N-linked glycosylation of platelet P_2Y_{12} ADP receptor is essential for signal transduction but not for ligand binding or cell surface expression

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Received 13 October 2003; revised 12 January 2004; accepted 29 January 2004

First published online 1 March 2004

Edited by Felix Wieland

Abstract P_2Y_{12} receptor is a G_i-coupled adenosine diphosphate (ADP) receptor with a critical role in platelet aggregation. It contains two potential N-linked glycosylation sites at its extra cellular amino-terminus, which may modulate its activity. Studies of both tunicamycin treatment and site-directed mutagenesis have revealed a dispensable role of the N-linked glycosylation in the receptor's surface expression and ligand binding activity. However, the non-glycosylated P_2Y_{12} receptor is defective in the P_2Y_{12}-mediated inhibition of the adenylyl cyclase activity. Thus the study uncovers an unexpected vital role of N-linked glycans in receptor's signal transducing step but not in surface expression or ligand binding.

Key words: Adenosine diphosphate receptor; N-linked glycosylation; G protein-coupled receptor; Adenylyl cyclase; Surface expression; Signal transduction

1. Introduction

Extracellular nucleotides act as potent signaling molecules that trigger a broad range of physiological responses including cardiac muscle contraction, chloride secretion, pain initiation, and platelet aggregation [1–3]. These nucleotides can be released from various cell types including nerve cells, platelets, chromaffin cells, and endothelial cells. They then activate two types of P2 purinergic receptors at the cell surface: P2X and P2Y receptors [1–4]. The P2X receptors are non-selective cation channels gated by adenosine triphosphate (ATP) [5] while P2Y receptors are G protein-coupled receptors. Eight mammalian P2Y receptors have been identified thus far including P_2Y_{1-2}, P_2Y_4, P_2Y_6, P_2Y_{11-14} [6]. Pharmacologically, P2Y receptors can be subdivided into the adenine nucleotide-preferring receptors mainly responding to adenosine diphosphate (ADP) and ATP (P_2Y_1, P_2Y_{11-13}), the uracil nucleotide-preferring receptors responding to uridine triphosphate (UTP) or uridine diphosphate (UDP) (P_2Y_4 and P_2Y_6), receptors of mixed selectivity (P_2Y_2), and recent UDP-glucose receptor (P_2Y_{14}) [6]. P2Y receptors can also be subdivided into five G_q-coupled subtypes (P_2Y_1, P_2Y_2, P_2Y_4, P_2Y_6, and P_2Y_{11}) and three G_i-coupled subtypes (P_2Y_{12}, P_2Y_{13}, P_2Y_{14}) [7]. Additionally, the P_2Y_{11} receptor can couple to G_s and activate adenylyl cyclase [8].

The P_2Y_{12} receptor [9,10], expressed mainly in brain and platelets, is one of the four purinergic receptors that can be activated selectively by ADP. Together with the G_q-coupled P_2Y_1 receptor [11,12], the G_i-coupled P_2Y_{12} receptor responds to ADP with inhibition of stimulated adenylyl cyclase and plays an essential role in the ADP-induced platelet aggregation [11–15]. An autosomal deletion in the coding region of the P_2Y_{12} gene was found in a family with a bleeding disorder and impaired platelet aggregation [10], confirming its function in vivo. The P_2Y_{12} receptor has also been shown to be the target of clopidogrel, a potent anti-thrombotic drug that inhibits platelet aggregation [13,16].

N-linked glycosylation has been shown to be essential for the surface expression and ligand potency of P2X receptors such as P_2X_1 [17], P_2X_2 [18,19] and P_2X_4 receptor [20]. However, roles of the N-linked glycans of P2Y receptors remain unknown. In this study, we have investigated the involvement of N-linked glycosylation in the surface expression, ligand binding and signaling function of the P_2Y_{12} receptor. Both tunicamycin treatment and site-directed mutagenesis approaches have demonstrated that N-linked glycosylation is not essential for the surface trafficking and ligand binding of the receptor. Nonetheless, we have found that the N-glycan modification of the receptor is important for the signal transduction function upon ligand binding.

