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Intracellular Trafficking of the Muscarinic Acetylcholine Receptor: Importance of Subtype and Cell Type

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SUMMARY

Agonist-induced decrease of surface muscarinic receptor number occurs in a number of cell lines. Recent work has suggested that some muscarinic receptor subtypes undergo internalization, whereas others do not. We investigated the agonist-induced trafficking of various muscarinic receptor subtypes transfected into CHO cells and compared it with the trafficking of receptors expressed natively in neuronal cells, fibroblasts, or epithelial cells. SH-SY5Y neuroblastoma cells, which express predominantly the m3 receptor subtype, show qualitatively similar changes in surface receptor number in response to agonist stimulation to those occurring in NG108–15 cells, which express predominantly the m4 subtype. The rate constants for internalization, however, were considerably different, indicating that receptors in SH-SY5Y cells show a much faster turnover than those in NG108–15 cells. In the transfected cells, the muscarinic receptor subtypes m1 and m3, which are coupled to second messenger systems via $G_{q/11}$, showed little agonist-induced loss of surface receptors. In contrast, the muscarinic receptor subtypes m2 and m4, which are coupled via G_i or G_o, showed a substantial loss of surface receptors after treatment with agonist. An interesting implication of this result is that agonist-induced receptor trafficking can still occur efficiently, even at very high receptor densities. Significant agonist-induced internalization also occurs in a fibroblast line (HeLa) and an epithelial cell line (HT29), both of which express predominantly m3 receptors. Our results suggest that the extent and rate of the loss of receptors from the cell surface in response to agonist stimulation are governed by both the receptor subtype and the cell type in which it is expressed.

Agonist stimulation of muscarinic receptors causes desensitization of the cellular response in some, but not all, cell types. In the short term, the desensitization process is believed to involve two main mechanisms: a conformational change in the receptor leading to decreased G protein coupling, and removal of receptors from the cell surface to an internal compartment. In the long term, changes in total receptor number occur due to increased degradation and changes in the rate of synthesis of the receptor. The contribution made by receptor internalization to the desensitization process remains controversial because in some instances desensitization occurs without apparent loss of surface receptor number (1), whereas in other systems, the loss of surface receptor number correlates well with the extent of desensitization (2, 3). Furthermore, there are some cells in which there is an apparent loss of surface receptor number with no concurrent desensitization (4, 5).

There are a number of mechanisms that could account for these differences, including the presence of spare receptors, differences in receptor trafficking in particular cell types, differential sorting of the receptors and their associated G proteins, and differences in the trafficking properties of the different receptor subtypes. There are five subtypes of muscarinic receptor, designated m1–m5, which couple to different second messenger systems; m1, m3, and m5 stimulate phosphoinositide hydrolysis, and m2 and m4 inhibit adenylate cyclase. The subtypes also show differential sensitivity to phosphorylating enzymes, which are believed to affect their function. For example, activation of protein kinase C in stably transfected adrenal cells causes internalization of the m1 receptor but not the m2 receptor (6). Methodological considerations are also important because the trafficking properties of the receptor are affected by temperature and can depend on whether the cells are in suspension or in a monolayer (7).

Internalization of the muscarinic receptor in response to agonist stimulation is generally observed as a 20–80% reduction in the binding of the membrane-impermeant radioligand [^{3}H]NMS (8). In the short term (up to 1 hr), there is no reduction in total receptor number, as measured by the binding of [^{3}H]scopolamine or [^{3}H]quinuclidinylbenzilate. We have derived mathematical expressions based on a widely accepted model (9), which describes the changes in surface and endosomal receptor numbers and the rates of receptor movement. The most important result of this work is that we have been able to estimate how quickly receptors are cycling

ABBREVIATIONS: DMEM, Dulbecco's modified Eagle's medium; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; NMS, *N*-methylscopolamine; PrBCM, propyl-benzilylcholine mustard.

to and from the cell surface (10). For example, during a 13-min incubation of NG108-15 cells with a saturating concentration of carbachol, a typical receptor will go through one internalization-and-recycling cycle. During this same period, there is a reduction in surface receptor binding of only 40%. In the first minute of incubation with agonist, 12% of surface receptors are internalized (1,380 receptors internalized from a control surface receptor density of 11,500 receptors), but there is no significant change in overall surface receptor number. After agonist incubation for 30 min, the rate of change of surface receptors reaches a plateau, but the rate of internalization of surface receptors is 606 receptors/min and the rate of recycling is 560 receptors/min, with the difference accounted for by degradation.

In the present study, we analyzed the trafficking of four muscarinic receptor subtypes (m1, m2, m3, and m4) transfected into CHO cells and the trafficking of the m3 subtype naturally expressed in SH-SY5Y cells [human neuroblastoma (11)], HeLa cells [human fibroblasts (12)], and HT29 [human gut epithelial cells (13)]. Our results show that the trafficking properties depend on both the receptor subtype and the cell type in which it is expressed.

Materials and Methods

Cell culture. SH-SY5Y cells were a gift of Prof. G. Henderson (Department of Pharmacology, University of Bristol, UK) and were grown at 37° in DMEM 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. CHO cells transfected with the m1, m2, m3, and m4 human muscarinic receptor subtypes were kindly provided by Dr. N. J. Buckley (MRC Cell Biology Centre, University College, London, UK) and were grown in DMEM supplemented with minimal nonessential amino acids, 5% fetal bovine serum, and 5% newborn bovine serum. HeLa cells were a gift of Dr. J. M. Young (Department of Pharmacology, University of Cambridge, UK) and were grown in DMEM supplemented with 5% fetal bovine serum and 5% newborn calf serum. HT29 cells were a gift of Dr. L. Sellars (Glaxo Institute of Applied Pharmacology, University of Cambridge, UK) and were grown in DMEM supplemented with 10% fetal bovine serum. Cells were detached from culture flasks for passaging by a brief incubation with trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) in PBS. Cells were passaged every 3–4 days. For experiments, cells were grown on 24-well plates. The CHO cells, which expressed receptors at high surface density, were used at densities of <100,000 cells/well to avoid radioligand depletion during binding experiments. The other cell types were used at confluence.

Analysis of receptor trafficking. Muscarinic receptor trafficking was monitored through changes in surface receptor number. Surface receptors were quantified through the binding of [^{3}H]NMS. Delivery of receptors to the plasma membrane was detected through the recovery of [^{3}H]NMS binding after alkylation of surface receptors. Receptor internalization (e.g., in response to agonist stimulation) was seen as a reduction in [^{3}H]NMS binding.

