Endocytosis of agonist-activated G protein-coupled receptors (GPCRs) is required for both resensitization and recycling to the cell surface as well as lysosomal degradation. Thus, this process is crucial for regulation of receptor signaling and cellular responsiveness. Although many GPCRs internalize into clathrin-coated vesicles in a dynamin-dependent manner, some receptors, including the M₄ muscarinic acetylcholine receptor (mAChR), can also exhibit dynamin-independent internalization. We have identified five amino acids, located in the sixth and seventh transmembrane domains and the third intracellular loop, that are essential for agonist-induced M₄ mAChR internalization via a dynamin-independent mechanism in JEG-3 choriocarcinoma cells. Substitution of these residues into the M₁ mAChR, which does not internalize in these cells, is sufficient for conversion to the internalization-competent M₄ mAChR phenotype, whereas removal of these residues from the M₄ mAChR blocks internalization. Cotransfection of a dominant-negative isoform of dynamin has no effect on M₄ mAChR internalization. An internalization-incompetent M₄ mutant that lacks a subset of the necessary residues can still internalize via a G protein-coupled receptor kinase-2 and β-arrestin-dependent pathway. Furthermore, internalization is independent of the signal transduction pathway that is activated. These results identify a novel motif that specifies structural requirements for subtype-specific dynamin-independent internalization of a GPCR.

Members of the superfamily of G protein-coupled receptors are exquisitely regulated by processes that attenuate signaling following activation by agonists (1). These processes can be grouped according to their time course and functional effects into three main categories: desensitization, internalization, and down-regulation. Desensitization, the most rapid of these processes, occurs within seconds to minutes of receptor activation and involves uncoupling of the activated receptor from G proteins, thereby preventing further signaling. For many GPCRs, this process occurs when agonist-bound receptors are phosphorylated by the G protein-coupled receptor kinase (GRK) family (2), which promotes the association of proteins known as β-arrestins (3). Receptor-β-arrestin complexes are then targeted to clathrin pits (4, 5), leading to their internalization or sequestration from the cell surface into an intracellular compartment. Down-regulation typically occurs following prolonged exposure to agonist (hours) and can result from a combination of receptor degradation, decreased receptor expression, and posttranscriptional mechanisms (6).

The internalization of G protein-coupled receptors plays an important role in the temporal regulation of receptor function and can be a major determinant in the regulation of signaling specificity and coupling to downstream effector molecules. Following internalization, desensitized receptors generally enter into early endosomes and can be sorted into a pathway that leads either to resensitization followed by recycling to the cell surface or to entry into lysosomes and degradation (1, 7). For example, following activation, β₂-adrenergic receptors are phosphorylated, which in turn promotes desensitization and internalization. The internalized receptors enter into the recycling pathway where the lower pH promotes agonist dissociation and dephosphorylation by a vesicular phosphatase (8). These resensitized receptors are then returned to the cell surface where they can again be activated. In contrast, internalization appears to attenuate resensitization of M₄ mAChRs (9). Furthermore, internalization of some GPCRs may allow coupling to a different complement of effector molecules, thereby contributing to the temporal and spatial regulation of intracellular signaling. This was demonstrated by the finding that internalization appears to be required for the activation of mitogen-activated protein kinases by the β₂-adrenergic receptor via a poorly understood mechanism (10, 11).

Clathrin-mediated endocytosis is dependent upon the GTPase activity of dynamin. Receptor activation leads to the association of cytosolic dynamin with coated pits, where it induces the budding of pits and the formation of clathrin-coated vesicles (12, 13). For most GPCRs that have been examined, internalization occurs by this dynamin-dependent mechanism and can be blocked by treatments that either destabilize clathrin-coated pits or block the actions of dynamin (1, 14). GTPase defective mutants of dynamin act as dominant-negative inhibitors of dynamin activity and block coated-vesicle formation and agonist-stimulated GPCR internalization (15, 16). Recently, a number of GPCRs have been identified that exhibit dynamin-independent internalization. These include the AT₁, angiotensin receptor (16), the D₃ dopamine receptor (17), the secretin receptor (18), and the M₄ mAChR (19, 20).
ingly, the M₂ mAChR can exhibit both dynamin-dependent and -independent internalization, depending upon the cellular context in which it is expressed. Internalization of the M₂ mAChR in HEK-293 cells appears to be completely dynamin-independent, whereas internalization in COS-7 cells is partially dynamin-dependent (19, 20). In addition, overexpression of arrestin-2 and -3 in HEK-293 cells enhances M₂ mAChR internalization in a manner that is blocked by coexpression of dominant-negative dynamin (19). The molecules involved in the dynamin-independent internalization pathway(s) and the structural determinants that allow GPCRs to enter into dynamin-independent pathways have not been identified.

