The Role of N53Q Mutation on the Rat μ-Opioid Receptor Function

A. Rostami,[1,] M. Rabbani,[2] and M. Mir-Mohammad-Sadeghi[2]*

[1]Department of Physiology and Pharmacology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran; and [2]Department of Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Glycosylation of the μ-opioid receptor may play an important role on its function. Using nested PCR, N53Q mutation was prepared in the N-terminal region of the rat μ-opioid receptor cDNA and cloned into the pcDNA3 vector. The plasmids containing the wild-type and mutated receptor cDNA were transfected into the COS-7 cells. Intracellular cAMP was measured in the morphine-treated and untreated transfected cells using an ELISA kit. Plasmid expression was evaluated using X-gal staining. Intracellular concentration of cAMP in the N53Q-mutated cells was not significantly different from the wild-type. The expression of the transfected plasmids was confirmed. Therefore, based on these results, it seems that glycosylation at the N53 site of the rat μ-opioid receptor does not influence the function of this receptor significantly.

KEY WORDS: cAMP, glycosylation

INTRODUCTION

Opioid receptors belong to the superfamily of G-protein-coupled receptors (GPCRs), which consists of over 1000 members.[1] They have seven transmembrane domains and one extracellular amino-terminal (N-terminal) with multiple glycosylation sites.[2]

Post-translational modifications such as glycosylation and palmitoylation frequently occur in these receptors, affecting their structure and function.[1] Investigating these modifications can lead to a better understanding of the receptor properties; it also can be a guide to the design of better drugs and understanding inter-individual variations in drug response and may propose new strategies for prevention of tolerance and dependence.[3–7]

N-glycosylation is a common post-translational protein modification,[8–10] which occurs on the amide group of an asparagine that is part of the consensus sequence of N-X-S/T (N, asparagine; S, serine; T, threonine; X, any amino acid except proline), known as the glycosylation consensus.[8,11–16] Glycosylation has been shown to influence GPCRs differently, ranging from a decrease to an increase in the function of these proteins.[3,13] Regarding opioid receptors, to our knowledge, only two site-directed mutagenesis studies have been performed in their amino terminal glycosylation sites.[3,13] In both of these studies, asparagines (N) at position 40 was mutated to aspartate (D), i.e., N40D substitution, performed in human μ-opioid receptor cDNA.[13] In one of these studies,[13] the substitution led to an increase in affinity of β-endorphin and also potency of the ligand in activating potassium channels. The study also established that the N40D mutation was the most frequent one amongst heroin addicts.[3] In the second study,[3] it was reported that the N40D mutation did not cause any changes in affinity or potency of several μ-opioid receptor agonists or signal transduction. N40 is one of the five potential sites of glycosylation in the human μ-opioid receptor.[3]

Position 40 in the rat μ-opioid receptor (RMOR) is not a glycosylation site; thus, in the present study, the mutant receptor cDNA at the position of 53 (N53Q) in RMOR was constructed because of the proximity to N40 in the human μ-opioid receptor gene. Asparagine 53 of the RMOR was mutated to glutamine to evaluate the role of this glycosylation on the function of the receptor. We preferred to alter asparagine to glutamine (N to Q substitution), as these two amino acids are structurally very similar, contrary to asparagine and aspartic acid residues (N to D substitution used in the previous studies).[8,12,15–17] Studying this mutation can not only help to clarify the differences observed in previous studies but can also shed more light on the role of glycosylation on μ receptor function.

MATERIALS AND METHODS

PCR buffer (10×) and DNA Taq polymerase enzyme were from Bio-tools (Spain). dNTP was from Eurobio (France), and DNA ligase enzyme, glycerin, MOPS, agarose, alkaline

*ADDRESS CORRESPONDENCE TO: A. Rostami, Department of Physiology and Pharmacology, School of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran (Fax: +98-241-4249553; E-mail: rostami@zums.ac.ir).

phosphatase, and all of the restriction enzymes were from Roche (Germany). Chloroquine diphosphate, DEAE-dextran, forskolin, HBSS, 3-isobutyl-1-methylxanthine (IBMX), and penicillin–streptomycin were from Sigma-Aldrich (St. Louis, MO, USA). Ampicillin, DMEM, FBS, and trypsin were obtained from Gibco (Scotland). Bromophenol blue was from Fluka (St. Louis, MO, USA). λ DNA marker was purchased from Fermentas (Poland). Trypton and yeast extract were from Liofilchem (Italy). Ethidium bromide, boric acid, and loading dye buffer were from Cinnagen (Iran). Other materials were purchased from Merck (Germany) in molecular grade.

cAMP enzyme immunoassay (EIA) kit was from Assay Designs (Ann Arbor, MI, USA, USA), gel extraction kit from Qiagen (Germany), Midi plasmid preparation kit from Bio-Rad (Hercules, CA, USA), and TansformAid transformation kit from Fermentas.

