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Pharmacological basis for functional selectivity of partial muscarinic receptor agonists

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Abstract

Muscarinic receptor agonists activate phosphoinositide hydrolysis and adenylate cyclase in Chinese hamster ovary cells transfected with cDNAs encoding the human muscarinic m1 and m_3 receptors. Whereas carbachol activates similarly both receptor subtypes, 4-[3-chlorophenyl-carbamoyloxy]-2-butynyltrimethyl ammonium chloride (McN-A-343) preferentially activates the m1 subtype over m3, in regard to both phosphoinositide hydrolysis and adenylate cyclase activity. On the other hand, oxotremorine activates phosphoinositide hydrolysis to a similar extent in both cell lines, but it activates preferentially adenylate cyclase in m1 versus m3 receptor expressing cells. Relative to carbachol, both McN-A-343 and oxotremorine activate preferentially phosphoinositide hydrolysis over adenylate cyclase in both cell lines. Prolonged incubation of cells with either carbachol, McN-A-343, or oxotremorine down-regulated the m1 receptors. This was accompanied by a parallel decrease in adenylate cyclase activity, whereas phosphoinositide hydrolysis remained relatively high. Inactivation of the receptors by alkylation with acetylethylcholine mustard, or by blocking with atropine, reduced carbachol-stimulated adenylate cyclase activity more effectively than carbachol-induced phosphoinositide hydrolysis in both m1 and m3 receptor expressing cells. These findings imply that the receptor reserve in these cell lines is greater for phosphoinositide hydrolysis response than for adenylate cyclase response. Yet, the receptor reserve for each of these responses is similar in both m1 and m3 receptor expressing cells. Since the binding affinities of McN-A-343 and of oxotremorine to m1 and m3 receptors are very similar, and since both cell lines contain similar amounts of spare receptors, we propose that the preferential activation of muscarinic m1 over m3 receptor by partial agonists is related to differences in the abilities of the two receptor subtypes to undergo conformational changes following agonist binding. This hypothesis is supported by results showing that the muscarinic m1 but not m3 receptor exhibits two affinity states in a competition binding assay.

Keywords: Muscarinic receptor; Acetylcholine; Spare receptor; Adenylate cyclase; Selectivity

1. Introduction

Five subtypes of the muscarinic acetylcholine receptor have been established by molecular cloning (Kubo et al., 1986; Bonner et al., 1987). The pharmacological characterization of these receptor subtypes rests mainly on the use of antagonists which demonstrate only limited selectivity (Eglen and Whiting, 1990; Dorje et al., 1991; Schwarz et al., 1993). According to pharmacological characterization, the m1, m2 and m3 receptor gene products correspond to the pharmacologically defined M_1, M_2 and M_3 receptors, respectively. The two additional subtypes (m4 and m5) share some pharmacological features with either the M_1, M_2 or the M_3 receptor subtypes. In contrast to antagonists, agonists seem to discriminate poorly among the muscarinic acetylcholine receptor subtypes (Schwarz et al., 1993). In this context, we have previously reported (Pittel et al., 1990) that 4-[3-chlorophenyl-carbamoyloxy]-2-butynyltrimethyl ammonium chloride (McN-A-343) and oxotremorine, respectively, demonstrate M_1 and M_2 receptor selectivity when tested in cerebrocortical membranes

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(rich in M_1 receptors) and cerebellar membranes (rich in M_2 receptors). However, it cannot be ruled out that tissue rather than receptor specificity may account for the differential binding observed with these two agonists.

The identification of multiple subtypes of muscarinic acetylcholine receptor has spurred the search for agonists with selectivity for a given receptor subtype. In particular, selective agonists for the muscarinic m1 receptor are of special interest, since such agents are of potential use in the therapy of senile dementia of Alzheimer's type (Fisher et al., 1989; Gray et al., 1989; McKinney and Coyle, 1991; Whitehouse, 1993). Consequently, in recent years there have been intense efforts in the development of centrally active muscarinic receptor agonists selective for the M_1/m1 subtype (Fisher et al., 1989, 1991; Hargreaves et al., 1992; Shapiro et al., 1992). The pharmacological evaluation of such potentially selective agonists requires a biological system that will enable to evaluate selectivity reliably.

Cell lines transfected with each of the muscarinic acetylcholine receptor cDNA and expressing a single receptor subtype constitute a useful tool for investigating the selectivity of muscarinic receptor agonists at both the recognition and the functional levels (Wang and El Fakahany, 1993). Recently we have demonstrated in such transfected cells that some agonists, like (±)-*cis*-2-methyl-*spiro*(1,3-oxathiolane-5′,3′) quinuclidine (AF102B), activate the m1 but not the m3 receptor subtype, and that they also differentially activate distinct signal transduction pathways (Gurwitz et al., 1994). By comparison, non-selective ligands, such as carbachol, activate simultaneously several signal transduction pathways mediated by a given muscarinic acetylcholine receptor subtype. For example, in cells expressing the rat muscarinic m1 receptor, carbachol stimulates phosphoinositide hydrolysis, arachidonic acid release and adenylate cyclase activity (Gurwitz et al., 1994).