In JEG-3 human choriocarcinoma cells, the G₁-coupled M₂ mAChRs undergo agonist-induced internalization, whereas the G₂-coupled M₁ mAChR does not (21). We previously used chimeric M₁–M₂ mAChR constructs to show that the region necessary for M₂ mAChR-specific internalization in JEG-3 cells is between Glu-382 and Leu-442, which includes six amino acids that are critical for coupling of the M₂ mAChR to this pathway. The results demonstrate that the M₂-specific internalization pathway exhibits a novel sequence requirement for these five amino acids in three topologically distinct regions of the M₂ mAChR.

**Experimental Procedures**

**Materials**—[³H]Quinuclidinyl benzilate ([³H]QNB, 47 Ci/mmol), N-[³H]methylscopolamine ([³H]NMS, 79–84 Ci/mmol), and [³H]inositol (16 Ci/mmol) were purchased from Amersham Pharmacia Biotech. Dulbecco’s modified Eagle’s medium, penicillin-streptomycin, and fetal bovine serum were purchased from Life Technologies, Inc. Restriction enzymes were from New England Biolabs, Inc. Forskolin was obtained from Calbiochem, and n-luciferin-potassium salt was from Analytical Luminescence Laboratory (Ann Arbor, MI). Carbamylcholine chloride (carbachol), atropine, and all other reagents were purchased from Sigma.

**DNA Constructs**—The M₁ and M₂ mAChR expression vectors and generation of the M₂/M₁(6) receptor have been described previously (21). The M₂/M₁(6) is a M₂ mAChR chimera in which TM6 has been converted to the M₁ mAChR sequence. Using mutagenic primers and sequential PCR with Ffu polymerase (Stratagene), M₁(VTILa) was created from the wild-type M₁ mAChR and then used to create M₁(VTILa) as described (21). M₁(Ma) was generated from M₁(VTILa) by subcloning a 328-base pair XcmI/BstEI fragment containing the M1416A mutation back into the wild-type M₁ mAChR. Wild-type dynamin-1 and dominant-negative dynamin-K44E were generously provided by Dr. R. B. Vale (University of California, San Francisco) and DRK2 and β-arrestin-1 were provided by Dr. R. J. Lefkowitz (Duke University). The M₂(VTILa–AALS) receptor (22, 23) was obtained from Dr. J. Wess (National Institutes of Health, Bethesda, MD). All other constructs have been described elsewhere (24).

**Cell Culture and Transfection**—JEG-3 and COS-7 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified 10% CO₂ environment. Unless stated otherwise, transiently transfected cells were subcultured 20–24 h after transfection and analyzed 44–48 h after transfection. Transfection on 24-well plates and subsequent assays of luciferase and β-galactosidase activity were performed essentially as described, except that the amount of receptor cDNA was increased to 50 ng/well for determination of PI hydrolysis, whereas for determination of PI hydrolysis, COS-7 cells on 100-mm plates were transfected with 5 μg of receptor DNA, whereas for determination of PI hydrolysis, COS-7 cells on 100-mm plates were transfected with 5 μg receptor DNA.

