0026-895X/98/040759-07$3.00/0

MOLECULAR PHARMACOLOGY, **53:**759–765 (1998).

The Relationship between Agonist Intrinsic Activity and the Rate of Endocytosis of Muscarinic Receptors in a Human Neuroblastoma Cell Line

PHILIP G. SZEKERES, JENNIFER A. KOENIG, and J. MICHAEL EDWARDSON

Department of Pharmacology (P.G.S, J.A.K., J.M.E.) and Glaxo Institute of Applied Pharmacology (J.A.K.), University of Cambridge, Cambridge, CB2 1QJ, United Kingdom

Received October 22, 1997; Accepted December 11, 1997

This paper is available online at http://www.molpharm.org

ABSTRACT

The molecular mechanisms underlying the internalization of G protein-coupled receptors are still poorly understood. Normally agonists but not antagonists cause internalization (defined here as a reduction in the number of receptors at the cell surface), suggesting a functional relationship between agonist activity and internalization. In this study we investigated the effects of eight muscarinic ligands on the rate constants for endocytosis and recycling of m3 muscarinic acetylcholine receptors in human SH-SY5Y neuroblastoma cells. We found that there was a linear correlation between the intrinsic activity of the ligand and its ability to increase the rate constant for endocytosis, suggesting that the same active conformation of the receptor is responsible for stimulating both second messenger generation and receptor endocytosis. In contrast, the rate constant for recycling did not depend on which agonist had triggered receptor endocytosis, suggesting that recycling is a purely constitutive process. Because receptor internalization depends on the rate constants for both endocytosis and recycling, the relationship between internalization and intrinsic activity is nonlinear. In particular, mathematical modeling of receptor trafficking revealed that under certain conditions very small (3% or less) increases in the rate constant for endocytosis are sufficient to cause substantial receptor internalization. An important implication of this analysis is that extremely weak partial agonists (which may in practice be indistinguishable from antagonists) may produce significant receptor internalization.

In unstimulated cells, G protein-coupled receptors are found predominantly at the plasma membrane. After agonist stimulation, the receptors are often efficiently endocytosed and delivered to intracellular compartments, from where they may be either recycled to the plasma membrane or transported to lysosomes for degradation. In many cases, receptor function has been shown to be regulated by this intracellular transport (Pippig *et al.*, 1995; Bogatkewitsch *et al.*, 1996), and so the unraveling of the mechanisms involved in receptor endocytosis is crucial to our understanding of receptor function.

The molecular and cellular processes involved in receptor endocytosis and recycling remain poorly understood. Normally, agonists, but not antagonists, cause an increase in the rate of endocytosis of receptors, suggesting that the activated conformation of the receptor, which couples to G proteins, is also responsible for initiating receptor endocytosis. The involvement of G protein activation and second messenger generation in the triggering of endocytosis, however, remains controversial (Clark *et al.*, 1985; Cheung *et al.*, 1990; Thompson *et al.*, 1991; Benya *et al.*, 1994; Hunyady *et al.*, 1994). Furthermore, studies of the relationship between the activity of agonists and their ability to cause receptor internalization have generated conflicting results. Indeed, it has recently been reported that an antagonist at the cholecystokinin receptor can stimulate internalization (Roettger *et al.*, 1997). Part of the problem may relate to confusion of the terms "endocytosis," which refers to the actual process of removal of receptors from the plasma membrane, and the process that is often measured, internalization (the reduction in number of receptors at the cell surface), which is a function of the rate constants for both endocytosis and recycling (Koenig and Edwardson, 1997).

In the present study, we compared the ability of a series of muscarinic ligands acting at m3 muscarinic receptors in human neuroblastoma SH-SY5Y cells (Wall *et al.*, 1991) to generate second messenger and to modify receptor trafficking. Using a two-compartment model for receptor trafficking (Koenig and Edwardson, 1994), we analyzed the effects of these ligands on the kinetics of receptor endocytosis and recycling. We demonstrate that there is a linear relationship between the increase in the rate constant for receptor endocytosis and the intrinsic activity of the ligand, but no effect of the stimulating ligand on the rate constant for recycling. Furthermore, using mathemat-

This work was supported by the Wellcome Trust.

ABBREVIATIONS: Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; k_e, rate constant for receptor endocytosis; k_r, rate constant for receptor recycling; NMS, *N*-methylscopolamine; PrBCM, propyl-benzilylcholine mustard; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

ical simulations we show that the level of internalization elicited by a drug is dependent not only on drug efficacy, but also varies with cell type-specific factors, such as the kinetics of receptor endocytosis and recycling. This analysis demonstrates that, under certain conditions, compounds with extremely low efficacy can cause significant receptor internalization.

Experimental Procedures

Cell culture. SH-SY5Y cells (passage 5–25) were grown at 37° in Dulbecco's modification of Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum in 5% CO_2/95% humidified air. Serum was heat-inactivated by incubation at 56° for 30 min. Cells were detached from the culture flasks for passaging every 3–5 days by brief (<2 min) incubation with trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) in phosphate-buffered saline.

Measurement of Ins(1,4,5)P_3 mass. Confluent cultures of SH-SY5Y cells were harvested and reseeded at an equivalent density in 24-well multidishes. After overnight incubation to allow cell attachment, cells were washed twice with 250 μl of medium at 37° and left for 10 min at 37°. Experiments were initiated by the removal of the wash buffer and its immediate replacement with 150 μl of buffer with or without agonist. Incubations were terminated by the addition of 150 μl of ice-cold 1 M trichloroacetic acid. Multiwell plates were then put on ice for 15 min. A 160-μl aliquot of the incubate was added to 40 μl of 10 mM EDTA, followed by 200 μl of a 1:1 (v/v) mixture of tri-*n*-octylamine and 1,1,2-trichlorotrifluoroethane. After vortexing, the samples were left at room temperature for 15 min and subsequently centrifuged at 13,000 × *g* for 3 min. The upper aqueous phase (100 μl) was taken, and 50 μl of 25 mM $NaHCO_3$ were added. Samples were then stored at −20° until assay of Ins(1,4,5)P_3.

