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Trafficking of green fluorescent protein-tagged muscarinic M_4 receptors in NG108-15 cells

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Abstract

The muscarinic M_4 receptor for acetylcholine was tagged at its C terminus with green fluorescent protein (GFP) and expressed in NG108-15 cells, which normally express this receptor subtype. The binding affinity of the antagonist *N*-methylscopolamine was not significantly affected by the presence of the GFP tag, whereas the affinity of the receptor for the agonist carbachol was reduced by four-fold. Stimulation of the tagged receptor resulted in inhibition of adenylyl cyclase. Following agonist stimulation, the tagged receptor was slowly internalized, and became partially co-localized with the endosomal marker Texas Red–transferrin after 30 min. There was little co-localization with the lysosomal marker lgp120 even after 60 min of internalization. Finally, the tagged receptor, unlike the endogenous receptor, failed to recycle to the plasma membrane on removal of the agonist. We conclude that the GFP-tagged muscarinic M_4 receptor does not traffic normally in NG108-15 cells, most likely because of its gross overexpression.

Keywords: Muscarinic receptor; Green fluorescent protein; Intracellular trafficking; NG108-15 cell

1. Introduction

Agonist stimulation of G protein-coupled receptors causes extensive intracellular receptor trafficking, which in turn has profound effects on cell responsiveness (Koenig and Edwardson, 1997; Tsao et al., 2001). Consequently, there is great interest in defining the trafficking routes traversed by the receptors and the molecular mechanisms that underlie intercompartmental transport. We have previously applied a kinetic analysis to the trafficking of the muscarinic M_4 receptor for acetylcholine that is endogenously expressed in the mouse neuroblastoma × rat glioma cell line NG108-15 (Koenig and Edwardson, 1994). In unstimulated NG108-15 cells, the vast majority of the muscarinic M_4 receptors reside at the plasma membrane. Receptor degradation, presumably in lysosomes, proceeds at a low rate, and the plasma membrane pool is topped up by a slow linear delivery of newly synthesized receptors. On agonist stimulation, the rate of receptor endocytosis is dramatically increased; receptors are removed from the plasma membrane by an exponential process and delivered to an endocytotic compartment. From this compartment, the receptors can either be sent on to a degradative compartment, or, on agonist removal, recycled to the plasma membrane in another exponential process. In NG108-15 cells, the receptor internalization appears to play a role in the desensitization of the response to agonists (Holroyd et al., 1999). For instance, the time courses of internalization and desensitization are almost identical, and manipulation of the rate of internalization (i.e. enhancement by G protein-coupled receptor kinase 2, or inhibition by hyperosmolar sucrose) has corresponding effects on desensitization.

The analysis described above relies on radioligand binding to measure the number of receptors present at the cell surface. Although this method provides valuable insights into the rates at which receptors are moved into and out of the plasma membrane, it gives little information about the intracellular compartments through which the receptors traffic. An alternative method of following receptor trafficking is to tag the receptor with green fluorescent protein (GFP). The number of receptors to which this technique has been applied is growing rapidly (e.g. Barak et al., 1997; reviewed by Kallal and Benovic, 2000), and it has

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generally been assumed that the tagged receptor will provide information about the behaviour of the wild-type receptor. However, since it is known that even small modifications to the intracellular domains of G protein-coupled receptors can have major effects on receptor behaviour (see, e.g. Cao et al., 1999), it is important to determine whether the addition of the large (27 kDa) GFP tag affects the receptor property that is being studied. In fact, effects on the intracellular trafficking of β-adrenoceptors as a consequence of the addition of a GFP tag have recently been reported (McLean and Milligan, 2000). Another potential complication is the possibility that receptor overexpression itself might perturb the behaviour of the receptor. In the present study, we have expressed a muscarinic M_4 receptor tagged at its C terminus with GFP in NG108-15 cells, and compared the ligand-binding and trafficking kinetics of the tagged and endogenous receptors, before proceeding to characterize the compartments through which the tagged receptor traffics in response to agonist stimulation.

2. Materials and methods

2.1. Construction of the GFP-tagged muscarinic M_4 receptor

The sequence of the mouse muscarinic M_4 receptor in the vector pCD-PS (kindly provided by C.J. van Koppen, Essen, Germany) was amplified by polymerase chain reaction (PCR) using an *Eco*RI forward primer, 5′-TTCGAATTCACCATGGCGAACTTCACACCT-3′, and a *Bam*HI reverse primer, 5′-GGTGGATCCCGCCTGGCTGTGCCGATGTT-3′. The PCR product was digested with *Eco*RI and *Bam*HI and ligated into the vector pEGFP-N1. This construct directs the production of a muscarinic M_4 receptor tagged at its C terminus with a red-shifted variant of GFP (muscarinic M_4 receptor–GFP).

2.2. Cell culture

NG108-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS), 100 μM hypoxanthine, 1 μM aminopterin, 16 μM thymidine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human embryonic kidney (HEK)-293 tsA201 cells were cultured in DMEM containing 10% (v/v) FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were detached from the culture flasks for passaging every 2–4 days by brief (<2 min) incubation with trypsin (0.5 mg/ml) and EDTA (0.2 mg/ml) in phosphate-buffered saline (PBS). For experiments, cells were grown on either poly-D-lysine-treated (100 μg/well) 24-well plates or poly-D-lysine-treated glass cover slips.

2.3. Transient transfection of cells

Both NG108-15 and HEK-293 tsA201 cells were transiently transfected with purified plasmids using a calcium phosphate transfection kit. For mock transfections, plasmid DNA was omitted, but the procedure used was otherwise identical. For radioligand-binding experiments, cells were seeded into 162 cm^2 tissue culture flasks at approximately 50% confluence 24 h before transfection. DNA (54 μg) was mixed with the modified calcium phosphate reagents and incubated with the cells. After 8 h, the cells were washed with warm PBS and fresh medium was added to the flasks. After a further 16 h, the transfected cells were transferred to 24-well plates. Cell numbers were counted using a hemocytometer, and equal numbers of transfected and mock-transfected cells were plated out. Radioligand binding was then determined after a further 24 h. For immunofluorescence experiments, transfection was carried out in six-well plates, as described above, using 0.5 μg of DNA per well.