Alkylation of receptors at the plasma membrane. Muscarinic receptors at the plasma membrane were irreversibly alkylated with PrBCM. The pharmacologically active species of PrBCM is the aziridinium ion, which was prepared according to the method of Young *et al.* (14). PrBCM (0.1 mM) was allowed to cyclize in PBS, pH 7.4, at room temperature for 1 hr, and the reaction was stopped by dilution to 10^{-7} M. This procedure gives a yield of ~91% (14). Cells were washed at 20° in 1 ml PBS containing 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS-Ca/Mg) and incubated in 2 ml of the same buffer containing 10^{-7} M PrBCM for 30 min at 15–20°. [^{3}H]NMS binding remaining after PrBCM treatment was <10% of the original binding for transfected CHO cells and less than 5% for SH-SY5Y cells. All subsequent values were corrected for this residual binding. We have shown previously that the delivery of receptors to the plasma membrane occurring during the 30-min incubation with PrBCM at 20° is negligible (8). After removal of PrBCM, cells were washed with 2 ml PBS-Ca/Mg and incubated in HEPES-buffered DMEM containing 0.2% bovine serum albumin (DMEM-HEPES) at 37° for various times to allow receptor delivery to the plasma membrane to proceed. The medium was removed, and the cells were washed with 2 ml PBS-Ca/Mg before quantification of receptor number at the plasma membrane by [^{3}H]NMS binding.

Quantification of receptors at the plasma membrane. Cells were incubated with 1 ml/well (SH-SY5Y cells), 2 ml/well (CHO cells), or 0.5 ml/well (HeLa, HT29 cells) of 0.1–0.4 nM [^{3}H]NMS in PBS-Ca/Mg for 16 hr at 4° (5 hr at 15° for HT29 cells). Radioligand binding reaches equilibrium under these conditions.[1] Nonspecific binding was defined with the use of *N*-methylatropine (1 μM). Incubations were terminated by two washes with 2 ml PBS-Ca/Mg. For saturation analysis, nine (SH-SY5Y) or eight (CHO, HeLa, HT29 cells) concentrations were used of [^{3}H]NMS over the range of 0.01–2 nM. At concentrations of [^{3}H]NMS around the dissociation constant (K_D), specific binding represented 92–99% of total binding.

After termination of each binding assay, the cells were solubilized in 0.2 ml 0.5% Triton X-100, and the contents of each well were transferred to vials for liquid scintillation counting. For SH-SY5Y cells, the contents of two wells were combined in one scintillation vial; this was subsequently called one sample. For HeLa and HT29 cells, three wells were combined for each sample. Cell density was determined by removing cells from the wells with 0.2 ml PBS containing EGTA (1 mM) and trypsin (50 mg/ml) and counting the cells with a Neubauer hemocytometer. Cell densities were: SH-SY5Y cells, $2.8 \pm 0.4 \times 10^6$ cells/sample (eight experiments); CHO-m1 cells, $1.8 \pm 0.9 \times 10^4$ cells/sample (three experiments); CHO-m2 cells, $1.1 \pm 0.3 \times 10^5$ cells/sample (three experiments); CHO-m3 cells, $1.4 \pm 0.4 \times 10^5$ cells/sample (11 experiments); CHO-m4 cells, $1.3 \pm 0.3 \times 10^5$ cells/sample (11 experiments); HeLa cells, $3.8 \pm 0.4 \times 10^6$ cells/sample (three experiments); and HT29 cells, $3.1 \pm 0.9 \times 10^6$ cells/sample (three experiments).

Modulation of receptor trafficking. Where appropriate, cells were pretreated at 37° with the muscarinic agonist carbachol, the protein synthesis inhibitor cycloheximide, or both. Carbachol stimulation was at 0.5 mM in DMEM-HEPES for various times; carbachol was then removed by two washes with 2 ml of the same medium. Cycloheximide pretreatment (20 μg/ml in DMEM-HEPES) lasted 1 hr, and the reagent was included in all subsequent buffers, including wash buffers, until determination of [^{3}H]NMS binding.

Materials. [^{3}H]NMS (specific activity, 81–85 Ci/mmol) was obtained from Amersham International (Amersham, UK). Cell culture medium was purchased from ICN Ltd. (High Wycombe, UK), and fetal bovine serum was purchased from GIBCO (Uxbridge, UK). PrBCM and *N*-methylatropine were generous gifts of Dr. J. M. Young (Department of Pharmacology, University of Cambridge, UK). All other reagents were obtained from Sigma (Poole, UK).

Data analysis. Within each experiment, values were determined in triplicate. All values are mean ± standard error. The relative replicate error ranged between 2% and 10%. Data for internalization and surface-delivery assays are expressed as a percentage of control values where the control is defined as the specific binding to cells that had been through all of the same washing procedures but were not exposed to any pharmacological agents. These values can be converted to units of receptors/cell/min or receptors/cell by multiplying the value in percent/min or percent by the maximal number of binding sites per cell. The average specific binding was ~4,000 dpm at 0.2 nM [^{3}H]NMS for SH-SY5Y, HeLa, and HT29 cells and 20,000 dpm at 0.2 nM [^{3}H]NMS for CHO cells. Saturation data were analyzed using the program LIGAND (15). Internalization and surface-delivery data were analyzed by nonlinear regression using the pro-

[1] J. A. Koenig and J. M. Edwardson, unpublished observations.

gram FigP (Elsevier Biosoft, Cambridge, UK). F tests (significant at $p < 0.05$) were used for comparison of nonlinear curve fitting of the data to different models.

Results

Experimental strategy. We applied a full kinetic analysis to the trafficking of the m3 muscarinic receptor subtype in SH-SY5Y cells, where it occurs naturally, and of the m3 and m4 subtypes in transfected CHO cells. For comparison, we also investigated the effect of agonist stimulation on the number of receptors at the surface of CHO cells expressing the m1 and m2 subtypes and of HeLa cells and HT29 cells, both of which naturally express predominantly the m3 subtype.

Characteristics of [^{3}H]NMS binding. Saturation analysis of [^{3}H]NMS binding showed that the affinity of the radioligand for the muscarinic receptor differed only 5-fold among the various cell types, whereas the density of receptors at the cell surface varied over almost a 1000-fold range. Scatchard parameters are given in Table 1.