Ins(1,4,5)P_3 mass was measured using a kit from Amersham (Buckinghamshire, UK) following the manufacturer's instructions. In some experiments the supplied Ins(1,4,5)P_3-binding protein was replaced with a porcine platelet membrane preparation (Cullen *et al.*, 1995).

Experiments were performed in duplicate, and results are means ± standard error from at least three separate experiments. Dose-response curves were fitted to a standard four-parameter logistic equation using SigmaPlot (SPSS Inc., Chicago, IL).

Determination of receptor binding affinities. The binding affinities of the muscarinic ligands were determined by the displacement of 0.25 nM [^{3}H]-NMS in SH-SY5Y cells in Dulbecco's modification of Eagle's medium/HEPES buffer containing 0.5 M sucrose to block receptor internalization. Incubations were for 1 hr at 37°. Displacement curves were analyzed by EBDA/LIGAND (Elsevier Biosoft, Cambridge, UK) to obtain Hill slopes and binding affinities (K_i). The dissociation constant (K_d) for [^{3}H]NMS was obtained under the same conditions as the agonist binding assays.

Analysis of receptor internalization. Changes in surface muscarinic receptor number were quantified through the binding of the polar radioligand [^{3}H]NMS to adherent SH-SY5Y cells grown in 24-well plates, as described previously (Koenig and Edwardson, 1996). In experiments to determine the concentration dependence of receptor internalization, curves were fitted to a standard four-parameter logistic equation using SigmaPlot (SPSS Inc., Chicago, IL).

Mathematical modeling of receptor trafficking. In a two-compartment model, receptors cycle between the cell surface and endosomes. The rate of change of the number of surface receptors is given by the rate of receptors arriving by recycling less the rate leaving by endocytosis

$$\frac{dR_s}{dt} = k_r R_e - k_e R_s \quad (1)$$

where R_s is the number of surface receptors, R_e is the number of endosomal receptors and k_r and k_e are the recycling and endocytotic rate constants, respectively. The total number of receptors, $R_s + R_e$, is equal to the number of receptors at time $t = 0$; i.e., $R_s + R_e = R_{s0} + R_{e0}$. Substituting $R_e = R_{s0} + R_{e0} - R_s$, we can rewrite eq. 1 as

$$\frac{dR_s}{dt} = k_r(R_{s0} + R_{e0}) - (k_e + k_r)R_s$$

which is now of the form $dy/dt = b - ay$. This can be integrated to $ay - b = Ce^{-at}$, where $b = k_r(R_{s0} + R_{e0})$; $a = k_e + k_r$ and C is the constant of integration. Rearrangement results in an equation describing the number of surface receptors as a function of time:

$$R_s = \frac{1}{k_e + k_r}[k_r(R_{s0} + R_{e0}) + (k_e R_{s0} - k_r R_{e0})e^{-(k_e + k_r)t}] \quad (2)$$

Receptor recycling curves make use of eq. 2 with k_e and R_{s0} set to zero. Then, R_s as a function of time is given by

$$R_s = R_{e0}(1 - e^{-k_r t}) \quad (3)$$

Thus, k_r can be determined from a plot of R_s against t. At large values of t, the exponential terms in eq. 2 approach zero, and therefore the steady state number of surface receptors ($R_{s,ss}$) may be written as

$$R_{s,ss} = \frac{k_r(R_{s0} + R_{e0})}{k_r + k_e} \quad (4)$$

Eq. 4 allows experimentally determined changes in $R_{s,ss}$ to be converted to changes in the endocytotic rate constant k_e, if k_r is known.

Materials. [^{3}H]Ins(1,4,5)P_3 (43 Ci/mmol) was from Amersham (Buckinghamshire, UK) and [^{3}H]NMS (85 Ci/mmol) was from DuPont-NEN (Stevenage, UK). Oxotremorine-M was from Research Biochemicals International (St. Albans, UK). McN-A-343 and propylbenzilylcholine mustard (PrBCM) were generous gifts of Dr. J. M. Young (Department of Pharmacology, University of Cambridge, UK). All other materials were from Sigma (Poole, UK).

Results

Intrinsic activity and potency of muscarinic ligands. The intrinsic activity and potency of the eight muscarinic ligands used in this study were assayed through their ability to stimulate the production of Ins(1,4,5)P_3. Addition of muscarinic agonists caused a biphasic Ins(1,4,5)P_3 response, with a rapid initial increase that reached a peak 10 sec after addition of the drug, followed by a reduction over the next 120 sec to a lower but sustained phase (data not shown) (Willars and Nahorski, 1995). Dose-response curves measuring peak Ins(1,4,5)P_3 responses were constructed for all eight muscarinic ligands (Fig. 1 and Table 1). Carbachol, methacholine, and oxotremorine-M were all full agonists, whereas bethanechol, arecoline, and pilocarpine were partial agonists, eliciting 51.6 ± 11.0%, 26.1 ± 3.9%, and 8.4 ± 1.2% of the maximal response to carbachol, respectively. Both *N*-methylatropine and McN-A-343 were antagonists and without effect in this assay.