2.4. Measurement of inhibition of adenylyl cyclase activity in response to receptor stimulation

The ability of carbachol to inhibit forskolin-stimulated adenylyl cyclase activity in transfected HEK-293 tsA201 cells was assessed as follows. Cells growing on 24-well plates were preincubated at 37 °C for 30 min in HEPES-buffered DMEM, pH 7.4, containing the protein synthesis inhibitor cycloheximide (20 μg/ml) and the cyclic AMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (0.5 mM). Where appropriate, carbachol (1 mM) and forskolin (final concentration, 50 μM) were then added as prewarmed aliquots, to achieve a final volume of 300 μl. The incubation was allowed to proceed at 37 °C for a further 5 min. Incubations were terminated by addition of 20 μl of 10 M HCl. Samples were neutralized by addition of 20 μl of 10 M NaOH, and then buffered with 200 μl of 1 M Tris–HCl, pH 7.0. Samples were centrifuged at $21{,}000 \times g$ for 5 min to pellet cell debris. The cyclic AMP content of the supernatants was then assayed through their ability to displace [^{3}H]cyclic AMP from protein kinase A. All steps in the cyclic AMP assay were carried out on ice. Samples of cell supernatant (100 μl) were incubated with 100 μl of [^{3}H]cyclic AMP (approximately 25,000 dpm) in 100 mM NaCl, 50 mM Tris–HCl (pH 7.0), 5 mM EDTA, and 100 μl of protein kinase A (32 μg/ml) in the same buffer containing bovine serum albumin (2.5 mg/ml). Incubation was for 2 h on ice and was followed by filtration through polyethyleneimine-treated GF/B filters. The retained radioactivity was determined by liquid scintillation counting. A standard curve was constructed using known concentrations of cyclic AMP, and the data were fitted to a logistic expression using GraphPad Prism. Cyclic AMP concentrations were read off this curve. Cellular

cyclic AMP concentrations were calculated, taking account of the volumes taken and the dilutions made during the assay.

2.5. Analysis of muscarinic receptor trafficking

Cell-surface receptor number was quantitated through the binding of the membrane-impermeant radioligand [^{3}H]*N*-methylscopolamine to cells growing on 24-well plates. All experiments were performed on cells that had been pretreated for 30 min at 37 °C with cycloheximide (20 μg/ml) to block protein synthesis. To measure receptor recycling to the plasma membrane, cells were first treated with 1 mM carbachol for 30 min at 37 °C to cause maximal receptor internalization. Cells were then washed twice with 2 ml of HEPES-buffered DMEM, pH 7.4, at 12 °C to remove the agonist, and recycling was allowed to proceed at 37 °C for various times, up to 60 min, in agonist-free buffer. Cells were washed twice with 2 ml of HEPES-buffered DMEM, pH 7.4, at 12 °C to terminate receptor trafficking, and then incubated with 1 ml of 0.4 nM [^{3}H]*N*-methylscopolamine in this buffer for 2 h at the same temperature. We have shown previously (Koenig and Edwardson, 1994) that binding reaches equilibrium under these conditions. At the end of the incubation, cells were washed twice more with HEPES-buffered DMEM, pH 7.4, at 12 °C, and solubilized by addition of 200 μl of 0.5% (v/v) Triton X-100. Wells were rinsed with a further 200 μl of Triton X-100, and cell-associated radioactivity was determined by liquid scintillation counting. Nonspecific binding was determined in the presence of *N*-methylatropine (1 μM).

To measure receptor internalization, cells were incubated at 37 °C with carbachol (usually at 1 mM) for various times, up to 30 min. Cells were then washed twice with HEPES-buffered DMEM, pH 7.4, at 12 °C, before measurement of [^{3}H]*N*-methylscopolamine binding, as above.

All determinations were carried out in triplicate. Specific binding at each time point was calculated by subtraction of the nonspecific binding from the total binding. At each time point, values for GFP-tagged receptors were obtained by subtraction of the specific binding in mock-transfected cells from the specific binding in transfected cells. (In a typical experiment, binding in transfected cells was approximately double the binding in mock-transfected cells.) For each experiment, binding values were expressed as a percentage of the value at time zero, which represents the number of receptors initially present at the cell surface. Data shown are means ± S.E.M. for several independent experiments.

A similar subtractive procedure was used to distinguish GFP-tagged receptors from endogenous receptors for the determination of antagonist (*N*-methylscopolamine) and agonist (carbachol) binding affinities.

2.6. Mathematical modelling of receptor trafficking

It was assumed that receptor trafficking could be adequately described by a two-compartment model, in which receptors cycle between the cell surface and endosomes. Such a model assumes that delivery of receptors to the plasma membrane along the synthetic route and receptor degradation are both negligible over the time course of the experiment. We have previously derived an equation that describes the variation in the number of receptors at the cell surface as a function of time (Koenig and Edwardson, 1994):

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

where R_s is the number of receptors at the cell surface at time t, R_{s0} is the surface receptor number at time zero, R_{e0} is the number of receptor in endosomes at time zero, and k_e and k_r are the rate constants for endocytosis and recycling, respectively. After the removal of agonist when internalization is complete, k_e can be set to zero, so that receptor recycling ($R_s - R_{s0}$) is given by:

$$R_s - R_{s0} = R_{e0}(1 - e^{-k_r t}) \quad (2)$$

A plot of $R_s - R_{s0}$ against t therefore allows k_r to be determined. It has been shown previously (Koenig and Edwardson, 1994) that there is no detectable endosomal pool of muscarinic receptors in unstimulated NG108-15 cells. Hence, during agonist-triggered receptor internalization, R_{e0} can be set at zero, so that R_s is given by:

$$R_s = \frac{R_{s0}}{k_e + k_r}\left[k_r + k_e e^{-(k_e + k_r)t}\right] \quad (3)$$

A plot of R_s against t now allows k_e to be determined, since k_r is already known. All curve fittings were performed using GraphPad Prism (GraphPad Software).

