Altered trafficking and unfolded protein response induction as a result of M₃ muscarinic receptor impaired N-glycosylation

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The human M₃ muscarinic acetylcholine receptor is present in both the central and peripheral nervous system, and it is involved in the pathophysiology of several neurodegenerative and autoimmune diseases. We suggested a possible N-glycosylation map for the M₃ muscarinic receptor expressed in COS-7 cells. Here, we examined the role that N-linked glycans play in the folding and in the cell surface trafficking of this receptor. The five potential asparagine-linked glycosylation sites in the muscarinic receptor were mutated and transiently expressed in COS-7 cells. The elimination of N-glycan attachment sites did not affect the cellular expression levels of the receptor. However, proper receptor localization to the plasma membrane was affected as suggested by reduced [³H]-N-methylscopolamine binding. Confocal microscopy confirmed this observation and showed that the nonglycosylated receptor was primarily localized in the intracellular compartments. The mutant variant showed an increase in phosphorylation of the α-subunit of eukaryote initiation factor 2, and other well-known endoplasmic reticulum stress markers of the unfolded protein response pathway, which further supports the proposal of the improper intracellular accumulation of the nonglycosylated receptor. The receptor devoid of glycans showed more susceptibility to events that culminate in apoptosis reducing cell viability. Our findings suggest up-regulation of pro-apoptotic Bax protein, down-regulation of anti-apoptotic Bcl-2, and cleavage of caspase-3 effectors. Collectively, our data provide experimental evidence of the critical role that N-glycan chains play in determining muscarinic receptor distribution, localization, as well as cell integrity.

Keywords: apoptosis / endoplasmic reticulum stress / muscarinic receptor / N-glycosylation / unfolded protein response

Introduction

The human M₃ muscarinic acetylcholine receptor (M₃R) is a member of the G-protein-coupled receptor (GPCR) superfamily and is present in the central and peripheral nervous system. This receptor is one of the five muscarinic subtypes (M₁–M₅) that mediate important cellular functions and has been linked to several neurodegenerative and autoimmune diseases including diabetes type 2, Sjögren’s syndrome, chronic obstructive pulmonary disease, overactive bladder, obesity, irritable bowel syndrome, gastrointestinal spasms, wound healing and cancer (Peretto et al. 2009).

As a membrane receptor protein, newly synthesized M₃R undergoes important modifications such as N-glycosylation and disulfide bond formation during its biosynthesis and translational processes to the endoplasmic reticulum (ER) membrane (Markkanen and Petaja-Repo 2008). Only receptors properly folded and assembled are transported from the ER to the cis-Golgi complex in a process termed “quality control” (Schroder and Kaufman 2005). This surveillance mechanism ensures that unfolded or misfolded proteins are retained in the ER lumen by molecular chaperones or carried to 26S proteasome for degradation through either ER-associated degragation or autophagy (Malhotra and Kaufman 2007; Ron and Walter 2007).

The excessive accumulation of unfolded or misfolded proteins aggregating into insoluble structures causes ER stress and it is associated with various human diseases such as metabolic alterations, atherosclerosis, neurodegenerative diseases and ischemia (Kaufman 2002; Rao and Bredesen 2004; Rasheva and Domingos 2009). When the survival and adaptive mechanism to overcome the ER stress is not sufficient to restore ER homeostasis, a persistent unfolded protein response (UPR) signaling occurs and the cell could enters into programmed cell death (Zhang and Kaufman 2008). Multiple UPR pathways have been proposed to contribute to ER-stress-induced cell apoptosis, although the mechanism...
remains mostly unknown (Zuppini et al. 2002; Takizawa et al. 2004). Recently, this apoptotic process has been reported to be mediated by factors, including C/EBP homologous protein (CHOP/GADD153), apoptosis signal-regulating kinase 1 (ASK1) and caspase-12 (Oyadomari and Mori 2004). Although N-linked glycosylation is the most common post-translational modification of GPCRs with ~70–90% of occurrence in the consensus sequences, only a few studies, with differing results, have been reported to date for their role in muscarinic acetylcholine receptors (mAChRs; Nathanson 2008). The molecular mechanism underlying the role of N-glycosylation in maturation and transport to the plasma membrane of mAChRs remains to be clarified. With this aim, we studied a mutant receptor where the five putative glycosylation sites were mutated [N-glycosylation-deficient mutant receptor (nGly-M3R)]. We determined relevant features of the role of N-linked glycosylation in human M3R expressed in COS-7 cells by means of biochemical, ligand binding and cellular approaches. Our findings suggested a role of N-glycan chains in the correct cellular processing of M3R. Furthermore, lack of glycosylation would make the receptor more prone to intracellular accumulation and could trigger events culminating in cell apoptosis and reduced cell viability.

