Essential Role of Dynamin in Internalization of M₂ Muscarinic Acetylcholine and Angiotensin AT₁A Receptors*

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Most G protein-coupled receptors (GPCRs), including the M₂ muscarinic acetylcholine receptor (mAChR), internalize in clathrin-coated vesicles, a process that requires dynamin GTPase. The observation that some GPCRs like the M₂ mAChR and the angiotensin AT₁A receptor (AT₁A) internalize irrespective of expression of dominant-negative K44A dynamin has led to the proposal that internalization of these GPCRs is dynamin-independent. Here, we report that, contrary to what is postulated, internalization of M₂ mAChR and AT₁A R in HEK-293 cells is dynamin-dependent. Expression of N272 dynamin, which lacks the GTP-binding domain, or K535M dynamin, which is not stimulatable by phosphatidylinositol 4,5-bisphosphate, strongly inhibits M₁ mAChR internalization. In contrast, M₂ mAChR internalization is not (or is only slightly) reduced by expression of these constructs or treatment with PP1 or genistein. Thus, dynamin GTPases are not only essential for M₁ mAChR but also for M₂ mAChR and AT₁A R internalization in HEK-293 cells. Our findings also indicate that dynamin GTPases are differentially regulated by c-Src-mediated tyrosine phosphorylation.

For most G protein-coupled receptors (GPCRs), receptor internalization is thought to be initiated by phosphorylation of the receptor by G protein-coupled receptor kinases and binding of the cytosolic protein β-arrestin to the phosphorylated receptor (1). β-Arrestin then sterically inhibits further interaction of the receptor with heterotrimeric G proteins and binds with high affinity to clathrin heavy chains (1). Through this interaction, GPCRs are believed to be targeted to clathrin-coated pits. Following transformation of the clathrin-coated pit into a clathrin-coated vesicle, the clathrin-coated vesicle pinches off from the plasma membrane. This process is catalyzed by the 100-kDa GTPase dynamin, which probably activates (as yet largely unknown) effectors of the fission machinery (2). Three closely related mammalian dynamin isoforms have been identified: neuronal dynamin-1, ubiquitously expressed dynamin-2, and dynamin-3, which is expressed in testes, neurons, and lung (3). Comparison of the primary sequence shows that all three dynamin isoforms contain three highly conserved GTP-binding motifs (i.e. elements I, II, and III). A Lys⁴⁴→Ala substitution in the first of the three putative GTP-binding motifs yields a dominant-negative dynamin mutant, which displays strongly impaired GTPase activity and is predicted to have a greatly reduced GTP binding affinity (4). The two other GTP-binding motifs in dynamin are likely to be involved in GTP binding as well. Mutation of the third GTP-binding motif (substitution Lys⁵⁰⁶→Asp in element III) or removal of all three GTP-binding motifs (amino acids 1–271 in dynamin-1) drastically reduces clathrin-coated vesicle-mediated internalization (4–6).

A second important regulator of dynamin function is phosphatidylinositol 4,5-bisphosphate (PIP₂) (6–9). All three dynamin isoforms contain a pleckstrin homology domain that is able to bind PIP₂. Binding of PIP₂ to dynamin not only strongly increases the GTPase activity of dynamin but may also serve to target dynamin to the plasma membrane, allowing subsequent dynamin self-assembly at the neck of the clathrin-coated vesicle (6–9). Expression of the dynamin mutant K535M, which is not stimulatable by PIP₂, effectively blocks transferrin receptor internalization in clathrin-coated vesicles (6).