Delivery of receptors to the cell surface. The general equation describing changes in cell surface receptor number as a function of time is defined with a series of parameters that correspond to the rate constants for each of the trafficking steps (10). This equation (eq. 1) is:

$$r_s = \frac{1}{k_2 + k_3}\left[\left\{k_1 + k_2 r_{eo} + k_2\frac{(k_4 - k_1)}{(k_2 + k_3)}\right\}(1 - e^{(k_2 + k_3)t}) + r_{so}(k_2 + k_3 e^{-(k_2 + k_3)t}) - k_2(k_4 - k_1)t\right] \quad (1)$$

where k_1 is rate of delivery to the cell surface due to new synthesis, k_2 is rate constant for recycling from endosomes to the surface, k_3 is rate constant for internalization from the surface to endosomes, k_4 is rate of degradation, r_{eo} is number of endosomal receptors at zero time, and r_{so} is number of surface receptors at zero time. The general equation can be simplified by manipulation of the experimental conditions. For example, alkylation of receptors at the surface with PrBCM can be used to set r_{so} to zero. If it is assumed for simplicity that k_3 and k_4 are negligible in PrBCM-treated cells in the absence of carbachol, then the general equation simplifies to the following (eq. 2):

$$r_s = r_{eo}(1 - e^{-k_2 t}) + k_1 t \quad (2)$$

which represents delivery of receptors from a finite internal pool [$r_{eo}(1 - e^{-k_2 t})$]) and from linear new synthesis ($k_1 t$).

The recovery of [^{3}H]NMS binding after exposure to PrBCM in untreated SH-SY5Y cells could be resolved into two components: an exponential, cycloheximide-resistant component representing delivery of receptors to the cell surface from an internal pool, and a linear, cycloheximide-sensitive component resulting from the surface delivery of newly synthesized receptors. Curve-fitting of the data for cycloheximide-treated cells gave an estimate of k_1 that was not significantly different from zero. This parameter was therefore fixed at zero in further calculations. It was also assumed that neither the recycling rate constant k_2 (0.038 ± 0.008 min^{-1}) nor the endosomal pool size r_{eo} (12.1 ± 1.0%, or 1620 receptors/cell) would be affected by cycloheximide treatment. These values were then used to calculate the rate of delivery of receptors to the cell surface in untreated cells (k_1 = 0.092%/min, corresponding to 12 receptors/cell/min; Fig. 1 and Table 2). The delivery of receptors to the cell surface in cycloheximide-treated cells was increased after stimulation with carbachol. The internal receptor pool in carbachol-treated cells was 30.0 ± 1.8% of the number initially at the surface (4020 receptors/cell) compared with 1620 receptors/cell in unstimulated cells. The rate constant for recycling, k_2, was also increased after carbachol stimulation, to a new value of 0.074 ± 0.014 min^{-1}.

In unstimulated CHO-m3 cells, the rate of surface delivery as a consequence of new receptor synthesis was similar to the corresponding value for SH-SY5Y cells when expressed as a percentage of the number of receptors at the cell surface (k_1 = 0.083% min^{-1}; Fig. 2A and Table 3), and the size of the endosomal receptor pool was smaller (r_{eo} = 5.5%). However, because of the large B_{max} value in CHO-m3 cells, these values translate into much larger numbers of receptors (k_1 = 670 receptors/cell/min; r_{eo} = 44,300 receptors/cell). Unlike the result with SH-SY5Y cells, cycloheximide and carbachol did not greatly modify the delivery of receptors to the surface of CHO-m3 cells. Cycloheximide treatment of CHO-m3 cells did not significantly affect any of the three parameters k_1, k_2

TABLE 1

Parameter estimates for saturation analysis of [^{3}H]NMS binding

Cell type	K_D	B_{max}	No. of experiments
	nM	*receptors/cell*	
SH-SY5Y	0.39 ± 0.08	13,400 ± 1,400	3
HeLa	0.052, 0.082	1,020, 1,820	2
HT29	0.099, 0.109	2,640, 2,590	2
CHO-m1	0.073 ± 0.001	1,272,000 ± 550,000	3
CHO-m2	0.27 ± 0.05	47,900 ± 6,800	3
CHO-m3	0.15 ± 0.03	806,000 ± 201,000	3
CHO-m4	0.12 ± 0.03	990,000 ± 290,000	3

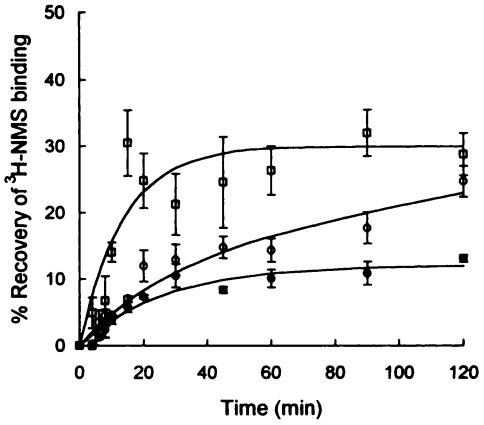

Fig. 1. Delivery of receptors to the plasma membrane in SH-SY5Y cells. Cells were either untreated (○) or pretreated with cycloheximide (20 μg/ml) for 1 hr at 37°. Cycloheximide-treated cells were either unstimulated (●) or stimulated with carbachol (0.5 mM) for 30 min at 37° (□). Cells were incubated with PrBCM (10^{-7} M for 30 min at 20°) and then allowed to recover for various times at 37° before determination of [^{3}H]NMS binding. Recovery is expressed as a percentage of control specific binding, with the residual binding after PrBCM treatment subtracted. Values are mean ± standard error from four separate experiments, each performed in triplicate. Graphs show lines of best fit from nonlinear regression analysis: untreated, k_1 = 0.092 ± 0.009% min^{-1}, k_2 = 0.038 min^{-1} (fixed), r_{eo} = 12.1% (fixed); cycloheximide-treated, k_1 = 0% min^{-1} (fixed), k_2 = 0.038 ± 0.008 min^{-1}, r_{eo} = 12.1 ± 1.0%; cycloheximide-treated and carbachol-stimulated, k_1 = 0% min^{-1}, k_2 = 0.074 ± 0.014 min^{-1}, r_{eo} = 30.0 ± 1.8%.

TABLE 2

Parameter estimates for the delivery of receptors to the cell surface in SH-SY5Y and NG108–15 cells

Cycloheximide	Carbachol	k_1	k_2	r_{eo}	Correlation coefficient
		receptors/cell/min	*min*$^{-1}$	*receptors/cell*	
SH-SY5Y					
–	–	12 ± 1	0.038 (fixed)	1620 (fixed)	0.96
+	–	0 (fixed)	0.038 ± 0.008	1620 ± 130	0.93
+	+	0 (fixed)	0.074 ± 0.014	4020 ± 240	0.92
NG108–15[a]					
–	–	22 ± 1	0	0	0.98
+	+	0	0.12 ± 0.02	3220 ± 120	0.92

[a] Data from Ref. 9, for comparison.