Results
M3R expressed in COS-7 cells is glycosylated
M3R contains five-consensus sequences for N-linked glycosylation as deduced from the amino acid sequence. Western blot analysis suggested that four of them, transiently expressed in COS-7 cells, were glycosylated (N5, N15, N41 and N48) (Supplementary data, Figure S1). In addition, nondrastic changes in [3H]-N-methylscopolamine ([3H]-NMS) binding properties, determined by a saturation binding assay, were detected when point mutations were introduced into the receptor sequence at the N-glycosylation sites (Supplementary data, Figure S2A). However, both the binding assay and confocal microscopy results showed that the lack of all glycosylation sites, produced by the treatment with tunicamycin, caused a significant decrease in the transport of the receptor to the plasma membrane, suggesting intracellular perinuclear localization (Supplementary data, Figure S2B). Here, the role of the M3R N-glycosylation on receptor structure and function was investigated by mutating all potential N-glycosylation sites. The asparagine residues at positions N5, N6, N15, N41 and N48 of the wild-type receptor (WT-M3R) were replaced by glutamine generating a fully nonglycosylated receptor (nGly-M3R). Chemical blockage of M3R N-glycosylation by tunicamycin was used as a control. Solubilized membrane proteins from tunicamycin-treated COS-7 cells expressing WT-M3R, and cells expressing nGly-M3R without tunicamycin treatment, were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by western blot analysis using an anti-M3R antibody. Solubilized M3R, with presumed native N-glycosylation, yielded a band at approximately 80 kDa. Immunoblots showed a decrease in the apparent molecular mass of the M3R band, in both the tunicamycin-treated WT-M3R and nGly-M3R, which was consistent with the lack of N-glycosylation, suggesting that the uppermost band represents the fully glycosylated form (Figure 1A). In addition, WT-M3R and nGly-M3R were expressed in COS-7 cells, and the cellular distribution was analyzed by immunocytochemistry and confocal microscopy. Different staining patterns were observed in cells expressing

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** The human M3 muscarinic receptor is glycosylated and the lack of attached N-glycan chains promotes intracellular receptor accumulation. (A) Tunicamycin-treated COS-7 cells transiently expressing WT-M3R, or cells expressing nGly-M3R without tunicamycin treatment, were lysed in Celllytic™ buffer. Solubilized proteins were then resolved by SDS–PAGE and immunoblotted for M3R using anti-M3R antibody and HRP-conjugated goat anti-rabbit IgG as a secondary antibody. (B) Subcellular localization of WT-M3R and nGly-M3R transiently expressed in COS-7 cells. After fixation and permeabilization, the cells were immunostained with rabbit anti-M3R polyclonal antibody followed by FITC-conjugated goat anti-rabbit (green). Nuclei were stained with Hoechst 33342 (blue). Images for indirect immunofluorescence were acquired with a confocal microscope. The background signal obtained from non-transfected cells stained with both primary and secondary antibodies was used as a control (control-I). Cells transfected with WT-M3R stained with the secondary antibody (absence of the primary antibody) was also used as a control (control-II). Images are representative of three separate experiments. Scale bar equals 50 µm.
Impaired N-glycosylation of M₃ muscarinic acetylcholine receptor

Table I. Ligand binding data of WT-M₃R and nGly-M₃R transiently expressed in COS-7 cells

<table>
<thead>
<tr>
<th>Receptor</th>
<th>[³H]-NMS K_D (pM)</th>
<th>B_max (fmol/mg)</th>
<th>[³H]-QNB_total (without stringent wash) K_D (pM)</th>
<th>B_max (fmol/mg)</th>
<th>[³H]-QNB_intracellular (with stringent wash) K_D (pM)</th>
<th>B_max (fmol/mg)</th>
</tr>
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<tbody>
<tr>
<td>WT-M₃R</td>
<td>185 ± 42</td>
<td>1658 ± 214</td>
<td>384 ± 32</td>
<td>1850 ± 71</td>
<td>320 ± 43</td>
<td>573 ± 88</td>
</tr>
<tr>
<td>nGly-M₃R</td>
<td>466 ± 35***</td>
<td>452 ± 129****</td>
<td>547 ± 21**</td>
<td>1647 ± 108*</td>
<td>2897 ± 27***</td>
<td>1964 ± 155***</td>
</tr>
</tbody>
</table>

[^3H]-NMS and [³H]-QNB binding was performed on intact COS-7 cells. Receptor expression levels were checked after COS-7 cells transfection with variable amounts of cDNA up to 2 μg as indicated in Materials and methods (Table I shows data obtained for 2 μg cDNA). Nonlinear curve-fitting using GraphPad PRISM 5.0 determined dissociation constants (K_D) and maximal binding capacities (B_max). Data are presented as the mean ± SEM; n = 6 in triplicate.