**Receptor Internalization Assays**—Determination of mAChR internalization in transiently transfected JEG-3 cells was accomplished using the binding of the membrane-impermeable muscarinic ligand [³H]NMS to intact cells using a previously published method (24). Briefly, 20–24 h after transfection, JEG-3 cells from one 150-mm culture dish were subcultured onto three 6-well plates and allowed to attach for an additional 24 h. The cells were treated with various concentrations of carbachol for 0–60 min as described in the figure legends (3 wells/condition), washed three times with 3 ml of ice-cold phosphate-buffered saline (PBS: 4.3 mM NaH₂PO₄, 1.4 mM KH₂PO₄, 137 mM NaCl, 5.0 mM KCl, 5.6 mM dextrose, pH 7.4), and labeled with approximately 1 nM [³H]NMS in PBS for 4–5 h at 4 °C. Nonspecific binding was determined in the presence of 1 μM atropine. Labeled cells were washed three times with 3 ml of ice-cold PBS, solubilized in 0.5 ml 1% Triton X-100, and combined with scintillation mixture before the determination of radioactivity by scintillation counting. Data are expressed as the percent of total receptors not accessible to the membrane-impermeable ligand.

**Receptor Down-regulation Assays**—Determination of mAChR down-regulation in transiently transfected JEG-3 cells was accomplished using the binding of the membrane-impermeable muscarinic ligand [³H]QNB to intact cells similar to that described (26). Briefly, 20–24 h after transfection, JEG-3 cells from one 150-mm culture dish were subcultured onto three 6-well plates and allowed to attach for an additional 24 h. The cells were treated with various concentrations of carbachol for 0–4 h, as described in the figure legends (3 wells/condition), washed 3 times with QNB assay buffer (116 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.81 mM MgSO₄, 1.0 mM NaH₂PO₄, 25 mM glucose, 25 mM HEPS, pH 7.4), incubated for 15 min at 37 °C, and then labeled with approximately 0.6 nM [³H]QNB for 90 min at 37 °C. Nonspecific binding was determined in the presence of 1 μM atropine. Cells were washed with cold PBS, washed with ice-cold PBS, and then filtered using N/F/C filters (Whatman). The filters were washed four times with ice-cold PBS, transferred to scintillation vials, and combined with scintillation mixture before determination of radioactivity by scintillation counting. Data are expressed as the percent of total receptors down-regulated.

**cAMP Regulation Assays**—Changes in cAMP levels were measured using a luciferase reporter gene under the transcriptional control of a cAMP-regulated promoter (CRE-luciferase) (24, 27, 28). The Rous sarcoma virus β-galactosidase gene was included to correct for minor variations in transfection efficiency. All drug treatments occurred 44–48 h after transfection. Cells cotransfected with the Ga₃-2 expression construct were stimulated with 0.4 μM forskolin to allow the measurement of inhibitory coupling following receptor activation by the concentrations of carbachol indicated in the figure legend. To measure stimulation of cAMP production by mAChRs, cells were transfected as described, except that the amount of receptor cDNA was increased to 100 ng/well and Ga₃-2 was not included. Cells for these experiments were treated with 1 mM carbachol without forskolin. In all cases, drug treatments were performed for 5 h at 37 °C and terminated by washing the cells with ice-cold PBS. For some experiments, cells were treated with 300 nM pertussis toxin (PTX) for 24 h before drug treatment. After drug treatment, cells were lysed and assayed for luciferase and β-galactosidase activities as described previously (27). Data were normalized for transfection efficiency and corrected for the background activity seen in untransfected cells.

**Determination of PI Hydrolysis**—COS-7 cells grown on 100-mm dishes were transiently transfected with 5 μg of receptor DNA. COS-7 cells were used because they have a lower endogenous PI response to carbachol treatment than JEG-3 cells.² Approximately 24 h after transfection, cells were subcultured onto two 6-well tissue culture dishes and allowed to attach for 4 h. One plate was incubated for an additional 16–20 h and

² M. L. Schlador and N. M. Nathanson, unpublished observations.
then subjected to whole cell binding assays using \(^{3}H\)NMS as described above. To the remaining plate, the growth medium was supplemented with 1 \(\mu\)Ci/ml myo-\(^{3}H\)inositol, and the cells incubated for an additional 16–20 h. The cells on this latter plate were washed three times with physiological saline solution (118 mM NaCl, 4.7 mM KCl, 3 mM CaCl\(_2\), 1.3 mM MgSO\(_4\), 1.2 mM KH\(_2\)PO\(_4\), 10 mM glucose, 0.5 mM EDTA, 20 mM HEPES, pH 7.4) containing 10 mM LiCl and incubated for 30 min at 37°C, and then triplicate wells were stimulated without (control) or with 1 mM carbachol for 20 min at 37°C. Total inositol phosphates were isolated using ion-exchange chromatography (Bio-Rad AG 1-X8, 100–200 mesh, formate form) as described (29). Data are expressed as fold-induction above untreated controls after correcting for receptor expression levels.

