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Functional partial agonism at cloned human muscarinic acetylcholine receptors

Hans Bräuner-Osborne [a,1], Bjarke Ebert [a,2], Mark R. Brann [b,c], Erik Falch [a], Povl Krogsgaard-Larsen [a,*]

[a] *PharmaBiotec Research Center, Department of Medicinal Chemistry, Royal Danish School of Pharmacy, 2100 Copenhagen, Denmark*

[b] *Molecular Neuropharmacology Section, Departments of Psychiatry and Pharmacology, College of Medicine, University of Vermont, Burlington, VT 05405, USA*

[c] *Receptor Technologies Inc., 276 East Allen Street, Winooski, VT 05404, USA*

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Abstract

We have previously defined the concept of functional partial agonism as the partial agonist responses recorded in brain slices after administration of full ionotropic glutamate receptor agonists and competitive antagonists at fixed ratios. Functional partial agonism can be established at any level of maximal response, depending on the molar ratio of agonist and antagonist used. Using recombinant human muscarinic acetylcholine receptors (m1 and m5) and the functional assay, receptor selection and amplification technology (R-SAT), we have now shown that co-administration of the full agonist, carbachol, and a competitive antagonist, atropine or pirenzepine, at fixed ratios display functional partial agonism. The levels of apparent intrinsic activity of the functional partial agonist responses were shown to be dependent of the receptor density and G-protein concentration in the same manner as that determined for the true partial muscarinic agonist, 4-[*N*-(3-chlorophenyl)carbamoyloxy]-2-butynyltrimethylammonium chloride (McN A-343). Thus, functional as well as true partial agonist responses became more efficacious and potent with increasing receptor and G-protein levels. The level of maximal functional partial agonist response, which is dependent on the agonist/antagonist ratio, is predictable from the Waud equation, describing competitive receptor/ligand interactions. In agreement with the relative antagonist potencies of pirenzepine at m1 and m5, a 10 : 1 ratio of carbachol and pirenzepine produced very low-efficacy functional partial agonism, approaching full antagonism, at m1 but virtually full agonism at the m5 subtype.

Keywords: Muscarinic acetylcholine receptor; Cloned human receptor; Receptor selection and amplification technology; Functional partial agonism; Functional receptor selectivity

1. Introduction

Partial agonists, which are receptor ligands possessing both agonistic and antagonistic properties, have therapeutic interest in a number of diseases, where receptor activation or inhibition is considered appropriate (Williams et al., 1995). However, for a number of receptor systems, no partial agonists are available with the right level of efficacy and/or selectivity. One such system is the (*RS*)-2-amino-3-(3-hydroxy-5-methyl-4-isoxazolyl)propionic acid (AMPA) receptor for which only one partial agonist has been reported, (*RS*)-2-amino-3-(3-hydroxy-5-phenyl-4-isoxazolyl)propionic acid (APPA) (Christensen et al., 1989). APPA has recently been resolved to give (*S*)-APPA, which is a full AMPA receptor agonist, and (*R*)-APPA, showing competitive AMPA receptor antagonism (Ebert et al., 1994a,b). Thus, the partial agonism produced by racemic APPA, a 1 : 1 mixture of (*S*)- and (*R*)-APPA, is only apparent (Ebert et al., 1994a). These aspects were further analyzed and, in agreement with theory for competitive ligand-receptor interaction, it was demonstrated that co-administration of (*S*)- and (*R*)-APPA to a rat brain slice preparation at different fixed ratios produced partial

* Corresponding author. PharmaBiotec Research Center, Department of Medicinal Chemistry, Royal Danish School of Pharmacy, 2 Universitetsparken, 2100 Copenhagen, Denmark.

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agonism at different levels of maximal response (Ebert et al., 1994a,b). Co-administration of other pairs of agonist and competitive antagonist at AMPA or *N*-methyl-D-aspartic acid (NMDA) receptors gave similar effects, and this new principle of establishing partial agonism at any desired level of maximal response has been termed functional partial agonism (Ebert et al., 1995, 1996). A similar pharmacological principle, named artificial partial agonism, has previously been used to study spare receptors in the adrenergic system (Feuerstein et al., 1994).