A large number of recent studies indicate that most GPCRs, including M₁, M₂, and M₃ muscarinic acetylcholine receptors (mAChRs) in HEK-293 cells, internalize in clathrin-coated vesicles in a dynamin-dependent manner. This evidence is primarily based on the inhibitory effect of the dominant-negative inhibitor of dynamin-mediated internalization, K44A dynamin (10–13). In contrast, M₂ mAChRs internalize in a clathrin-independent manner and irrespective of expression of K44A dynamin in HEK-293 cells (10, 12). Likewise, internalization of angiotensin AT₁A receptors (AT₁A Rs) (13), dopamine D₂ receptors (14), and secretin receptors (15) is also insensitive to expression of K44A dynamin. This has led to the proposal that internalization of these GPCRs is dynamin-independent. However, in light of the notion that the binding of GTP to dynamin probably involves binding to all three GTP-binding motifs in the GTP-binding pocket, we reasoned that a dynamin mutant lacking all three GTP-binding motifs might be a more appropriate dominant negative dynamin mutant to determine whether internalization of a particular GPCR is dynamin-dependent. Indeed, we here demonstrate that internalization of M₂ mAChR and AT₁A R is strongly inhibited by expression of N272 dynamin, which lacks the complete GTP-binding domain. Also, expression of K535M dynamin, which lacks PIP₂-stimulated GTPase activity, significantly blocks internalization of these GPCR species.
**Dynamin-regulated M2 mAChR and AT1R Internalization**

**EXPERIMENTAL PROCEDURES**

**Materials—**N-[1H]Methylsphingosine (82 Ci/mmol), [32P]ATP (3000 Ci/mmol) were purchased from NEN Life Science Products. PPl and genistein were purchased from Biomol and Calbiochem, respectively. The cDNA constructs encoding wild-type c-Src and K298M c-Src were gifts from Dr. T. Parsons. Rat wild-type dynamin-1aa and rat E535M dynamin-1aa (100 units/μl) were generously provided by Dr. J. Albanesi. Rat Y231F, Y597F dynamin-1aa in pCMV96-7 (6) was a gift from Dr. R. Lefkowitz. The mouse AT1R cDNA in pB72121 was a gift from Dr. S. Schmid. Dynamin-1aa N272 was generated by digestion of rat wild-type dynamin-1aa in pCMV96-7 with BglII and EcoRV. The product was filled in with Pfu DNA polymerase (Stratagene). The complete receptor DNA sequence was verified by dideoxy DNA sequencing and subcloned in pcDNA3 plasmid vector. The authenticity of the N272 dynamin mutant was confirmed by dyeoxy DNA sequencing and Western blot analysis. Rabbit anti-c-src polyclonal antibody (N-89), mouse anti-Tyr(P) antibody (PY20), and goat anti-dynamin antibody (clone 41), and peroxidase-conjugated goat anti-mouse antibody were obtained from Calbiochem, Transduction Laboratories, and Dianova, respectively. Peroxidase-conjugated rabbit anti-goat antibody, peroxidase-conjugated goat anti-rabbit antibody, and fluorescein isothiocyanate-labeled anti-mouse antibody were from Sigma.

**Cell Culture, Plasmid Construction, and DNA Transfection—**HEK-293 tsA201 and HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)/F-12 medium supplemented with 10% fetal calf serum, penicillin G (100 units/ml), streptomycin (100 μg/ml), and 25 mm HEPES-buffered DMEM/F-12 medium buffer containing 1 mg/ml BSA with 100 mM NaH2PO4 at 4 °C as described in detail previously (10). Expression levels of M1 and M2 mAChRs varied between 100 and 750 fmol/mg intact cells in 25 mM HEPES-buffered saline, pH 7.4 (HBS), containing 100 mM NaCl, 3 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 10 mM glucose, and 50 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Permeabilization of the plasma membrane with 0.5% saponin, followed by resuspension in 85 μl of serum-free medium. After 16 h, cells were preincubated in 25 mM HEPES-buffered DMEM/F-12 medium for 30 min and then stimulated for 5 min with 100 μM carbachol. After rapid suction of medium from the plates, the 1.5 ml of ice-cold radioimmune precipitation buffer and once with ice-cold buffer A (110 mM NaCl, 3 mM KCl, 7 mM Na2HPO4, 2 mM KH2PO4, and 20 mM NaF, pH 7.4). The pellets were resuspended in 30 μl of 2× Laemmli sample buffer. After boiling for 5 min, samples were centrifuged, and protein in the supernatant was analyzed on a 10% SDS-polyacrylamide gel and Western blotting with anti-Tyr(P) antibody (0.2 μg/ml) and, after stripping, with anti-dynamin antibody (clone 41; 0.125 μg/ml). Immunoreactivity was visualized with peroxidase-conjugated goat anti-mouse antibody.