**RESULTS**

**Identification of Multiple Epitopes Necessary for M\(_{2}\)-specific Internalization**—There is a high degree of sequence identity between the M\(_{1}\) and M\(_{2}\) mAChRs in the membrane-proximal regions of the I3 loop, TM6 and TM7 (Fig. 1B). Our previous studies demonstrated that sequences from the C-terminal region of I3 as well as from TM6 and TM7 were necessary for M\(_{2}\)-specific internalization in JEG-3 cells (21). Because sequences at the membrane-cytoplasmic interface seemed likely to be responsible for these differences in internalization, we tested the role of these residues in M\(_{2}\)-specific internalization. Molecular modeling suggests that the \(a\)-helix of TM6 extends into I3, and four of the unique M\(_{2}\) mAChR residues in this region, termed the VTIL sequence (Val-385, Thr-386, Ile-389, and Leu-390) at the I3/TM6 interface, are pre-dashes denote amino acid identity with M\(_{1}\). The M\(_{1}\)(VTIL), M\(_{1}\)(VTILA), and M\(_{1}\)(A) receptors were generated from the M\(_{1}\) mAChR, whereas the M\(_{2}\)/M\(_{1}\)(6) receptor was generated from the M\(_{2}\) mAChR.

Identification of Multiple Domains Mediate M\(_{2}\) mAChR Internalization—We sought to determine whether the M\(_{2}\)-specific pathway in JEG-3 cells utilizes dynamin. Treatment with 0.1 mM carbachol for 60 min typically leads to internalization of up to 50% of cell surface receptors. Cotransfection of either wild type (Dyn-1) or dominant-negative (Dyn-K44E) dynamins (expression confirmed by immunoblot analysis) had negligible effects on M\(_{2}\) internalization and our previous studies did not test the potential involvement of TM7, we tested if residues in TM7 opposed to the VTIL sequence in TM6 were involved. There are three differences between the M\(_{1}\) and M\(_{2}\) mAChRs in this region: two valine/isoleucine substitutions near the middle of TM7 and a methionine/alanine substitution nearer to the cytoplasmic end of TM7 (Met-416 in M\(_{1}\) versus Ala-438 in M\(_{2}\)) (Fig. 1B). The proximity of Ala-438 to the cytoplasmic-TM7 interface led us to test its effects on internalization by making Met-416 \(\rightarrow\) Ala substitutions in the wild type M\(_{1}\) and M\(_{1}\)(VTILA) receptors to create the M\(_{1}\)(A) and M\(_{1}\)(VTILA) receptors, respectively.

The Met-416 \(\rightarrow\) Ala mutation alone had only slight effects on the internalization of the M\(_{1}\) mAChR (Fig. 2, A and B), leading to an approximately 10-fold increase in the initial rate of internalization in the presence of carbachol (Fig. 2B, inset, and Table 1). In stark contrast, this mutation in combination with the AALS \(\rightarrow\) VTIL substitution created a receptor whose internalization profile closely resembled that of the M\(_{2}\) mAChR. Both the dose dependence and time course of internalization of the M\(_{1}\)(VTILA) receptor were enhanced (Fig. 2, A and B) compared with the M\(_{1}\)(A) and M\(_{1}\)(VTIL) receptors. Although the maximal extent of internalization for the M\(_{1}\)(VTILA) receptor was slightly less than the M\(_{2}\) mAChR (Fig. 2B), the initial rate of internalization for the two receptors was almost identical (66-fold increase for M\(_{1}\)(VTILA) versus 75-fold increase for M\(_{2}\)) (Fig. 2B, inset, and Table 1). Together, these data suggest that multiple structural elements must come together within the M\(_{2}\) mAChR in order for it to become internalization-competent in JEG-3 cells. These structural elements appear to be provided by Val-385, Thr-386, Ile-389, and Leu-390 at the I3/TM6 interface in combination with Ala-438 in TM7.