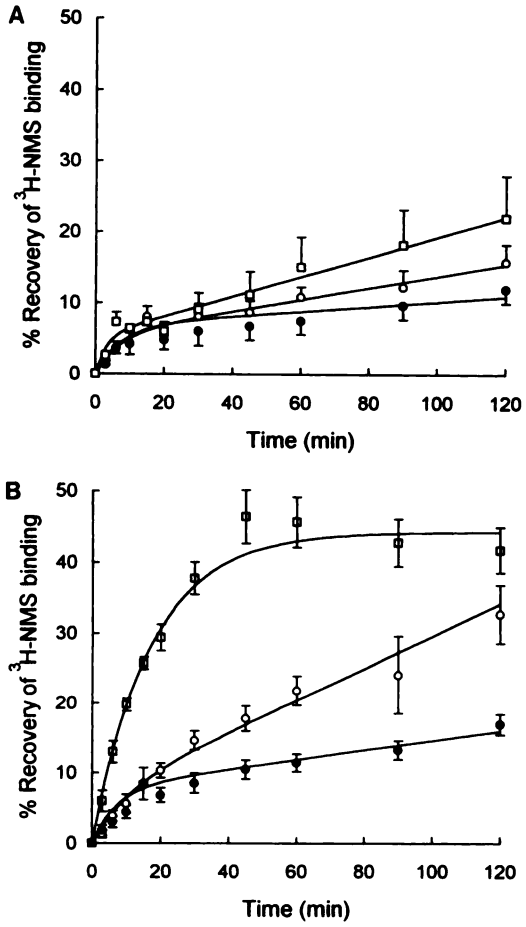

Fig. 2. Delivery of receptors to the plasma membrane in CHO-m3 (A) and CHO-m4 (B) cells. Cells were either untreated (○) or pretreated with cycloheximide (20 μg/ml) for 1 hr at 37°. Cycloheximide-treated cells were either unstimulated (●) or stimulated with carbachol (0.5 mM) for 30 min at 37° (□). Cells were incubated with PrBCM (10^{-7} M for 30 min at 20°) and then allowed to recover for various times at 37° before determination of [^{3}H]NMS binding. Recovery is expressed as a percentage of control specific binding, with the residual binding after PrBCM treatment subtracted. Values are mean ± standard error from five (untreated and cycloheximide-treated) or three (cycloheximide-treated and carbachol-stimulated) separate experiments, each performed in triplicate. Graphs show lines of best fit from nonlinear regression analysis. A, Untreated, k_1 = 0.083 ± 0.027% min^{-1}, k_2 = 0.14 ± 0.08 min^{-1}, r_{eo} = 5.5 ± 1.7%; cycloheximide-treated, k_1 = 0.035 ± 0.020% min^{-1}, k_2 = 0.13 ± 0.04 min^{-1}, r_{eo} = 6.7 ± 1.2%; cycloheximide-treated and carbachol-stimulated, k_1 = 0.14 ± 0.03% min^{-1}, k_2 = 0.31 ± 0.04 min^{-1}, r_{eo} = 5.3 ± 0.5%. B, Untreated, k_1 = 0.23 ± 0.04% min^{-1}, k_2 = 0.10 ± 0.05 min^{-1}, r_{eo} = 6.6 ± 2.3%; cycloheximide-treated, k_1 = 0.069 ± 0.016% min^{-1}, k_2 = 0.14 ± 0.05 min^{-1}, r_{eo} = 7.7 ± 1.0%; cycloheximide-treated and carbachol-stimulated, k_1 = 0% min^{-1}, k_2 = 0.059 ± 0.005 min^{-1}, r_{eo} = 44.3 ± 1.8%.

(0.14 min^{-1} in untreated cells), and r_{eo}. k_1 and k_2 were increased after carbachol stimulation of cycloheximide-treated cells, although, again, r_{eo} was unchanged.

In CHO-m4 cells, k_1 was ~3-fold larger than in CHO-m3 cells (2280 ± 400 receptors/cell/min; Fig. 2B and Table 3), although k_2 and r_{eo} were not significantly different. In contrast to the result for CHO-m3 cells, cycloheximide significantly reduced k_1 in CHO-m4 cells (to 680 ± 160 receptors/cell/min) but did not affect r_{eo} (65,300 ± 22,800 receptors/cell without and 76,200 ± 9,900 receptors/cell with cycloheximide). The most striking difference between the behavior of CHO-m3 and CHO-m4 cells was the dramatic increase in the size of the endosomal receptor pool (to 438,600 ± 17,800 receptors/cell) caused by stimulation of CHO-m4 cells by carbachol. Agonist stimulation also caused a further reduction in k_1, to zero.

Internalization of cell-surface receptors. The rate constant for internalization of receptors, k_3, and the rate of receptor degradation, k_4, were estimated with the use of nonlinear curve fitting of the experimental data to eq. 1, with the parameters k_2, k_1, r_{eo}, and r_{so} fixed at the values determined above. The number of receptors at the surface of untreated or cycloheximide-treated SH-SY5Y cells decreased slowly with time (Fig. 3). Cycloheximide treatment did not affect either k_3 or k_4 (k_3 = 0.018 ± 0.003 min^{-1} without and 0.017 ± 0.002 min^{-1} with cycloheximide; k_4 = 28 ± 8 receptors/cell/min and 25 ± 5 receptors/cell/min, respectively; Table 4). Stimulation of cycloheximide-treated SH-SY5Y cells with carbachol induced a dramatic loss of receptors from the cell surface. The agonist-induced internalization rate constant, k_3, was 0.28 ± 0.01 min^{-1}, and the corresponding degradation rate, k_4, was 84 ± 13 receptors/cell/min.

The number of receptors at the surface of untreated CHO-m3 cells remained constant over a 2-hr period (Fig. 4A). Treatment of the cells with cycloheximide did not cause a significant loss of surface receptors over this period. Neither k_3 (0.006 ± 0.003 min^{-1} without and 0.002 ± 0.003 min^{-1} with cycloheximide; Table 5) nor k_4 (480 ± 140 receptors/cell/min without and 890 ± 160 receptors/cell/min with cycloheximide) was significantly affected. Stimulation of cycloheximide-treated cells with carbachol caused a more rapid loss of receptors from the cell surface, but this effect also was minor, taking 3 hr to become significant (not shown on graph). However, both k_3 (0.021 ± 0.004 min^{-1}) and k_4 (2500 ± 80 receptors/cell/min) were increased by carbachol stimulation.

