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Short communication

# Distinct mechanisms for the endocytosis of muscarinic receptors and $G_{q/11}$

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**Abstract**

To determine whether the agonist-mediated endocytosis of muscarinic receptors and $G_{q/11}$ are mechanistically related events, the internalization of $G_{\alpha q/11}$ was monitored under conditions established to prevent muscarinic receptor endocytosis. Incubation of SH-SY5Y neuroblastoma cells with oxotremorine-M resulted in the translocation of both muscarinic receptors and $G_{q/11}$ into a 'light vesicle' membrane fraction. Although muscarinic receptor translocation was prevented by either the depletion of phosphoinositides or by disruption of clathrin assembly, the endocytosis of $G_{q/11}$ was unaffected. These results demonstrate that the agonist-induced internalization of muscarinic receptors and $G_{q/11}$ proceed via distinct mechanisms. 

*Keywords:* G-protein coupled receptor; Wortmannin; Phosphoinositide; Clathrin

## 1. Introduction

Prolonged agonist occupancy of G-protein coupled receptors results in a number of adaptive responses, one of which is the translocation of the receptor from the cell surface into a more lipophilic cell compartment. This process, termed endocytosis, can be monitored as the appearance of the receptor in a 'light vesicle' membrane fraction following centrifugation of hypotonic cell lysates. Receptor endocytosis may serve to both limit the duration of receptor signaling and permit dephosphorylation of the receptor (Bohm et al., 1997). In addition to the G-protein coupled receptor, its associated heterotrimeric G-protein can also undergo endocytosis in response to agonist addition, as demonstrated for $G_{q/11}$ following the activation of muscarinic receptors present in brain, chinese hamster ovary cells and SH-SY5Y neuroblastoma cells (Ho et al., 1991; Svoboda and Milligan, 1994; Sorensen et al., 1997). The activation of the thyrotropin releasing hormone receptor has also been reported to induce the endocytosis of $G_{11}$ (Drmota et al., 1998).

Studies of the mechanisms underlying the endocytosis of G-protein coupled receptors have indicated that several of these receptors, including the $M_3$ muscarinic receptor, appear to be internalized via a clathrin-coated pit mechanism (Slowiejko et al., 1996). In addition, we have recently demonstrated that ongoing phosphoinositide synthesis is a prerequisite for the endocytosis of muscarinic receptors in SH-SY5Y neuroblastoma cells (Sorensen et al., 1998). However, whether the internalization of $G_{q/11}$ occurs via the same or different mechanism(s) remains unknown. To address this question, in the present study we have monitored the internalization of $G_{q/11}$ in SH-SY5Y cells using two experimental paradigms that have been established to block muscarinic receptor endocytosis: (i) pretreatment of the cells with wortmannin, an agent which inhibits phosphoinositide synthesis, or (ii), by rendering the cells hypertonic, a condition under which clathrin assembly is disrupted (Slowiejko et al., 1996; Sorensen et al., 1998). Unexpectedly, the results demonstrate that, unlike the agonist-induced internalization of the muscarinic receptor, the internalization of $G_{q/11}$ remains robust under both of these experimental conditions and suggest that the endocytosis of the G-protein occurs independently of the receptor.

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## 2. Materials and methods

### 2.1. Materials

Detection agents for enhanced chemiluminescence were purchased from Amersham (Arlington Heights, IL, USA). [$^3$H]Quinuclidinyl benzilate (45.4 Ci/mmol) was obtained from New England Nuclear (Boston, MA, USA). Atropine and wortmannin were obtained from Sigma (St. Louis, MO, USA). 2-Butyn-1-ammonium, *N*,*N*,*N*-trimethyl-4-(2-oxo-1-pyrrolidinyl) iodide (oxotremorine-M) was purchased from Research Biochemicals International (Natick, MA, USA). Polyclonal antibodies to the α subunits of $G_{q/11}$ and $G_s$, and peroxidase-conjugated goat anti-rabbit immunoglobulin G were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Tissue culture supplies were purchased from Corning Glass Works (Corning, NY, USA) and Sarstedt (Newton, NC, USA). Powdered Dulbecco's modified Eagle medium was obtained from Gibco (Grand Island, NY, USA). Fetal calf serum was obtained from Summit Biotechnology (Fort Summit, CO, USA). Human SH-SY5Y neuroblastoma cells were obtained form Dr. June Biedler (Sloan-Kettering Institute, New York, NY, USA).