The molecular basis for the discrete activation of signal transduction pathways by selective agonists is unknown. Moreover it is not clear whether the preferential activation of the transduction signalling of the m1 receptor versus the m3 receptor, is due to a differential recognition of the ligand by the specific receptor subtype as has been suggested by Hu and El-Fakahany (1990), or due to differences in the efficacy of the selective ligands in activating certain signal transduction pathways (Fisher et al., 1993a, b). In the present study we addressed these questions by employing Chinese hamster ovary (CHO) cells transfected with the human cDNAs for either the m1 or the m3 receptor subtypes. We examined binding parameters and functional selectivity (in activating adenylate cyclase and phosphoinositide hydrolysis) of the putative muscarinic m1 receptor agonist, McN-A-343, and the putative m2 receptor agonist, oxotremorine (Pittel et al., 1990). We suggest that the distinctive activation of signal transduction pathways within a single receptor subtype by selective agonists, is accounted for by differences in spare receptors available for phosphoinositide and for adenylate cyclase pathways. On the other hand, the differential activation of the muscarinic m1 versus m3 receptor by selective agonists is most probably related to differences between the abilities of the two receptor subtypes to undergo conformational changes necessary for the induction of the relevant signal transduction mechanisms.

2. Materials and methods

2.1. Cell cultures

CHO cells stably transfected with the human muscarinic m1 receptors (Hm1 cells) and the human muscarinic m3 receptors (Hm3 cells) were obtained as previously described (Buckley et al., 1989). Cells were grown under an atmosphere of 5% CO_2 and 95% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 200 μg/ml geneticin. Expression of muscarinic acetylcholine receptors in cells maintained under these conditions, was stable for at least 90 passages. For experiments a split of 1 : 6 was performed following trypsinization and cells were grown in either 6-well or 24-well plates in growth medium without geniticin.

2.2. Binding studies

To determine the number of the muscarinic acetylcholine receptors expressed by the cells, we have measured the binding of [^{3}H]*N*-methylscopolamine (73.1 Ci/mmol from NEN, Du-Pont) at a concentration of 1 nM. Binding studies in intact cells were performed in 24-well plates. The [^{3}H]*N*-methylscopolamine was added to each well in 0.5 ml DMEM containing 1 mg/ml bovine serum albumin and 20 mM Hepes buffer pH 7.4. Following incubation of 60 min at 37°C and four rapid washes (with 1 ml DMEM each), cells were lysed in 1% sodium dodecylsulfate and the lysate transferred to scintillation vials for radiometric determination of the bound ligand. Non-specific binding was measured in the presence of 10 μM atropine and was generally less than 1% of the specific binding. We found that 1 nM [^{3}H]*N*-methylscopolamine completely saturated the muscarinic binding sites and gave a reliable indication of the B_{max} values. When binding was measured with membrane preparations, cells were collected by scraping, homogenized in 50 mM Tris buffer, pH 7.4, containing 2 mM ethylenediamine tetraacetic acid (sodium salt), centrifuged at 30000 × g for 10 min, resuspended in the Tris buffer and assayed for ligand binding by incubation (20 μg membrane protein per test tube) with 1 nM [^{3}H]*N*-methylscopolamine for 1 h at 37°C. The membranes were filtered and washed over GF/B glass filter (Whatman)

using a cell harvester (Brandell) and the bound ligand determined radiometrically.

2.3. Phosphoinositide hydrolysis

Cells grown in 6-well plates were labeled overnight with 1 μCi/ml [^{3}H]inositol (NEN, Du Pont) in growth medium. The cells were then washed with DMEM and incubated for 30 min at 37°C in DMEM containing 10 mM LiCl and 20 mM Hepes pH 7.4, as well as the tested agonist, which was added 10 min after the initiation of the incubation. Incubation was terminated by scraping the cells into 0.5 ml water and total inositol phosphates were analyzed by ion exchange column chromatography as described by Berridge (1983).

2.4. Adenylate cyclase activity in intact cells

Cells in 24-well plates were labeled for 3 h with 2 μCi/ml [^{3}H]adenine (Rotem Industries, Israel) in growth medium, washed and incubated for an additional 20 min in DMEM containing 20 mM Hepes, 0.1 mM of the phosphodiesterase inhibitor, isobutyl 1-methylxanthine and the tested agonist. The incubation was terminated by aspirating the incubation medium and addition of 0.5 ml 2.5% perchloric acid containing 0.1 mM cAMP. After 30 min at 4°C the cell extracts were collected, titrated with KOH to ca. pH 7 and potassium perchlorate precipitated by centrifugation. Labeled cAMP was collected and counted following a two step column separation as described by Johnson and Salomon (1991) and modified by Vogel et al. (1993).