The rank order of potency for stimulation of Ins(1,4,5)P_3 production was oxotremorine-M > methacholine > (carbachol ≈ arecoline ≈ pilocarpine) ≫ bethanechol (Table 1). Comparison of EC_{50} values with receptor affinities determined by the displacement of [^{3}H]NMS binding (Table 1) shows that, as expected, the dose-response curves for the full agonists carbachol and oxotremorine-M lie 2–3-fold to the left of the receptor occupancy curves and the dose-response curve for methacholine overlies the receptor occupancy curve. In

contrast, the dose-response curves for the partial agonists bethanechol, arecoline, and pilocarpine lie 2.9-, 9.5-, and 8.7-fold to the right of their respective receptor-occupancy curves. At the moment, we cannot fully explain this surprising behavior of the dose-response curves for the partial agonists. One possibility is that the measurements of Ins(1,4,5)P_3 mass are being complicated by its metabolism (by Ins(1,4,5)P_3 5-phosphatase and/or Ins(1,4,5)P_3 3-kinase). It has recently been shown that at least Ins(1,4,5)P_3 3-kinase is activated through a receptor-dependent mechanism (Communi *et al.*, 1997). Such a dual effect of the activated receptor on both the generation and metabolism of Ins(1,4,5)P_3 would undoubtedly complicate any dose-response relationship, and might produce the sort of unexpected picture seen here with the partial agonists.

Measurement of muscarinic receptor recycling rates after removal of agonist. Upon agonist stimulation, muscarinic receptors are internalized and cycle continuously between the cell surface and intracellular compartments (Koenig and Edwardson, 1994, 1996). The quantification of muscarinic receptor trafficking therefore requires the determination of rate constants for both the endocytosis of receptors from the plasma membrane (k_e) to an intracellular compartment and their recycling to the cell surface (k_r). It is possible to measure the rate of recycling of endocytosed receptors in isolation from the effect of receptor endocytosis after removal of agonist, because in the absence of agonist k_e is so small that it may be considered insignificant (Koenig and Edwardson, 1994; Morrison *et al.*, 1996). k_r was determined by treating cells with carbachol (1 mM; 30 min) to achieve maximal endocytosis (Koenig and Edwardson, 1996). Cells were then washed free of agonist and exposed to the muscarinic receptor alkylating agent PrBCM (100 nM) to alkylate any receptors still remaining at the cell surface. After removal of PrBCM, the rate of receptor reinsertion into the plasma membrane was measured through the binding of the polar radioligand [^{3}H]NMS (Fig. 2 and Table 2). Modeling of these data using eq. 3 indicates that muscarinic receptors were recycled to the cell surface with first order kinetics, giving a rate constant (k_r) of 0.054 ± 0.004 min^{-1}.

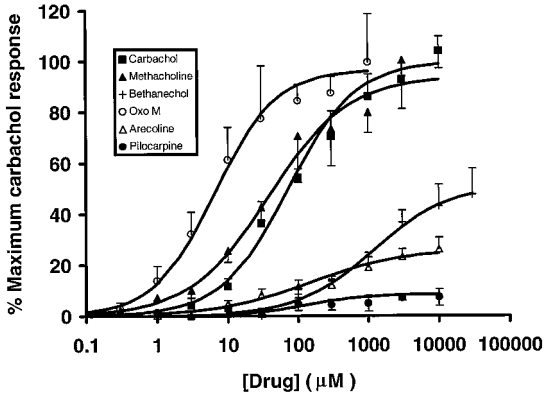

Fig. 1. Dose-response relationships for peak changes in Ins(1,4,5)P_3 mass (determined at 10 sec) in adherent SH-SY5Y cells. Data are expressed as a percentage of the maximal response seen with carbachol. Resting [Ins(1,4,5)P_3] was 3.8 ± 1.5 pmol/106 cells, and peak [Ins(1,4,5)P_3] after a maximal dose of carbachol was 46.4 ± 7.1 pmol/106 cells. McN-A-343 and *N*-methylatropine did not stimulate the production of Ins(1,4,5)P_3, and the data for these compounds are not shown for the purpose of clarity.

In further experiments, receptors were endocytosed in response to five muscarinic agonists of varying intrinsic activity. After washout of these compounds, receptors recycled back to the plasma membrane with identical kinetics (Table 2), showing that the rate of receptor recycling does not depend on the agonist used to cause endocytosis. The rate of receptor recycling after internalization with pilocarpine could not be measured because of the low levels of internalization observed with this compound.

Receptor endocytosis initiated by full and partial muscarinic agonists. To establish whether agonist intrinsic activity plays a role in determining the rate of receptor endocytosis, we measured receptor internalization in response to increasing concentrations of eight muscarinic ligands of varying intrinsic activity. Cells were stimulated for 30 min with the appropriate agonist, after which time receptor cycling to and from the plasma membrane has reached steady state (data not shown) (Koenig and Edwardson, 1996), and then thoroughly washed before quantifying the amount of receptors left at the cell surface (Fig. 3A and Table 1). The full agonists carbachol, methacholine, and oxotremorine-M all induced maximal receptor internalization, causing the loss of ~90% of surface receptors to intracellular compartments at maximal concentrations. Bethanechol, arecoline, and pilocarpine, which were partial agonists in the

TABLE 1

Binding affinity constants of the muscarinic ligands used in this study, and a comparison of their ability to stimulate Ins(1,4,5) P_3 production, internalization of receptors and the endocytotic rate constant, k_e. Values are means ± standard error from three or four experiments

The ability of the muscarinic ligands to stimulate Ins(1,4,5) P_3 production and internalization of receptors was measured as described in the text. Transformation of internalization data to yield increases in the endocytotic rate constant k_e was performed using eq. 4.