In order to investigate whether the principle of functional partial agonism can be extended to the G-protein-coupled receptors, we have studied the effect of fixed ratios of full agonists and antagonists at cloned human muscarinic acetylcholine receptors. Furthermore, we have studied the principle into greater detail by comparing the effects of changing receptor/G-protein level on functional partial agonists and a true partial agonist. Finally, we here demonstrate that the principle of functional partial agonism can be applied to obtain receptor subtype selectivity.

2. Materials and methods

2.1. Receptor selection and amplification technology

Receptor selection and amplification technology (R-SAT) was performed as described earlier (Bräuner-Osborne et al., 1995; Messier et al., 1995; Bräuner-Osborne and Brann, 1996). NIH 3T3 (ATCC No. CRL 1658) cells were maintained in a 37°C humidified 5% CO_2 incubator in Dulbecco's modified Eagle's media (Gibco, Paisley, UK) supplemented with 10% calf serum (HyClone, UT, USA).

1 day prior to transfection, cells were plated in a density of 2×10^6 cells/15-cm tissue culture dish. Cells were transfected by the calcium phosphate-DNA precipitation method (Wigler et al., 1977). In experiments with 'low' and 'medium' receptor/G-protein level, cells were transfected with 2 or 10 μg receptor DNA (Bonner et al., 1987, 1988), respectively, whereas cells with 'high' receptor/G-protein level were transfected with 10 μg receptor DNA and 2 μg Gq DNA (Conklin et al., 1993). All plates were also transfected with 10 μg p-SV-β-galactosidase DNA (Promega, WI, USA) and 40 μg salmon sperm DNA (Sigma, MO, USA).

1 day after transfection, media were changed and the next day cells were divided into the wells of two 96-well plates. Ligands were added into a final volume of 200 μl/well. After 4 days in the presence of ligands, media were exchanged with 200 μl β-galactosidase substrate consisting of 3.5 mM *o*-nitrophenyl-β-D-galactopyranoside and 0.5% nonidet P-40 (both Sigma) in phosphate-buffered saline (PBS). After 16 h of incubation at room temperature, plates were read at 420 nm on a plate reader (Molecular Devices).

2.2. Data analysis

Data from R-SAT experiments were fitted to the simple mass action equation:

$$R = \frac{R_{max}}{1 + (EC_{50}/[A])} + R_{basal} \quad (1)$$

where [A] is the concentration of agonist and R the response. Curves were generated by non-weighted least-squares fits using the program, KaleidoGraph 2.1 (Abelbeck Software) for the Macintosh computer.

Data from R-SAT were also fitted to the Waud equation (Waud, 1975):

$$R = \frac{R_{max}}{\left(1 + \left\{\frac{EC_{50} \times \left(\frac{[B]^s}{K_b} + 1\right)}{[A]}\right\}\right)^b} + R_{basal} \quad (2)$$

where [A] and [B] are the concentrations of agonist and antagonist, respectively, EC_{50} is the concentration of agonist causing 50% of its maximal response, K_b is the antagonist equilibrium dissociation constant and R is the response. The Schild slope parameter(s) for the antagonist and the Hill slope factor (b) for the agonist were both set to unity. Data from each experiment using four different ratios of agonist and antagonist were fitted simultaneously to the equation (using the program GraFit 3.0 from Erithaus Software, Staines, UK) producing the EC_{50} of the agonist and the K_b for the antagonist.

3. Results

R-SAT is a functional assay of ligand-receptor interaction which is based on the observation that agonists stimulate cellular proliferation and induce the formation of macroscopic colonies called foci in NIH 3T3 cells transfected with a muscarinic acetylcholine receptor coupled to phosphoinositide turnover (Gutkind et al., 1991). In R-SAT, we co-transfect the receptor with the marker gene β-galactosidase and measure the agonist-induced cellular proliferation by a color response. We have previously shown that R-SAT results correlates very well with results using second messenger assays and isolated tissues (Bräuner-Osborne and Brann, 1996; Messier et al., 1995). The NIH 3T3 cells are transfected using a calcium phosphate-DNA precipitation method (Wigler et al., 1977), which has a transfection efficiency of 1–2% (results not shown). Due to the low transfection efficiency it is not possible to measure an increase in receptor or G-protein level. Thus, the terms 'low', 'medium' and 'high' receptor/G-protein level is based on the assumption that the

level of expression is reflecting the amount of DNA used for transfection.