As in CHO-m3 cells, surface receptor numbers in untreated CHO-m4 cells remained constant over a 2-hr period (Fig. 4B). Treatment with cycloheximide again caused a slow decline in surface receptor numbers that became significant

TABLE 3
Parameter estimates for the delivery of receptors to the cell surface in CHO-m3 and CHO-m4 cells

Cycloheximide	Carbachol	k_1	k_2	r_{eo}	Correlation coefficient
		receptors/cell/min	*min*$^{-1}$	*receptors/cell*	
CHO-m3					
−	−	670 ± 220	0.14 ± 0.08	44,300 ± 13,700	0.96
+	−	280 ± 160	0.13 ± 0.04	54,000 ± 9,700	0.86
+	+	1,130 ± 240	0.31 ± 0.08	42,700 ± 4,000	0.96
CHO-m4					
−	−	2,280 ± 400	0.10 ± 0.05	65,300 ± 22,800	0.98
+	−	680 ± 160	0.14 ± 0.05	76,200 ± 9,900	0.96
+	+	0	0.059 ± 0.005	438,600 ± 17,800	0.98

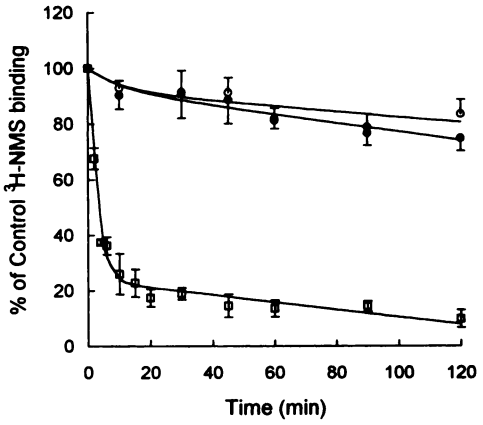

Fig. 3. Internalization of receptors in SH-SY5Y cells. Cells were incubated for various times under control conditions (○), with cycloheximide (20 μg/ml; ●) or with carbachol (0.5 mM at 37°) after a 1-hr pretreatment with cycloheximide (□). The number of surface receptors was then measured through the binding of [^{3}H]NMS. Data are expressed as a percentage of control binding. Values are mean ± standard error from three (untreated and cycloheximide-treated) and four (cycloheximide-treated and carbachol-stimulated) experiments, each performed in triplicate. Graphs show lines of best fit from nonlinear regression analysis. Parameters fixed are: k_1 = 0.092% min^{-1} (untreated), 0% min^{-1} (cycloheximide-treated and cycloheximide-treated/carbachol-stimulated), k_2 = 0.074 min^{-1}, r_{eo} = 12.1%, r_{so} = 100%. Parameters estimated are: untreated, k_3 = 0.018 ± 0.003 min^{-1}, k_4 = 0.21 ± 0.06% min^{-1}; cycloheximide-treated, k_3 = 0.017 ± 0.002 min^{-1}, k_4 = 0.19 ± 0.04% min^{-1}; cycloheximide-treated and carbachol-stimulated, k_3 = 0.28 ± 0.01 min^{-1}, k_4 = 0.63 ± 0.10% min^{-1}.

at 2 hr. This effect was largely a result of a reduction in k_1 (from 2280 ± 400 receptors/cell/min without to 680 ± 160 receptors/cell/min with cycloheximide; Table 3). Both k_3 (0.014 ± 0.003 min^{-1} without and 0.014 ± 0.005 min^{-1} with cycloheximide; Table 5) and k_4 (2380 ± 300 receptors/cell/min without and 2480 ± 300 receptors/cell/min with cycloheximide) were unaffected. Unlike the behavior of CHO-m3 cells, however, stimulation of cycloheximide-treated CHO-m4 cells with carbachol caused a rapid and extensive internalization of receptors. This was accompanied by an increase in k_3 to 0.071 ± 0.006 min^{-1}; k_4 (3270 ± 300 receptors/cell/min) was not significantly affected.

We extended our previous model (10) to determine the rate of receptor movement to and from the cell surface. The rate constants for internalization and recycling can be used to determine the extent and rate of receptor trafficking in the presence and absence of agonist. The rate of receptor internalization is given by the following (eq. 3):

$$\frac{dr_s}{dt} = r_s \times k_3 \quad (3)$$

and the rate of receptor recycling is given by the following (eq. 4):

$$\frac{dr_s}{dt} = -r_e \times k_2 \quad (4)$$

r_s can be determined by eq. 1 by using appropriate values for each of the parameters. A similar equation has been derived for endosomal receptor number (10). Fig. 5 shows the predicted rates of recycling and internalization in SH-SY5Y and NG108–15 cells. Fig. 6 shows the predicted rates for CHO-m3 and CHO-m4 cells.

The results presented so far suggest that m3 receptors expressed in "foreign" cells behave differently than those that are naturally present and that m3 and m4 receptors expressed in the same cell line, CHO, also behave differently. To assess the wider significance of these observations, we applied a less complete analysis to the trafficking of muscarinic receptors in four additional cell lines. The internalization of receptors in response to carbachol stimulation was much greater in CHO cells expressing the m2 subtype than in those expressing the m1 receptor subtype (Fig. 7), which perhaps indicates a correlation between agonist-induced receptor trafficking and the second messenger system activated (i.e., m1 and m3, which stimulate phosphoinositide hydrolysis, show little receptor internalization, whereas m2 and m4, which inhibit adenylate cyclase, show pronounced internalization). The other two cell lines tested, HeLa and HT29, which natively express predominantly the m3 receptor subtype, showed a reduction in surface receptor number in response to carbachol stimulation that was reminiscent of that seen in SH-SY5Y cells (Fig. 8).