2.7. Confocal laser scanning microscopy

Experiments were carried out 24 h after transfection. NG108-15 cells growing on glass cover slips were washed twice with 120 mM sodium phosphate buffer, pH 7.4, at 37 °C, and then fixed by incubation in 4% paraformaldehyde in the same buffer at 4 °C for 60 min. Cells were washed twice for 5 min with 120 mM sodium phosphate buffer, twice for 5 min with low-salt PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) and twice for 5 min with high-salt PBS (500 mM NaCl, 20 mM sodium phosphate, pH 7.4). Cells were then permeabilized by a 30-min incubation in low-salt PBS containing 0.2% (w/v) fish gelatin and 0.1% (w/v) saponin. Cells were incubated with the appropriate primary antibody, in permeabilization buffer, for 2 h on Parafilm. Antibodies used were HPC-1,

an anti-syntaxin I mouse monoclonal antibody (Barnstable et al., 1985; kindly provided by R. Jahn, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), and an anti-lgp120 mouse monoclonal antibody (Howe et al., 1988; kindly provided by Dr. P. Luzio, Department of Clinical Biochemistry, University of Cambridge, UK). Antibody dilutions were 1:500. Cells were then washed three times for 10 min with high-salt PBS, and incubated for 90 min with an anti-mouse secondary antibody coupled to Cy3 (1:500). Cells were washed twice for 10 min with high-salt PBS, once for 10 min with 120 mM sodium phosphate buffer, and once for 5 min with 5 mM sodium phosphate buffer. Cover slips were mounted on glass slides in Vectashield™ mounting medium. Cells were visualized using a Leica NT-TCS confocal laser scanning microscope, using a ×100 objective lens with 1.0 numeric aperture. Images were collected with appropriate filters: GFP was excited using the 488-nm line of a krypton/argon laser and imaged with a 510- to 540-nm band-pass filter. Cy3 and Texas Red™ were excited with the 568-nm line and imaged with a long-pass 590-nm filter. Images were processed and annotated using Adobe Photoshop 5.0.

2.8. Materials

[5,8-^{3}H]cyclic AMP (specific activity 41 Ci/mmol) and [^{3}H]*N*-methylscopolamine (specific activity 81 Ci/mmol) were purchased from Amersham International. The vector pEGFP-N1 and calcium phosphate transfection kits were from Clontech. Restriction enzymes were from New England Biolabs. Powdered HEPES-buffered DMEM was from GibcoBRL. Texas Red–transferrin was from Molecular Probes. VectaShield mounting solution was from Vector Labs. All other reagents were from Sigma.

3. Results

3.1. Ligand-binding properties of the muscarinic M_4 receptor–GFP expressed in NG108-15 cells

The polar radioligand [^{3}H]*N*-methylscopolamine is membrane impermeant, and can therefore be used to quantify cell-surface muscarinic acetylcholine receptors in intact cells. We have shown previously (Koenig and Edwardson, 1994) that in unstimulated NG108-15 cells, the vast majority of the muscarinic M_4 receptors reside at the plasma membrane; a typical NG108-15 cell has 11,500 cell-surface receptors. In the present study, transient transfection of the cells with the muscarinic M_4 receptor–GFP construct typically resulted in a doubling of the maximum binding of [^{3}H]*N*-methylscopolamine to intact cells. The percentage of the cells that were transfected was determined by counting the number of cells expressing green fluorescence, and comparing this with the total number of cells, visualized by staining of the nuclei with 4′,6-diamidino-2-phenylindole. It was found that approximately 5% of the cells were transfected. Attempts to increase the efficiency of transfection by using other transfection reagents (e.g. FuGENE, Roche) were unsuccessful. A two-fold increase in total [^{3}H]*N*-methylscopolamine binding equates to approximately 200,000 muscarinic M_4 receptor–GFP binding sites per transfected cell. Essentially, then, each transfected cell population contains two types of cell: the majority (95%) expressing 11,500 endogenous receptors at the cell surface, and a minority (5%) expressing 11,500 endogenous and 200,000 tagged receptors. The properties and behaviour of the GFP-tagged receptors can be determined by subtracting the [^{3}H]*N*-methylscopolamine binding value for the mock-transfected cells from the corresponding value for the transfected cells. The fact that only a small proportion of the cells was transfected means that even if the overexpression of GFP-tagged receptors has an effect on the behaviour of the endogenous receptors in the transfected cells, this will have a negligible effect on the total population of endogenous receptors.

[^{3}H]*N*-methylscopolamine binding curves were constructed for both the endogenous muscarinic M_4 receptor and the muscarinic M_4 receptor–GFP. Fitting of the data to a hyperbolic function yielded values for the dissociation constant for [^{3}H]*N*-methylscopolamine of 55 pM for the endogenous receptor and 80 pM for the tagged receptor. These values are very close to each other and to the reported value of 110 pM (Koenig and Edwardson, 1994). Hence, the addition of the GFP tag to the receptor does not have a major effect on antagonist binding affinity.

Carbachol inhibited the binding of [^{3}H]*N*-methylscopolamine (100 pM) to the muscarinic M_4 receptors in a concentration-dependent manner. The IC_{50} values for carbachol were 8.5 μM for the endogenous receptor and 25 μM for the tagged receptor (mean values from four experiments). Using the values for the dissociation constant of [^{3}H]*N*-methylscopolamine given above, dissociation constants for carbachol were calculated as 3.0 μM for the endogenous receptor and 11.1 μM for the tagged receptor. Hence, carbachol has an approximately four-fold higher affinity for the endogenous muscarinic M_4 receptor than for the transiently expressed muscarinic M_4 receptor–GFP. Addition of GFP to the C terminus, therefore, appears to reduce the agonist binding affinity of the muscarinic M_4 receptor.