Compound	Peak Ins(1,4,5)P_3 response		Binding affinity constants		Maximal internalization of receptors		Maximal increase in endocytotic rate constant	
	R_{max}[a]	EC_{50}	K_i	Hill slope	I_{max}	EC_{50}	$k_{e(max)}$	EC_{50}
	%	μM	μM		%	μM	min^{-1}	μM
Carbachol	100	73.9 ± 1.9[b]	155 ± 20	0.88 ± 0.02	90.4 ± 0.7	3.5 ± 0.2[b]	0.545 ± 0.045	23.7 ± 2.0[b]
Oxotremorine-M	96.7 ± 2.5	6.57 ± 2.95[b]	23.2 ± 3.3	0.74 ± 0.01	90.3 ± 1.7	0.75 ± 0.12[b]	0.553 ± 0.092	6.68 ± 2.09[b]
Methacholine	94.0 ± 11.8	38.7 ± 7.0	57.9 ± 20.1	0.84 ± 0.07	89.1 ± 1.1	4.8 ± 1.3[b]	0.486 ± 0.059	40.3 ± 15.5
Bethanechol	51.6 ± 11.0	1197 ± 238[c]	407 ± 32	0.97 ± 0.08	82.0 ± 0.6	128 ± 19[b]	0.261 ± 0.011	552 ± 87
Arecoline	26.1 ± 3.9	147 ± 35[c]	15.5 ± 0.9	0.90 ± 0.04	78.4 ± 2.3	6.9 ± 2.4[b]	0.184 ± 0.012	38.1 ± 22.3
Pilocarpine	8.4 ± 1.2	127 ± 63	14.6 ± 1.4	0.99 ± 0.04	18.7 ± 0.7	57.2 ± 10.1[c]	0.013 ± 0.001	68.5 ± 11.4[c]
McN-A-343	No effect		35.6 ± 9.0	0.96 ± 0.14	No effect		No effect	
N-Methylatropine	No effect		0.00172 ± 0.00089	0.91 ± 0.10	No effect		No effect	

[a] R_{max} = % of maximal response elicited by carbachol.
[b] Significantly less than K_i, $p < 0.05$ by Student's t test.
[c] Significantly greater than K_i, $p < 0.05$ by Student's t test.

Ins(1,4,5)P_3 mass assays, also exhibited partial agonism in internalization assays, causing the maximal removal of 82.0 ± 0.6%, 78.4 ± 2.3%, and 18.7 ± 0.7%, respectively, of surface receptors at steady state, whereas McN-A-343 and *N*-methylatropine were again without effect. All agonists except the very weak partial agonist pilocarpine caused internalization with EC_{50} values that were significantly less than their K_i values (Table 1), suggesting that maximal receptor internalization does not require the presence of saturating concentrations of agonist.

One feature which is immediately clear from comparison of Figs. 1 and 3A is that, although the rank order of intrinsic activity is the same in both assays (carbachol = methacholine = oxotremorine-M > bethanechol > arecoline > pilocarpine > McN-A-343 = *N*-methylatropine), the ability of the three partial agonists to cause receptor internalization is much greater than one would expect from their effect on Ins(1,4,5)P_3, and the two effects are clearly not related in a simple linear fashion (Fig. 4A). Eq. 4 allows the calculation of k_e from steady state levels of surface receptors. Transformation of the best fit internalization curves shown in Fig. 3A to increases in k_e are shown in Fig. 3B. Comparison of Fig. 1 and Fig. 3B reveals that the ability of muscarinic ligands to stimulate the production of Ins(1,4,5)P_3 in SH-SY5Y cells and their ability to increase k_e show a striking correlation (Fig. 4B). Furthermore, the potency with which the muscarinic agonists increase k_e is of the same order of magnitude as that measured in the Ins(1,4,5)P_3 assays (Table 1), which contrasts with the approximately 10-fold more potent values obtained in the internalization assays (Fig. 3A and Table 1). The rank order of potency observed for increases in the rate of endocytosis is oxotremorine-M > (carbachol ≈ methacholine ≈ arecoline ≈ pilocarpine) ≫ bethanechol, which is the same as that seen for Ins(1,4,5)P_3 production.

Our data therefore suggest that the rate of endocytosis of receptors is modified by the addition of agonist. Once endocytosed, however, receptors follow a default pathway back to the cell surface, and the rate at which they proceed to the plasma membrane is independent of the agonist that drove them inside the cell. The number of receptors remaining at the cell surface after agonist stimulation is dependent on both k_e and k_r (eq. 4). Because only one of these parameters is agonist-dependent, the amount of internalization caused by a compound is not a linear function of its effect on k_e. Furthermore, the number of receptors internalized in re-

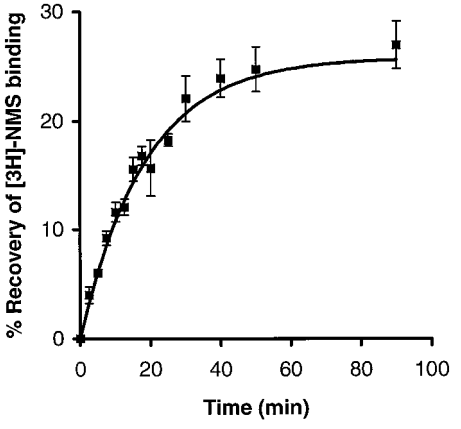

Fig. 2. Delivery of muscarinic receptors to the plasma membrane. Cells were stimulated with 1 mM carbachol for 30 min at 37°C to induce endocytosis of muscarinic receptors. After washing, cells were incubated with PrBCM (100 nM for 30 min at 15°C) to alkylate remaining surface receptors, washed again, and then incubated for various times at 37°C before determination of [^{3}H]NMS binding. Recovery is expressed as a percentage of control specific binding (i.e., binding in untreated cells), with the residual binding after PrBCM subtracted. Data are means ± standard error from three separate experiments. The recycling rate constant, k_r, was 0.054 ± 0.004 min^{-1}.

TABLE 2

Rate constants for recycling of muscarinic receptors after endocytosis in response to stimulation by various agonists

Cells were treated with agonist for 30 min at 37°C to induce receptor endocytosis. They were then washed free of agonist, and remaining surface receptors were alkylated with PrBCM at 15°C. PrBCM was washed away and the cells were warmed to 37°C to allow internal receptors to recycle, before measuring surface muscarinic receptor number using [^{3}H]NMS, as in Fig. 2. Curves were fitted to eq. 3.