**Dynamin Immunoprecipitation and c-Src Kinase Activity Assay—**Immunoprecipitates were washed five times with lysis buffer and once in c-Src kinase buffer (100 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1 mM EDTA, 20 mM NaF, 0.1% Nonidet P-40, 1 mM dithiothreitol, 2.5 mM/ml leupeptin, 2.5 mM/ml aprotinin, and 100 μM phenylmethylsulfonyl fluoride). From this lysate, c-Src was immune precipitated with anti-Src antibody N-16 (1.5 μg) with 50 μl of Protein A Plus/Protein G-agarose beads. Immunoprecipitates were washed five times with lysis buffer and once in c-Src kinase buffer (100 mM Tris-HCl, pH 7.4, 125 mM MgCl2, 25 mM MnCl2, 2 mM EDTA, 250 μM Na2VO4, and 0.4 mM orthovanadate), followed by resuspension in 5 μl of Src kinase buffer with prepared c-Src substrate peptide and [γ-32P]ATP (125 μM, 10–20 μCi/μl) according to the manufacturer’s instructions (Upstate Biotechnology, Inc., Lake Placid, NY). The mixture was incubated at 30 °C for 10 min while shaking, and the reaction was stopped by the addition of 40 μl of 40% trichloroacetic acid. Thirty-microliter aliquots of the reaction mixture were spotted on P81 phosphocellulose paper in duplicate, washed five times for 5 min each with 0.75% phosphoric acid and once with acetone for 3 min, followed by radioactivity counting.

**Receptor Internalization Assays—**Internalization of mAChRs was determined 48 h after DNA transfection by [3H]HINMS binding assays to intact cells in 25 mM HEPES-buffered saline, pH 7.4 (HBS), containing 113 mM NaCl, 6 mM glucose, 3 mM KCl, 3 mM MgCl2, 2 mM MgSO4, and 1 mM Na2HPO4, at 4 °C as described in detail previously (10). Expression levels of M1, and M2 mAChRs varied between 100 and 750 fmol/mg of protein. Where indicated, transfected HEK-293 tsA201 cells were serum-starved for an additional 16 h in DMEM/F-12 medium. AT1R internalization was measured following incubation in 25 mM HEPES-buffered DMEM/F-12 medium buffer containing 1 mg/ml BSA with 1 μM unlabeled human angiotensin II (Sangstat, South San Francisco, CA) for 60 min at 37 °C. Coverslips were washed twice with HBS, twice with ice-cold 20 mM morpholinoethanesulfonic acid, 300 mM NaCl (pH 5.0); and twice with ice-cold HBS buffer (3 min each) to remove angiotensin II from receptor. Thereafter, cells were incubated in HBS buffer (with 1 mg/ml BSA) at 4 °C with 4–5 μCi [3H]Tyr-Sar-Ile-angiotensin II with or without 1 μM angiotensin II to determine nonspecific and total binding, respectively. After 4 h, cells were washed three times with HBS buffer and stained with 3% paraformaldehyde in 200 mM sodium phosphate buffer, pH 7.4, for 15 min. After several washes with distilled water, the cells were dehydrated in an ethanol series, and coverslips were mounted using Mowiol (Calbiochem). Immunofluorescence was detected using a Zeiss Axioskop fluorescence microscope equipped with standard fluorescein filter. Immunofluorescence was marginal in nontransfected cells and cells expressing wild-type M1 mAChRs (without Myc tag), as well as in Myc-tagged M2 mAChR-expressing cells with the first antibody and second antibody.
Dynamin-regulated \( M_2 \) mAChR and AT1AR Internalization