2.5. Receptor alkylation

Alkylation of muscarinic acetylcholine receptors in intact living cells was accomplished by incubating the cells in 24-well plates for 30 min at 37°C with various concentrations (0.1 μM to 1 mM) of acetylethylcholine azyridinium. Acetylethylcholine azyridinium was prepared immediately prior to the alkylation reaction from acetylethylcholine mustard precursor (synthesized in our laboratory at > 99% purity) as previously described (Spalding et al., 1994). After the alkylation, the cells were washed 3 times with DMEM and triplicate sets of plates were assayed in parallel for binding of [^{3}H]*N*-methylscopolamine, phosphoinositide hydrolysis and adenylate cyclase activity. Loading of the cells with [^{3}H]inositol or [^{3}H]adenine (see above) was performed prior to the alkylation procedure.

3. Results

Activation of the muscarinic acetylcholine receptor induced increases in both adenylate cyclase activity and phosphoinositide hydrolysis in Hm1 and Hm3 cells. Concentration-response curves for these transduction pathways

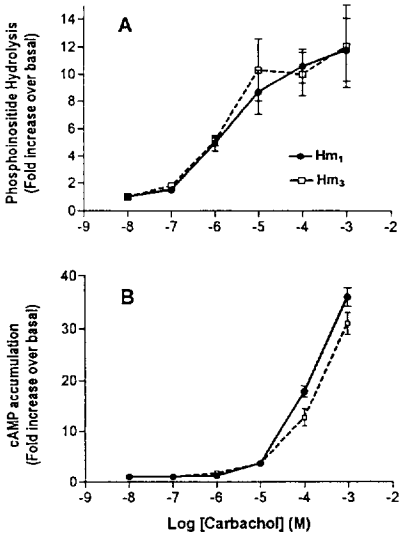

Fig. 1. Concentration-response curves for carbachol-induced phosphoinositide hydrolysis (A) and carbachol-induced adenylate cyclase activity (B) in Hm1 and Hm3 cells. Each point represents an average ± S.E.M. of three to five different experiments performed in triplicate.

are described in Fig. 1. Carbachol was equipotent in Hm1 and Hm3 cells in inducing phosphoinositide hydrolysis (Fig. 1A) and also in inducing increase in adenylate cyclase activity (Fig. 1B). However, in each of these cell lines, carbachol was more efficacious in activating phosphoinositide hydrolysis than in activating adenylate cyclase. Thus, higher concentrations of carbachol were required to activate adenylate cyclase than those needed to induce phosphoinositide hydrolysis. In fact, the concentration-response curves for the activation of phosphoinositide hydrolysis reached saturation at about 10 μM, whereas those for the activation of adenylate cyclase did not reach saturation even at 1 mM carbachol (Fig. 1). The agonists McN-A-343 and oxotremorine showed different patterns of relative efficacies. Oxotremorine was more efficacious than McN-A-343 and as efficacious as carbachol in stimulating phosphoinositide hydrolysis in Hm1 cells (Fig. 2A) but in Hm3 cells carbachol was more efficacious than oxotremorine, and oxotremorine still more efficacious than McN-A-343 (Fig. 2B). Yet, stimulation of adenylate cyclase by oxotremorine or by MCN-A-343 was more pronounced in Hm1 than in Hm3 cells (Fig. 3). By comparison, at 100 μM, McN-A-343 was more active in stimulating both phosphoinositide hydrolysis and adenylate cyclase activity in Hm1 as compared to Hm3 cells, but was less active than oxotremorine in stimulating either of these transduction pathways (Fig. 3). Similar results were obtained with a concentration of 1 mM of the agonists (data not shown).

In an attempt to examine whether the preferential activation of Hm1 versus Hm3 cells originates from differences between the affinities of McN-A-343 or oxotremorine to muscarinic m1 and m3 receptor subtypes, we carried out competition binding studies in which we used

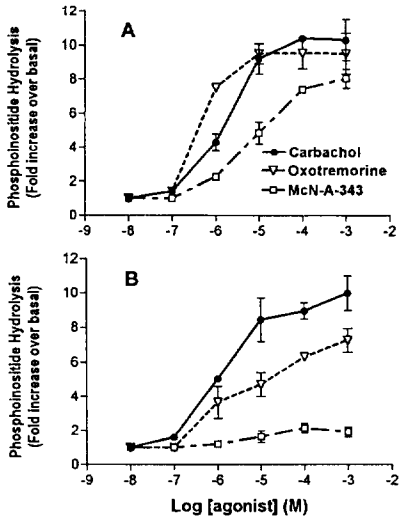

Fig. 2. Concentration-response curves of several muscarinic receptor agonists for the activation of phosphoinositide hydrolysis in Hm1 (A) and Hm3 (B) cells. Each point represents an average ± S.E.M. of two to eight different experiments performed in either duplicate or triplicate.