**Dynamin Independence of M\(_{2}\) mAChR Internalization in JEG-3 Cells**—Stimulation of transiently transfected JEG-3 cells with muscarinic agonist leads to the rapid internalization of M\(_{1}\) but not M\(_{2}\) mAChRs (21). Many GPCRs internalize via clathrin-coated vesicles (34). Phosphorylation of activated receptors by GRKs leads to the binding of \(\beta\)-arrestins, which act as clathrin adapters. Budding of clathrin-coated pits is regulated by dynamin and can be inhibited by expression of a dominant-negative dynamin mutant (e.g. Dyn-K44E) (14). Because the M\(_{2}\) mAChR can internalize via dynamin-dependent and -independent internalization pathways depending on the nature of the molecules that are coexpressed (19, 20), we sought to determine whether the M\(_{2}\)-specific pathway in JEG-3 cells utilizes dynamin.

To answer this question, JEG-3 cells were cotransfected with M\(_{2}\) mAChR cDNA and either wild type or dominant-negative isoforms of dynamin-1. Treatment with 0.1 mM carbachol for 60 min typically leads to internalization of up to 50% of cell surface receptors. Cotransfection of either wild type (Dyn-1) or dominant-negative (Dyn-K44E) dynamins (expression confirmed by immunoblot analysis) had negligible effects on M\(_{2}\).
mACHR internalization (Fig. 3A), indicating that this process is dynamin-independent. In addition, internalization of the M2 mACHR by the M2-specific pathway appears to be independent of GRKs and arrestins. Because the GRK phosphorylation sites in the M2 mACHR are in the middle of I3 (35) and not necessary for internalization (36), it is unlikely that GRK phosphorylation is involved with the M2-specific internalization pathway in JEG-3 cells. Substitution of TM6 from the M1 mAChR into the M2 mAChR (M2/M1(6)) ablates agonist-induced internalization (36), it is unlikely that GRK phosphorylation sites are in the middle of I3 (35) and not necessary for internalization by an alternative mechanism, JEG-3 cells were cotransfected with constructs encoding the M1 (solid squares), M2 (solid circles), M1(VTILA) (open circles), and M1(A) (open diamonds) receptors and then treated for 30 min with the concentration of carbachol (CCh) indicated (A) or with 0.1 mM carbachol for the times indicated (B). The inset shows the data for disappearance from the cell surface fit to a first-order exponential decay curve. Cell-surface receptors were measured using the membrane-impermeable muscarinic antagonist [3H]NMS as described under “Experimental Procedures.” Data are expressed as the percent of receptors internalized and represent the means ± S.E. of three to six independent experiments, each performed in triplicate.

### Table 1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Initial rate</th>
<th>Fold above M1</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>-0.34</td>
<td>1</td>
</tr>
<tr>
<td>M2</td>
<td>-2.12</td>
<td>75</td>
</tr>
<tr>
<td>M1(VTILA)</td>
<td>-0.61</td>
<td>21</td>
</tr>
<tr>
<td>M1(A)</td>
<td>-0.34</td>
<td>12</td>
</tr>
<tr>
<td>M1(VTILA)</td>
<td>-1.88</td>
<td>66</td>
</tr>
</tbody>
</table>

Whether the internalization-competent mutant M1 receptors were sorting into a similar endocytic pathway, the extent of receptor down-regulation after prolonged agonist treatment was determined. All of the wild type and mutant mACHR constructs underwent a time-dependent decrease in total receptor number following exposure to carbachol. Down-regulation of the M2 mACHR was approximately 2-fold greater than the M1 mAChR following both 1- and 4-h agonist treatments (Fig. 4). The extent of M1(VTILA) receptor down-regulation was very similar to that of the M2 mACHR, whereas both the M1(A) and M1(VTILA) receptors were between that of the M1 and M2 mAChRs (Fig. 4; M1 was significantly different from both M2 and M1(VTILA) at both times; M1(VTILA) was significantly different from M2 at 1 h and both M1 and M2 at 4 h). This pattern is similar to the differences seen for agonist-induced internalization (Fig. 2B) and suggests that the M1(VTILA) and M2 receptors sort into the same endocytic pathway.