### 2.2. Subcellular fractionation

SH-SY5Y cells were grown under conditions previously described (Sorensen et al., 1998). Cells were detached and then resuspended in 5.0 ml of buffer A (142 mM NaCl, 5.6 mM KCl, 2.2 mM $CaCl_2$, 3.6 mM $NaHCO_3$, 1 mM $MgCl_2$, 5.6 mM D-glucose, and 30 mM HEPES, pH 7.4). In some experiments, buffer A was made hypertonic (> 600 mosM) by the addition of sucrose. Cells were pretreated as indicated prior to the addition of either oxotremorine-M or vehicle for 30 min at 37°C. A light membrane fraction ($V_1$) was isolated from cell homogenates as previously described (Slowiejko et al., 1996).

### 2.3. Radioligand binding

After subcellular fractionation, $V_1$ fractions (100–200 μg of protein) were incubated in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) with 1 nM [$^3$H]quinuclidinyl benzilate at 37°C for 90 min as previously described (Sorensen et al., 1998).

### 2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis / immunoblot analysis of $G_{\alpha q/11}$

Aliquots (25 μg) of $V_1$ fractions were electrophoresed through 10% sodium dodecyl sulfate polyacrylamide gels, proteins transferred to polyvinylidene fluoride membranes and quantitative immunoblot analysis performed for $G_{\alpha q/11}$ as previously described (Sorensen et al., 1997).

### 2.5. Data analysis

Data are expressed as means ± S.E.M. for the number of separate experiments performed. Student's two-tailed *t*-tests were used to evaluate the statistical differences between the mean values of paired sets of data.

## 3. Results

### 3.1. Wortmannin prevents the endocytosis of muscarinic receptors, but not of $G_{q/11}$

In this study, the agonist-induced endocytosis of muscarinic receptors or $G_{q/11}$ was monitored as the appearance