*Significantly different compared with cell expressing WT-M₃R (P < 0.05) by Student’s t-test.

**Significantly different compared with cells expressing WT-M₃R (P < 0.01) by Student’s t-test.

***Significantly different compared with cells expressing WT-M₃R (P < 0.001) by Student’s t-test.

nGly-M₃R compared with WT-M₃R (Figure 1B). nGly-M₃R showed a predominantly intracellular signal, in contrast to WT-M₃R which displayed localization in the plasma membrane (Supplementary data, Figure S1B and Figure S3).

Loss of N-glycan chains promoted intracellular receptor accumulation

Saturation binding assays were performed in order to determine the WT-M₃R and the nGly-M₃R expression levels and pharmacological properties. Radioligand binding experiments were carried out with intact COS-7 cells expressing WT-M₃R or nGly-M₃R. The total M₃R expression level (both surface and internal) was measured using the membrane-permeant antagonist [³H]-quinuclidinyl benzilate ([³H]-QNB), and the intracellular level was determined by [³H]-QNB binding dissociation from the cell surface with a stringent wash buffer. M₃R at the cell surface was measured using the membrane-impermeable antagonist [³H]-NMS. The [³H]-QNB binding studies, without stringent wash after incubation, showed only minor differences in the total number of nGly-M₃R when compared with the number of WT-M₃R at 48 h. However, upon [³H]-QNB ligand dissociation from the cell surface, different receptor expression levels were detected. The mutant showed a 3-fold higher receptor level at intracellular compartments when compared with WT-M₃R, suggesting intracellular accumulation (Table I and Supplementary data, Figure S4).

Furthermore, clear differences in receptor density (B_max) were observed by means of [³H]-NMS binding measurements. [³H]-NMS binding data showed that nGly-M₃R was expressed at the cell surface at over 60% lower levels (B_max) when compared with WT-M₃R (Table I and Supplementary data, Figure S5). nGly-M₃R displayed a slight change in binding affinity (K_D) for the [³H]-NMS antagonist when compared with the WT-M₃R. An effect on K_D for [³H]-QNB was also observed (Table I). We tried to ensure ligand access to internal receptors; however, some membrane-permeant ligands like [³H]-QNB are not equally able to label internal as well as surface receptors, perhaps because the endosomal compartment is at a low pH, or other factors promoting the radioligand’s often lower affinity in intact cell experiments (Koenig and Edwardson 1994). For this reason, we did not discuss the K_D values, but only B_max values, although [³H]-QNB shows high affinity for the muscarinic receptor.

N-glycosylation-deficient mutant was able to activate the G protein

We decided to examine two classical steps in the signaling pathway of activated M₃R, activation of the G protein and triggering of the mitogen-activated protein kinase (MAPK) ERK1/2 pathway. Membrane homogenates were prepared from COS-7 cells expressing WT-M₃R or nGly-M₃R, and [³H]-NMS binding was performed to guarantee equal pmol receptor/mg of protein in all samples. G-protein activation was carried out by using a [³H]-GTP[γS] (guanosine 5′-[γ-³²P]-thiotriphosphate) filter-binding assay (Borroto-Escuela et al. 2010). Carbachol stimulated WT-M₃R and nGly-M₃R with similar potency (EC₅₀ values: WT-M₃R, 4.80 ± 0.17 μM and nGly-M₃R, 6.55 ± 0.27 μM, n = 3). We found that nGly-M₃R could activate the G protein with the same ability as the WT-M₃R (Figure 2A). In addition, COS-7 cells expressing the nGly-M₃R mutant receptor, activated upon agonist stimulation, were able to increase the p-ERK1/2 level to a similar extent to that of WT-M₃R. Interestingly, nonstimulated cells expressing nGly-M₃R showed a high level of p-ERK1/2 (Figure 2B). This finding can be linked with the previous report that this activation pathway may be associated with up-regulation of ER-resident molecular chaperones (Zhang et al. 2009).