**Functional Analysis of Wild Type and Mutant mACHRs**—The VTILA sequence has been implicated in coupling of the M2 mAChR to the Gq family of G proteins (22, 23), whereas the corresponding residues in the M2 mAChR (Ala-489, Ala-489, Leu-492, and Ser-493; AALS) have been implicated in coupling to the Gi family of G proteins (37). Like the M2 mAChR, the Gq-coupled M1 mAChR also contains the AALS sequence in the corresponding location. Alanine 438 is located within the highly conserved NPXYXY motif, which is involved in agonist binding and GPCR signaling (38, 39). For these reasons, we tested if the differences in the internalization of the wild type and mutant M1 and M2 mAChRs were related to differences in their G protein coupling. Because the M1 mAChR is a prototypical Gq-coupled receptor, we first examined the ability of the mutant M1 receptors to activate phospholipase C (PLC). In cells transiently transfected with the M1 mAChR, maximal stimulation with carbachol led to an approximately 5-fold stimulation of PLC activity, whereas in cells transfected with the M2 mAChR, maximal stimulation of PLC activity was approximately 2-fold above unstimulated controls. Treatment of cells transfected with any of the mutant receptors (e.g. M1(VTILA), M1(VTILA), and M1(A)) led to maximal stimulation of PLC activity that was very similar to that seen with the wild type M1 mAChR (Fig. 5). These data indicate that in the M1 mAChR, the AALS sequence is not required for coupling to PLC, a predominantly Gq-coupled response. Furthermore, they
suggest that each of the mutant receptors is fully functional and capable of stimulating a functional response that is essentially equivalent to the wild type M1 mAChR.

To measure coupling of the mutant receptors to Gi proteins, changes in intracellular cAMP were determined using a luciferase reporter gene under the control of a cAMP-regulated promoter (CRE-luciferase). We have previously used this method to measure coupling of the M2 and M1 mAChRs to Gi proteins (when cotransfected with Goi-2) as well as ectopic coupling of all mAChR subtypes to stimulation of adenyl cyclase (AC) via Gi2 (27). As demonstrated previously, the M1 mAChR coupled to transfected Goi-2, inhibiting forskolin-stimulated AC activity and CRE-luciferase expression in a dose-dependent fashion (Fig. 6). Consistent with previous work from our laboratory (27), the M1 mAChR did not couple to Gi proteins and stimulated AC directly in a dose-dependent fashion, leading to increased CRE-luciferase expression in cells even when Goi-2 is overexpressed (Fig. 6). Under these same conditions, the M1(A) receptor stimulated AC to levels similar to that of the M1 mAChR. Unexpectedly, the mutant M1 receptors that contain the VTIL sequence (e.g. M1(VTIL) and M1(VTILA)) did not appear to regulate AC activity (Fig. 6), which suggests that the VTIL sequence is not sufficient to confer Gi coupling but might be sufficient to interfere with ectopic Gi coupling. We also found that conversion of the VTIL sequence in the M2 mAChR to the corresponding amino acids in the M1 mAChR (e.g. M2(VTIL→AALS)) resulted in a complete loss of coupling to inhibition of CRE-luciferase expression. These data are consistent with the work of others (22, 23), which demonstrated that the VTIL sequence is necessary for M2 mAChR coupling to the Goi-2 protein family but differ in that the VTIL sequence does not affect AC activity. 3 There are two obvious explanations for the apparent inability of the M2(VTIL) and M1(VTILA) receptors to regulate AC activity: 1) these receptors can couple to neither Gi nor Go proteins or 2) these receptors can couple to both Gi and Go proteins so that their effects on AC activity counteract each other. To examine these hypotheses, we first tested the mutant M1 receptors for the ability to stimulate AC activity in cells lacking transfectect Goi subunits. In the absence of transfectect Goi, treatment of JEG-3 cells expressing the M2 mAChR with
carbachol in the absence of forskolin stimulates AC activity but to a lower extent than in cells expressing the M1 mAChR (27), (Fig. 7, A and B). In general, all of the receptors that contain the VTIL sequence (M2, M1(VTIL), and M1(VTILA)) were able to stimulate AC activity but to a lesser extent than receptors that contained the AALS sequence (M1 and M1(A)) (Fig. 7, A and B). Next, we tested if any of the wild type or mutant receptors might be coupling to both stimulatory and inhibitory G proteins by treating transfected cells with PTX to inactivate G alpha proteins and then stimulating with carbachol in the absence of forskolin. Inactivation of Goalpha subunits with PTX led to increased stimulation of AC by carbachol in cells expressing the M1(VTIL) and M1(VTILA) receptors that was approximately 2-fold greater than that seen in cells stimulated with carbachol alone (Fig. 7B). Stimulation of AC activity by the M1 and M1(A) receptors was unaffected by PTX treatment, whereas PTX treatment alone had only minor effects on AC activity and CRE-luciferase expression. Increased stimulation of AC activity following PTX treatment is due to decreased inhibitory coupling and suggests that these receptors can couple to endogenous G alpha proteins. Together, these data indicate that the M1(VTIL) and M1(VTILA) receptors can couple to both stimulation and inhibition of AC activity and that the VTIL sequence is necessary but not sufficient for efficient coupling to G alpha. Most importantly, the similar functional properties of the internalization-incompetent M1(VTIL) receptor and the internalization-competent M1(VTILA) receptor indicate that internalization by the M2-specific pathway is independent of the signaling specificity of the receptor.