[^{3}H]*N*-methylscopolamine as the labeled probe. We found that oxotremorine has higher affinity than McN-A-343 for both receptor subtypes (Fig. 4) and that McN-A-343 (Fig. 4A) and oxotremorine (Fig. 4B) showed similar affinities to both muscarinic m1 and m3 receptors (K_i values for McN-A-343 in Hm1 cells = 5.0 μM and in Hm3 cells = 5.5 μM; K_i values for oxotremorine in Hm1 cells = 0.46 μM and in Hm3 cells = 0.97 μM). Therefore, the preferential activation of the muscarinic m1 versus m3 receptor by McN-A-343 and oxotremorine does not seem to be related to differences in affinities of the ligands to these receptor subtypes. In contrast to McN-A-343 and oxotremorine, carbachol (which did not show any selectivity in activating the muscarinic m1 versus m3 receptor) showed different profiles of competition binding curves for Hm1 and Hm3 cells. In Hm1 cells carbachol exhibited a competition binding curve composed of two binding sites (K_H = 0.85 μM; K_L = 228 μM), whereas in Hm3 cells carbachol displayed a single (K_i = 12.2 μM) binding site (Fig. 4C). Thus, it seems that in the presence of partial agonists, such as McN-A-343 and oxotremorine, both muscarinic m1 and m3 receptors demonstrate only a single binding site. However, in the presence of efficacious muscarinic receptor agonist such as carbachol, the m1 receptor shows two binding sites, whereas the m3 receptor shows a single binding site.

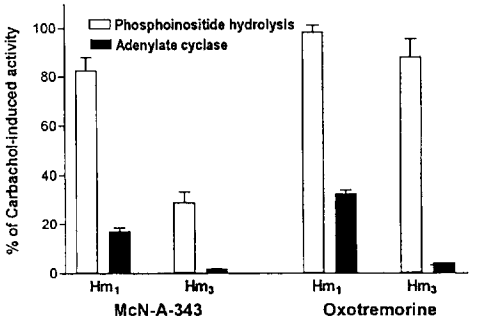

Fig. 3. Stimulation of phosphoinositide hydrolysis and adenylate cyclase activity by 100 μM McN-A-343 or 100 μM oxotremorine relative to activities induced by 100 μM carbachol. Each bar represents an average ± S.E.M. of at least three different experiments performed in triplicate.

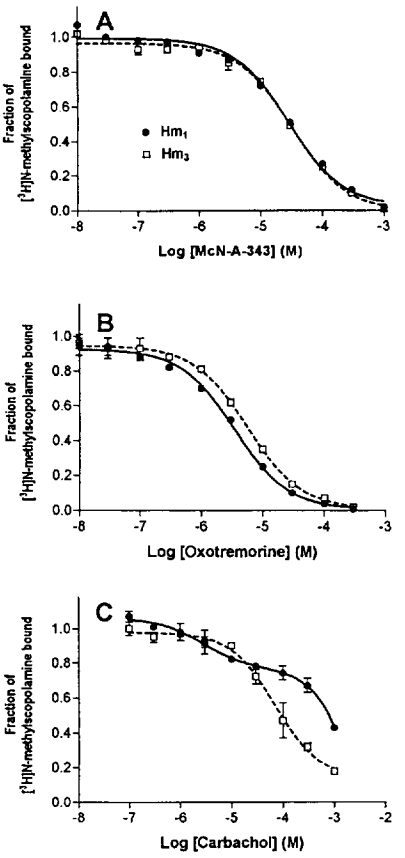

Fig. 4. Competition between [^{3}H]*N*-methylscopolamine and the non-labeled muscarinic receptor agonists, McN-A-343 (A), oxotremorine (B), or carbachol (C), for binding to intact Hm1 or Hm3 cells. The concentration of the [^{3}H]*N*-methylscopolamine was 0.5 nM. Various concentrations (as indicated) of the non-labeled ligands were added together with [^{3}H]*N*-methylscopolamine to cells grown in 24-well plates and binding was measured as described in Methods. Each point represents an average ± S.E.M. from two experiments performed in duplicate. Data are expressed as fractions of total [^{3}H]*N*-methylscopolamine bound in the absence of non-labeled ligand (defined as 1).

It is possible that non-efficacious agonists may need to occupy a larger number of receptors than more efficacious agonists in order to trigger a specific biochemical response. Therefore, we examined whether the preferential activation of the Hm1 cells over the Hm3 cells by McN-A-343 could originate from differences in the number of the receptors between these two cell lines. We found that similar amounts of [^{3}H]*N*-methylscopolamine were bound to intact Hm1 and Hm3 cells as well as to membrane preparations from these cell lines, when saturating concentration of the ligand (1 nM) was used in the binding assay (Table 1). Thus, differences in receptor density between

Hm1 cells and Hm3 cells do not seem to account for the functional selectivity described above.