Discussion

Our results show that the agonist-induced decrease in surface muscarinic receptor number is greater in CHO cells expressing either m2 or m4 muscarinic receptor subtypes than in those expressing m1 or m3 receptors. This partly confirms other work that suggested that m3 receptors expressed in CHO cells do not show an agonist-induced decrease in surface receptor density (1), whereas m2 receptors expressed in either BHK-21 or COS-7 cells do undergo internalization (16). We were interested to determine whether this resulted from a property that was inherent in the receptor or was influenced by the cell type in which the receptor is expressed. Comparison of agonist-induced changes in muscarinic receptor density at the cell surface in a variety of cell lines, including neuronal cells (SH-SY5Y), human gut epithelial cells (HT29), and human fibroblasts (HeLa), showed similar decreases in surface receptor density. This suggested

TABLE 4

Parameter estimates for the internalization and degradation of receptors in SH-SY5Y and NG108–15 cells

Cycloheximide	Carbachol	k_3	k_4	Correlation coefficient
		min^{-1}	*receptors/cell/min*	
SH-SY5Y	–	0.018 ± 0.003	28 ± 8	0.79
+	–	0.017 ± 0.002	25 ± 5	0.92
+	+	0.28 ± 0.01	84 ± 13	0.99
NG108–15[a]				
–	–	0.002 ± 0.004	26 ± 6	0.98
+	+	0.13 ± 0.01	55 ± 7	0.92

[a] Data from reference (9), for comparison.

that the differences seen in the transfected cells were not typical of cells that natively express the receptor.

We have shown previously that agonist-induced changes in surface receptor density are determined by both the rate of internalization and the rate of recycling of the receptor (10). Given that there were marked differences between the trafficking properties of the receptors in different cell types, we were interested to determine whether the kinetic properties varied between receptor subtype and cell type. We therefore applied a kinetic analysis, initially used on NG108–15 cells (10), to the trafficking of the muscarinic receptor in SH-SY5Y cells (m3 subtype) and in CHO cells transfected with cDNA encoding either the m3 or the m4 receptor subtype.

We found that SH-SY5Y cells show similar receptor trafficking properties to NG108–15 cells. There is a small internal pool of receptors in unstimulated cells, as demonstrated by the recovery of [^{3}H]NMS binding after PrBCM treatment of unstimulated cells in which protein synthesis had been inhibited by pretreatment with cycloheximide. This recovery amounts to 12.1% of the number of surface receptors in control cells, or 1620 receptors/cell. Subtraction of this recovery from that obtained in the absence of cycloheximide revealed that the rate of new receptor synthesis was 12 receptors/cell/min. This value is slightly lower than the rate of new synthesis in NG108–15 cells (22 receptors/cell/min). The endosomal receptor pool in SH-SY5Y cells was increased from 12.1% of control surface receptor number to 30.0% (4020 receptors/cell) by stimulation with carbachol. This increase is smaller than that seen in NG108–15 cells (28% in carbachol-stimulated cells compared with 0% in unstimulated cells).

The loss of surface receptor number after agonist stimulation is much greater in SH-SY5Y cells than in NG108–15 cells. Our mathematical analysis shows that the level of the plateau phase is determined by both k_2 and k_3, i.e., when time is >20 min, the exponential terms in eq. 1 approach zero and the equation can be simplified to the following (eq. 5):

$$r_s = r_{so}\left(\frac{k_2}{k_2 + k_3}\right) \quad (5)$$

Therefore, the greater apparent loss of surface receptors in SH-SY5Y cells could be due to either a decrease in k_2 or an increase in k_3 compared with the corresponding values for NG108–15 cells. k_2 is smaller in SH-SY5Y cells than in NG108–15 cells (0.074 min^{-1} versus 0.12 min^{-1}), and curve-fitting of the data for agonist-induced loss of surface receptor number yielded values for k_3 of 0.28 min^{-1} in SH-SY5Y cells and 0.13 min^{-1} in NG108–15 cells. Therefore, differences in both internalization and recycling rate constants contribute to the differences in trafficking kinetics between the two cell types.

Despite the quantitative differences described above, the be-

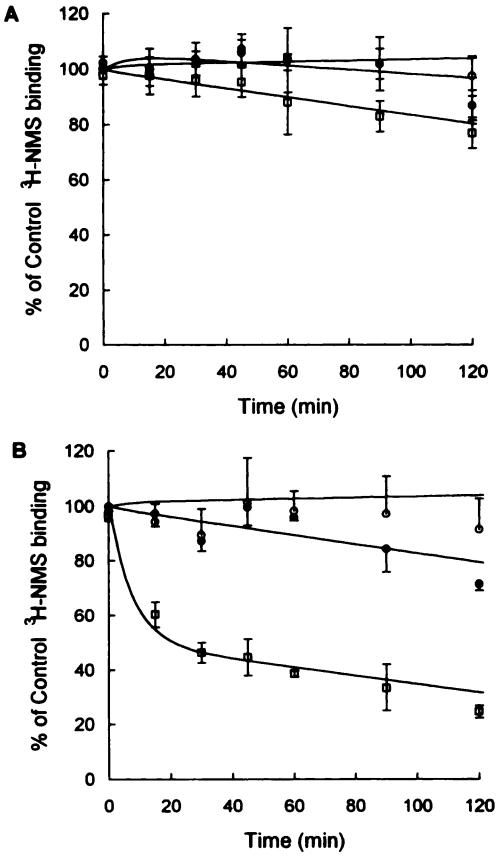

Fig. 4. Internalization of receptors in CHO-m3 (A) and CHO-m4 (B) cells. Cells were incubated for various times under control conditions (○), with cycloheximide (20 μg/ml; ●) or with carbachol (0.5 mM at 37°) after a 1-hr pretreatment with cycloheximide (□). The number of surface receptors was then measured through the binding of [^{3}H]NMS. Data are expressed as a percentage of control binding. Values are mean ± standard error from three experiments, each performed in triplicate. Graphs show lines of best fit from nonlinear regression analysis. A, Parameters fixed are: untreated, k_1 = 0.083% min^{-1}, k_2 = 0.14 min^{-1}, r_{eo} = 5.5%, r_{so} = 100%; cycloheximide-treated, k_1 = 0.035% min^{-1}, k_2 = 0.13 min^{-1}, r_{eo} = 6.7%, r_{so} = 100%; cycloheximide-treated and carbachol-stimulated, k_1 = 0.14% min^{-1}, k_2 = 0.31 min^{-1}, r_{eo} = 5.3%, r_{so} = 100%. Parameters estimated are: untreated, k_3 = 0.006 ± 0.003 min^{-1}, k_4 = 0.060 ± 0.017% min^{-1}; cycloheximide-treated, k_3 = 0.002 ± 0.003 min^{-1}, k_4 = 0.11 ± 0.02% min^{-1}; cycloheximide-treated and carbachol-stimulated, k_3 = 0.021 ± 0.004 min^{-1}, k_4 = 0.31 ± 0.01% min^{-1}. B, Parameters fixed are: untreated, k_1 = 0.23% min^{-1}, k_2 = 0.10 min^{-1}, r_{eo} = 6.6%, r_{so} = 100%; cycloheximide-treated, k_1 = 0.069% min^{-1}, k_2 = 0.14 min^{-1}, r_{eo} = 7.7%, r_{so} = 100%; cycloheximide-treated and carbachol-stimulated, k_1 = 0% min^{-1}, k_2 = 0.059 min^{-1}, r_{eo} = 7.7%, r_{so} = 100%. Parameters estimated are: untreated, k_3 = 0.014 ± 0.003 min^{-1}, k_4 = 0.24 ± 0.03% min^{-1}; cycloheximide-treated, k_3 = 0.014 ± 0.005 min^{-1}, k_4 = 0.25 ± 0.03% min^{-1}; cycloheximide-treated and carbachol-stimulated, k_3 = 0.071 ± 0.006 min^{-1}, k_4 = 0.33 ± 0.03% min^{-1}.

