Carbachol-activated muscarinic (M1 and M3) receptors transfected into Chinese hamster ovary cells inhibit trafficking of endosomes

(fluid-phase endocytosis/G proteins/calcium/fusion/signal transduction)

KAZUTAKA HARAGUCHI* AND MARTIN RODBELL

Section on Signal Transduction, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

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ABSTRACT We examined the effects of isoproterenol and carbachol on fluid-phase endocytosis by Chinese hamster ovary (CHO) cells transfected with β -adrenergic, M1, or M3 cholinergic receptors. Isoproterenol increased cAMP production and carbachol increased intracellular Ca, indicating successful expression of the receptor genes and coupling to typical signal transduction pathways. Carbachol inhibited the uptake of horseradish peroxidase (HRP) or Lucifer yellow (markers of fluid-phase endocytosis) in both M1- and M3-containing cells but not in wild-type cells, whereas isoproterenol did not affect pinocytosis in cells transfected with β -adrenergic receptors. Carbachol inhibited the transit of HRP from an exchangeable pool to a nonexchangeable pool by a latent process requiring minimally 5 min of incubation. During the latent period, only one peak of low-density HRP-containing vesicles was found on Percoll gradients; after 5 min, HRP appeared in both high- and low-density vesicles. Carbachol-treated cells contained less HRP in the high-density fraction enriched in lysosomal markers. Early endosomes from CHO cells labeled for 5 min with HRP underwent fusion to make a more dense population of vesicles in the presence of ATP and KCl at 37°C but not at 4°C. The fused material contained increased levels of G proteins as detected either by ADP ribosylation with appropriate toxins or by immunoblotting with specific antibodies. These findings suggest that GTP binding proteins are internalized in endocytic vesicles and enter into a complex trafficking process involving fusion with other vesicular compartments. Trafficking of endosomes to these compartments is inhibited by activated M1 and M3 muscarinic receptors in CHO cells.

Endocytosis is a fundamental property of eukaryotic cells and is the means by which cell membrane proteins and solutes are internalized and delivered to endosomal compartments from which they are recycled back to the cell surface or routed to lysosomes. Great strides have been made in understanding the complex sorting and trafficking of molecules taken up adsorptively (receptor mediated) and nonadsorptively (fluid-phase endocytosis or pinocytosis) (1–7). Internalized molecules are first observed in early tubulovesicular endosomal elements (early endosomes) from which receptors recycle rapidly back to the cell surface, whereas molecules destined to be degraded are transported to late endosomes that also have a complex vesicular and multivesicular appearance. Late endosomes are frequently observed in the perinuclear region where lysosomes are found (ref. 8 and references therein). Our interest in vesicular trafficking stemmed from observations that hormones that stimulate the production of cAMP in rat adipocytes enhanced the fluid-phase uptake of horseradish peroxidase (HRP), a marker for pinocytosis, into undefined endosomal compartments (9, 10). Because GTP binding proteins (G proteins) (G_s , G_i) and

adenylyl cyclase were present in HRP-containing endosomes (termed pinosomes) in activatable forms, it was speculated that the pinosomes could be a source of cAMP and therefore a means of disseminating signals initiated at the surface membrane by hormones (10). Although an intriguing possibility, investigations of the mechanism of isoproterenol-induced pinocytosis proved difficult. Increased production of unesterified fatty acids in response to the lipolytic hormone may be partially responsible since the levels of pinosomes seemed related to the concentration of accumulated internal fatty acids (9, 10). An added difficulty is the paucity of adipocyte endocytic vesicles required for investigating their biochemical properties.

As a means of obviating some of these difficulties, we have investigated the effects of hormones on pinocytosis in Chinese hamster ovary cells (CHO) that have been transfected with genes for muscarinic and β -adrenergic receptors. Techniques for investigating the biochemical aspects of vesicle trafficking *in vitro* have been developed in these cells (11, 12). An additional incentive for using cells containing transfected receptors is that advantage can be taken of the known signaling mechanisms for each type of transfected receptor; transfection with two or more receptors having different signaling mechanisms potentially can be used for examining “cross-talk” between receptor-signaling mechanisms. In this study, we report effects of muscarinic agents on the uptake of HRP by CHO cells transfected with either M1 or M3 muscarinic receptors.