UPR occurred in cells expressing nonglycosylated mutant M₃R

Our previous findings led to the hypothesis that the nGly-M₃R is associated with ER stress. In eukaryotic cells, UPR consists of three distinct signaling pathways. In the last decade, numerous studies have described in detail the inositol-requiring kinase 1, double-stranded RNA-activated protein kinase-like ER kinase and activating transcription factor 6 signaling cascades (Schroder and Kaufman 2005; Malhotra and Kaufman 2007; Ron and Walter 2007). In order to determine if abnormal accumulation of the nGly-M₃R is able to induce UPR in COS-7 cells, we performed western blot and in-cell western immunofluorescent assays to detect the expression of ER-stress markers. We found evidence for activation of UPR, marked by elevated levels of phosphorylated α-subunit of eukaryote initiation factor 2 (p-eIF2α) and an increased expression of ER-localized chaperone proteins, which were consistent with our earlier proposal. p-eIF2α was detected at a lower level in cells expressing WT-M₃R and completely...
abolished by cell incubation with a p-eIF2α inhibitor peptide used as a control (Figure 3A). However, cells expressing nGly-M3R showed an increased level of p-eIF2α compared with those expressing WT-M3R. A similar result was observed in tunicamycin-treated cells, an ER-stress inducer used as a control (Figure 3A). Furthermore, nGly-M3R was able to significantly increase the GRP78/Bip levels in COS-7 cells, and to a lower extent, in cells expressing WT-M3R. Also, calnexin and calreticulin levels were found to be increased (Figure 3B). Similar results were observed when compared with those obtained for the ER-stress inducer, tunicamycin (Figure 3B). In addition, the CHOP expression level was minimal in cells expressing WT-M3R and up-regulated in cells expressing nGly-M3R similar to what could be observed for cells treated with tunicamycin (Figure 3C). These results agree with the idea that CHOP is induced at the transcription level in response to ER stress.

**Prolonged UPR by mutant M3R induces ER stress and promotes cell apoptosis**

Cells accumulating misfolded nonglycosylated proteins are unable to restore homeostasis through UPR, leading to cell death, basically via apoptosis (Hauptmann et al. 2006). Multiple UPR pathways can contribute to ER stress-induced cell apoptosis and autophagy (Zuppini et al. 2002; Takizawa et al. 2004; Zhang and Kaufman 2008). Recently, it has been reported that apoptosis is mediated by factors, including CHOP/GADD153, ASK1 and caspase-12 (Morishima et al. 2002; Gotoh et al. 2004; Nakayama et al. 2010). The occurrence of ER stress in COS-7 cells expressing nGly-M3R led us to investigate the possibility that mutated cells could have an increased susceptibility to undergo apoptosis. Events ending in apoptosis were assessed by measuring protein levels of anti-apoptotic or pro-apoptotic regulators of the Bcl-2 family, via western blot analysis (Figure 4A), and activation of caspase-3, which was determined by the appearance of cleaved fragments of caspase-3 (Figure 4B). Our results showed nonsignaling apoptosis in cells transfected with WT-M3R. In contrast, apoptosis seemed to be elicited in cells expressing nGly-M3R. Furthermore, a robust increase in the activated caspase-3 level was achieved in the case of nGly-M3R when the classical apoptosis-inducer hydrogen peroxide was used (Figure 4B). The increased expression level of pro-apoptotic Bax corroborates the above results (Figure 4A). Similarly, Bcl-2 levels were significantly down-regulated by deglycosylated mutant, suggesting a loss of the survival response by a persistent UPR (Figure 4A). These findings suggest that nGly-M3R predispose cells to apoptosis through caspase-3 activation counter-balanced with an early marked anti-apoptotic signal of Bcl-2 family proteins. This signaling would be unbalanced with time, by a chronic UPR, making cells expressing nGly-M3R more susceptible to cell disruption. In addition, cell growth was measured by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay. We found a significant decrease in cell viability for COS-7 cells expressing the mutated receptor compared with cells expressing WT-M3R (Figure 4C).

**Discussion**

The presence of consensus sequences for N-glycosylation in most members of the GPCR family suggests that this post-translational modification may play important roles in receptor expression, structure and/or function. Increasing evidence supports the notion that core N-glycan contributes not only to
receptor folding, but also to the cell surface transport (Duvernay et al. 2005; Lanctot et al. 2006; Markkanen and Petaja-Repo 2008; Roy et al. 2010). All mAChRs contain putative asparagine-linked glycosylation sites but only scarce reports, with differing results, have been published for N-glycosylation of mAChRs (Nathanson 2008). In our current study, we analyzed the role of N-glycan chains of the human M3R transiently expressed on COS-7 cells, in both receptor folding and cell surface trafficking. Using samples treated with the N-glycosylation blocking agent tunicamycin as a control, western blot analysis of a receptor with all five sites for asparagine-linked glycosylation in mutated WT-M3R provides compelling evidence that M3R is glycosylated at four of the five potential N-glycosylation sites. In addition, we found that N-glycan chains are required for proper receptor cell surface trafficking. [3H]-NMS binding results, together with confocal microscopy observations, showed that lack of N-glycosylation resulted in altered cell surface receptor expression. Simultaneously, [3H]-QNB binding suggested intracellular accumulation of nGly-M3R.