The differential activation of the two second messenger pathways by McN-A-343 and oxotremorine may result from differences in the number of spare receptors for the adenylate cyclase and the phosphoinositide pathways. To examine this possibility, we reduced the number of receptors in Hm1 cells, and then examined the ability of carbachol to induce phosphoinositide hydrolysis and activation of adenylate cyclase. The number of muscarinic m1 receptors was reduced by prolonged incubation with an agonist, resulting in receptor down-regulation (Hu et al., 1991; Maloteaux and Hermans, 1994). Fig. 5A shows that exposure of Hm1 cells to 0.1 mM carbachol for 2 h decreased the number of [^{3}H]*N*-methylscopolamine binding sites by ca. 20%. Under these conditions, a similar decrease was observed in adenylate cyclase activity. By contrast, under the same conditions phosphoinositide hydrolysis was not reduced. These results were even more pronounced after 24 h incubation with carbachol. Under these conditions a profound decrease (ca. 75%) in the number of muscarinic m1 receptors was observed, and adenylate cyclase activity was reduced to a much larger extent than phosphoinositide hydrolysis (Fig. 5A). Similar results were obtained when receptor down-regulation was induced by preincubation of Hm1 cells with 0.1 mM McN-A-343 (Fig. 5B) or with oxotremorine (Fig. 5C). These results suggest that Hm1 cells have apparently more spare receptors for the phosphoinositide pathway than for the adenylate cyclase pathway, and that these two pathways are distinct.

Preincubation of Hm3 cells with 0.1 mM carbachol for 2 or 24 h resulted in only negligible (ca. 5%) or small (ca. 15%) receptor down-regulation, respectively. Under these conditions no decrease in phosphoinositide hydrolysis was evident in Hm3 cells preincubated with carbachol. Similar results were obtained with McN-A-343 and oxotremorine. Thus, we could not test the existence of spare receptors in Hm3 cells by down-regulating the receptors. Moreover, treatments that lead to receptor down-regulation, may influence other components of the signalling pathways (e.g. G-protein subunits). We have therefore resorted to a different method in which we partially inactivated the receptors on the cell surface by either alkylating them with acetylethylcholine azyridinium or by blocking the receptors with the antagonist atropine (which non-selectively binds to both muscarinic m1 and m3 receptors). Alkylation of the receptors with increasing concentrations of acetylethylcholine azyridinium resulted in a progressive reduction in [^{3}H]*N*-methylscopolamine binding to Hm1 and Hm3 cells. In parallel, it led to a reduction in carbachol-induced phosphoinositide hydrolysis and adenylate cyclase activity. Fig. 6 describes the reduction in carbachol-induced phosphoinositide hydrolysis and adenylate cyclase activity as a function of the fraction of the alkylated receptors (calculated from the data of the [^{3}H]*N*-methylscopolamine binding). The results clearly show that the response of adenylate cyclase to carbachol is much more sensitive to the number of the remaining [^{3}H]*N*-methylscopolamine binding sites than the phosphoinositide hydrolysis response. Both muscarinic m1 and m3 receptors were similarly affected with regard to the two responses.

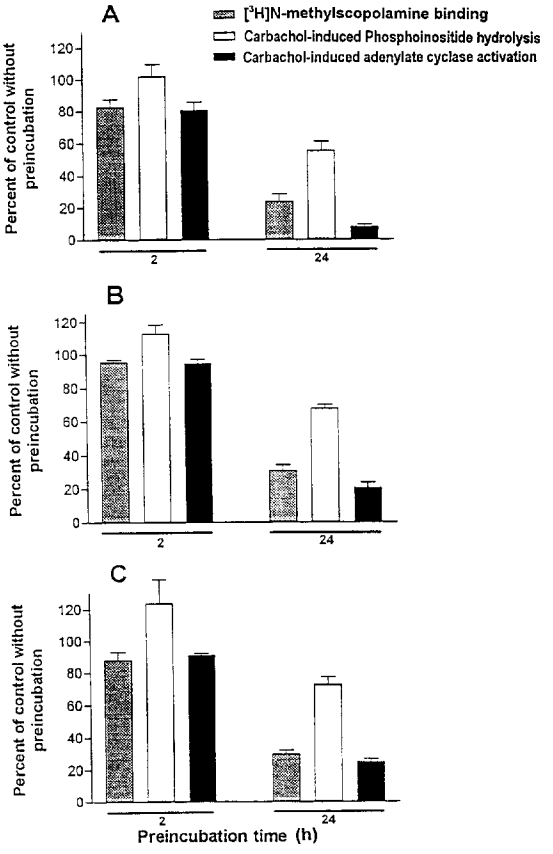

Fig. 5. Effect of prolonged incubation of Hm1 cells with carbachol (A), McN-A-343 (B), or oxotremorine (C) on [^{3}H]*N*-methylscopolamine binding, carbachol-induced phosphoinositide hydrolysis and carbachol-induced stimulation in adenylate cyclase activity. Following preincubation with the specified agonist for either 2 or 24 h, the cells were washed and tested in parallel for [^{3}H]*N*-methylscopolamine binding using 1 nM of the labeled ligand (to determine B_{max}), for phosphoinositide hydrolysis and adenylate cyclase activity in presence and absence of 100 μM carbachol. In each experiment data were normalized according activity measured in control cells (defined as 100%) which were not exposed to preincubation. Percentages of control were calculated for each experiment separately, and bars represent averages ± S.E.M. from two to three experiments.