MATERIALS AND METHODS

Materials. Buffers, salts, HRP, Lucifer yellow, creatine phosphate, creatine phosphokinase, and bovine serum albumin were obtained from Sigma and Boehringer Mannheim. Sera used for growing CHO cells were obtained from GIBCO. cAMP assay kits were from Amersham. [32 P]NAD was obtained from New England Nuclear. Plates (24 wells) were purchased from Costar. The ball-bearing homogenizer was purchased from Berni-tech Engineering (Saratoga, CA); tungsten-carbide ball bearings (clearance, 0.0325 mm) were from Industrial Tectonics (Ann Arbor, MI).

Cells. CHO cells transfected with muscarinic (M1 and M3) receptors and β -adrenergic receptors have been described (13, 14) and were generously supplied by Claire Fraser (National Institute of Neurological Disorders and Stroke). M1 receptors were expressed at a concentration of 800 fmol per mg of membrane protein; M3 receptors were expressed in separate CHO cells at a concentration of 450 fmol per mg of membrane protein. Cells were maintained in Ham's F-12

Abbreviations: G proteins, GTP binding proteins involved in signal transduction; HRP, horseradish peroxidase; NEP, nonexchangeable pool of HRP taken up in cells.

*Present address: The Third Department of Internal Medicine, University of Yamanashi Medical School, Tamaho, Yamanashi-Ken 409-38, Japan.

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medium supplemented with 10% fetal calf serum and genetin (10 μ g/ml). For studies of the effects of muscarinic agonists on pinocytosis, cells were seeded on 24-well plates and were grown in F-12 medium supplemented with 10% calf serum; generally, cells were taken after 2 days of growth and washed with phosphate-buffered saline and then with F-12 without serum prior to experiments. Preliminary studies under various growth conditions indicated that cells grown rapidly to confluency (such as observed with cells grown in medium supplemented with fetal calf serum) displayed considerably smaller rates of pinocytosis and the effects of muscarinic agonists were slight. In studies of the cell-free characteristics of pinosomes, considerably greater concentrations of CHO cells (5×10^5 cells per ml) were required. This was achieved by growing them in 500-ml spinner culture bottles in F-12 medium supplemented with 10% calf serum.

Assay for Pinocytosis. Prior to the experiments, cells were washed in Puck's saline G (127 mM NaCl/5.3 mM KCl/1 mM CaCl_2 /0.625 mM MgSO_4 /6.1 mM glucose/2.1 mM sodium phosphate) containing 10 mM Hepes (pH 7.3). Pinocytosis was generally assayed by measuring the uptake of HRP; in a few cases, assays were carried out using Lucifer yellow as a marker (15). In studies of the effects of hormones or other agents on uptake, cells were incubated first with these agents for 30 min. HRP uptake was found to be proportional to HRP concentration up to 8 mg/ml and uptake was linear for at least 3 hr. This allowed variation of the initial concentration of HRP according to the length of the incubation so that HRP activity was nearly the same in most experiments. For example, HRP was added at 2 mg/ml for a 1-hr incubation and at 6 mg/ml when incubations with HRP were carried out for 15 min or less. All incubations were at 37°C and were terminated by rapidly immersing the 24-well plates five times successively into beakers containing 1 liter of cold Puck's saline (16) within 5 min. After washing, 50 mM sodium phosphate buffer containing 0.05% Triton X-100 was added to each well. After 10 min, the contents were dispersed with a 200- μ l pipette. Total uptake represents the total HRP content in the cell. The exchangeable pool was calculated by subtracting the nonexchangeable pool (NEP) from total uptake. NEP was measured as follows: the cells were allowed to take up HRP (pulse) and then were again exposed to incubation medium lacking HRP at 37°C (chase). The cells lose peroxidase activity rapidly from the exchangeable pool. By 40 min, the rate of disappearance of HRP slowed and nearly equaled that at later times; this may represent degradation of HRP taken up by lysosomes. Thus, HRP activity remaining in the cell after 40 min of chase is termed NEP.

Other Assays. Peroxidase activity was measured at 465 nm in a Hitachi spectrophotometer. Units are defined as ng of peroxidase taken up per mg of protein. Protein concentrations were determined by the method of Bradford (17). cAMP production was determined on plated CHO cells incubated in the presence of isobutylmethylxanthine at 37°C in the presence or absence of hormones. Incubations were stopped by discarding the medium and washing plates twice as described above in 10 mM Tris-HCl, pH 7.4/5 mM EDTA/150 mM NaCl. The plates were placed on top of a boiling water bath and 0.5 ml of hot Tris/EDTA buffer was added to each well. cAMP was assayed according to the procedures provided in the Amersham kit. Cells plated on glass slides were assayed for cytoplasmic calcium concentration as described (18).