Construction of Expression Vector Containing Mutant μ-Opioid Receptor cDNA

The full-length cDNA clone encoding the RMOR (GenBank Accession Number L13069), subcloned in the pcDNA3 vector, was kindly provided by Professor Graeme Henderson (School of Medical Sciences, University of Bristol, Bristol, UK). The N53Q mutant (AAC to CAA substitution in the 157–158 position) was constructed using nested PCR as described previously.[18]

Transient Expression of μ-Opioid Receptors in COS-7 Cells

COS-7 cells (Pasteur Institute, I.R. Iran) were grown in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cell cultures were maintained at 37°C in a humidified 5% CO_2 incubator.

pcDNA3 vector containing the cDNA of the wild-type or the mutant μ-opioid receptor was transfected into the cells by performing the modified method of Luthman and Magnusson[19] using DEAE-dextran.[20]

Quantification of Intracellular cAMP

To investigate μ-opioid receptor-mediated inhibition of forskolin-stimulated cAMP formation, COS-7 cells expressing μ-opioid receptors were seeded onto 24-well plates. On the experiment day, culture medium was removed, and cells were washed with serum-free medium. Then, cell cultures were incubated for 20 min at 37°C with 1 mM IBMX and 100 μM forskolin in the presence or absence of 10 μM morphine sulfate, a μ-opioid receptor agonist. The reaction was terminated by removing the media, the cells were then detached and lysed using 0.25% trypsin and 0.1 M HCl, and the cAMP level was determined using the cAMP EIA kit (Assay Designs). The cAMP kit uses a competitive immunoassay for the quantitative determination of the intracellular cAMP in the samples. The kit uses a polyclonal antibody to cAMP to bind competitively to the cAMP in the standard, sample, or the cAMP attached covalently to an alkaline phosphatase molecule. All of the standards and samples were run in duplicate. The OD of the samples and standards were read at $\lambda = 405$ nm. The intensity of the bound yellow color was inversely proportional to the concentration of the cAMP in standards or samples. The measured OD was used to calculate the concentration of cAMP.

The average net OD bound was calculated for each standard and sample by subtracting the average nonspecific binding (NSB) OD from average OD bound: average net OD = average bound OD − average NSB OD.

The curve of average net OD versus concentration of cAMP for the standards was obtained, a straight line was approximated through the points, and then, the concentration of cAMP in the unknown samples was determined by interpolation. Microsoft Excel software was used to analyze