3.2. Functional activity of the muscarinic M_4 receptor–GFP

The presence of endogenous M_4 muscarinic receptors in NG108-15 cells makes it difficult to devise an experiment to test the functional activity of the tagged receptor. Consequently, we decided to express the receptor in another cell line (HEK-293 tsA201) that has no endogenous muscarinic receptors (data not shown). The activity of the

tagged receptor was assessed through the ability of carbachol to inhibit adenylyl cyclase activity in transiently transfected HEK-293 tsA201 cells. As shown in Fig. 1, forskolin caused an approximately eight-fold rise in the intracellular concentration of cyclic AMP in both transfected and mock-transfected cells. Carbachol (1 mM) reduced the effect of forskolin by approximately 50% in the transfected cells but had no significant effect in the mock-transfected cells. Because of the large variation in the fluorescence intensity of cells in transfected populations, it was difficult to determine accurately the percentage of transfected cells in these experiments, although the value was of the order of 50%. Hence, carbachol caused an almost complete inhibition of the forskolin-induced generation of cyclic AMP in the cells that were transfected. The GFP-tagged receptor is therefore functionally active.

3.3. Kinetics of the trafficking of endogenous and GFP-tagged muscarinic M_4 receptors in response to agonist stimulation

When mock-transfected NG108-15 cells were stimulated with a saturating concentration (1 mM) of the carbachol, muscarinic M_4 receptors were rapidly internalized, with maximal internalization being reached after 30 min. When the agonist was removed after a 30-min treatment, receptors recycled to the plasma membrane (Fig. 2A). Fitting of the recycling data to Eq. (2) yielded values for the rate constant for recycling, k_r, of 0.12 ± 0.01 min^{-1}, and for the size of the recyclable pool of receptors of $35 \pm 1\%$ of the number of receptors originally at the cell surface, in agreement with previously reported values (k_r, 0.12 min^{-1}; recyclable pool, 28%; Koenig and Edwardson, 1994). In contrast, the muscarinic M_4 receptor–GFP did not recycle significantly upon agonist removal; for instance, the number of receptors at the cell surface after 60 min of recycling was not significantly different from the number at the surface at time zero (Fig. 2A).

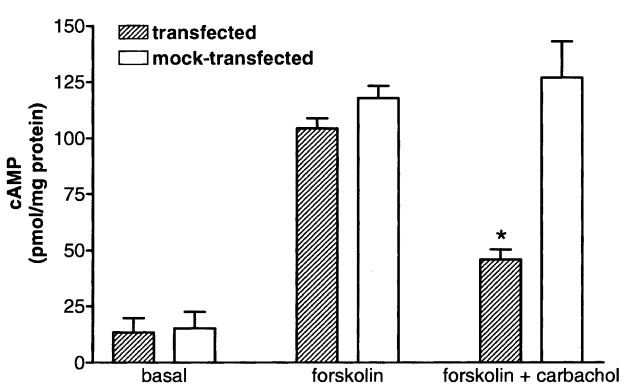

Fig. 1. Inhibition of adenylyl cyclase through carbachol stimulation of GFP-tagged muscarinic M_4 receptors expressed in HEK-293 tsA201 cells. Cells were either transfected with the muscarinic M_4 receptor–GFP construct or mock transfected, and the ability of carbachol (1 mM) to inhibit forskolin-stimulated adenylyl cyclase activity was determined. Data are means ± S.E.M. from three experiments, and are normalized to cell protein content. $^{*}P < 0.001$, different from forskolin treated.

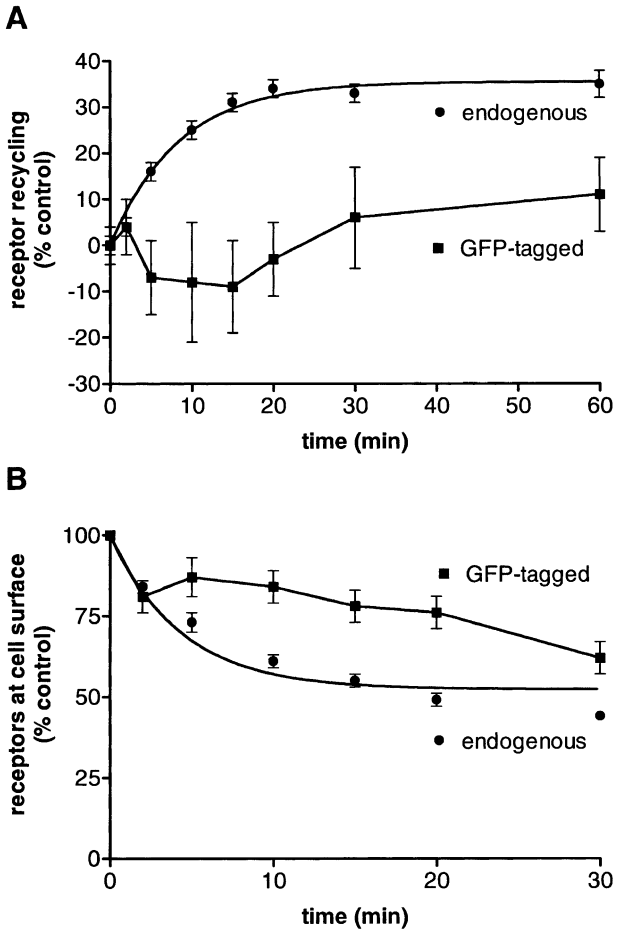

Fig. 2. Comparison of the trafficking kinetics of endogenous and GFP-tagged muscarinic M_4 receptors in NG108-15 cells. (A) Cells were either transfected with the muscarinic M_4 receptor–GFP construct or mock transfected. Receptor internalization was induced by treatment with carbachol (1 mM) for 30 min. The agonist was then washed away, and recycling was allowed to proceed at 37 °C for various times, up to 60 min. Surface receptor number was determined through the binding of [^{3}H]*N*-methylscopolamine. Data are means ± S.E.M. from 15 (endogenous receptor) and 5 (GFP-tagged receptor) experiments, and were fitted to Eq. (2). (B) Cells were incubated at 37 °C with a saturating concentration of carbachol (1 mM) for various times, up to 30 min. Surface receptor number was determined through the binding of [^{3}H]*N*-methylscopolamine. Data are means ± S.E.M. from 15 (endogenous receptor) and 7 (GFP-tagged receptor) experiments, and were fitted to Eq. (3), with k_r set at 0.12 min^{-1}.