Subcellular Fractionation. Cells grown in spinner culture (5×10^5 cells per ml) were harvested by low speed (500 rpm) centrifugation and suspended in Puck's saline G containing HRP (6 mg/ml) to give 5×10^7 cells per ml. At the indicated times, 3 ml of cell suspension was aliquoted into tubes containing 5 ml of crushed frozen Puck's saline. The cells were spun down at 500 rpm and washed (with resuspension) three times with the same medium. The final wash was with

10 mM sodium phosphate buffer (pH 7.0) containing 5 mM EDTA and 150 mM NaCl. The suspension was again centrifuged and suspended in 2 ml of cold homogenizing buffer (20 mM Hepes, pH 7.0/250 mM sucrose/0.5 mM EGTA). Cells were broken at 4°C by nitrogen cavitation (30 psi; 1 psi = 6.9 kPa) for 15 min followed by homogenization in a ball-bearing homogenizer using eight strokes. This procedure yielded 60% cell breakage. Unbroken cells, nuclei, and debris were removed by spinning at $800 \times g$ for 10 min at 4°C. The supernatant was applied on 10% Percoll containing 0.25 M sucrose with a 2-ml cushion of 65% sucrose. The tubes were spun at 18,000 rpm in a Beckman 70 Ti rotor for 100 min. Fractions were collected by aspiration from bottom to top and were assayed for their content of HRP and β -hexoaminidase (a lysosomal marker enzyme) as described by Rome *et al.* (19). Stability of the pinosomes was assessed by measuring HRP activity before and after treatment with 0.05% Triton X-100. By this criterion, $\approx 60\%$ of the pinosomes were unbroken.

Pinosome Redistribution. The postnuclear supernatant described above was diluted to a concentration of 0.8–1.0 mg of protein per ml in medium containing 1 mM ATP, 1.5 mM MgCl_2 , 50 mM KCl, 1 mM dithiothreitol, 8 mM creatine phosphate, and 100 μ g of creatine phosphokinase per ml. The final sucrose concentration was 0.25 M. Fractionation of the suspension of pinosomes was carried out by the procedure of Diaz *et al.* (20). Briefly, the suspension was centrifuged at 4°C in a Beckman tabletop ultracentrifuge (rotor 100.3) at five different speeds and times: pellet 1, $5400 \times g$ for 2 min; pellet 2, $12,200 \times g$ for 1 min; pellet 3, $34,000 \times g$ for 1 min; pellet 4, $66,000 \times g$ for 1 min; pellet 5, $265,000 \times g$ for 10 min. Each pellet was washed once with homogenizing buffer and suspended in 300 μ l of 50 mM sodium phosphate buffer (pH 7.0). The suspension was used both for assay of HRP activity and for determination of content of G proteins by pertussis toxin-catalyzed ADP ribosylation (21) and/or by immunoblotting with specific antibodies to G-protein subunits as described (22).

RESULTS

Effects of Muscarinic Agonists. In preliminary studies, we examined whether CHO cells transfected with M1 or M3 muscarinic receptors and with β -adrenergic receptors would yield expressed receptors as evidenced by signaling pathways seen in cells normally containing these receptor types. In CHO cells transfected with M1 and M3 muscarinic receptors, carbachol, as found typically in cells containing muscarinic receptors (23), stimulated an increase in cytoplasmic calcium with nearly equal potency (half-maximal, $\approx 10 \mu\text{M}$) in medium containing EGTA, indicating mobilization of internal calcium stores (data not shown). Basal cAMP levels were not changed by carbachol in M1 transfected CHO cells. In M1 cells cotransfected with β -adrenergic receptors, isoproterenol (10^{-9} to 10^{-4} M) stimulated cAMP production indicating expression of this receptor. No effect of isoproterenol was observed on HRP uptake in these cells (data not shown), suggesting that cAMP was not directly involved in the uptake or processing of pinosomes (see *Discussion*). Accordingly, the remainder of these studies concentrated on the actions of carbachol on HRP uptake.