RESULTS

Subcellular Redistribution of \( M_2 \) mAChR in HEK-293 Cells in Response to Carbachol—Internalization of \( M_2 \) mAChRs in HEK-293 cells was monitored by indirect immunofluorescence of \( M_2 \) mAChRs tagged with a c-Myc epitope at the extracellular amino terminus. For this, we used stably \( M_2 \) mAChR-expressing cells instead of transiently expressing HEK-293 tsA201 cells. In the latter cell type, there was a significant preexisting intracellular pool of \( M_2 \) mAChRs that did not permit unequivocal demonstration of receptor translocation from the plasma membrane into the cytosol upon carbachol treatment. Control \(^{[3]H}\)NMS binding experiments demonstrated that the Myc-tagged \( M_2 \) mAChRs sequestered with similar characteristics as the wild-type \( M_2 \) mAChRs in either HEK-293 tsA201 or HEK-293 cells (data not shown). As shown in Fig. 1A, \( M_2 \) mAChRs in untreated cells were found predominantly at the cell surface. During 60 min of incubation with 1 mM carbachol, \( M_2 \) mAChRs translocated into the cytoplasm (Fig. 1B). These results indicate that \( M_2 \) mAChRs like \( M_2 \) mAChRs (18) internalize into the cell interior of HEK-293 cells.

Effect of N272 Dynamin on \( M_1 \)-\( M_2 \) mAChR Internalization in HEK-293 Cells—To investigate the role of dynamin in \( M_2 \) mAChR internalization, N-terminal deletion dynamin-1 mutant N272 was expressed with either \( M_1 \) or \( M_2 \) mAChR in HEK-293 tsA201 cells. Fig. 2 shows the overexpression of the various transfected dynamin constructs used in this study. Expression of all dynamin forms (with the exception of N272 dynamin) was determined with a dynamin antibody recognizing the N-terminal part of dynamin-1 and dynamin-2. N272 dynamin-1, which migrates with an apparent molecular mass of \( \sim 72 \) kDa instead of \( \sim 100 \) kDa, lacks the greater part of this antibody-binding epitope. Expression of N272 dynamin was therefore detected by a dynamin-1 antibody that specifically recognizes a C-terminal domain of dynamin-1. Fig. 3 shows the effect of expression of N272 dynamin on \( M_1 \) and \( M_2 \) mAChR internalization in HEK-293 tsA201 cells. Expression of N272 dynamin inhibited internalization of \( M_1 \) and \( M_2 \) mAChRs in response to receptor stimulation with 100 or 10 \( \mu \)M carbachol for 60 min by 68 and 55%, respectively. Also, sequestration of \( M_2 \) and \( M_1 \) mAChRs in response to 100 or 10 \( \mu \)M carbachol for 60 min was reduced from 20 \( \pm \) 5 to 1 \( \pm \) 1% and from 35 \( \pm \) 3 to 12 \( \pm \) 4%, respectively, by co-expression of N272 dynamin in HEK-293 tsA201 cells (\( n \) = 3 experiments; data not shown). In contrast, as reported earlier by us (10) and others (12), expression of K44A dynamin inhibited \( M_1 \) but not \( M_2 \) mAChR internalization (Fig. 3).

Role of c-Src in Dynamin-mediated mAChR Internalization in HEK-293 Cells—The results presented above strongly suggested that dynamin is not only required for internalization of \( M_1 \), \( M_3 \), and \( M_4 \) mAChRs but also essential for \( M_2 \) mAChR internalization in HEK-293 tsA201 cells. We therefore set out to analyze whether dynamin function in the \( M_1 \) and \( M_2 \) mAChR internalization pathways is differentially regulated. Recently, it was reported that internalization of \( \beta_2 \)-adrenergic

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receptors in HEK-293 cells requires c-Src-mediated phosphorylation of dynamin on two tyrosine residues (i.e. Tyr<sup>231</sup> and Tyr<sup>597</sup>) (16). c-Src is activated by β-arrestin, which is bound to the agonist-occupied β<sub>2</sub>-adrenergic receptor and targets the receptor to the clathrin-coated pit (16). As M<sub>1</sub> mAChRs in HEK-293 tsA201 cells internalize into clathrin-coated vesicles transiently expressing M<sub>1</sub> or M<sub>2</sub> mAChR, similar images document equivalent dynamin loading (lower panel). The filter shown in the upper panel with phosphorylation of dynamin was detected by immunoblotting (IB) subjected to SDS-polyacrylamide gel electrophoresis, and tyrosine phosphorylation of dynamin was monitored without (Con) and with 100 μM carbachol (Car) for 5 min. Src kinase was immunoprecipitated from cell lysates, and c-Src kinase activity was determined with [γ-<sup>32</sup>P]ATP and Src substrate peptide. PP1 (1 μM) was included in the reaction mixture where indicated 15 min before the addition of [γ-<sup>32</sup>P]ATP. The presence of dimethyl sulfoxide as vehicle (final concentration of 0.01%) did not affect c-Src activity. Data are the mean ± S.E. of four (M<sub>1</sub> mAChRs) or six (M<sub>2</sub> mAChRs) experiments.