Agonist	Recycling rate constant, k_r	r^2
	min^{-1}	
Carbachol (1 mM)	0.054 ± 0.004	0.997
Methacholine (1 mM)	0.053 ± 0.004	0.998
Oxotremorine-M (1 mM)	0.064 ± 0.008	0.999
Bethanechol (10 mM)	0.055 ± 0.002	0.999
Arecoline (1 mM)	0.061 ± 0.004	0.999

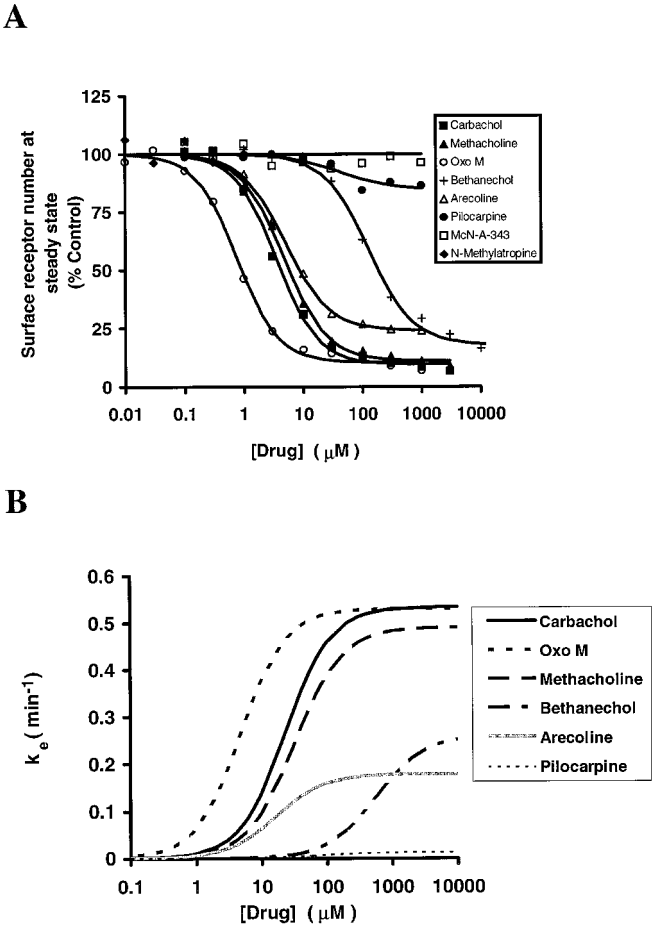

Fig. 3. A, Dose-response curves for the internalization of muscarinic receptors in SH-SY5Y cells. Cells were treated for 30 min at 37°C with the indicated concentration of drug and then washed thoroughly before determination of the number of muscarinic receptors remaining at the cell surface using [^{3}H]NMS. Data are means of three or four separate experiments. Error bars have been omitted for clarity. B, Transformation of steady state cell surface receptor number to increases in the endocytotic rate constant k_e. Best fit lines generated in A were transformed using eq. 4.

sponse to any agonist in a particular cell will depend on the recycling rate of the receptor in that cell, and on the maximum possible stimulation of the rate constant of endocytosis, k_e(max).

Fig. 5 shows a series of theoretical curves that demonstrate how k_e and k_r determine the level of receptor internalization seen with a very weak partial agonist, possessing only 3% of the intrinsic activity of a full agonist. Fig. 5A shows that the level of internalization is critically dependent on k_r. If k_r is large, then endocytosed receptors are quickly recycled back to the surface of the cell, and no loss of surface receptors is seen. However, as k_r falls, an increasing number of receptors are seen inside the cell at steady state, even when they are being stimulated by a very weak partial agonist. Fig. 5B shows the effect of varying k_e(max) on the level of receptor internalization. When k_e(max) is small, no internalization is seen, even in the presence of saturating doses of the agonist. However, as k_e(max) rises, a greater proportion of receptors is found inside the cell at steady state. Thus, in a cell line where k_r is small and k_e(max) is large, even a very weak partial agonist can cause significant levels of internalization of cell surface receptors.

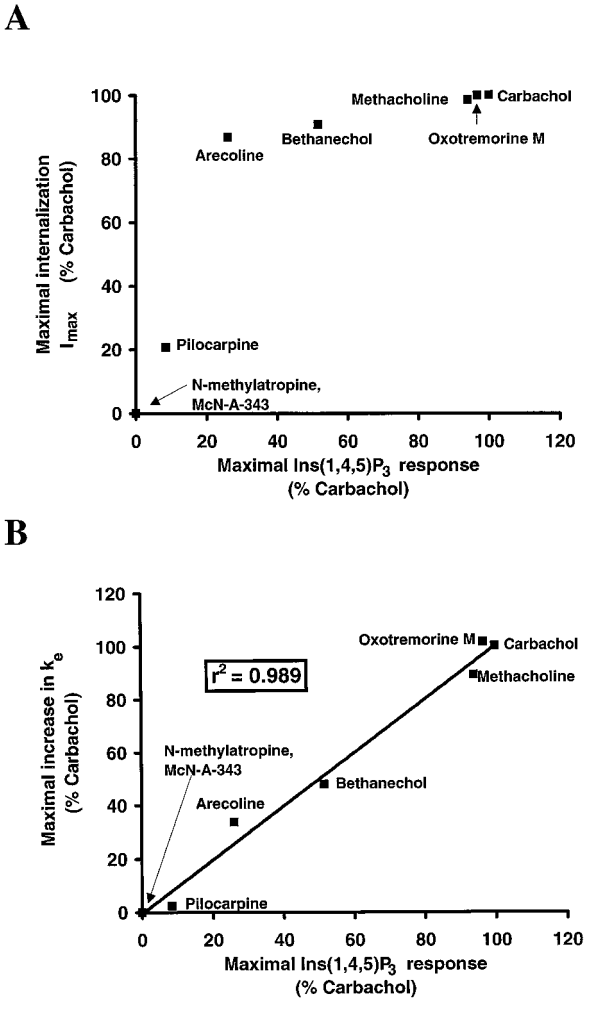

Fig. 4. Correlation between the ability of muscarinic ligands to stimulate the production of Ins(1,4,5)P_3 and their ability to A, cause internalization of muscarinic receptors, and B, increase k_e.