As shown in Fig. 1A, carbachol (0.1 mM) added simultaneously with HRP inhibited total HRP uptake; inhibition was apparent at 10 min of incubation. Even when the cells were preincubated with carbachol for 1 hr before adding HRP, inhibition was not evident until after 5 min of incubation. Once the exchangeable pool (calculated by subtracting the NEP from total uptake) reached its plateau at 5 min, the rate of total uptake was the same as that of NEP and remained constant for as long as 3 hr. Hence, carbachol did not affect

FIG. 1. (A) Time course of HRP uptake by CHO cells containing M1 receptors. Cells were incubated in 0.4 ml of prewarmed Puck's saline containing HRP (6 mg/ml), with (circles) or without (squares) 10^{-4} M carbachol. After washing, cells from two plates were assayed for total HRP content (solid symbols). Cells in the other two plates were further incubated with 0.5 ml of Puck's saline G per well at 37°C for another 40 min. HRP remaining in the cells represents NEP (open symbols). (B) Effect of atropine. CHO (M1) cells were incubated as in A without (▲) or with (■, □, ●) carbachol (3×10^{-4} M) for the indicated times. At 30 or 60 min of incubation, as indicated by the arrows, the cells were washed once and incubated with the same buffer with (■, ●) or without (□, ▲) atropine (20 μM). Each point represents the mean of quadruplicate determinations.

the initial uptake of HRP. Its major effect was to cause inhibition of HRP transfer to NEP; this was equivalent with both M1 and M3 transfected CHO cells.

To test for specificity and reversibility of the carbachol-inhibited process, M1 cells were incubated with 0.1 mM carbachol and the cells were challenged with atropine, a specific blocker of muscarinic receptors. Atropine (10^{-6} M) itself slightly increased total HRP uptake but, as depicted in Fig. 1B, blocked the inhibitory action of carbachol when added either simultaneously with the agonist or after agonist-induced inhibition of HRP uptake. Note the rapid return to the normal rate of HRP uptake with added atropine. At any time of incubation, the exchangeable pool remained constant and was not altered by carbachol. One-half of the exchangeable pool (calculated from the peroxidase activity remaining in the washed cells) was replaced within 2.5–3 min depending on the batch of cells and showed no difference in carbachol-treated and nontreated cells (data not shown). The $t_{1/2}$ of HRP in NEP, which probably reflects the degradation of HRP in lysosomes, was ≈ 4 hr with either control or carbachol-treated cells.

Several known inhibitors of pinocytosis, such as 10 mM NaF, 1 mM KCN, 10 μM colchicine, and 0.1 mM dibucaine, also inhibited HRP uptake by 65%, 25%, 50%, and 36%, respectively; incubation at low temperature (4°C) inhibited pinocytosis nearly completely; NH₄Cl was without effect (data not shown). The same inhibitory effect of carbachol on

Lucifer yellow uptake was seen with M1 cells (data not shown).

The dose-dependent inhibition of HRP uptake (total or NEP) by carbachol was nearly the same (half-maximal, ≈ 10 μM) with both M1 and M3 transfected cells (data not shown). No effect of carbachol was detected on wild-type, nontransfected cells.

Properties of Early Endosomes. As a means of investigating the nature and properties of the HRP-containing vesicles, CHO cells grown in spinner cultures were incubated with HRP for 5 min with or without carbachol. Homogenates, after removal of nuclei and cell debris by low-speed centrifugation, were fractionated on 10% Percoll gradients and the vesicles containing HRP were identified by their peroxidase activity. By 5 min of incubation, peroxidase activity in the early pinosomes, representing the exchangeable pool, was detected in a single peak having a specific gravity of 1.04 g/ml (Fig. 2, pulse). Note that carbachol did not change either the distribution or the amount of HRP on the gradient. By contrast, when the cells were incubated with HRP for 30 min (pulse) and then washed for another 40 min (chase), HRP appeared in fractions having a specific gravity of around 1.28 g/ml (measured by weight) near the bottom of the Percoll gradient (Fig. 2, chase). This peak of HRP activity in the dense fractions, representing the nonexchangeable HRP pool, cofractionated with β-hexosaminidase activity, a lysosomal marker enzyme; the peak of β-hexosaminidase activity

FIG. 2. Gradient fractionation of HRP-containing vesicles. Spinner cultured cells (8×10^7 cells per ml) were washed once and incubated with the same buffer containing 2 mg of HRP per ml for 5 min with (▲) or without (□) 10^{-4} M carbachol. The cells were washed and either homogenized immediately (pulse) or incubated in buffer without HRP for another 40 min (chase) and then homogenized and subjected to Percoll gradient fractionation. Aliquots were collected from the bottom (fraction 32) to the top (fraction 1) and assayed for HRP content.

was identical in both control and carbachol-treated M1 cells (data not shown).