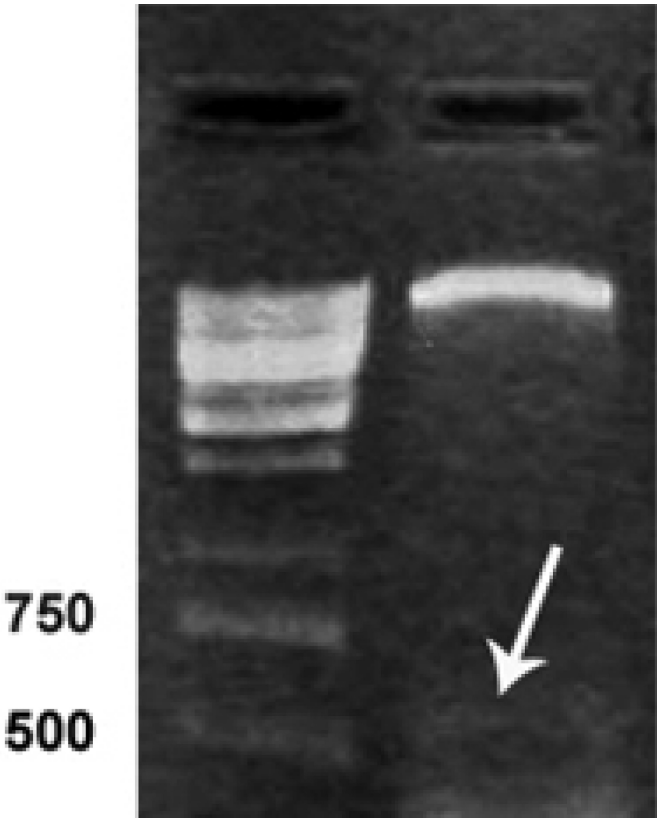

FIGURE 1

Gel electrophoresis of the plasmid suspected to have the N53Q mutant. The prepared plasmid was digested using *Hin*dIII together with *Bam*HI restriction enzymes that resulted in the appearance of a band of approximately 500 bp (white arrow). The obtained products were gel-electrophoresed (0.7% agarose), and their sizes were determined using a 250-bp DNA weight marker.

the dose-response curve for μ-opioid receptor-mediated inhibition of adenylyl cyclase activity.

Statistics

All results are expressed as mean ± SEM. The independent sample Student's *t*-test (two-tailed) was used to determine whether the difference was statistically significant ($P = 0.05$).

RESULTS

N53Q Mutation

The mutation was produced using nested PCR, and the mutated cDNA was inserted into the pcDNA3 plasmid. Digestion of this construct resulted in the appearance of the expected insert size of about 500 bp (Fig. 1).

Transfection

After transfection of COS-7 cells with the wild-type or mutated plasmids, X-gal staining was performed to detect expression of the receptor in the transfected cells. Nearly all of the transfected cells showed blue color, indicating that transfection was successful (Fig. 2A and B). No visible difference in cell staining between the wild-type and N53Q mutant-transfected cells was observed. These results are in agreement with what has been reported by other investigators .[21–23]