The time course of internalization of the endogenous muscarinic M_4 receptor is shown in Fig. 2B. Fitting of the data to Eq. (3) using a value for k_r of 0.12 min^{-1}, gave a value for k_e of 0.11 ± 0.01 min^{-1}, which once again is very similar to the previously reported value (0.13 min^{-1}; Koenig and Edwardson, 1994). The muscarinic M_4 receptor–GFP was internalized more slowly than the endogenous receptor (Fig. 2B), and the data could not be fitted to Eq. (3) because a value for k_r could not be obtained from the recycling data.

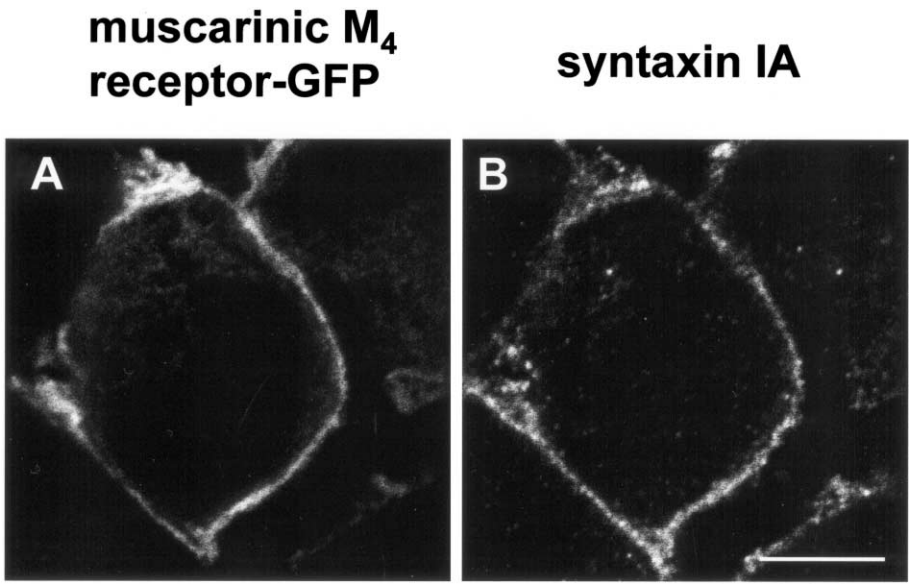

Fig. 3. Cell-surface localization of GFP-tagged muscarinic M_4 receptors in unstimulated NG108-15 cells. (A) Muscarinic M_4 receptor–GFP; (B) syntaxin IA. NG108-15 cells growing on glass cover slips were transiently transfected with the muscarinic M_4 receptor–GFP construct and used 48 h later. The primary antibody was a mouse monoclonal antibody against syntaxin I (1:500); the secondary antibody was Cy3-conjugated anti-mouse IgG (1:500). Transfected cells were visualized using confocal microscopy. Bar, 10 μm.

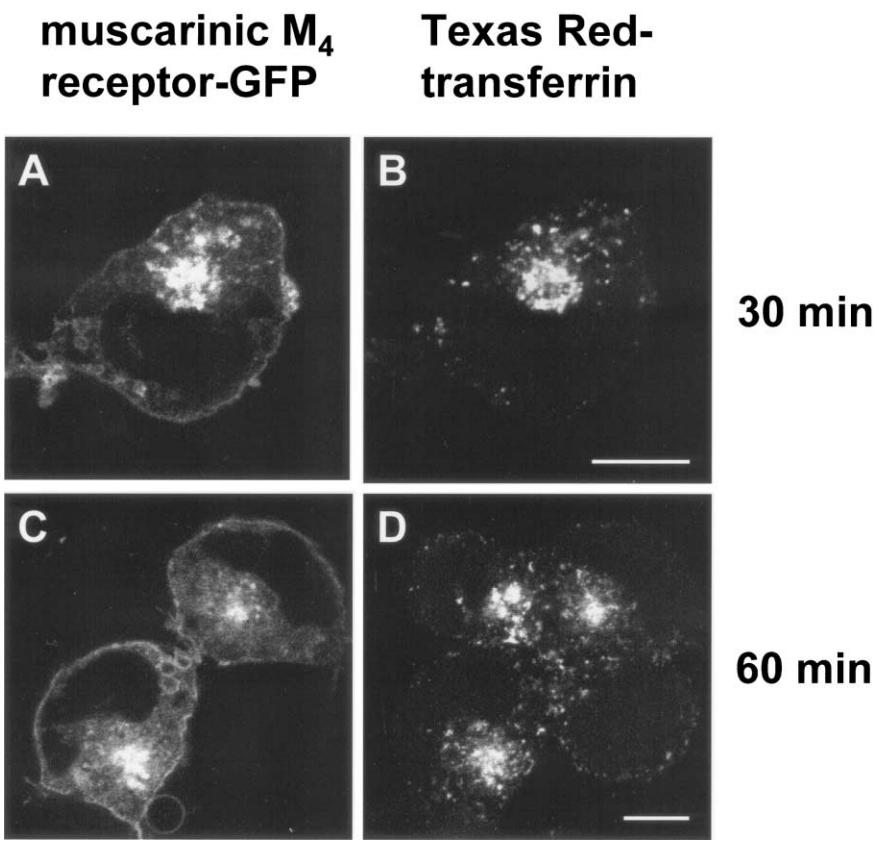

Fig. 4. Endocytosis of GFP-tagged muscarinic M_4 receptors in response to agonist stimulation. (A,C) Muscarinic M_4 receptor–GFP; (B,D) Texas Red–transferrin. NG108-15 cells growing on glass cover slips were transiently transfected with the muscarinic M_4 receptor–GFP construct and used 48 h later. Cells were incubated with a saturating concentration of carbachol (1 mM) for the indicated times. Texas Red–transferrin (25 μg/ml) was present throughout the carbachol incubation period. Transfected cells were visualized using confocal microscopy. Bars, 10 μm.