TABLE 5

Parameter estimates for the internalization and degradation of receptors in CHO-m3 and CHO-m4 cells

Cycloheximide	Carbachol	k_3	k_4	Correlation coefficient
		min^{-1}	*receptors/cell/min*	
CHO-m3				
−	−	0.006 ± 0.003	480 ± 140	0.32
+	−	0.002 ± 0.003	890 ± 160	0.61
+	+	0.021 ± 0.004	2500 ± 80	0.97
CHO-m4				
−	−	0.014 ± 0.003	2380 ± 300	0.15
+	−	0.014 ± 0.005	2480 ± 300	0.84
+	+	0.071 ± 0.006	3270 ± 300	0.97

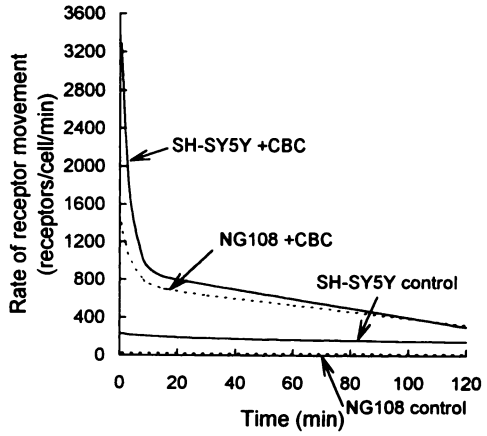

Fig. 5. Predicted rates of agonist-induced internalization and recycling in SH-SY5Y cells and NG108–15 cells. *Solid lines*, SH-SY5Y cells; *broken lines*, NG108–15 cells. Rates of agonist-induced internalization and recycling were predicted by calculating the rate of internalization ($r_s \times k_3$) using r_s as defined by eq. 1 and parameters determined experimentally. These parameters were: SH-SY5Y cells, k_1 = 16 receptors/cell/min, k_2 = 0.1 min^{-1}, k_3 = 0.28 min^{-1}, k_4 = 84 receptors/cell/min, r_{eo} 1250 receptors/cell, r_{so} = 13,400 receptors/cell; NG108–15 cells, k_1 = 22 receptors/cell/min, k_2 = 0.12 min^{-1}, k_3 = 0.12 min^{-1}, k_4 = 64 receptors/cell/min, r_{eo} = 0 receptors/cell, r_{so} = 11,500 receptors/cell. NG108–15 data are from Ref. 9.

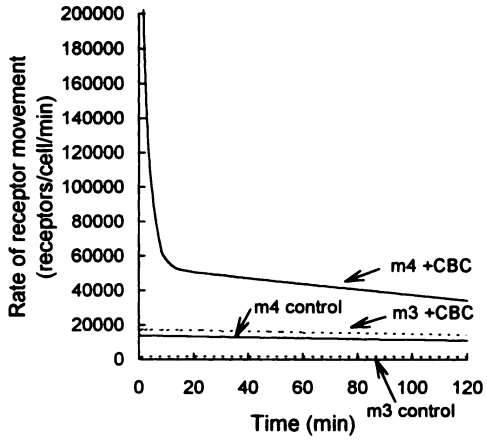

Fig. 6. Predicted rates of agonist-induced internalization and recycling in CHO-m3 and CHO-m4 cells. *Broken lines*, CHO-m3 cells; *solid lines*, CHO-m4 cells. Rates of agonist-induced internalization and recycling were predicted by calculating the rate of internalization ($r_s \times k_3$) using r_s as defined by eq. 1 and parameters determined experimentally. These parameters were: CHO-m3 cells, control k_1 = 280 receptors/cell/min, carbachol-stimulated (*CBC*) k_1 = 1130 receptors/cell/min, control k_2 = 0.13 min^{-1}, CBC k_2 = 0.31 min^{-1}, control k_3 = 0.002 min^{-1}, CBC k_3 = 0.021 min^{-1}, control k_4 = 890 receptors/cell/min, CBC k_4 = 2500 receptors/cell/min, r_{eo} 54,000 receptors/cell, r_{so} = 806,000 receptors/cell; CHO-m4 cells, control k_1 = 680 receptors/cell/min, CBC k_1 = 0 receptors/cell/min, control k_2 = 0.14 min^{-1}, CBC k_2 = 0.059 min^{-1}, control k_3 = 0.014 min^{-1}, CBC k_3 = 0.33 min^{-1}, control k_4 = 2480 receptors/cell/min, CBC k_4 = 3270 receptors/cell/min, r_{eo} = 76,200 receptors/cell, r_{so} = 990,000 receptors/cell.

havior of the muscarinic receptors in SH-SY5Y cells and NG108–15 cells is essentially similar, even though the two receptor subtypes predominantly expressed in these cells couple to different second messenger systems. When we broadened our analysis to include CHO cell lines expressing only m3 or m4 receptors, however, the picture became more complicated. Although the affinities of the receptors in the CHO cell lines for the radioligand [^{3}H]NMS were similar to those of the receptors in SH-SY5Y and NG108–15 cells, the numbers of receptors at the cell surface were much larger in the CHO cell lines. Interestingly, the presence of such huge numbers of receptors did not by itself cause significant changes in receptor trafficking as the behavior of the m4 receptor in the CHO cells was similar to that in the NG108–15 cells. In particular, agonist stimulation of CHO-m4 cells caused rapid removal of receptors from the cell surface into an intracellular compartment, from which they could then be recycled after agonist removal. This, incidentally, is in contrast to previously reported results with epidermal growth factor receptors that show saturation of the endocytic pathway at high receptor occupancy in A431 cells, which naturally express high levels of epidermal growth factor receptors (17). As in SH-SY5Y cells, agonist stimulation of CHO-m4 cells caused increases in the internalization rate constant, k_3, and the endosomal pool size, r_{eo}.