Fig. 1 shows the concentration-response curves for the

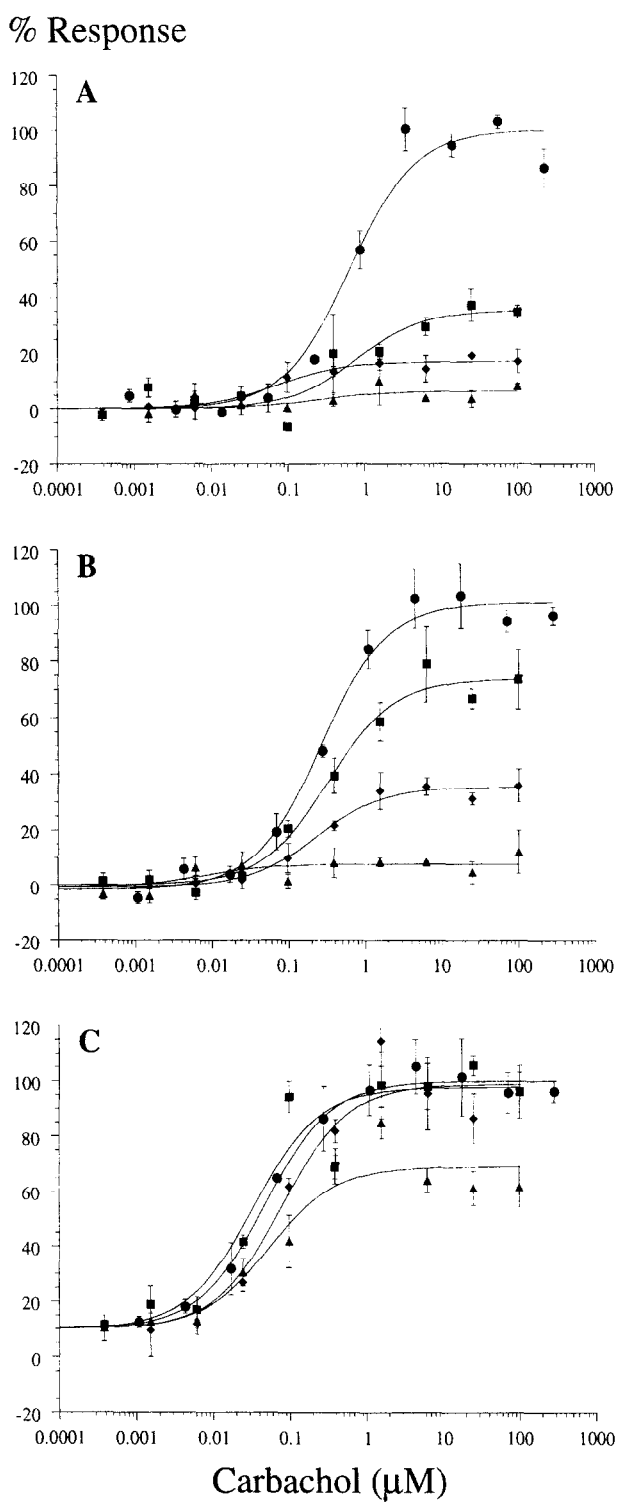

Fig. 1. Pharmacological profile determined by R-SAT of the full agonist carbachol (●) and a functional partial agonist consisting of carbachol/atropine in the ratios (1000:1, ■), (1000:3, ◆) and (100:1, ▲). 2×10^6 NIH 3T3 cells in a 15-cm tissue culture dish were transfected with 10 μg β-galactosidase DNA and (A) 2 μg m5 DNA, (B) 10 μg m5 DNA or (C) 10 μg m5 DNA and 2 μg Gq DNA to give 'low', 'medium' and 'high' receptor/G-protein levels, respectively.