Table 1
Number of [^{3}H]*N*-methylscopolamine binding sites in intact cells and membrane preparations from Hm1 and Hm3 cells

Cell type	[^{3}H]*N*-Methylscopolamine binding	
	Membrane preparations (fmol/mg protein ± S.E.M.) [a]	Intact cells (binding sites per cell ± S.E.M.) [b]
Hm1	1927 ± 203	767759 ± 15515
Hm3	2014 ± 156	818538 ± 19698

[a] Data represent averages from two experiments. [b] Data represent averages from five experiments.

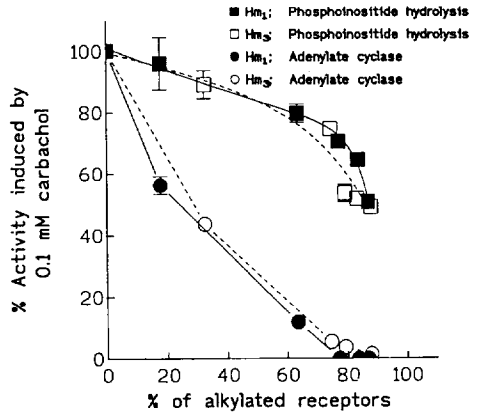

Fig. 6. Reduction in carbachol-induced phosphoinositide hydrolysis and carbachol-induced adenylate cyclase activity as a function of the degree of receptor alkylation. Receptors in Hm1 and Hm3 cells were inactivated by alkylation with 0.1 μM to 1 mM acetylethylcholine azyridinium as described in Methods. The two activities were determined in cells after alkylation and percentages of control were calculated based on the activities determined in cells that were not exposed to the alkylating agent. The activities determined at each concentration of the alkylating agent were plotted as a function of the percent of the alkylated receptors (determined by the reduction in the binding of 1.5 nM [^{3}H]*N*-methylscopolamine to parallel wells). The values are means ± S.E.M. of triplicate determinations in a representative experiment.

Thus, reduction in the number of receptors by 50% (in both Hm1 and Hm3 cells) led to a 75% decrease in carbachol-induced adenylate cyclase activity, and to less than 15% reduction in carbachol-induced phosphoinositide hydrolysis. Similar results were also observed when the m1 and m3 receptors were blocked with the reversible antagonist, atropine. As shown in Fig. 7, in both Hm1 and Hm3 cells, atropine inhibited carbachol-induced adenylate cyclase activity at much lower concentrations than those needed to inhibit carbachol-induced phosphoinositide hydrolysis. These results suggest that both Hm1 and Hm3 cells contain more spare receptors for phosphoinositide

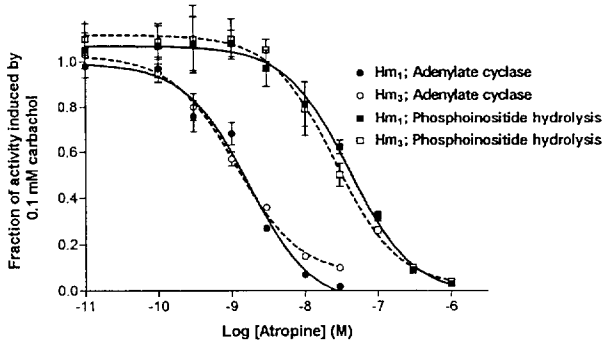

Fig. 7. Inhibition of carbachol-induced phosphoinositide hydrolysis and carbachol-induced adenylate cyclase activity by atropine in Hm1 or Hm3 cells. Atropine sulphate at various concentrations (as indicated) was added simultaneously with 100 μM carbachol and phosphoinositide hydrolysis as well as adenylate cyclase activity were measured as described in Methods. Each point represents an average ± S.E.M. from two to three experiments.

hydrolysis than for adenylate cyclase activation. Moreover, the data also suggest that similar amounts of spare receptors exist in Hm1 and Hm3 cells for each of the respective transduction pathways.

4. Discussion

In the present study we have investigated the mechanism underlying agonist selectivity in muscarinic m1 and m3 receptors. We demonstrated two phenomena of selectivity: (a) differential activation of phosphoinositide hydrolysis over adenylate cyclase in both Hm1 and Hm3 cells; (b) preferential activation of the muscarinic m1 receptor subtype over the m3 subtype by functionally selective agonists. Our results suggest that each of these functional selectivity phenomena is a consequence of a different mechanism.