It is known that improperly folded proteins are recognized by UDP-glucose glycoprotein glucosyltransferase that catalyzes the re-glucosylation of immaturely processed glycoprotein with ER retention prompting another round of calnexin/calreticulin-assisted folding (Pearse et al. 2010) which could be associated with ER stress. On the other hand, our [3H]-QNB data could be interpreted as reflecting partially impaired receptor folding. However, although [3H]-QNB shows high affinity for the muscarinic receptor, radioligand labeling to internal receptors, in intact cells, can be affected by several factors which shows lower affinities than those expected (Koenig and Edwardson 1994). In any case, calnexin/calreticulin binds to a specific lectin structure during glycoprotein maturation promoting proper protein folding and disulfide bond formation by interacting with the protein disulfide isomerase ERp57 (Zapun et al. 1998; Lanctot et al. 2006; Markkanen and Petaja-Repo 2008). Proper M3R conformation is important for receptor cell surface transport (Zeng et al. 1999). However, mutating the glycosylation sites did not drastically affect the pharmacological properties of the receptor, as shown by only a slight change in the dissociation constant for [3H]-NMS in membrane homogenates. M3R association in dimeric/oligomeric complexes (Alvarez-Curto et al. 2010) may allow other receptors to act as scaffold proteins helping receptor transport to the plasma membrane (Van Craenenbroeck et al. 2011), and this could explain the presence of a functional receptor at the cell surface. Collectively, our findings suggested that preventing attachment of oligosaccharides to M3R resulted in a decreased receptor level at the cell surface probably due to defects in the
nGly-M3R with tunicamycin treatment (ANOVA; ++ and +++, significantly different compared with cells expressing nGly-M3R without tunicamycin treatment (P < 0.05 and P < 0.001) by two-way ANOVA; ## and ###, significantly different compared with cells expressing WT-M3R with tunicamycin treatment (P < 0.01) by two-way ANOVA; ++ and +++, significantly different compared with cells expressing nGly-M3R with tunicamycin treatment (P < 0.01 and P < 0.001) by two-way ANOVA.

trafficking process by which glycan structures contribute to receptor transport through the secretory pathway without drastic conformational changes.

At some point in the ER-stress mechanism, cells activate UPR in an effort to maintain ER homeostasis (Malhotra and Kaufman 2007). Phosphorylation of eIF2α is a well-characterized mechanism for down-regulation of protein synthesis under ER stress (Schroder and Kaufman 2005), whereas up-regulation of ER-resident chaperones and induction of transcriptional factors have been previously reported in ER stress (Ron and Walter 2007). The unexpected activation of ERK1/2 in nonagonist-stimulated cells expressing nGly-M3R suggests that this pathway is associated with nGly-M3R-induced ER stress by intracellular mutant accumulation. This view is in agreement with previous reports showing that this is associated with GRP78 up-regulation (Zhang et al. 2009) and therefore we determined the levels of p-eIF2α by immunoblotting. To further validate ER-stress induction, we assessed the levels of GRP78/Bip and p-eIF2α.

The results showed strong intracellular localization of nGly-M3R compared with WT-M3R and concomitant induction of the ER-stress markers GRP78/Bip and p-eIF2α. Furthermore, a slight increased expression level of ER resident molecular chaperones, which facilitate proper glycoprotein folding during biosynthesis like calnexin and calreticulin, was detected. Thus, our results suggest that UPR is activated in the case of the expression of nGly-M3R.

CHOP is also known to be induced at the transcriptional level in response to ER stress (Nakayama et al. 2010). We found CHOP induction, in tunicamycin-treated COS-7 cells expressing WT-M3R or nGly-M3R, to a similar extent to cells expressing nGly-M3R without tunicamycin treatment. It had been previously shown that the ER stress-CHOP apoptosis signal is transmitted to mitochondria through Bax translocation (Gotoh et al. 2004), presumably mediated by Bim, a pro-apoptotic BH3-only type Bcl-2 family member (Puthalakath et al. 2007). This early evidence could sustain our observed Bax increased levels. In addition, Bcl-2 levels were promoted in either tunicamycin-treated cells expressing WT-M3R and nGly-M3R suggesting survival response. It should be noted that tunicamycin affects not only the first step of muscarinic receptor glycosylation but of all other glycoproteins as well, and this may lead to marked stimulation of Bc-2. This finding is consistent with previous results that propose the importance of Bc-2 not only at the mitochondria but also at the ER (Schinzl et al. 2004). However, Bc-2 levels were appreciably down-regulated during nGly-M3R-induced ER stress suggesting loss of the survival response by a persistent UPR.