3.4. Cell-surface localization of the muscarinic M_4 receptor–GFP in unstimulated cells

NG108-15 cells expressing the muscarinic M_4 receptor–GFP showed a strong green fluorescent signal that was photostable and resistant to fixation (Fig. 3). In unstimulated cells, the tagged receptor gave a ring-like fluorescence that corresponded with the staining given by the syntaxin IA, a neuronal-specific protein that is anchored in the plasma membrane with the bulk of the protein facing the cytoplasm (Inoue and Akagawa, 1993). This result indicates that almost all of the GFP-tagged receptors are present on the cell surface.

3.5. Endocytosis of the muscarinic M_4 receptor–GFP in response to agonist stimulation

Carbachol application elicited a redistribution of muscarinic M_4 receptor–GFP fluorescence from the plasma membrane to an intracellular compartment, as shown in Fig. 4. Tagged receptors extensively co-localized with Texas Red–transferrin, a protein that is endocytosed together with constitutively trafficking cell-surface transferrin receptors. Transferrin is selectively sorted into early and recycling endosomes, and is an established marker for this subcellular compartment (Woods et al., 1989; Von Zastrow and Kobilka, 1992). Confirmation that endocytosis of the muscarinic M_4 receptor–GFP and Texas Red–transferrin proceed via the same pathway was sought by testing the ability of 0.6-M sucrose to inhibit endocytosis. Both tagged receptor and Texas Red–transferrin failed to be endocytosed in the presence of hyperosmolar sucrose, an agent known to perturb clathrin-coated pit formation (Hansen et al., 1993; Fig. 5).

3.6. Trafficking of the endocytosed muscarinic M_4 receptor–GFP

To determine the extent of delivery of the muscarinic M_4 receptor–GFP to lysosomes, the pattern of green fluorescence was compared with the staining for the lysosomal glycoprotein, lgp120 (Howe et al., 1988). Fig. 6 shows that although the majority of the endocytosed GFP-tagged receptors were present in the same region of the cells as the lysosomes after either 30 or 60 min of carbachol treatment, they were not predominantly co-localized with the lysosomal marker, although a few areas of partial overlap were observed (indicated by the arrows). Furthermore, the initial ring-like fluorescence at the plasma membrane observed for untreated cells did not reappear even after incubation of the cells in the absence of agonist for up to 60 min (data not shown). This result indicates that few, if any, of the

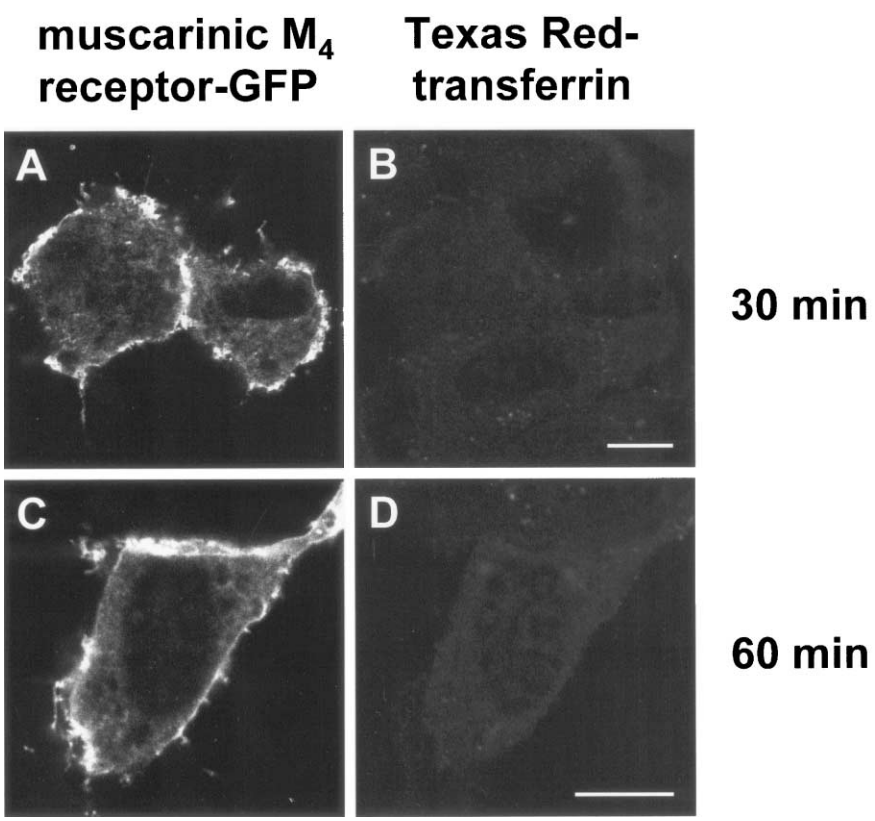

Fig. 5. Effect of hyperosmolar sucrose on endocytosis. (A,C) Muscarinic M_4 receptor–GFP; (B,D) Texas Red–transferrin. NG108-15 cells growing on glass cover slips were transiently transfected with the muscarinic M_4 receptor–GFP construct and used 48 h later. Cells were incubated with a saturating concentration of carbachol (1 mM) in the presence of 0.6 M sucrose for the indicated times. Texas Red–transferrin (25 μg/ml) was present throughout the carbachol incubation period. Transfected cells were visualized using confocal microscopy. Bars, 10 μm.