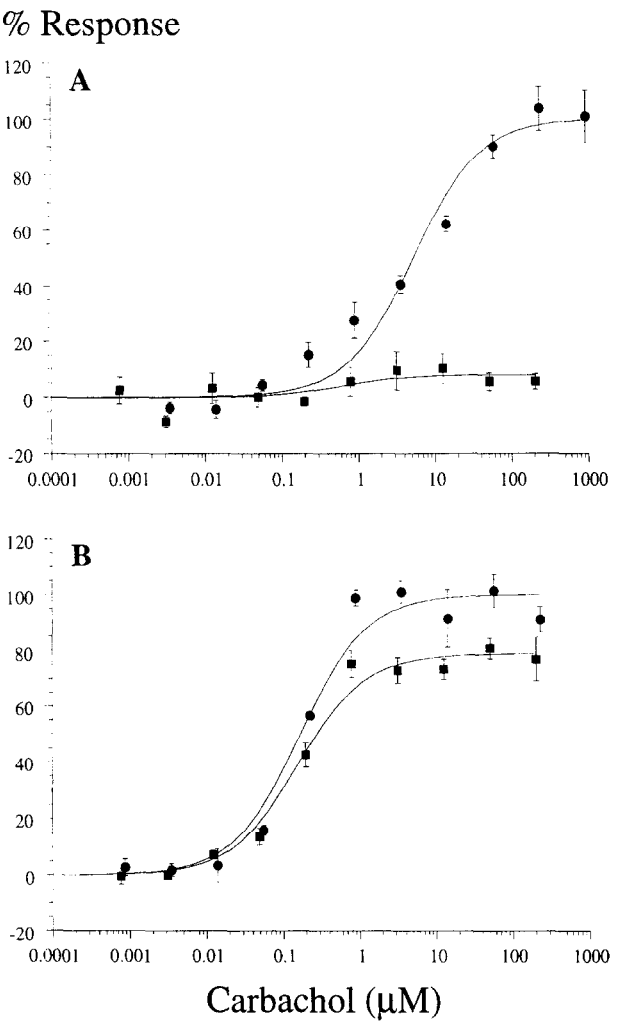

Fig. 2. Pharmacological profile determined by R-SAT of the full agonist carbachol (●) and a functional partial agonist consisting of carbachol/pirenzepine in a 10:1 ratio (■). 2×10^6 NIH 3T3 cells in a 15-cm tissue culture dish were transfected with 10 μg β-galactosidase DNA and (A) 10 μg m1 DNA or (B) 10 μg m5 DNA.

agonist, carbachol, with various ratios of the competitive antagonist, atropine. In agreement with our previous results on ionotropic glutamic acid receptors (Ebert et al., 1994b, 1995, 1996), the maximal response diminished as the agonist/antagonist ratio decreased. The mixed agonist/antagonist thus behaved like a partial agonist, a behavior we have previously defined as functional partial agonism (Ebert et al., 1994b, 1995, 1996). Fig. 1 also shows how the apparent intrinsic activity and potency of the functional partial agonists depends on the receptor/G-protein level. When the receptor/G-protein level was reduced from 'medium' to 'low', by decreasing the amount of m5 receptor DNA used for transfection, the potency and intrinsic activity of the partial agonist, McN A-343, and the potency and apparent intrinsic activity of the functional partial agonists was approximately halved, respectively (Table 1). When the receptor/G-protein level was increased from 'medium' to 'high' by co-transfecting the m5 receptor with the G-protein Gq, the potency was increased

Table 1
Pharmacological parameters of muscarinic agonists and antagonists at human muscarinic acetylcholine receptor subtype m5 determined by receptor selection and amplification technology (R-SAT)