Our results revealed that prolonged UPR in COS-7 cells expressing nGly-M3R led to measurable activation of caspase-3. These cells were more susceptible to disruption, indicated by strong apoptosis signal response to hydrogen peroxide treatment. Our findings, including hypothetical cytochrome c release by Ca2+ increase resulting from ER stress (Boya et al. 2002), can be interpreted as nGly-M3R promoting ER stress-induced events which leads to cell apoptosis by caspase activation through a mitochondrial-dependent pathway. However, it was previously shown that, in ER stress-induced apoptosis, caspase-12 was able to activate caspase-9 thus promoting cleavage of procaspase-3 by a non-dependent cytochrome c pathway (Morishima et al. 2002).
The possibility that nGly-M₃R may also use this pathway to active caspase-3 should also be considered and would require further research. Our results suggested that nGly-M₃R can promote events ending in apoptotic cell death, which is consistent with previous evidence supporting this possibility (Hauptmann et al. 2006).

Overall, our findings suggested the M₃R N-glycosylation-deficient mutant to promote impairment of receptor trafficking, and elicit ER stress, and increase susceptibility to cell disruption.

Materials and methods

**Generation of M₃R N-glycosylation-deficient mutant**

To generate cDNA constructs encoding nGly-M₃R, asparagine residues in each glycosylation consensus sequence of WT-M₃R were replaced with glutamine residues using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutations were confirmed by DNA sequencing. The primers (from Sigma Aldrich, St Louis, MO) used for mutagenesis (5′→3′) were as follows: M₃RN5Q: 5′-gAgAgTCACAAATgACCTtgCACCAgAAGAATACACgCCTTTg-3′; M₃RN6Q: 5′-gAgAgTCACAAATgACCTtgCACCAAATgACCTTTg-3′; M₃RN15Q: 5′-CCCTgCTTTTgTTTCCACAgATCAgCTCTCCTgg-3′; M₃RN41Q: 5′-CATTTCCggCAGgCTCCAagTggCTggC-3′; M₃RN48Q: 5′-CTCtgAggCAGgCTgCTggC-3′.

**Cell culture and transfection**

COS-7 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 2 mM l-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin and 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C and in atmosphere of 5% CO₂. For transfection, cells were grown in 6-well dishes at a concentration of 1 × 10⁶ cells/well or in 75 cm² flasks and cultured overnight before transfection. Cells were transiently transfected using either linear Polyethyleneimine transfection reagent (Polysciences Inc., Warrington, PA) or Fugene® HD transfection reagent (Roche Applied Science, Indianapolis, IN), according to the manufacturer’s protocol. As an ER-stress control, cells were incubated, at 4 h post-transfection, in the absence or the presence of 5 µg/mL tunicamycin (Sigma Aldrich) and cells were harvested after 48 h. Apoptosis was induced in grown COS-7 cells by the addition of hydrogen peroxide at a concentration of 100 µM for 15 h before assays.

**Cell membrane preparation and solubilization**

About 48 h after transfection, COS-7 cells were washed twice with ice-cold phosphate-buffered saline (PBS), scraped in ice-cold buffer containing 50 mM Tris–HCl, pH 7.4, 5 mM MgCl₂ and protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Samples were subjected to centrifugation for 5 min at 500 × g, and pellets were homogenized using a Polytron tissue homogenizer (for 30 s, five times each 30 s on ice) followed by centrifugation at 10000 × g for 5 min at 4°C to remove unbroken cells and nuclei. The supernatant fraction was removed and passed through a 25-gauge needle 10 times before being transferred to ultracentrifuge tubes and subjected to centrifugation at 40,000 × g for 30 min at 4°C. The resulting membrane pellets were resuspended in buffer containing 10 mM Tris–HCl, pH 7.4, 5 mM MgCl₂. In some cases, membrane pellets were incubated in solubilization buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 1% of glycerol, protease inhibitor cocktail and a mixture of n-dodecyl-β-D-maltoside and Nonidet P40 (1%) during 1 h at 4°C. Solubilized membrane proteins were recovered by collection of the supernatant, after ultra-centrifugation at 100,000 × g for 30 min at 4°C. Membrane homogenates and solubilized membrane protein concentrations were determined by means of a Bradford protein assay kit (Bio-Rad, Hercules, CA), using bovine serum albumin (BSA) as standard. Samples were immediately used or stored at −80°C until required.

For p-eIF2α blocking experiments, used as a control, twice the volume of blocking peptide (10 µg/mL) was incubated with one volume of an antibody against p-eIF2α (Cell Signaling Technology, Danvers, MA, USA). After 45 min at room temperature, p-eIF2α was subjected to immunoblotting as described in SDS-PAGE and western blotting section.