As can be seen from the results, muscarinic receptor agonists activate phosphoinositide hydrolysis as well as adenylate cyclase in both Hm1 and Hm3 cells. Muscarinic activation of adenylate cyclase is not an episode limited to transfected cells, as it has been previously shown that in the olfactory bulb muscarinic acetylcholine receptors mediate increases in adenylate cyclase (Olianas and Onali, 1991). In a previous report (Gurwitz et al., 1994) we suggested that the muscarinic activation of phosphoinositide hydrolysis and the muscarinic activation of adenylate cyclase are mediated by two different G-proteins. This conclusion was based, in part, on the finding that in CHO cells expressing the muscarinic m1 receptor, carbachol activated both transduction pathways, whereas in CHO cells expressing muscarinic m3 receptor, carbachol did not activate adenylate cyclase while it activated phosphoinositide hydrolysis to the same extent as in cells expressing the muscarinic m1 receptor. These previous results were obtained with cells transfected with the rat cDNA encoding for muscarinic acetylcholine receptors. Interestingly, in CHO cells transfected with the human cDNA encoding muscarinic m3 receptor, carbachol activates both phosphoinositide hydrolysis and adenylate cyclase. The fact that both human muscarinic m1 and m3 receptors mediate stimulation of phosphoinositide hydrolysis and of adenylate cyclase activity, makes the cell lines transfected with these human receptor subtypes adequate for studying the pharmacological basis underlying the selective activation of both transduction pathways. Our present findings that the concentrations of carbachol which are needed to activate adenylate cyclase in both cell lines are much higher than those needed to activate phosphoinositide hydrolysis, are consistent with our previous conclusion (Gurwitz et al., 1994) that activation of phosphoinositide hydrolysis and stimulation of adenylate cyclase are supported by two different mediators. Also consistent with this conclusion is our finding that carbachol-induced phosphoinositide hydrolysis and carbachol-induced increase of adenylate cyclase

activity are differentially affected when the receptors are down-regulated. These results are contrary to the suggestion by Felder et al. (1989) who proposed that stimulation of adenylate cyclase is mediated via phosphoinositide hydrolysis. In fact, similar to our findings for the muscarinic acetylcholine receptor, it has been recently reported that the β-adrenoceptor is also coupled to both adenylate cyclase and phospholipase C via distinct G-protein α subunits (James et al., 1994).

Since the Hm1 and the Hm3 cells contain similar numbers of receptors per cell (see Table 1), a reliable comparison (Wang and El Fakahany, 1993) can be made between the two transduction pathways in each of these cell lines, and between the muscarinic m1 and m3 receptors, in regard to agonist selectivity. We demonstrated in the present study that the muscarinic receptor agonists, McN-A-343 and oxotremorine, activate preferentially phosphoinositide hydrolysis over adenylate cyclase in both Hm1 and Hm3 cells. Moreover, we also observed that pilocarpine, which has been reported to be a selective muscarinic receptor agonist (Wang and El Fakahany, 1993; Leff et al., 1993), also activates preferentially phosphoinositide hydrolysis over adenylate cyclase (Gurwitz et al., 1994; Heldman et al., 1993). In fact, all the functionally selective agonists tested in our laboratory showed the same tendency (Fisher et al., 1992, 1993b). In addition, McN-A-343, oxotremorine, pilocarpine and many other functionally selective muscarinic receptor agonists antagonize the stimulatory effect of carbachol on adenylate cyclase in cells expressing muscarinic m1 (Gurwitz et al., 1994) and m3 (E. Heldman, unpublished observations) receptors. Thus, many of the functionally selective muscarinic receptor agonists seem to fall within the category of 'partial agonists' according to the definition by Hoyer and Boddeke (1993) who have defined as a partial agonist a 'compound which displays a large range of intrinsic activities at the same receptor'. We suggest that the feature of being partial agonists renders these compounds with their property of being functionally selective agonists. This conclusion is consistent with the study reported by Freedman et al. (1993), who showed that decreasing the efficacy of agonists resulted in compounds with functional selectivity.

The preferential activation of the phosphoinositide hydrolysis over the stimulation of the adenylate cyclase by partial agonists may result from differences in the degree of coupling between the receptor and the relevant G-proteins. When efficient coupling exists between a G-protein and a receptor, maximal activity may be obtained by both full and partial agonists, since high concentrations of partial agonists may activate enough G-protein-coupled receptors to yield the maximal response. However, when poor coupling exists between the G-protein and the receptor, partial agonists may not be able to activate enough G-protein-coupled receptors in order to induce maximal response. Thus, a higher degree of coupling may reflect a larger population of spare receptors, and partial agonists should activate preferentially transduction pathways with larger amount of spare receptors. To validate this assumption, it is necessary to determine whether the phosphoinositide hydrolysis pathway has relatively more spare receptors than the adenylate cyclase pathway.