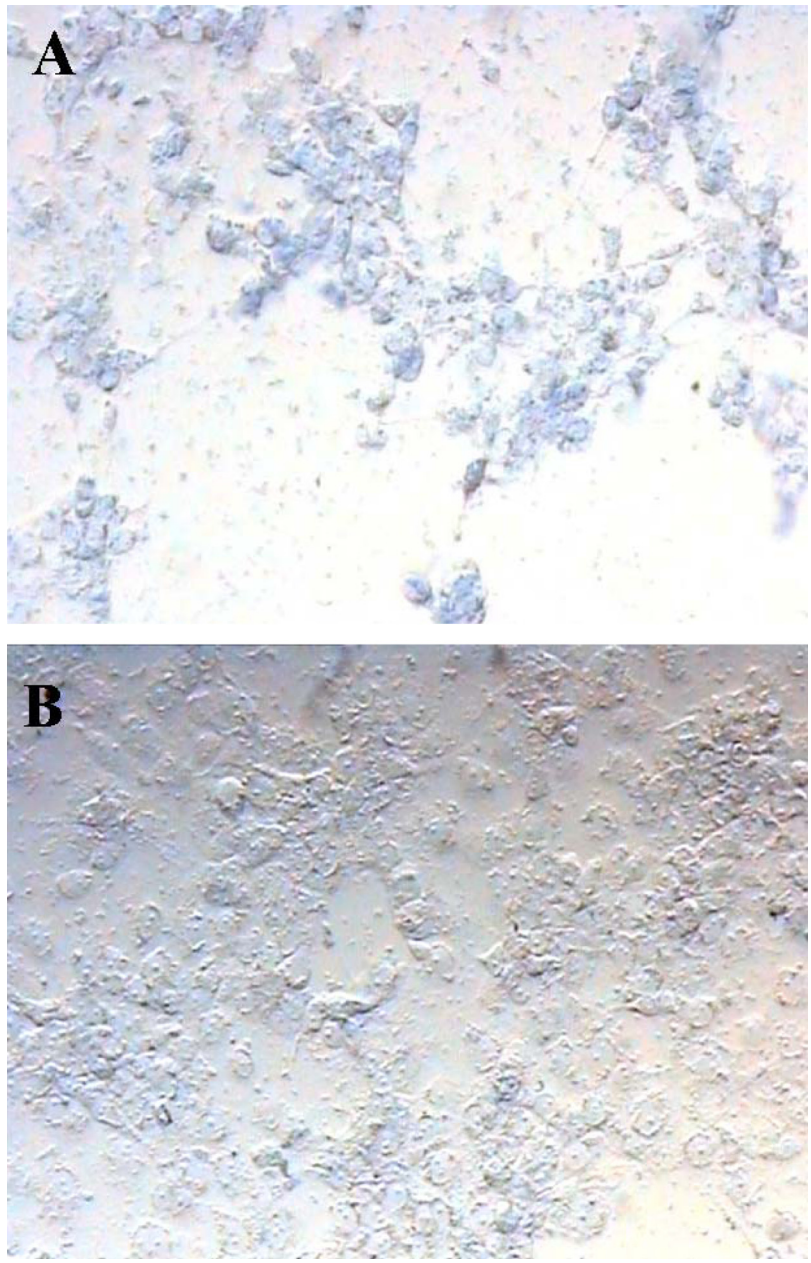

FIGURE 2

X-Gal staining of the COS-7 cells transfected with the plasmid containing (A) the wild-type (original magnification, ×40) and (B) mutated μ receptor cDNA (original magnification, ×40). Blue staining of the transfected cells is visible in the cell cytoplasm.

Functional Assay

COS-7 cells were transiently transfected with the wild-type or N53Q mutant RMOR plasmids. Forty-eight hours after transfection, the cells were treated with IBMX (1 mM for 20 min) and then forskolin (100 μM) plus morphine sulfate (10 μM) for another 20 min. cAMP concentration in the wild-type group was greater than the N53Q mutant-transfected group (0.062 compared with 0.035 pmol/ml, respectively), but this difference was not statistically significant (Fig. 3).

DISCUSSION

The aim of the present study was to evaluate the role of glycosylation on the function of the RMOR. Transfection was successful according to the X-gal-staining method. Intracellular cAMP concentration was measured using a commercial kit and reported as pmol/ml, similar to other studies.[24,25] The cAMP concentration in mutant group was not significantly different from the cells transfected with the wild-type receptor. This means that glycosylation at the N53 position may have no obvious effect on the function of the RMOR. On the contrary, Bond et al.[13] showed that N40D substitution in human μ-opioid receptor increased the affinity of β-endorphin and also potency of the ligand in activating potassium channels as a target of the receptor activation. They used the Xenopus oocyte expression system. In the study,[13] β-endorphin, a large endogenous opioid peptide, was used, and in the present study, morphine, a smaller alkaloid agonist, was used. Another difference is that potassium channel activity was used as their functional assay,[13] and in the present study, reduction in cAMP production was measured. Additionally, cell lines used in the two studies were different; thus, altogether, these differences may contribute to the observed results.

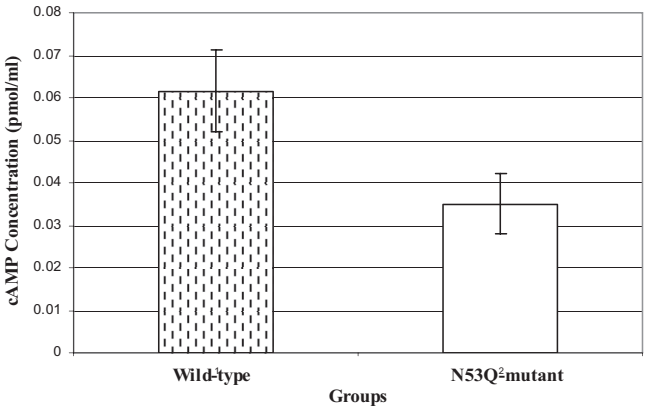

FIGURE 3

cAMP concentrations in the morphine-treated groups. The cells were transfected with 1 μg of the DNA encoding the wild-type RMOR or N53Q mutant RMOR. Forty-eight hours after transfection, the cells were treated with 10 μM morphine as described. cAMP concentrations were determined using an EIA commercial kit in duplicates. Data are expressed as the mean ± SE; $n = 9$ in the wild-type group; $n = 10$ in the N53Q mutant group.