**SDS–PAGE and western blotting**

Proteins resolved by 12% SDS–PAGE were subjected to immunoblotting onto PVDF membranes (Millipore, Bedford, MA) using a semidry transfer system. Membranes were blocked for 1 h in 5% BSA or nonfat dry milk, Tris-buffered saline, pH 7.4, with 0.1% Tween-20 prior to overnight incubation with primary antibody under mild agitation at 4°C. Proteins were detected with the following primary antibody: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cell Signaling Technology). Membranes were washed and developed, using the enhanced Super Signal chemiluminescence’s detection kit (ECL; Pierce Biotechnology, Rockford, IL), and HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ) using ChemiDocTM XRS (Bio-Rad). The primary antibodies used were as follows: rabbit anti-M₃ (Santa Cruz Biotechnology, Santa Cruz, CA), GRP78/Bip, p-eIF2α and total eIF2α, Bax, Bcl-2 and caspase-3 were all from Cell Signaling Technology.

**Immunoblotting for total and phosphorylated ERK1/2**

COS-7 cells transiently expressing WT-M₃R and nGly-M₃R were rendered quiescent by serum starvation overnight prior to ERK phosphorylation. Subsequently, an additional 2 h pre-incubation step, in a fresh serum-free medium, was performed to minimize basal activity before cells were challenged with the muscarinic agonist carbachol for the time and concentration indicated (100 µM for 5 min). Next, a rapid rinsing with ice-cold PBS was performed in order to finish stimulation, and before, cells were lysed with 500 µL ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, protease and phosphatase inhibitor cocktail) during 10 min. The cellular debris was removed by centrifugation at 10,000 × g for 5 min at 4°C, and the total protein content was measured using BCA Protein Assay Reagent (Pierce Biotechnology). Aliquots corresponding to 20 µg of protein were mixed with SDS–PAGE sample buffer, separated by
12% SDS–PAGE and analyzed by immunoblotting. Membranes were blotted for total-44/42 MAPK (Erk1/2) and phosphorylated-44/42 MAPK (p-Erk1/2) using polyclonal antibodies, followed by HRP-conjugated goat anti-rabbit IgG as a secondary antibody as described (all from Cell Signalling Technology). Immunoreactivity bands were visualized as described in Figure 2 and then measured by quantitative densitometry.

In-cell western immunofluorescent assay
The CHOP level was measured by in-cell western blot. COS-7 cells were seeded onto poly-d-lysine-coated 96-well plates (Corning, Corning, NY) in DMEM and were transiently transfected as described in cell culture and transfection section with WT-M3R or nGly-M3R. Cells treated with tunicamycin 4 h after transfection, and harvested 48 h post-transfection, were used as an ER-inducer control. Following fixation, permeabilization and blocking with LI-COR Odyssey Blocking Buffer® (LI-COR Bioscience, Cambridge, UK), cells were incubated overnight at 4°C with monoclonal antibody against CHOP (Cell Signaling Technology). After further washing, cells were incubated with an infrared secondary antibody (goat anti-mouse, 1:1500 dilution, LI-COR Biosciences) in LI-COR Odyssey Blocking Buffer® and plates were scanned by Odyssey infrared scanner. Total number was normalized using DRAQ5/Sapphire 700 staining agents. Results are presented as arbitrary units.

Immunocytochemistry staining
Cells were cultured on 35-mm glass cover slips coated with 0.1 mg/mL poly-d-lysine (Sigma Aldrich) in 6-well dishes as described in cell culture and transfection section. Cells were grown at a concentration of 1 × 10⁶ cells/well and transfected as described. After 48 h, cells were cultured in PBS and fixed with 3.7% formaldehyde solution for 20 min at room temperature followed by two washes with PBS containing 20 mM glycine (buffer A). Then, after permeabilization with buffer A containing 0.2% (v/v) Triton X-100/PBS for 5 min at room temperature, cells were treated with buffer A containing 5% BSA. After 1 h, cells were labelled with rabbit anti-M3R antibody (Santa Cruz Biotechnology) for 1 h at 37°C, extensively washed and stained with goat anti-rabbit IgG-fluorescein isothiocyanate (FITC)-conjugated (Santa Cruz Biotechnology) in the same manner. Washed samples were rinsed and mounted onto glass slides using fluorescent mounting medium (Dako, Barcelona, Spain). Microscope observations were performed with a Leica TCS-SL confocal microscope (Leica, Bannockburn, IL).