Molecular Model of the VTILA Motif—To better understand the role that the VTILA motif plays in G protein coupling and internalization, we generated three-dimensional models of the M2 mAChR helical bundle (Fig. 8) based on a published model of rhodopsin (30). In rhodopsin, a methionine (corresponding to Ile-392 in the M2 mAChR) near the region analogous to the VTIL sequence is situated between TM6 and TM7 and tightly packed with side chains of the NPXXY motif (40). Because Ala-438 is within the NPXXY motif, this would position the VTIL sequence very near the region that contains Ala-438 in the M2 mAChR. Analysis of the molecular model for the region containing TM6 and TM7 of the M2 mAChR demonstrates that the VTIL sequence is oriented toward the center of the helical bundle, and Ala-438 is oriented outward (Fig. 8). This suggests that Ala-438 does not interact with side chains of the VTIL sequence but may interact with other molecules to facilitate endocytosis.

**DISCUSSION**

Stimulus-dependent internalization performs a critical role in the regulation of GPCR function. Entry of receptors into early endosomes allows them to be sorted into pathways leading either to resensitization or down-regulation (1, 7). Regulation of these intracellular sorting pathways provides an additional mechanism to modulate receptor function and can ultimately determine the signaling strength of a receptor system. For some GPCRs, internalization also allows coupling to additional second messenger pathways (10, 11). For these receptors, desensitization followed by internalization does not block signaling entirely but instead alters the receptor-effector coupling profile and leads to the activation of a different subset of signals, thereby providing for precise temporal regulation of multiple signaling pathways by a single receptor system.

Each member of the mAChR family can undergo agonist-induced internalization if expressed in the appropriate cell type. For example, the M1 mAChR does not rapidly internalize when expressed in JEG-3 cells (21) but readily internalizes following agonist activation when expressed in Y1 cells (41) or