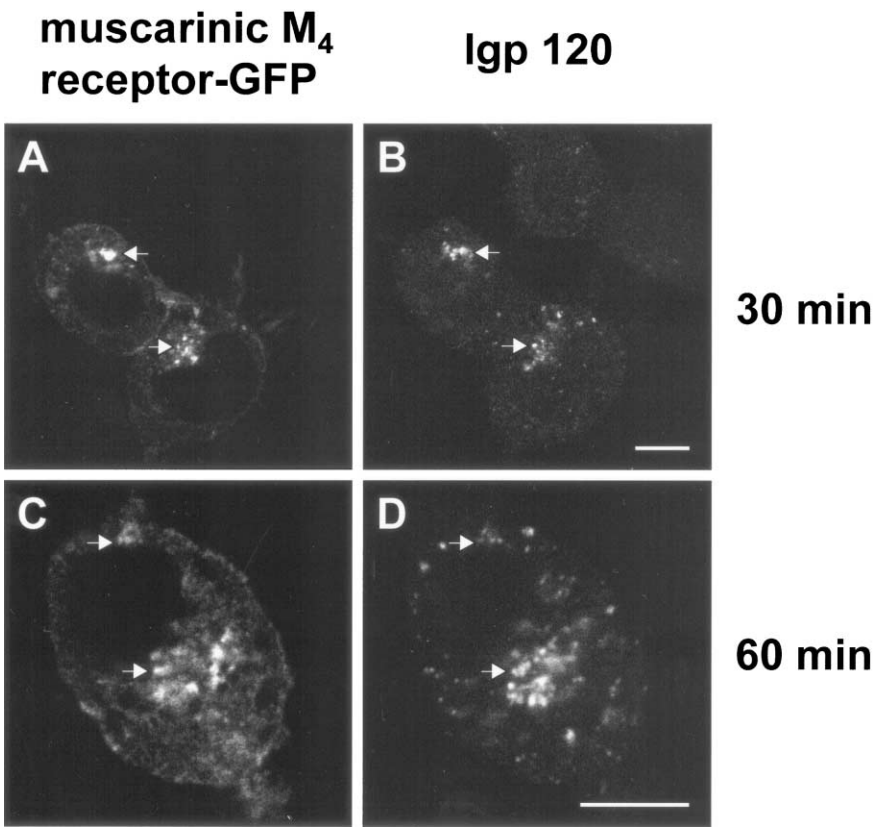

Fig. 6. Comparison of the distribution of internalized GFP-tagged muscarinic M_4 receptors and lysosomes. (A,C) Muscarinic M_4 receptor–GFP; (B,D) lgp120. NG108-15 cells growing on glass cover slips were transiently transfected with the muscarinic M_4 receptor–GFP construct and used 48 h later. Cells were incubated with a saturating concentration of carbachol (1 mM) for the indicated times. The primary antibody was a mouse monoclonal antibody against lgp120 (1:500); the secondary antibody was Cy3-conjugated anti-mouse IgG (1:500). Transfected cells were visualized using confocal microscopy. Arrows indicate areas of partial overlap of fluorescence in the corresponding panels. Bars, 10 μm.

tagged receptors were returning to the surface after agonist removal, in agreement with the radioligand binding data. We conclude, therefore, that once internalized, the GFP-tagged receptors traffic only slowly, if at all, out of endosomes.

4. Discussion

The addition of a C-terminal GFP tag is now a commonly used approach to the study of agonist-triggered trafficking of G protein-coupled receptors (reviewed by Kallal and Benovic, 2000). In most cases, the fundamental properties of the receptors, such as ligand binding and second messenger generation, have been shown to be largely unaffected by addition of the tag. In the present study, we found that whereas the binding affinity of the antagonist *N*-methylscopolamine to the muscarinic M_4 receptor was not significantly affected by the presence of the C-terminal GFP tag, the affinity of the agonist carbachol was reduced by four-fold. It is well known that the binding of agonists (but not antagonists) to G protein-coupled receptors is affected by the interaction between receptor and G protein, and that dissociation of the G protein causes a significant reduction in agonist binding affinity (Birnbaumer et al., 1990). It is possible, therefore, that the GFP tag is causing a reduction in the affinity of the muscarinic M_4 receptor by interfering with receptor–G protein coupling. However, the fact that stimulation of the GFP-tagged receptor elicits a significant fall in cellular cyclic AMP levels indicates that the receptor is able to couple to G_i/G_o efficiently enough to inhibit adenylyl cyclase, despite the presence of the 27-kDa GFP tag at its C terminus.

It has been reported recently (McLean and Milligan, 2000) that GFP-tagged β_1- and β_2-adrenoceptors expressed in HEK-293 cells are endocytosed more slowly than their wild-type counterparts, and that the extent of degradation of the β_2-adrenoceptor during maintained exposure of the cells to agonist is substantially reduced by the presence of the GFP tag. In our study, we found a similar slowing of the endocytosis of the GFP-tagged muscarinic M_4 receptor, and also a complete block of recycling on agonist removal. It is clear, therefore, that the trafficking of the tagged receptor is abnormal. There are two possible explanations for this aberrant behaviour: either the modification of the C terminus of the receptor could perturb its interaction with proteins involved in

receptor trafficking (see, e.g. Cao et al., 1999), or the huge overexpression of the receptor in the transfected cells could cause a saturation of the trafficking machinery. In fact, we have also investigated the trafficking of a muscarinic M_4 receptor that has been tagged at its N terminus with the smaller hemagglutinin (HA) epitope (Madziva and Edwardson, unpublished observations). This receptor, like the muscarinic M_4 receptor–GFP, is overexpressed by approximately 20-fold in transfected cells but has identical intracellular domains to the endogenous receptor. We found that the HA-tagged receptor, like the GFP-tagged receptor, was internalized more slowly than the endogenous receptor and showed no recycling on removal of agonist. We therefore conclude that it is the overexpression of the receptor, and not the modification of its C terminus, that causes the reduction in the trafficking rates of the muscarinic M_4 receptor–GFP. To confirm this conclusion, it will be necessary to manipulate receptor levels within the cells, either through the use of a vector that allows inducible expression of the receptor, or through the generation of stably transfected cell lines.

Receptor overexpression is most probably reducing the rate of agonist-triggered receptor endocytosis by saturating components of the endocytotic machinery, such as β-arrestin (Lohse et al., 1990) and dynamin (Werbonat et al., 2000), that are likely to be expressed in amounts necessary to deal with much smaller numbers of receptors. We have seen a similar phenomenon previously, when we compared the rate constant for endocytosis of the M_4 receptor expressed endogenously in NG108-15 cells ($0.13\ min^{-1}$) with the rate constant for endocytosis of the same receptor expressed at approximately 100-fold higher levels in Chinese hamster ovary cells ($0.071\ min^{-1}$; Koenig and Edwardson, 1996). A more significant effect of receptor overexpression was observed in A431 cells, which naturally express very high levels of epidermal growth factor (EGF) receptors; here, the rate of EGF endocytosis fell dramatically at high receptor occupancies (Wiley, 1988), again, presumably, because of the saturation of the cellular endocytotic machinery.