Discussion

We have shown previously (Koenig and Edwardson, 1997) that the number of receptors internalized in response to agonist stimulation depends not only on the rate constant for endocytosis, k_e, but also on the rate constant for recycling, k_r. In the present study, we compared the abilities of various muscarinic ligands, ranging from full agonists through partial agonists to antagonists, to stimulate Ins(1,4,5)P_3 production and receptor internalization. Ins(1,4,5)P_3 production was used as an index of agonist intrinsic activity because this was considered to be the parameter that would most closely reflect receptor activation, being the first measurable point in the signal transduction cascade in whole cells.

We analyzed the internalization data to dissect out the effects of the ligands on k_e and k_r, and found that the intrinsic activities of the ligands correlate directly with their ability to increase k_e. In contrast, k_r is independent of the ligand that caused the endocytosis. The constitutive nature of the recycling process is in agreement with a previous report that the recycling rate of β_2-adrenergic receptors is not affected by the

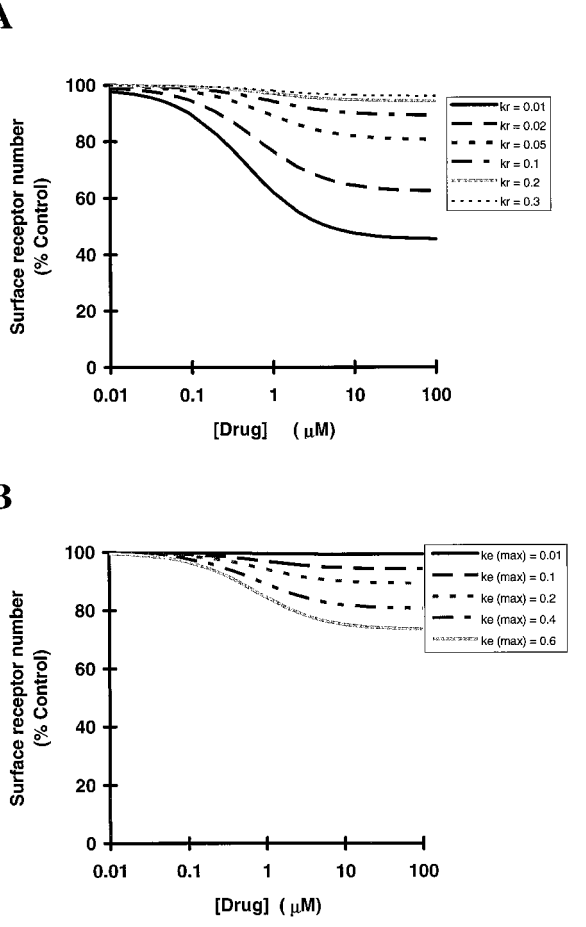

Fig. 5. Theoretical internalization curves for a weak partial agonist generated from eq. 4, assuming that the k_e(max) is related linearly to the intrinsic activity of the agonist. A, Effect of varying the rate constant for recycling; B, effect of varying the rate constant for endocytosis. *Curves*, theoretical surface receptor number at steady state after stimulation with a weak partial agonist which has an intrinsic activity of only 3% of that of a full agonist. k_e is increased by the partial agonist with an EC_{50} of 1 μM and a Hill slope of 1. In A, the maximal value of k_e in the presence of a maximal dose of a full agonist (k_e(max)) was set at 0.4 min^{-1}, and k_r was varied between 0.01 and 0.3 min^{-1}. In B, the value of k_r was set at 0.05 min^{-1}, and k_e(max) was varied between 0.01 and 0.6 min^{-1}.

presence of agonist (Morrison *et al.*, 1996). It has also been shown (Mayor *et al.*, 1993) that transferrin receptors and membrane lipids pass through the endocytotic system with indistinguishable kinetics, suggesting that recycling of membrane proteins is the default pathway in the absence of specific lysosomal sorting motifs.

To measure k_r as accurately as possible, recycling was determined after alkylation of all receptors remaining at the surface with PrBCM at a relatively high concentration (100 nM). The extent of recycling observed under these conditions (approximately 25% of the number of receptors initially present at the surface) is much smaller than the loss of receptors from the surface in response to agonist stimulation (approximately 90%). In fact, the protocol used in this study results in an underestimation of the true extent of recycling, because PrBCM is apparently able to alkylate some internal receptors. Consequently, in these experiments we are underestimating the extent of recycling to measure k_r accurately. If the PrBCM step is omitted, the extent of recycling is approximately 70% of the initial cell-surface value (Szekeres PG, Koenig JA, and Edwardson JM, unpublished observations). Even in the absence of the PrBCM treatment, therefore, recycling is incomplete. The shortfall cannot be accounted for by receptor degradation,[1] indicating that some of the receptors are being translocated into a compartment from which they recycle very slowly or not at all. These findings suggest that a two-compartment model is an oversimplification, and we are currently developing a model that will more accurately reflect the real situation.

The correlation between intrinsic activity of the ligand and its effect on k_e extends the observations of a previous study (Thompson and Fisher, 1990), and strongly suggests that, at least in this case, the same receptor conformation is responsible for coupling to G proteins and stimulating endocytosis. Further evidence for this concept comes from the identical potency profiles observed for both production of $Ins(1,4,5)P_3$ and stimulation of the endocytotic rate constant, k_e. Usually, agonists but not antagonists cause internalization of G protein-coupled receptors. However, a recent report (Roettger *et al.*, 1997) indicated that antagonists at the cholecystokinin receptor may also cause receptor internalization, implying that different receptor conformations are required to stimulate second messenger production and endocytosis. Further evidence for separate receptor conformations has been obtained from recent observations of the μ-opioid and δ-opioid receptors in transfected cells and enteric neurons (Sternini *et al.*, 1996; Keith *et al.*, 1996), where enkephalins and etorphine stimulate receptor endocytosis, but the full agonist morphine does not. One interpretation of these results is that the opioid receptors contain multiple agonist recognition domains (Wang *et al.*, 1995; Kong *et al.*, 1993), leading to the possibility of multiple activated conformations upon treatment with different agonists. In contrast, muscarinic agonists are generally thought to interact with a conserved agonist binding site (Hulme *et al.*, 1990; Wess *et al.*, 1991). Our study certainly provides no evidence for the involvement of separate receptor conformations in initiating signal transduction and endocytosis of muscarinic receptors.