Muscarinic receptor radioligand binding assay
The binding of [3H]-NMS (81 Ci/mmol, Perkin-Elmer Life Sciences, Madrid, Spain) and [3H]-QNB (42 Ci/mmol; GE Healthcare, Barcelona, Spain) were performed according to a few modification of the methodologies described previously (Hoover and Toews 1989; Schmidt et al. 1995; Romero-Fernandez et al. 2011). In brief, COS-7 cells expressing WT-M3R or nGly-M3R were harvested by trypsinization followed by centrifugation and resuspension in iso-osmotic 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) buffer. Cells (100,000 cells/1 mL assay by hemocytometer counting) were then incubated with different concentrations of [3H]-NMS or [3H]-QNB in DMEM buffered with Hepes at 4°C, overnight. Nonspecific binding was assessed in the presence of 10 µM atropine. Only for [3H]-QNB ligand dissociation from cell surface, a stringent wash after ligand incubation was used [acid wash buffer: 10 mM 2-[N-morpholino] ethane sulfonic acid (MES), pH 5.0, 120 mM NaCl, 0.9 mM CaCl₂ and 0.5 mM MgCl₂]. Bound ligand was separated from free ligand by vacuum filtration over Whatman GF/B filters that had been pretreated with 0.5% polyethyleneimine for 3 h using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). The filters were washed three times with 5 mL of ice-H₂O. Cells were incubated in 0.5% (v/v) Triton X-100/PBS for 1 h, 4 mL of scintillant was added and the bound ligand was estimated by scintillation counting.

[^3]S-GTPγS binding assays
The[^3]S-GTPγS (1250 Ci/mmol; Perkin-Elmer Life Sciences) binding assays were conducted as a modification of the method described previously (Borroto-Escuela et al. 2010). Briefly, membrane homogenate fractions (100 µg protein/mL) were incubated with 0.3 nM[^3]S-GTPγS and 1 µM guanosine-5′-diphosphate (GDP; Sigma Aldrich), in the absence or in the presence of carbamoylcholine chloride (from 1 mM to 1 mM) in 1 mL of 10 mM Hepes, pH 7.4, 10 mM MgCl₂, 1 mM ethylene glycol tetraacetic acid (EGTA), 100 mM NaCl, 0.2% BSA and 10 µg/mL saponin. Reactions were incubated at 30°C for 1 h. Nonspecific binding was determined by incubation of samples in the presence of 10 µM unlabeled GTPγS. Reactions were stopped by addition of 5 mL of ice-cold assay buffer, immediately followed by rapid filtration through Whatman GF/C filters presoaked in the same buffer containing 0.5% polyethyleneimine. The washed filters were dry and resuspended in 4 mL scintillation cocktail, vortex mixed, and radioactivity was detected by liquid scintillation counting.

Cell viability assay
To estimate cell viability, COS-7 cells expressing WT or N-glycosylation-deficient receptors were seeded (5 × 10⁴) into 96-well plates (BD Biosciences, Stockholm, Sweden) in 200 µL of DMEM and incubated at 37°C, 5% CO₂. After culturing for 24 h, 20 µL of the CellTiter 96® Aqueous One Solution (MTS; Promega, Madison, WI) was added to each well and cells were then incubated for 1 h at 37°C, 5% CO₂; 25 µL of 10% SDS was added to stop the reaction, and absorbance was measured at 492 nm using POLARstar Optima plate reader (BMG Lab Technologies, Offenburg, Germany).

Statistical analysis
All binding data were analyzed using the commercial program GraphPad PRISM 5.0 (GraphPad Prism, San Diego, CA). Statistical analyses were performed using unpaired Student’s t-test or one-/two-way ANOVA. The number of samples in each experimental condition is indicated in figure legends. Differences were considered statistically significant when
P-values were <0.05. Values are expressed as the mean ± SEM.

Supplementary data
Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest
None declared.

Abbreviations
ASK1, apoptosis signal-regulating kinase 1; BSA, bovine serum albumin; CHOP, C/EBP homologous protein; DMEM, Dulbecco’s modified Eagle’s medium; EGTA, ethylene glycol tetraacetic acid; ER, endoplasmic reticulum; FITC, fluorescein isothiocyanate; GDP, guanosine 5'-diphosphate; GPCR, G-protein-coupled receptor; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [3H]-NMS, [3H]-N-methylscopolamine; [3H]-QNB, [3H]-quinuclidinyl benzilate; HRP, horseradish peroxidase; mAChRs, muscarinic acetylcholine receptors; MAPK, mitogen-activated protein kinase; MES, 2-[N-morpholino] ethane sulfonic acid; M₃R, human M₃ muscarinic acetylcholine receptor; MTS, (4S,4S)-4,4-dimethylthiazol-2-yl)-3-(4-carboxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium; nGly-M₃R, N-glycosylation-deficient mutant receptor; p-eIF2α, phosphorylated α-subunit of eukaryote initiation factor 2; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; [35S]GTPyS, guanosine 5'-[γ-35S]-thiotriphosphate; UPR, unfolded protein response; WT-M₃R, wild-type receptor.

References

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