**FIG. 4.** mACHR-induced activation of c-Src and tyrosine phosphorylation of dynamin in HEK-293 cells. A, HEK-293 cells stably expressing M<sub>1</sub> or M<sub>2</sub> mAChRs and serum-starved overnight were stimulated without (Con) and with 100 μM carbachol (Car) for 5 min. Src kinase was immunoprecipitated from cell lysates, and c-Src kinase activity was determined with [γ-<sup>32</sup>P]ATP and Src substrate peptide. PP1 (1 μM) was included in the reaction mixture where indicated 15 min before the addition of [γ-<sup>32</sup>P]ATP. The presence of dimethyl sulfoxide as vehicle (final concentration of 0.01%) did not affect c-Src activity. Data are the mean ± S.E. of four (M<sub>1</sub> mAChRs) or six (M<sub>2</sub> mAChRs) experiments. B, HEK-293 cells stably expressing M<sub>1</sub> mAChRs were stimulated without (Con) and with 100 μM carbachol (Car) for 5 min after serum starvation overnight. Dynamin immunoprecipitates (IP) were subjected to SDS-polyacrylamide gel electrophoresis, and tyrosine phosphorylation of dynamin was detected by immunoblotting (IB) with anti-phosphotyrosyl antibody (upper panel). The filter shown in the upper panel was stripped and blotted with anti-dynamin antibody to document equivalent dynamin loading (lower panel). Similar images were obtained in three other experiments.

**FIG. 5.** Effects of expression of wild-type c-Src, K298M c-Src, wild-type dynamin and Y231F,Y597F dynamin on M<sub>1</sub> and M<sub>2</sub> mAChR internalization in HEK-293 tsA201 cells. A, detection of c-Src expression in total lysates of control transfected HEK-293 tsA201 cells (pRK5) and cells overexpressing wild-type c-Src (WT Src) or K298M c-Src (K298M Src), wild-type dynamin (WT Dyn), or Y231F,Y597F dynamin (Y231,597F Dyn) were incubated with 100 μM (M<sub>1</sub> mAChR) or 10 μM (M<sub>2</sub> mAChR) carbachol for 60 min. Internalization of receptors was determined by specific [3H]NMS binding to intact cells. Data are the mean ± S.E. of five (M<sub>1</sub> mAChRs) and six (M<sub>2</sub> mAChRs) experiments each. *, p < 0.05 compared with response of empty pRK5 vector-transfected cells (two-tailed t test).
Effect of N272 and K535M Dynamin on AT1AR Internalization in HEK-293 Cells—Since internalization of AT1ARs in HEK-293 cells has been previously reported to be insensitive to overexpression of K44A dynamin (13), we also determined whether N272 dynamin or K535M dynamin blocks AT1AR internalization in HEK-293 tsA201 cells. As shown in Fig. 7, expression of N272 dynamin and K535M dynamin inhibited AT1AR internalization by 63 and 71%, respectively. In accordance with the aforementioned study on AT1AR internalization (13), expression of K44A dynamin did not affect AT1AR internalization (Fig. 7).