Contrary to the results of Bond et al.[13] and in support of our findings, Befort and colleagues[3] concluded that the N40D mutant had similar affinity, potency, signaling, and down-regulation when compared with the wild-type receptor. They used COS-7 cells for the expression and functional assays as our study.[3] In that study, several opioids (morphine, diprenorphine, [D-Ala^2,*N*-Me-Phe^4,Gly^5-ol]-enkephalin, and D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-amide) and opioid peptides (β-endorphin, [Met]enkephalin, and dynorphin A) were used and displayed similar binding affinities for the N40D mutant.[3,13]

Our study has an advantage over the investigations mentioned above, as N has been substituted to Q instead of D. Asparagine and glutamine are structurally more similar as compared with asparagine and aspartic acid.[6] Thus, it is expected that only a distinct glycosylation site of the RMOR be interrupted, and the rest of the receptor characteristics would be preserved. Therefore, from what has been discussed, it can be concluded that N53 glycosylation of μ-receptor does not seem to influence its function significantly.

Regarding other GPCRs, it was shown that two out of the three N-glycosylation sites of the melanin-concentrating hormone receptor 1 were not necessary for its activity.[6] This finding was similar to another study, in which the removal of three glycosylation sites of the gastrin-releasing peptide receptor did not alter receptor affinity and G-protein coupling.[12]

In contrast, Zhong et al. showed that the nonglycosylated P_2Y_{12} receptor is defective in the inhibition of adenylyl cyclase activity.[26] In another study, it was concluded that N-glycosylation of N4 or N16 was required for the expression of the functionally active tocopherol α isoform of the thromboxane A_2 receptor.[27] Other studies about the corticotropin-releasing factor receptor type 1, prostacyclin receptor, human thyrotropin receptor, human type 1 α metabotropic glutamate receptor, and somatostatin receptor have also shown that N-glycosylation has an important role in their function.[8,10,16,28,29]

In conclusion, in μ-opioid receptors, the glycosylation of the N53 position does not seem to influence the receptor function. However, based on the available data regarding other receptors and as the μ receptor has five glycosylation sites at its N terminal, the possibility of the importance of these sites on its expression and function cannot be ruled out. As some of the studies mentioned above indicate that a combination of mutations is needed to show the effects of glycosylation, it seems appropriate that in future studies,

this strategy be applied to advance our understanding of this post-translational modification on μ-receptor function.

ACKNOWLEDGMENTS

This work was supported by Isfahan University of Medical Sciences. The authors thank F. Moazzen and S. Sadeghi for their technical assistance.

