suggests M_5 receptors are confined to the CNS, there is evidence that they may be found on blood cells, specifically, microglial/macrophage cells express mRNA for the M_5 receptor subtype (Ferrari-DiLeo & Flynn, 1995). However, these transcripts were associated with brain tissue and it is unclear as to whether peripheral/circulating macrophages also express the M_5 receptor subtype. Circulating lymphocytes possess muscarinic receptors, but the subtype(s) has not yet been identified (Zalcman *et al*., 1981).

The recent generation of gene-targeted mice, lacking the M_5 receptor, has begun to shed light on the role of this receptor subtype (Yeomans *et al*., 1998). However, the development of

M_5 selective ligands and antibodies remains an important requirement for the identification of tissues and/or cell lines that express the native M_5 receptor protein. The present study has identified a number of muscarinic antagonists that, while lacking selectivity for the M_5 receptor subtype, should provide useful tools for separating M_5 receptors from M_3 receptors, because of their low affinity for the M_5 receptor subtype. High affinity M_5 selective ligands are currently not available. Nevertheless, through a combination of classical pharmacology, using those compounds that discriminate M_3 and M_5 receptors, and molecular biological techniques that demonstrate the presence of message or protein for the M_5 receptor subtype, it should be possible to identify a functional role for endogenous M_5 receptors.

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