Detection of G Proteins in Early Endosomes. When the postnuclear supernatant containing early pinosomes was incubated under conditions required for fusion (1 mM ATP/1.5 mM KCl at 37°C) (8), HRP activity increased in a time-dependent manner in a pellet (P1) obtained at the lowest speed of centrifugation (Fig. 3). HRP activity concomitantly decreased in the high-speed pellet (P5). The P1 fraction also showed an increase of $G\alpha_i$ as detected by pertussis toxin-catalyzed ADP ribosylation (Fig. 4 Upper). The amount of HRP and $G\alpha_i$ in P1 increased gradually with time and reached a constant value at 60 min at 37°C in the complete medium used for fusion. Removal of either ATP or KCl or decreasing the incubation temperature to 4°C prevented both the redistribution of HRP and $G\alpha_i$ seen at 37°C under complete conditions, as was shown in Fig. 3. Immunodetection of $G\alpha_s$ and $G\beta$ subunits with specific antisera revealed under identical conditions the same pattern of distribution seen with $G\alpha_i$ (data not shown). Early pinosomes (P5) prepared from cells treated with carbachol did not behave differently from those obtained from control cells under complete fusion conditions (data not shown). The possible role of microtubulin in the fusion process was tested by the addition of colchicine (1 μ M) to the complete medium; no effect of colchicine was observed (data not shown).

DISCUSSION

We have shown in this study that the muscarinic agonist carbachol inhibits the uptake of HRP or Lucifer yellow, both

FIG. 3. *In vitro* clustering ("fusion") of pinosomes: effects of ATP, KCl, and temperature. CHO (M1) cells were prepared and incubated in the presence of HRP (6 mg/ml) for 5 min at 37°C. The cells were homogenized and the postnuclear supernatant was prepared. The postnuclear supernatant was incubated in 0.3 ml of complete fusion medium at 37°C, complete medium at 4°C, complete medium minus ATP (medium contains 5 mM glucose and 30 units of hexokinase per ml to deplete endogenous ATP) at 37°C, and complete medium minus KCl at 37°C. Final protein concentration was 0.84 mg/ml. Incubations were terminated by adding 2 ml of cold homogenizing buffer (20 mM Hepes, pH 7.0/0.25 M sucrose/0.5 mM EGTA); subfractionation was performed as described. Pellet 5 was obtained at the longest time and highest speed of centrifugation; pellet 1 was obtained at the shortest time and lowest speed of centrifugation. Each pellet was suspended in 300 μ l of 10 mM Tris-HCl, pH 7.5/5 mM NaCl/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride/50 μ g of leupeptin per ml. Aliquots were analyzed for HRP activity.

FIG. 4. In the same experiments described in the legend to Fig. 3, a portion of the postnuclear supernatant material was incubated under complete medium conditions at 37°C or at 4°C for the indicated times and fractionated as described. Pellet 1 was analyzed both for HRP content and for its content of G proteins subject to pertussis toxin-catalyzed ADP ribosylation.

markers for fluid-phase endocytosis (pinocytosis), in CHO cells transfected with M1 and M3 muscarinic receptors. As observed in CHO and many other cell types (2, 8), endocytosis is affected by cytoskeletal-disrupting drugs such as colchicine by decreasing the incubation temperature and by a number of compounds that affect the energy-transducing mechanisms in cells. Kinetically, the major effect of carbachol was to lower HRP levels in NEP without altering the level of HRP taken up in an exchangeable vesicular pool, the net effect being a lowering of the net total uptake. Consistent with this interpretation was the finding that the accumulation of HRP in a fraction containing lysosomes and late endosomes, generally regarded as representative of NEP, was less in carbachol-treated cells. It would appear, therefore, that carbachol inhibits HRP uptake by a process involving the transit of early endosomes into a still undefined pool of intracellular vesicles that releases its contents poorly if at all.