**FIG. 7.** Stimulation of AC activity in JEG-3 cells transiently transfected with wild type and mutant receptor constructs in the absence of added Goalpha. JEG-3 cells were transiently transfected with constructs encoding the M1, M2, M1(VTIL), and M1(VTILA) receptors, or with empty vector as indicated (100 ng/well) in the absence of added Goalpha-2 and then treated with 1 mM carbachol (CCh) without (A) or with (B) pretreatment with pertussis toxin (PTX) as described under “Experimental Procedures.” Data are expressed as the fold stimulation (A) of luciferase expression measured in the absence of carbachol or the corrected level of luciferase expression (B) and represent the means ± S.E. of three to six independent experiments, each performed in triplicate.
Currently, very little is known about the mechanisms by which structurally similar receptors are differentially sorted. Recently, Vickery and von Zastrow (17) demonstrated that the D₁ and D₂ dopamine receptors are selectively internalized into dynamin-dependent and -independent pathways when expressed in HEK-293 cells. Following internalization, both the D₁ and D₂ receptors were rapidly recycled back to the cell surface. In a similar fashion, the M₁ and M₂ mAChRs are selectively internalized into dynamin-dependent and -independent pathways in HEK-293 cells (19, 20). In contrast to the D₁ and D₂ receptors, the M₂ mAChR returns very rapidly to the cell surface following internalization in HEK-293 cells but the M₂ mAChR does not (20). We have found that when expressed in JEG-3 cells, internalized M₂ mAChRs are also very slow to return to the cell surface. In the current study, we show that long term (4 h) treatment with agonist leads to moderately higher levels of down-regulation for the M₂ mAChR than for the M₁ mAChR (Fig. 4). Substitution of the VTIL sequence into the M₁ mAChR led to significantly higher levels of down-regulation (Fig. 4), which suggests that the M₁(VTIL) receptor is sorting into the same endocytic pathway as the M₂ mAChR. Given that recent work has demonstrated that dynamin-dependent endocytosis can also lead to down-regulation of GPCRs (43), it now seems likely that selective internalization into a particular pathway (dynamin-dependent versus -independent) does not commit receptors to a particular fate (resensitization versus down-regulation) and that intracellular sorting of internalized receptors must also be regulated.

The mechanism by which the VTIL motif mediates subtype-specific internalization of the M₂ mAChR remains to be elucidated. Because the VTIL sequence has been previously implicated in M₂ mAChR coupling to G proteins (22), whereas the corresponding AALS sequence was reported to be necessary for M₁ mAChR coupling to G proteins (37), we tested if the G protein coupling patterns of the mutant M₁ receptors correlated with their internalization phenotypes. We found that replacement of the AALS sequence of the M₁ mAChR with the VTIL sequence conferred partial coupling to inhibition of AC activity (Figs. 6 and 7) but did not appear to interfere with PLC coupling (Fig. 5). This contradicts previous studies that identified the VTIL sequence as being necessary and sufficient for coupling to G proteins (22). These authors examined the effects of AALS to VTIL substitutions in the M₃ mAChR, and it is possible that subtle differences in the M₁ and M₂ mAChRs are responsible for the differences in coupling specificity. Furthermore, the AALS sequence conferred only partial G₁α-coupling to the M₂ mAChR (37), whereas the effect of the loss of this sequence was not examined. Most importantly, our results demonstrate that the G protein coupling patterns of the wild type and mutant M₁ and M₂ mAChRs expressed in JEG-3 cells do not correlate with the internalization phenotype. Together, these observations suggest that coupling of the M₂ mAChR to the VTIL-dependent internalization machinery is independent of G protein-coupling and downstream signaling events.

To better understand how the VTIL motif might be involved in M₂ mAChR internalization, we generated a three-dimensional model of the M₂ mAChR based on a published model of rhodopsin (30). Based on our internalization data, we hypothesized that Ala-438 in TM7 likely interacts with side chains of the VTIL sequence. Examination of the model, however, shows that this is highly unlikely (Fig. 8). The VTIL residues are found on one side of the helix on the interior of the helical bundle, whereas Ala-438 lies on the outside of TM7. Given the putative interactions among the side chains of TM6 and TM7 (see “Results”), it is likely that changes in secondary structure within this region following agonist binding occur in
a coordinated fashion. Interestingly, immunological experiments demonstrate that the NPXXY motif in rhodopsin becomes exposed to the cytosol following activation (44), indicating that this region would be ideally suited to interact with activation-dependent endocytic machinery.

What the VTIIL motif might be interacting with is not known. Furthermore, little is known about dynamin-independent endocytosis and requires additional information present in TM7 of the M2 mAChR at Ala-438. Internalization of the M2 mAChR is a complex process that can be mediated by both dynamin-independent and -dependent pathways. Identification of the structural requirements for entry into a dynamin-independent endocytic pathway will allow further analysis of this mechanism and the role it plays in the intracellular sorting of GPCRs.

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