Whether G protein activation and second messenger production are themselves involved in triggering endocytosis is a question that has been addressed by many groups. The most extensively studied G protein-coupled receptor is the β_2-adrenergic receptor, which couples to G_s and activates adenylyl cyclase. Because β_2-adrenergic receptors are internalized in cells that do not express G_s (Clark *et al.*, 1985) and mutant β_2-adrenergic receptors that do not effectively couple to G_s are internalized to the same extent as wild-type receptors (Cheung *et al.*, 1990), it is likely that, at least for this receptor, G protein coupling and/or activation is not a prerequisite for receptor internalization.

It has been reported previously that, in permeabilized SH-SY5Y cells, both U-73122, an inhibitor of phospholipase C-dependent processes, and guanosine-5′-*O*-(2-thiodiphosphate), which blocks G protein activation, inhibit agonist-mediated muscarinic receptor internalization, but that omission of either ATP or Ca^{2+}, both of which are required for stimulation of phosphoinositide hydrolysis, was without effect (Thompson *et al.*, 1991; Slowiejko *et al.*, 1994). These results indicate that G protein activation is required for internalization of the m3 muscarinic receptor but that second messenger production is not. Conflicting results have been obtained for other G protein-coupled receptors. For example, Benya *et al.* (1994) produced two mutant gastrin-releasing peptide receptors and showed that one of these mutant receptors maintained intact G protein coupling and showed a robust internalization in response to agonist, whereas the other mutant was no longer coupled to G protein and did not internalize, suggesting a functional link between the two processes. On the other hand, the coupling of the thyrotropin-releasing hormone receptor to the G protein G_q was not required for receptor internalization (Petrou *et al.*, 1997), and there was no correlation between G protein coupling and internalization in a series of mutants of the angiotensin II receptor constructed by Hunyady *et al.* (1994), suggesting that endocytosis of these receptors is independent of G protein coupling. Finally, mutant muscarinic receptors have been constructed that show impaired internalization but normal G protein coupling (Moro *et al.*, 1993). Overall, the results of the above studies leave us with the possibility that there is no general rule governing the behavior of all members of the G protein-coupled receptor superfamily, and that the determinants of internalization may depend on both receptor type and cell type. One potentially confusing factor, which is never addressed in studies based upon receptor mutagenesis, is that the mutant receptors may have altered recycling rates. This may cause apparent differences in their internalization properties, because the number of receptors internalized will depend on both the rates of endocytosis and recycling. The results of these studies, therefore, need to be interpreted with some caution.

An important implication of our results is that the removal of receptors from the plasma membrane is not a linear function of k_e, and in addition will depend on both k_r and k_e(max), parameters that are cell type-specific (Koenig and Edwardson, 1996). Furthermore, agonist regulation of k_e, but not k_r, provides a means by which very weak stimulation of the receptor can result in substantial internalization. In the simulations shown here (Fig. 5), we consider the effects of a partial agonist, which possesses only 3% of the intrinsic activity of a full agonist. In our view, the detection of such a low level of agonist activity would be unlikely in most second messenger assays, and therefore this compound could easily be classified as an antagonist. Our analysis shows that when k_e and k_r are varied over a range often seen for G protein-

coupled receptors in mammalian cells (Koenig and Edwardson, 1994, 1996; Barak *et al.*, 1995; Pippig *et al.*, 1995; Morrison *et al.*, 1996), significant internalization of receptors can be achieved with a maximal dose of drug, under conditions where either k_r is low or k_e(max) is high, or both. Such values for the rate constants would give rise to a system where stimulation with a full agonist results in rapid internalization of a large proportion of surface receptors. It is interesting to note that in the one published example of antagonist-induced receptor internalization (Roettger *et al.*, 1997), full agonist occupation of the same receptor gave rise to a rapid internalization of 90% of the receptors, whereas antagonist caused the internalization of only 37% of receptors. Thus, the amplification effect of small agonist-induced increases in k_e producing large amounts of receptor internalization may provide an alternative explanation of apparent antagonist-triggered receptor internalization. Our results also raise the possibility that cells which have a low value of k_r and a high value of k_e(max) for a particular receptor type might provide a useful system in which to use receptor internalization to detect weak partial agonist activity.

Acknowledgments

We thank Andy Letcher (Department of Pharmacology, University of Cambridge, Cambridge, UK) for the generous gift of the porcine platelet membrane preparation and Dr. J. M. Young for providing us with PrBCM and McN-A-343. The expert technical assistance of Diane Hall is gratefully acknowledged. J.A.K. thanks Prof. P. P. A. Humphrey, the Glaxo Institute for Applied Pharmacology, and Magdalene College, Cambridge, for their support.

References

Barak LS, Ménard L, Ferguson SSG, Colapietro A-M, and Caron MG (1995) The conserved seven-transmembrane sequence $NP(X)_{2,3}Y$ of the G-protein-coupled receptor superfamily regulates multiple properties of the β_2-adrenergic receptor. *Biochemistry* **34**: 15407–15414.

Benya RV, Akeson M, Mrozinski J, Jensen RT, and Battey JF (1994) Internalization of the gastrin-releasing peptide receptor is mediated by both phospholipase C-dependent and independent processes. *Mol Pharmacol* **46**:495–501.