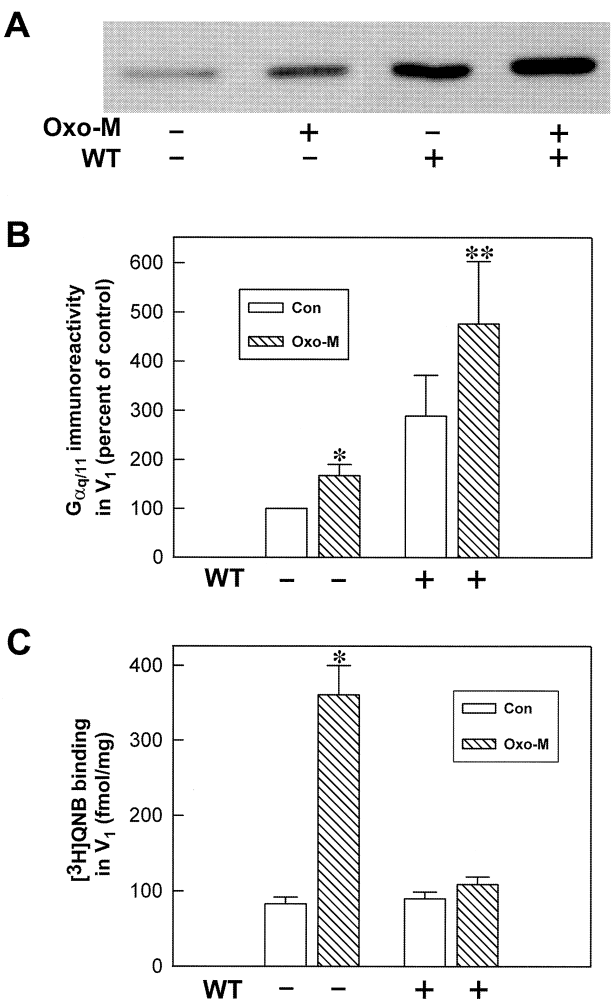


Fig. 1. Wortmannin blocks the endocytosis of muscarinic receptors, but not of $G_{q/11}$ to a 'light' membrane ($V_1$) fraction. SH-SY5Y cells were pretreated with 10 μM wortmannin (WT) or 0.1% DMSO for 15 min and incubated in the presence or absence of 1 mM oxotremorine-M (Oxo-M) for an additional 30 min at 37°C. Reactions were terminated with ice-cold buffer and hypotonic cell lysates were subjected to differential centrifugation as described in the text. (A) Equivalent aliquots (25 μg of protein) of $V_1$ fractions were immunoblotted for $G_{\alpha q/11}$. Blot shown is representative of 17 (−WT) or 8 (+WT) separate experiments. (B) Quantitative densitometry of $G_{\alpha q/11}$. Results shown are means ± S.E.M. for 8–17 separate experiments. (C) Muscarinic receptors present in the $V_1$ fraction were monitored by means of [$^3$H]quinuclidinyl benzilate binding. Values are expressed as the specific binding of [$^3$H]quinuclidinyl benzilate. Results shown are means ± S.E.M. for 8–10 experiments. * Different from control, $P < 0.001$, ** Different from wortmannin control, $P < 0.01$.

of either [$^3$H]quinuclidinyl benzilate binding sites or the α subunit of $G_{q/11}$ in a 'light' membrane fraction ($V_1$) obtained by differential centrifugation of hypotonic cell lysates (Sorensen et al., 1997). We have previously demonstrated that $V_1$ fractions obtained from quiescent SH-SY5Y cells possess $< 5\%$ of the total cellular complement of muscarinic receptors, $G_{q/11}$ immunoreactivity or [$^3$H]ouabain binding sites (a plasma membrane marker). Exposure of the cells to 1 mM oxotremorine-M for 30 min resulted in increases in both muscarinic receptor density ($444 \pm 84\%$ of control) and $G_{q/11}$ immunoreactivity ($167 \pm 23\%$ of control) in the $V_1$ fraction (Fig. 1B,C), which could be blocked by inclusion of 10 μM atropine (data not shown). No increase in $G_s$ immunoreactivity in $V_1$ is observed following muscarinic agonist indicating that the activation of the muscarinic receptors results in a selective translocation of $G_{q/11}$ (Sorensen et al., 1997). As observed previously (Sorensen et al., 1998), pretreatment of the cells with 10 μM wortmannin, an agent that inhibits phosphoinositide synthesis, essentially abolished the agonist-induced translocation of muscarinic receptors (Fig. 1C). Although pretreatment of the cells with wortmannin increased the immunoreactivity associated with $G_{q/11}$ in the $V_1$ fraction obtained from quiescent cells, this effect was non-specific since $G_s$ immunoreactivity was similarly increased (data not shown). Nonetheless, when wortmannin-pretreated cells were incubated in the presence of oxotremorine-M, an agonist-induced translocation of $G_{q/11}$ was still readily observed ($197 \pm 27\%$ of wortmannin control, Fig. 2B).