**DISCUSSION**

In the past few years, the question whether dynamin plays an essential role in the internalization of a particular GPCR has been mostly analyzed by using K44A dynamin as dominant-negative mutant. While internalization of most GPCRs is blocked by expression of K44A dynamin, some GPCRs like the M2 mAChR, D2 dopamine receptors, secretin receptors, and AT1ARs internalize irrespective of K44A dynamin expression, suggesting that internalization of these GPCRs is dynamin-independent (10, 12–15). We now report that, contrary to what is currently postulated, internalization of M2 mAChR and AT1AR is dynamin-dependent. Coexpression of the dominant-negative dynamin mutants N272 and K535M strongly inhibited M2 mAChR and AT1AR internalization in HEK-293 tsA201 cells. These findings imply that N272 and K535M dynamin are more appropriate dominant-negative dynamin mutants than K44A dynamin. In this context, it will be interesting to determine whether fluid-phase endocytosis (5, 20) and internalization of ricin (21) are affected by expression of N272 or K535M dynamin also, because these trafficking processes are not blocked by K44A dynamin and are thus considered to be dynamin-independent. It is intriguing that N272 dynamin, which lacks all three GTP-binding motifs, inhibits internalization of both M1 and M2 mAChRs, while K44A dynamin, which lacks only the first GTP-binding motif, blocks only M1 mAChR internalization. It is possible that K44A dynamin selectively sequesters away an essential component of the M1 but not of the M2 mAChR internalization pathway. Another potential explanation relates to the fact that K44A dynamin is able to coassemble with wild-type dynamin (22). Since dynamin assembly and interaction of dynamin with other proteins requires the C terminus of the dynamin, which varies among the dynamin isoforms (23), different internalization pathways may use different dynamin isoforms. As a result, different internalization pathways may display differential sensitivity toward interference of K44A dynamin. Perhaps assembled GTP-bound K44A dynamin is sufficiently active to catalyze the budding of M2 mAChR- and AT1AR-containing vesicles from the plasma membrane but is not able to support internalization of M1 mAChRs in clathrin-coated vesicles.
In the present study, we observed that mAChR and AT1AR internalization in HEK-293 cells is strongly inhibited by expression of K535M dynamin, a dynamin mutant, which lacks the putative PIP2 binding site (6). At present, it is unknown at which stage of the vesicle budding process PIP2 binding to dynamin is essential. It has been postulated that, after recruitment of dynamin to the clathrin-coated pit, dynamin’s interaction with the plasma membrane is strengthened by the binding of dynamin’s pleckstrin homology domain with PIP2 in the plasma membrane (8). In addition, PIP2 binding might promote self-assembly of the dynamin molecules at the neck of the clathrin-coated pit and stimulate dynamin’s GTPase activity (8, 9). An alternative possibility is that lysine 535 in the pleckstrin homology domain of dynamin serves to promote interaction of dynamin with proteins rather than with PIP2 (6). Regardless of the mechanism, our study clearly underscores the relevance of dynamin’s lysine 535 residue in GPCR internalization. Identification of the binding partners of dynamin’s pleckstrin homology domain with PIP2 in the membrane will be important for understanding the mechanisms of GPCR internalization.

In analogy with recent studies on the regulation of internalization of β2-adrenergic receptors in HEK-293 cells (16, 24), we show that internalization of M1 mAChRs is strongly reduced by inhibition of c-Src activity and by overexpression of Y231F,597F dynamin, which cannot be phosphorylated by c-Src. Since M1 mAChRs internalize in clathrin-coated vesicles in a β-arrestin-independent manner, we propose that, in analogy to β2-adrenergic receptors, internalization of M1 mAChRs involves β-arrestin-mediated targeting of receptor in the clathrin-coated pit and activation of c-Src by β-arrestin. c-Src then phosphorylates dynamin, a process that is required for M1 mAChR internalization in HEK-293 cells. Whether tyrosine phosphorylation activates dynamin or allows activation of dynamin by other molecules remains to be determined. In contrast, dynamin-mediated internalization of M2 mAChRs was found not to be inhibited by expression of kinase-defective K298M c-Src or treatment of the cells with the specific c-Src inhibitor PP1. Thus, c-Src does not play a role in M2 mAChR internalization. These findings are supported by recent studies showing that M2 mAChR internalization in HEK-293 cells is β-arrestin-independent (11, 12). However, treatment of the cells with the generic tyrosine kinase, genistein, or coexpression of the catalytic subunit of protein kinase A resulted in partial inhibition of HEK-293 cells with 0.45 M sucrose fully blocks M2 (and AT1AR) internalization. We have observed that pretreatment of the cells with the specific c-Src inhibitor PP1.

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