In contrast to the behavior of the m4 receptor, the number of m3 receptors at the surface in CHO cells was largely refractory to agonist stimulation. In fact, agonist stimulation increased k_3 in both CHO-m3 and CHO-m4 cells, and k_4 was also significantly elevated in CHO-m3 cells. Therefore, the stimulation of removal of receptors from the cell surface is occurring in both cell types, although to a considerably lesser extent in CHO-m3 than in CHO-m4. The lack of overall effect of agonist stimulation on receptor movement in CHO-m3 cells is a consequence of the balance of effects on all four rate constants. Although k_3 and k_4 are increased by agonist stimulation of CHO-m3 cells, k_1 and k_2 are also elevated, so there is no observable decrease in surface receptor number. (It is important to acknowledge, however, that recycling rate constants of more than ~0.2 min^{-1}, corresponding to a $t_{1/2}$ of <3.5 min, are very difficult to measure reliably with this methodology). In contrast, in CHO-m4 cells, the rise in k_3 is accompanied by decreases in both k_1 and k_2, which results in the observed rapid internalization. This result emphasizes the importance of measuring both internalization and recy-

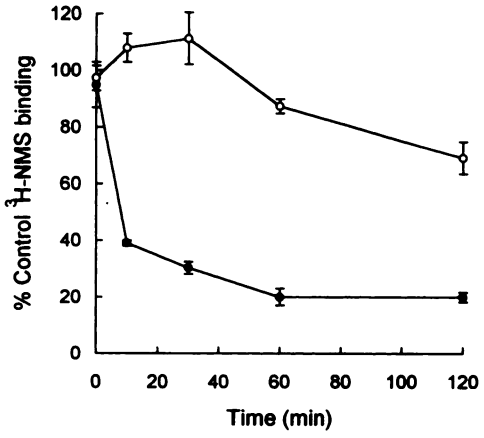

Fig. 7. Internalization of receptors in CHO-m1 cells (○) and CHO-m2 cells (●). Cells were incubated for various times with cycloheximide (20 μg/ml) or with carbachol (0.5 mM at 37°) after a 1-hr pretreatment with cycloheximide. The number of surface receptors was then measured through the binding of [^{3}H]NMS. Data are expressed as binding to carbachol and cycloheximide-treated cells as a percentage of binding in the presence of cycloheximide only. Values are mean ± standard error from three experiments, each performed in triplicate.

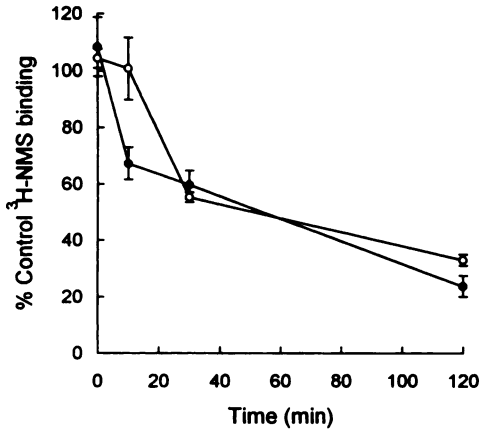

Fig. 8. Internalization of receptors in HeLa cells (○) and HT29 cells (●). Cells were incubated for various times with cycloheximide (20 μg/ml) or with carbachol (0.5 mM at 37°) after a 1-hr pretreatment with cycloheximide. The number of surface receptors was then measured through the binding of [^{3}H]NMS. Data are expressed as binding to carbachol and cycloheximide-treated cells as a percentage of binding in the presence of cycloheximide only. Values are mean ± standard error from three experiments, each performed in triplicate.

cling rates to determine mechanisms underlying changes in surface receptor density.

Extension of our earlier model of receptor trafficking (10) allows an estimation of the number of receptors moving within a given time. This analysis shows that the initial rate of internalization is greater in SH-SY5Y cells (0.28% min^{-1}) than in NG108–15 cells (0.13% min^{-1}). This means that in the first minute of agonist application, a greater number of receptors are internalized in SH-SY5Y cells (24.6%, or 3300 receptors) than in NG108–15 cells (12.2%, or 1400 receptors). Furthermore, in SH-SY5Y cells, it would take 7 min for all surface receptors to be internalized once compared with 12 min for NG108–15 cells. CHO-m4 cells show similar properties, with 28.3% of the receptors (280,200 receptors) internalized in the first minute and complete turnover in 6.5 min. However, CHO-m3 cells internalized only 2.1% of surface receptors (16,900 receptors) in the first minute, with a complete turnover taking 50 min.

The analysis used in the present study allowed us to appreciate how quickly receptors are moving from the surface to endosomes and back again. It also demonstrates the error inherent in the assumption that if the number of receptors at the surface does not change, then receptors are not being internalized. Understanding the dynamic nature of receptor trafficking can help us to understand the role that internalization and recycling might play in desensitization. Recent work has suggested that internalization and recycling are required for dephosphorylation and, therefore, resensitization of the receptor (18–20). For CHO-m3 cells, receptor internalization and recycling are slow, and it could therefore be expected that the receptor will be particularly liable to desensitization; this is the case (1). In contrast, m3 muscarinic receptors undergo rapid trafficking in SH-SY5Y cells. In a similar cell line (SK-N-SH), desensitization is greater when cycling is prevented between the surface and endosomes (21). If it is true that recycling is an absolute requirement for resensitization, then it is possible to estimate the contribution of internalization to desensitization. If recycling is prevented or if reactivation of the receptor is prevented but the inactive receptor is recycled, then the effective rate of change in active surface receptors is $dr_s/dt = -r_s k_3$. The integral of this is $r_s = r_{so}e^{-k_3 t}$. In the case of SH-SY5Y cells, where $k_3 = 0.28\ min^{-1}$, phosphorylation and subsequent internalization of receptors could result in a 50% reduction in responsiveness in 2.5 min and a 95% reduction in 10 min.

A number of other questions remain to be answered. The efficiency of dephosphorylation as well as the rate of trafficking are both likely to determine the rate of resensitization, but the relative importance of these mechanisms is not known. The importance of the rate of trafficking may also depend on whether the G protein and receptor are internalized and recycled together or separately and on whether recoupling is rate limiting. The effect of loss of surface receptor number may contribute in its own right, depending on the number of spare receptors. Therefore, an understanding the kinetics of the trafficking of receptors will assist in understanding the mechanisms underlying rapid desensitization.

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