CHO cells transfected with β -adrenergic receptors did not display changes in HRP uptake when challenged with isoproterenol, although cAMP levels were promptly and greatly augmented. We have also found that forskolin treatment results in even larger levels of cAMP that are further amplified by carbachol action (14). Despite this increase, no change was observed in HRP uptake during the first 10–20 min; only at longer times of incubation was significant inhibition of HRP uptake observed (unpublished observations). Carbachol treatment causes a slight but significant increase in cAMP levels in M1 but not in M3 cells (unpublished observations), yet there was little difference in the effects of carbachol on pinocytosis. Hence, in contrast to adipocytes (10), there is no evidence that the adenylyl cyclase pathway of signal transduction is directly involved in regulating fluid phase endocytosis in CHO cells. This difference from CHO cells may be due to the putative role of fatty acids in pinocytosis inferred from the effects of albumin on HRP uptake in adipocytes exposed to isoproterenol and other lipolytic agents (9, 10).

A prominent response to muscarinic receptors observed in many cell types is increased levels of intracellular calcium whether promoted through the release of internal stores via

the phosphatidylinositol/inositol trisphosphate transduction pathway (23) and/or by stimulation of surface calcium channels (24). We have confirmed findings (24) that transfected M1 and M3 receptors increase levels of calcium in CHO cells. Carbachol stimulated calcium uptake and inhibited pinocytosis over nearly the same concentration range. We have also found that the calcium ionophore A23187 inhibits HRP uptake in the presence of 0.1 mM extracellular calcium (unpublished observations). Although these observations impugn calcium ions in the inhibitory process, it should be emphasized that activated muscarinic receptors produce multiple signals, possibly through parallel and interactive pathways (25–28). Furthermore, the wide-ranging effects of calcium ions on cells prevent any facile interpretation of mechanism, particularly on complex processes involving endosome trafficking.

Before any interpretation of carbachol action can be made, clearly needed are means of identifying the endocytic pathway(s) affected in response to the muscarinic receptor agonist. Toward this goal, we separated a membrane fraction enriched in a lysosomal marker that was initially devoid of HRP and G proteins. Interaction (possibly fusion) required ATP and KCl and was inhibited at 4°C. In accord with other cell-free studies (11, 29), these requirements and the time dependency suggest that the dense fraction (pellet 1) contains a later endosomal compartment from which early endosomes are either routed for recycling to the plasma membrane or transported to a later stage (prelysosomes, lysosomes, perinuclear compartment, etc.). The lack of a colchicine effect on the interaction of the early pinosomes (pellet 5) with the dense fraction (pellet 1) suggests that their interaction is not mediated by microtubulin, which other studies indicate is involved in the sorting and trafficking to the lysosomal compartment (29). The nature of the more dense vesicles in pellet 1 that interact with the early endosomes remains unknown but might include a recently discovered population of large spherical vesicles that seem to mediate transport between the early and late endosomal compartments (29). Since the inhibitory effects of carbachol are restricted to the uptake of HRP into NEP and are blocked in the intact cell by colchicine, it is likely that carbachol exerts its effects on the trafficking and sorting processes that occur beyond the late endosomal processing stage.

We initiated these studies with the idea that CHO cells transfected with specific receptors might provide cellular models for investigating hormonal regulation of vesicle trafficking and the possible dissemination and targeting of signals during trafficking. To the extent that endosomes in CHO cells contain G proteins, this idea is still attractive. An additional possibility stems from the fact that activated muscarinic receptors inhibit the trafficking of endosomes to the lysosomal compartment. Theoretically, the net result of this phenomenon should be diversion to and maintenance of the levels of signal transduction systems (receptors, G proteins, effectors such as adenylyl cyclase and ion channels) in the surface membrane. In future studies of this type it will be necessary to determine whether not only G proteins but also receptors and effectors are contained in endosomal compartments. Activated muscarinic receptors appear to be taken up by non-clathrin-coated vesicles (possibly by fluid-phase endocytosis) in human fibroblasts (30, 31) as may be the case for β -adrenergic receptors (32) and the α -mating factor receptor in yeast (33). If this proves to be the case generally for receptors that act through G proteins, an interesting question is whether the signal transduction elements are initially taken up together in the same endocytic vesicles or whether assembly of the signal transduction elements occurs during the sorting, fusion, and trafficking of endosomes containing these elements as separate entities. The recent finding that G

proteins in the giant squid axon are transported in vesicles by fast axonal transport (34) lends credence to the possibility that endosome trafficking involving G proteins could be sufficiently rapid to allow for both processing and signaling to occur in the normal time frame (seconds) for physiological actions of hormones. The challenge for future research is to identify and isolate the endosomes carrying the signal transduction elements. Given the expanding number of experimental tools for studying endosome processing in cell-free extracts (35–38), it now seems possible to meet this challenge.

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