Ligand(s)	EC_{50} [μM] (% of maximal carbachol response)		
	'Low'	'Medium'	'High'
	receptor/G-protein level		
Carbachol	1.3 ± 0.3 (100%)	0.39 ± 0.07 (100%)	0.060 ± 0.026 (100%)
Carbachol/atropine (1000 : 1)	0.64 ± 0.12 (35 ± 3%)	0.28 ± 0.04 (66 ± 3%)	0.064 ± 0.016 (101 ± 10%)
Carbachol/atropine (1000 : 3)	0.20 ± 0.03 (16 ± 3%)	0.20 ± 0.02 (31 ± 2%)	0.044 ± 0.021 (96 ± 14%)
Carbachol/atropine (100 : 1)	No response	0.038 ± 0.027 (11 ± 3%)	0.054 ± 0.010 (74 ± 24%)
McN A-343	5.3 ± 2.2 (29 ± 7%)	3.8 ± 0.2 (61 ± 3%)	1.1 ± 0.6 (90 ± 9%)

2×10^6 NIH 3T3 cells with 'low' and 'normal' receptor reserve were transfected with 10 μg β-galactosidase and 2 or 10 μg m5 receptor DNA, respectively, per 15-cm tissue culture dish. Cells with 'high' receptor reserve were transfected with 10 μg m5 receptor, 10 μg β-galactosidase and 2 μg Gq DNA. 2 days after transfection, the cells were divided into the wells of two 96-well plates and ligands were added into a final volume of 200 μl/well. 4 days after ligand addition, the amount of β-galactosidase was assayed as described (Lim and Chae, 1989). Data represent the mean ± S.E.M. of 3 experiments.

approximately 4-fold and the intrinsic activity of the partial agonists as well as the apparent intrinsic activity of the functional partial agonists were increased dramatically (Table 1), which is in agreement with results, which we have published previously (Burstein et al., 1995). Also in agreement with our previous results (Burstein et al., 1995), there was a constitutive activation of the cells with 'high' receptor/G-protein level causing an elevated basal response of 10% (Fig. 1c), which could be suppressed by the competitive antagonist, atropine (data not shown).

To demonstrate that the principle of functional partial agonism can be used to obtain functional selectivity at receptor subtypes, we tested a functional partial agonist consisting of carbachol/pirenzepine in a 10 : 1 ratio (Fig. 2). As shown previously, carbachol was more potent at m5 ($EC_{50} = 0.39 \pm 0.07$, $n = 3$) (Table 1) than at m1 ($EC_{50} = 1.9 \pm 0.7$, $n = 3$), and the antagonist, pirenzepine is more potent at m1 ($pK_i = 7.7$) than at m5 ($pK_i = 6.9$), when assayed with R-SAT (Bräuner-Osborne et al., 1995; Bräuner-Osborne and Brann, 1996). Thus, as expected, pirenzepine blocked the carbachol response at the m1 receptor, while leaving the m5 response almost intact (Fig. 2).

We have previously shown that the concentration-response curves of functional partial agonists can be accurately simulated by the Waud equation (Eq. 2), by simply inserting the EC_{50} value of the agonist and the K_i value of the antagonist (Ebert et al., 1995, 1996). In order to investigate whether the Waud equation could also be used to describe the functional partial agonism observed at muscarinic acetylcholine receptors, we fitted the data from carbachol and the three functional partial agonists simultaneously to the Waud equation, thus calculating the EC_{50} value of the agonist and the K_b value of the antagonist. As seen in Table 2, the EC_{50} value of carbachol calculated from the fit to the Waud equation decreased as the receptor/G-protein level increased with values very close to the EC_{50} value of carbachol in Table 1. The K_b value of the antagonist atropine calculated from the fit to the Waud equation was similar to the previously published K_i value of 0.79 μM (Bräuner-Osborne and Brann, 1996). As expected, the K_b value of the antagonist did not change when the receptor/G-protein was altered.