Our results show that when the muscarinic m1 receptor is down-regulated, adenylate cyclase was the first pathway whose activity was profoundly decreased. The parallel drop in the number of receptors and the decrease in carbachol-induced stimulation of adenylate cyclase, suggests that there are small amounts of spare receptors for this biochemical pathway. On the other hand, phosphoinositide hydrolysis was reduced to a lesser extent than expected from the decrease in the number of receptors, suggesting a larger population of spare receptors for this biochemical pathway. Consistent with this conclusion, our results show that lower concentrations of carbachol are needed to activate phosphoinositide hydrolysis than to stimulate adenylate cyclase. We should take into consideration that receptor down-regulation may cause changes in intracellular components associated with the signalling pathways, and that these changes may be responsible for the differential effect on phosphoinositide hydrolysis and adenylate cyclase activity. However, Hu et al. (1991) showed that the major reason for the reduction in the carbachol-stimulated phosphoinositide hydrolysis following receptor down-regulation is the reduced number of receptors. Moreover, we have shown that prolonged exposure to oxotremorine did not result in changes in the immunoreactive concentrations of G-protein α subunits (Nah et al., 1993). Therefore, it can be concluded from the results which show a more pronounced decrease in adenylate cyclase activity than in phosphoinositide hydrolysis (when muscarinic receptors are down-regulated), that the latter pathway has a higher amount of spare receptors.

The relative amount of spare receptors could be best examined using the technique of receptor alkylation (Furchgott, 1966). In the present study we used acetylethylcholine mustard to alkylate the agonist binding site of the receptor. Acetylcholine mustard has been shown to specifically interact with the aspartate residue on the third transmembrane helix of the muscarinic acetylcholine receptor, resulting in receptor inactivation (Spalding et al., 1994). Here we demonstrate that partial inactivation of the muscarinic m1 and m3 receptors by alkylation with acetylethylcholine mustard results in a moderate decrease in carbachol-induced phosphoinositide hydrolysis, but in a sharp and pronounced decrease in carbachol-induced adenylate cyclase activity. Thus, our results show that alkylation of 50% of the cell surface receptors resulted in a 15% reduction in carbachol-induced phosphoinositide hydrolysis and in a 75% reduction in carbachol-induced adenylate cyclase activity. This experiment clearly indicates that the phosphoinositide hydrolysis response has a much higher receptor reserve than the adenylate cyclase response. Our

findings that higher concentrations of atropine were needed to inhibit the carbachol-stimulated phosphoinositide hydrolysis than those needed to inhibit carbachol-stimulated adenylate cyclase activity corroborate this conclusion. Interestingly, the reduction in both activities were similar in Hm1 and Hm3 cells, suggesting that for each of these activities there is a similar number of spare receptors in the m1 and the m3 receptor expressing cells which were used in the present study.

The fact that the two different methods that were used to reduce the number of functional receptors (receptor down-regulation and receptor inactivation) gave the same results, indicates that the reduction in the number of receptors indeed constitutes the major reason for the drop in the responsiveness of the cells to carbachol. We therefore conclude that the preferential activation of phosphoinositide hydrolysis as compared to adenylate cyclase activity, within a single receptor subtype, is most probably related to the differences in the spare receptors available for each of these transduction pathways.

As can be concluded from the results discussed above, the functional selectivity of partial agonists in preferentially activating the muscarinic m1 over the m3 receptor, cannot be related to differences in spare receptors between the Hm1 and Hm3 cells. Differences in receptor densities between Hm1 and Hm3 cells also do not explain the results, as these two cell lines contain similar numbers of receptors per cell. Hu and El-Fakahany (1990) suggested that differences in affinities of McN-A-343 to muscarinic m1 and m3 receptors may be the reason for its functional selectivity. However, our results show that both McN-A-343 and oxotremorine bind to the muscarinic m1 and m3 receptors with very similar affinities, and therefore, differences in receptor recognition cannot be responsible for the subtype selectivity demonstrated with these agonists. Why then McN-A-343 activates preferentially the muscarinic m1 versus m3 receptor subtype? The answer to this question may arise from considerations of the fitness of the agonist to the receptor binding site, and its ability to induce the conformational changes needed to form the active receptor/G-protein complex. The data show that both McN-A-343 and oxotremorine bind to the muscarinic acetylcholine receptor with higher affinity than carbachol (see Fig. 4). Therefore, we could assume that the differences between the efficacies of these partial agonists and carbachol are not associated with the binding step, but rather related to a step subsequent to ligand binding, namely the conformational change that occurs following agonist binding. We propose that the activation energy needed to transform the receptor from the resting state to the active state, is lower for muscarinic m1 receptor than for m3 receptor. A similar situation has been recently discussed for other receptors (Bond et al., 1995). Consistent with this hypothesis, we found that only the m1 receptor and not the m3 receptor showed a competition curve (with carbachol) which was composed of two binding sites. We assume that these two affinity states of the receptor represent the two conformational states. In summary, it could be argued that the preferential activation of the muscarinic m1 over m3 receptor by partial agonists, may be related to differences between the abilities of the two receptors to undergo conformational changes following agonist binding. In the term 'conformational adaptability', we referred to the ease in which a receptor undergoes conformational change following agonist binding. Our results suggest that the conformational adaptability of the muscarinic m1 receptor is greater than that of the muscarinic m3 receptor. Indeed, functionally selective agonists show m1 receptor selectivity.

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