Whether or not the observed lack of trafficking of the GFP-tagged receptor to lysosomes is a characteristic of the endogenous receptor expressed at normal levels remains an open question. In a previous study (Koenig and Edwardson, 1994), we used estimates of the rate constants for the various intracellular trafficking steps to obtain a predicted value of 20% receptor degradation over a 60-min period of agonist stimulation. Assuming that degradation occurs in lysosomes, 20% is therefore the maximum percentage of total receptor labeling that should appear in this compartment over 60 min. Realistically, the percentage of receptors actually delivered to lysosomes in the present study, where trafficking is clearly compromised, is likely to be lower. It is therefore possible that a small amount of delivery to lysosomes does occur, but is not apparent in our fluorescence-based experiments. Detection of receptors in lysosomes may, of course, be further hampered by ongoing proteolysis in this compartment.

In this study, we have been able to compare directly the trafficking characteristics of endogenous muscarinic M_4 receptors and overexpressed GFP-tagged receptors in the same cells. In the light of our results, we would urge caution in the interpretation of data on G protein-coupled receptor trafficking that have been obtained through the use of GFP-tagged receptors. If the behaviour of GFP-tagged muscarinic M_4 receptors in our system is representative, it is clear that no useful quantitative data on receptor trafficking can be gained. Indeed, the profound effects of overexpression on receptor trafficking would seem to suggest that only the most basic qualitative information can be provided by this approach.

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References

Barak, L.S., Ferguson, S.S.G., Zhang, J., Martenson, C., Meyer, T., Caron, M.G., 1997. Internal trafficking and surface mobility of a functionally intact β_2-adrenergic receptor–green fluorescent protein conjugate. Mol. Pharmacol. 51, 177–184.

Barnstable, C.J., Hofstein, R., Akagawa, K., 1985. A marker of early amacrine cell development in rat retina. Dev. Brain Res. 20, 286–290.

Birnbaumer, L., Abramowitz, J., Brown, A.M., 1990. Receptor–effector coupling by G proteins. Biochim. Biophys. Acta 1031, 163–224.

Cao, T.T., Deacon, H.W., Reczek, D., Bretscher, A., Von Zastrow, M., 1999. A kinase-regulated PDZ–domain interaction controls endocytic sorting of the β_2-adrenergic receptor. Nature 401, 286–290.

Hansen, S.H., Sandvig, K., van Deurs, B., 1993. Clathrin and HA2 adapters: effects of potassium depletion, hypertonic medium, and cytosol acidification. J. Cell Biol. 121, 61–72.

Holroyd, E.W., Szekeres, P.G., Whittaker, R.D., Kelly, E., Edwardson, J.M., 1999. Effect of G protein-coupled receptor kinase 2 on the sensitivity of M_4 muscarinic acetylcholine receptors to agonist-induced internalization and desensitization in NG108-15 cells. J. Neurochem. 73, 1236–1245.

Howe, C.L., Granger, B.L., Hull, M., Green, S.A., Gabel, C.A., Helenius, A., Mellman, I., 1988. Derived protein sequence, oligosaccharides, and membrane insertion of the 120-kDa lysosomal membrane glycoprotein (lgp120): identification of a highly conserved family of lysosomal membrane glycoprotein. Proc. Natl. Acad. Sci. U. S. A. 85, 7577–7581.

Inoue, A., Akagawa, K., 1993. Neuron specific expression of a membrane protein, HPC-1: tissue distribution, and cellular and subcellular localization of immunoreactivity and mRNA. Mol. Brain Res. 19, 121–128.

Kallal, L., Benovic, J.L., 2000. Using green fluorescent proteins to study G-protein-coupled receptor localization and trafficking. Trends Pharmacol. Sci. 21, 175–180.

Koenig, J.A., Edwardson, J.M., 1994. Kinetics of the trafficking of

muscarinic acetylcholine receptors between the plasma membrane and intracellular compartments. J. Biol. Chem. 269, 17174–17182.

Koenig, J.A., Edwardson, J.M., 1996. Intracellular trafficking of the muscarinic acetylcholine receptor: importance of subtype and cell type. Mol. Pharmacol. 49, 351–359.

Koenig, J.A., Edwardson, J.M., 1997. Endocytosis and recycling of G protein-coupled receptors. Trends Pharmacol. Sci. 18, 276–287.

Lohse, M.J., Benovic, J.L., Codina, J., Caron, M.G., Lefkowitz, R.J., 1990. β-Arrestin: a protein that regulates β-adrenergic receptor function. Science 248, 1547–1550.

McLean, A.J., Milligan, G., 2000. Ligand regulation of green fluorescent protein-tagged forms of the human β_1- and β_2-adrenoceptors; comparisons with the unmodified receptors. Br. J. Pharmacol. 130, 1825–1832.

Tsao, P., Cao, T., Von Zastrow, M., 2001. Role of endocytosis in mediating downregulation of G-protein-coupled receptors. Trends Pharmacol. Sci. 22, 91–96.

Von Zastrow, M., Kobilka, B.K., 1992. Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. J. Biol. Chem. 267, 3530–3538.

Werbonat, Y., Kleutges, N., Jakobs, K.H., van Koppen, C.J., 2000. Essential role of dynamin in internalization of M_2 muscarinic acetylcholine and angiotensin AT_{1A} receptors. J. Biol. Chem. 275, 21969–21974.

Wiley, H.S., 1988. Anomalous binding of epidermal growth factor to A431 cells is due to the effect of high receptor densities and a saturable endocytic system. J. Cell Biol. 107, 801–810.

Woods, J.W., Goodhouse, J., Farquhar, M.G., 1989. Transferrin receptors and cation-independent mannose-6-phosphate receptors deliver their ligands to 2 distinct subpopulations of multivesicular endosomes. Eur. J. Cell Biol. 50, 132–143.