REFERENCES

1. Petaja-Repo UE, Hogue M, Laperriere A, Walker P, Bouvier M. Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human δ opioid receptor. *J Biol Chem* 2000;275:13727–13736.
2. Chaturvedi K, Shahrestanifar M, Howells RD. μ Opioid receptor: role for the amino terminus as a determinant of ligand binding affinity. *Brain Res Mol Brain Res* 2000;76:64–72.
3. Befort K, Filliol D, Decaillot FM, Gaveriaux-Ruff C, Hoehe MR, Kieffer BL. A single nucleotide polymorphic mutation in the human μ-opioid receptor severely impairs receptor signaling. *J Biol Chem* 2001;276:3130–3137.
4. Bot G, Blake AD, Li S, Reisine T. Mutagenesis of a single amino acid in the rat μ-opioid receptor discriminates ligand binding. *J Neurochem* 1998;70:358–365.
5. Brillet K, Kieffer BL, Massotte D. Enhanced spontaneous activity of the μ opioid receptor by cysteine mutations: characterization of a tool for inverse agonist screening. *BMC Pharmacol* 2003;3:14.
6. Saito Y, Tetsuka M, Yue L, Kawamura Y, Maruyama K. Functional role of N-linked glycosylation on the rat melanin-concentrating hormone receptor 1. *FEBS Lett* 2003;533:29–34.
7. Wang HL, Chang WT, Hsu CY, Huang PC, Chow YW, Li AH. Identification of two C-terminal amino acids, Ser(355) and Thr(357), required for short-term homologous desensitization of μ-opioid receptors. *Biochem Pharmacol* 2002;64:257–266.
8. Assil IQ, Abou-Samra AB. N-glycosylation of CRF receptor type 1 is important for its ligand-specific interaction. *Am J Physiol Endocrinol Metab* 2001;281:E1015–E1021.
9. Lanctot PM, Leclerc PC, Clement M, et al. Importance of N-glycosylation positioning for cell-surface expression, targeting, affinity and quality control of the hAT 1 receptor. *Biochem J* 2005;390:367–376.
10. Nagayama Y, Nishihara E, Namba H, Yamashita S, Niwa M. Identification of the sites of asparagine-linked glycosylation on the human thyrotropin receptor and studies on their role in receptor function and expression. *J Pharmacol Exp Ther* 2000; 295:404–409.
11. Akiyama SK, Yamada SS, Yamada KM. Analysis of the role of glycosylation of the human fibronectin receptor. *J Biol Chem* 1989;264:18011–18018.
12. Benya RV, Kusui T, Katsuno T, et al. Glycosylation of the gastrin-releasing peptide receptor and its effect on expression, G protein coupling, and receptor modulatory processes. *Mol Pharmacol* 2000;58:1490–1501.
13. Bond C, LaForge KS, Tian M, et al. Single-nucleotide polymorphism in the human μ opioid receptor gene alters β-endorphin binding and activity: possible implications for opiate addiction. *Proc Natl Acad Sci USA* 1998;95:9608–9613.
14. Fenrick R, McNicoll N, De Lean A. Glycosylation is critical for natriuretic peptide receptor-B function. *Mol Cell Biochem* 1996; 165:103–109.
15. Lee KH, Ahn JI, Yu DH, et al. Effect of N-glycosylation on ligand binding affinity of rat V1a vasopressin receptor. *Biochem Biophys Res Commun* 2001;286:707–713.
16. Zhang Z, Austin SC, Smyth EM. Glycosylation of the human prostacyclin receptor: role in ligand binding and signal transduction. *Mol Pharmacol* 2001;60:480–487.
17. Wenzel-Seifert K, Seifert R. Critical role of N-terminal N-glycosylation for proper folding of the human formyl peptide receptor. *Biochem Biophys Res Commun* 2003;301:693–698.
18. Rabbani M, Sadeghi HM, Jafarian-Dehkordi A, Hajhashemi V, Rostami A. N53Q site directed mutagenesis of rat mu opioid receptor and its cloning using pcDNA3 vector. *Pak J Biol Sci* 2005;8:1115–1118.
19. Luthman H, Magnusson G. High efficiency polyoma DNA transfection of chloroquine treated cells. *Nucleic Acids Res* 1983; 11:1295–1308.
20. Sadeghi HM, Innamorati G, Birnbaumer M. An X-linked NDI mutation reveals a requirement for cell surface V2R expression. *Mol Endocrinol* 1997;11:706–713.
21. Avidor-Reiss T, Zippel R, Levy R, et al. κ-Opioid receptor-transfected cell lines: modulation of adenylyl cyclase activity following acute and chronic opioid treatments. *FEBS Lett* 1995; 361:70–74.
22. Massi P, Vaccani A, Rubino T, Parolaro D. Cannabinoids and opioids share cAMP pathway in rat splenocytes. *J Neuroimmunol* 2003;145:46–54.
23. Tso PH, Wong YH. G(z) can mediate the acute actions of μ- and κ-opioids but is not involved in opioid-induced adenylyl cyclase supersensitization. *J Pharmacol Exp Ther* 2000;295:168–176.
24. Ammer H, Christ TE. Identity of adenylyl cyclase isoform determines the G protein mediating chronic opioid-induced adenylyl cyclase supersensitivity. *J Neurochem* 2002;83:818–827.
25. Bailey CP, Connor M. Opioids: cellular mechanisms of tolerance and physical dependence. *Curr Opin Pharmacol* 2005;5:60–68.
26. Zhong X, Kriz R, Seehra J, Kumar R. N-linked glycosylation of platelet P2Y12 ADP receptor is essential for signal transduction but not for ligand binding or cell surface expression. *FEBS Lett* 2004;562:111–117.
27. Kelley LP, Kinsella BT. The role of N-linked glycosylation in determining the surface expression, G protein interaction and effector coupling of the α (α) isoform of the human thromboxane A(2) receptor. *Biochim Biophys Acta* 2003;1621:192–203.
28. Mody N, Hermans E, Nahorski SR, Challiss RA. Inhibition of N-linked glycosylation of the human type 1α metabotropic glutamate receptor by tunicamycin: effects on cell-surface receptor expression and function. *Neuropharmacol* 1999;38:1485–1492.
29. Nehring RB, Richter D, Meyerhof W. Glycosylation affects agonist binding and signal transduction of the rat somatostatin receptor subtype 3. *J Physiol Paris* 2000;94:185–192.