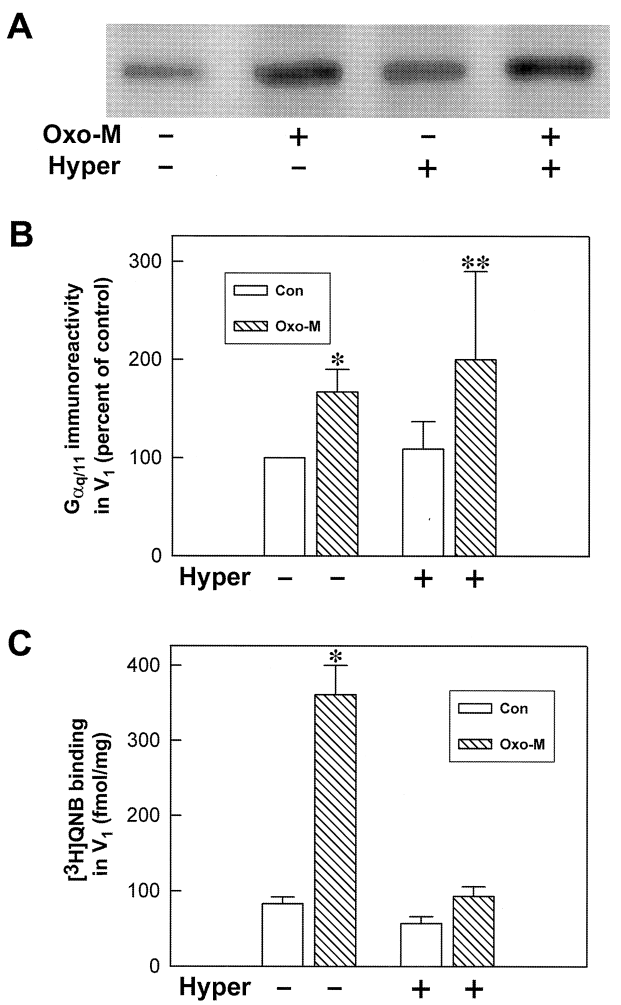


Fig. 2. Hypertonicity prevents the endocytosis of muscarinic receptors, but not the internalization of $G_{q/11}$. SH-SY5Y cells were incubated in isotonic (325 mosM) or hypertonic (Hyper, 600 mosM) buffer A for 15 min prior to incubation with 1 mM oxotremorine-M for 30 min at 37°C. $V_1$ fractions were isolated and assayed as described in the legend to Fig. 1. (A) Western blot analysis of $G_{\alpha q/11}$ shown is representative of 17 (−Hyper) or 4 (+Hyper) separate experiments. (B) Quantitative densitometric analysis of $G_{\alpha q/11}$. Results shown are means ± S.E.M. for 4–17 separate experiments. (C) [$^3$H]Quinuclidinyl benzilate binding data (means ± S.E.M. for 4–10 experiments). * Different from control, $P < 0.001$, ** Different from Hyper control, $P < 0.05$.

### *3.2. Differential effects of hypertonicity on the endocytosis of $G_{q/11}$ and muscarinic receptors*

When cells are made hypertonic (600 mosM) by the addition of sucrose, clathrin-coated pit formation is blocked (Heuser and Anderson, 1989). Hypertonicity had no effect on $G_{q/11}$ immunoreactivity associated with the $V_1$ fraction obtained from quiescent cells and did not prevent the oxotremorine-M-induced endocytosis of $G_{q/11}$ ($195 \pm 20\%$ of control, $n = 5$, Fig. 2A,B). In contrast, hypertonicity inhibited the endocytosis of muscarinic receptors by $>$ 90% (Fig. 2C), as previously reported (Slowiejko et al., 1996).

## 4. Discussion

Although the mechanism(s) underlying the endocytosis of G-protein coupled receptors has frequently been addressed, little information is currently available to indicate whether the internalization of the heterotrimeric G-protein occurs via similar or distinct mechanisms. The results obtained in the present study permit a clear dissociation to be made between the agonist-induced internalization of $G_{q/11}$ and of the muscarinic receptor in SH-SY5Y cells. Thus whereas receptor endocytosis is prevented under conditions of impaired phosphoinositide synthesis, or when cells are rendered hypertonic, the muscarinic agonist-induced internalization of $G_{q/11}$ remains robust under both of these experimental paradigms. The failure of hypertonicity to block the internalization of $G_{q/11}$ indicates that, unlike the muscarinic receptor, $G_{q/11}$ does not undergo endocytosis via a clathrin-coated pit mechanism. Additional support for the possibility that receptor and G-protein are internalized via distinct mechanisms was recently obtained by Drmota et al. (1998) who observed that the endocytosis of the thyrotropin releasing hormone receptor and $G_{11}$ exhibit distinctly different kinetic characteristics. Taken collectively, these results suggest that an uncoupling

of the heterotrimeric G-protein from its receptor occurs during the internalization process.

The precise functional significance of G-protein internalization remains to be defined. The possibility that the internalized G-protein couples to a functional receptor to mediate second messenger formation in an endosomal compartment appears to be unlikely since previous reports have shown the internalized muscarinic receptor to be uncoupled from its effector enzyme (Thompson and Fisher, 1991; Sorensen et al., 1997). Alternatively, G-protein internalization may serve as a prerequisite for its subsequent down-regulation (Milligan, 1993). As a result, internalization could serve to limit the availability of the G-protein and provide a mechanism for both homologous and heterologous regulation of G-protein coupled receptor-mediated signaling.

In conclusion, the present results have demonstrated that the agonist-induced internalization of $G_{q/11}$ persists under conditions in which the endocytosis of the muscarinic receptor is prevented. These results demonstrate that the internalization of the receptor and G-protein proceed via distinct mechanisms.

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