Table 2
Potency of the muscarinic ligands at the human muscarinic acetylcholine receptors subtype m5 as determined by simultaneously fitting of carbachol and three functional partial agonists to the Waud equation

Ligand(s)	'Low'	'Medium'	'High'
	receptor/G-protein level		
Carbachol (EC_{50} [μM])	1.1 ±0.2	0.35 ± 0.04	0.075 ± 0.026
Atropine (K_b [nM])	0.65 ± 0.19	0.48 ± 0.05	0.64 ±0.19

The potencies of the agonist carbachol and the antagonist atropine was determined by simultaneously fitting the pharmacological data of carbachol and three functional partial agonists (carbachol/atropine in the ratios 1000 : 1, 1000 : 3 and 100 : 1) to the Waud equation (Eqn. 2). Data represent the mean ± S.E.M. of 3 experiments.

4. Discussion

We have previously introduced the concept of functional partial agonism at the AMPA and NMDA receptor subtypes of ionotropic glutamic acid receptors on the basis of analyses of the pharmacological effects of agonists and competitive antagonists co-administered at fixed ratios to rat brain slices (Ebert et al., 1994b, 1995, 1996). Using R-SAT, a functional assay of ligand-receptor interaction (Bräuner-Osborne et al., 1995; Messier et al., 1995; Bräuner-Osborne and Brann, 1996), and recombinant hu-

man muscarinic acetylcholine receptors we have now shown that this new pharmacological principle can be extended to G-protein-coupled receptors. In agreement with our findings for AMPA and NMDA receptor ligands, we have been able to produce functional partial agonism at muscarinic acetylcholine receptors at any desired level of apparent intrinsic activity, calculated on the basis of the potency of agonist and antagonist.

In diseases, where receptor activation or inhibition is appropriate, the optimal levels of maximal pharmacodynamic response normally are unpredictable. Furthermore, design of true partial agonists on a rational basis is not possible, as yet. Thus, functional partial agonists may be a flexible therapeutic alternative to true partial agonists. It must, however, be stressed that functional partial agonism implies administration of two compounds, an agonist and a competitive antagonist, probably showing different pharmacokinetic and metabolic characteristics (Feuerstein et al., 1994). Prior to clinical studies, it will be necessary to establish agonist/antagonist molar ratio-response curves in animal experiments in order to estimate optimal levels of functional partial agonism in patients.

It is well established that the intrinsic activity and potency of a partial agonist depends on the amount of receptor and G-protein, the partial agonist appearing more efficacious and potent as the receptor/G-protein level is increased (Kenakin and Morgan, 1989). Interestingly as stated above, we found that the characteristics of functional partial agonism and true partial agonism are very similar. Thus, the effect of co-administered carbachol and atropine at a fixed ratio of 1000:1 is virtually indistinguishable from that of the partial agonist, McN A-343, indicating that the mechanisms underlying functional partial agonism and true partial agonism might be identical. This is in agreement with results showing that functional partial agonism produced by acetylcholine/atropine (1600:1) had very low disposition to cause receptor desensitization (Winding and Bindslev, 1993), a well-known characteristic of partial agonists (Hu et al., 1991; Pontzer and Crews, 1990; Thompson and Fisher, 1990).

In this and previous studies (Ebert et al., 1995, 1996), we have shown that the Waud equation (Waud, 1975) can be used to describe the concentration-response relationships of functional partial agonists. Thus, when the EC_{50} value of the agonist and the K_b value of the antagonist is known, it is possible to predict the concentration-response relationship of a functional partial agonist allowing one to choose an agonist/antagonist ratio that will give the desired response. By inserting the known potencies of carbachol (m5 over m1 selective) and pirenzepine (m1 over m5 selective) into the Waud equation, we simulated the concentration-response curves of various fixed ratios (data not shown), and since the two ligands show a reverse selectivity, we were able to predict a ratio of carbachol/pirenzepine (10:1) capable of blocking the m1 response while leaving the m5 response almost intact. In subsequent RSAT experiments, the results were exactly as expected (Fig. 2). This also demonstrates that the concept of functional partial agonism can be used to achieve functional selectivity between receptor subtypes. Such effects may be of clinical interest in Alzheimer's disease, where activation of postsynaptic and concomitant inhibition of presynaptic muscarinic acetylcholine receptors may be therapeutically beneficial (Whitehouse, 1993).

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