Bogatkewitsch GS, Lenz W, Jakobs KH, and Van Koppen CJ (1996) Receptor internalization delays m4 muscarinic acetylcholine receptor resensitization at the plasma membrane. *Mol Pharmacol* **50**:424–429.

Cheung AH, Dixon RAF, Hill WS, Sigal IS, and Strader CD (1990) Separation of the structural requirements for agonist-promoted activation and sequestration of the β-adrenergic receptor. *Mol Pharmacol* **37**:775–779.

Clark RB, Friedman J, Prashad N, and Ruoho AE (1985) Epinephrine-induced sequestration of the β-adrenergic receptor in cultured S49 WT and cyc^- lymphoma cells. *J Cyclic Nucleotide Protein Phosphorylation Res* **10**:97–119.

Communi D, Vanweyenberg V, and Erneux C (1997) D-*myo*-Inositol 1,4,5-trisphosphate 3-kinase A is activated by receptor activation through a calcium: calmodulin-dependent protein kinase II phosphorylation mechanism. *EMBO J* **16**:1943–1952.

Cullen PJ, Dawson AP, and Irvine RF (1995) Purification and characterization of an Ins(1,3,4,5)P_4 binding protein from pig platelets: Possible identification of a novel non-neuronal Ins(1,3,4,5)P_4 receptor. *Biochem J* **305**:139–143.

Hulme EC, Birdsall NJM, and Buckley NJ (1990) Muscarinic receptor subtypes. *Annu Rev Pharmacol Toxicol* **30:**633–673.

Hunyady L, Baukal AJ, Balla T, and Catt KJ (1994) Independence of type I angiotensin II receptor endocytosis from G protein coupling and signal transduction. *J Biol Chem* **269**:24798–24804.

Keith DE, Murray SR, Zaki PA, Chu PC, Lissin DV, Kang L, Evans CJ, and von Zastrow M (1996) Morphine activates opioid receptors without causing their rapid internalization. *J Biol Chem* **271**:19021–19024.

Koenig JA and Edwardson JM (1994) Kinetic analysis of the trafficking of muscarinic acetylcholine receptors between the plasma membrane and intracellular compartments. *J Biol Chem* **269**:17174–17182.

Koenig JA and Edwardson JM (1996) Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. *Mol Pharmacol* **49**: 351–359.

Koenig JA and Edwardson JM (1997) Endocytosis and recycling of G protein-coupled receptors. *Trends Pharmacol Sci* **18**:276–287.

Kong H, Raynor K, Yasuda K, Moe ST, Portoghese PS, Bell GI, and Reisine T (1993) A single residue, aspartic acid 95, in the δ opioid receptor specifies selective high affinity agonist binding. *J Biol Chem* **268**:23055–23058.

Mayor S, Presley JF, and Maxfield FR (1993) Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J Cell Biol* **121**:1257–1269.

Moro O, Lameh J, and Sadée W (1993) Serine- and threonine-rich domain regulates internalization of muscarinic cholinergic receptors. *J Biol Chem* **268**:6862–6865.

Morrison KJ, Moore RH, Carsrud NDV, Trial J, Millman EE, Tuvim M, Clark RB, Barber R, Dickey BF, and Knoll BJ (1996) Repetitive endocytosis and recycling of the β_2-adrenergic receptor during agonist-induced steady state redistribution. *Mol Pharmacol* **50**:692–699.

Petrou C, Chen L, and Tashjian AH Jr (1997) A receptor-G protein coupling-independent step in the internalization of the thyrotropin-releasing hormone receptor. *J Biol Chem* **272**: 2326–2333.

Pippig S, Andexinger S, and Lohse MJ (1995) Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization. *Mol Pharmacol* **47**:666–676.

Roettger BF, Ghanekar D, Rao R, Toledo J, Yingling J, Pinon D, and Miller LJ (1997) Antagonist-stimulated internalization of the G protein-coupled cholecystokinin receptor. *Mol Pharmacol* **51**:357–362.

Slowiejko DM, Levey AI, and Fisher SK (1994) Sequestration of muscarinic cholinergic receptors in permeabilized neuroblastoma cells. *J Neurochem* **62**:1795–1803.

Sternini C, Spann M, Anton B, Keith DE, Bunnett NW, von Zastrow M, Evans C, and Brecha NC (1996) Agonist-selective endocytosis of μ opioid receptor by neurons *in vivo. Proc Natl Acad Sci USA* **93**:9241–9246.

Thompson AK and Fisher SK (1990) Relationship between agonist-induced muscarinic receptor loss and desensitization of stimulated phosphoinositide turnover in two neuroblastomas: methodological considerations. *J Pharmacol Exp Ther* **252**: 744–752.

Thompson AK, Mostafapour SP, Denlinger LC, Bleasdale JE, and Fisher SK (1991) The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J Biol Chem* **266**:23856–23862.

Wall SJ, Yasuda RP, Li M, and Wolfe BB (1991) Development of an antiserum against m3 receptors: Distribution of m3 receptors in rat tissues and clonal cell lines. *Mol Pharmacol* **40:**783–789.

Wang WW, Shahrestanifar M, Jin J, and Howells RD (1995) Studies on μ and δ opioid receptor selectivity utilizing chimeric and site-mutagenized receptors. *Proc Natl Acad Sci USA* **92**:12436–12440.

Wess J, Gdula D, and Brann MR (1991) Site-directed mutagenesis of the m3 muscarinic receptor: Identification of a series of threonine and tyrosine residues involved in agonist but not antagonist binding. *EMBO J* **10:**3729–3734.

Willars GB and Nahorski SR (1995) Quantitative comparisons of muscarinic and bradykinin receptor-mediated Ins(1,4,5)P_3 accumulation and Ca^{2+} signalling in human neuroblastoma cells. *Br J Pharmacol* **114:**1133–1142.

Send reprint requests to: Dr. J. M. Edwardson, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, UK. E-mail: jme1000@cus.cam.ac.uk