2. Materials and methods

2.1. DNA constructs and site-directed mutagenesis

The N-terminal hemagglutinin (HA) tag (YPYDVPDYAL) and C-terminal 1D4 tag (NLETSQVAPA) were incorporated in polymerase chain reaction (PCR) primer sequences just before initiator methionine and termination codon of P_2Y_{12} receptor respectively. The site-directed mutagenesis of replacing Asn^6 and/or Asn^{13} by Gln was carried out with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). All PCR products were gel purified and subcloned into pSEMD2 vector behind murine CMV promoter. All constructs were verified by double strand sequencing.

2.2. Cell culture, transfection, tunicamycin treatment, and membrane preparation

All mammalian cell lines (CHO-DHFR minus and cos) were grown as described earlier [21]. Transfections were carried out using the lipofectamine method (Invitrogen, Carlsbad, CA, USA). Tunicamycin was prepared in sterile Me_2SO and added to a final concentration of 5 μg/ml, while the control cells received Me_2SO alone. Cells were

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Abbreviations: 2MeSADP, 2-methyl-thiol-adenosine diphosphate; 2MeSAMP, 2-methyl-thiol-adenosine monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PCR, polymerase chain reaction; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorter

0014-5793/04/$30.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(04)00191-7

analyzed after a 48-h incubation at 37°C. For membrane preparations, cells were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.5, 4 mM ethylenediamine tetraacetic acid (EDTA), pH 7.5, complete protease inhibitors), and homogenized in ice by Duall tissue grinder (Kontes, Vineland, NJ, USA). After spinning homogenates at $700\times g$, membranes were pelleted at $100\,000\times g$ (Beckman, Ultracentrifuge, rotor 70.1) for 30 min at 4°C. Membrane pellets were resuspended in membrane buffer (50 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 4 mM EDTA, 20% glycerol, complete protease inhibitors).

2.3. Radioligand binding

P_2Y_{12}-expressing CHO cells were incubated with different concentrations of [^{3}H]2-methyl-thiol-adenosine diphosphate (2MeSADP) (Moravek Biochemicals Inc, Brea, CA, USA) for 5 min at 25°C. After incubation, the reactions were stopped by rapid filtration through Whatman GF/B glass fiber filters. The filters were then rinsed with Tris-buffered saline (TBS), dried in air and counted with a liquid scintillation counter. The 2MeSADP specific binding was calculated by subtracting the non-specific binding defined with 150 μM unlabeled 2MeSADP. For membrane binding experiments, the reactions were incubated for 2 min at 25°C, and HAWP nitrocellulose filters (Millipore, Bedford, MA, USA) were used for rapid filtration. For the competition studies, 10 nM [^{3}H]2MeSADP was added to aliquots of cells that were incubated with several concentrations of 2MeSADP or 2-methyl-thiol-adenosine monophosphate (2MeSAMP) (Alexis Corp., San Diego, CA, USA).

2.4. Flow cytometry analysis and Western analysis

Approximately, 10^6 cells lifted with ethyleneglycol-bis-(β-amino-ethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and washed with phosphate-buffered saline (PBS), were incubated with rat monoclonal anti-HA 3F10 antibody or purified rat $IgG_{1\kappa}$ (10 μg/ml each) for 1 h at 4°C. After a second wash, cells were labeled with anti-rat IgG_1/IgG_{2a}-fluorescein isothiocyanate (FITC) (1:100 dilution) for 1 h at 4°C. Cells were then washed twice and analyzed with a fluorescence-activated cell sorter (FACS)-Calibur flow cytometer (Becton Dickinson, Mountain view, CA, USA). For Western analysis, samples were run on 4–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel (Invitrogen, Carlsbad, CA, USA) at 120 V and then transferred to nitrocellulose membrane. Anti-HA 3F10 antibody peroxidase conjugate was used in a 1:500 dilution. Goat anti-mouse IgG peroxidase conjugate was used in a 1:2000 dilution. The chemiluminescent signals (Supersignal kit, Pierce, Rockford, IL, USA) were detected through FluorChem Imaging (Alpha Innotech Corp, San Leandro, CA, USA).

2.5. Cellular cyclic AMP (cAMP) measurement

Wild-type or mutant HA-P_2Y_{12}-expressing cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) with 1 mM 3-isobutyl-1-methylxanthine (IBMX). The cells were exposed to various concentrations of 2MeSADP with 1 μM forskolin dissolved in DMEM containing 1 mM IBMX for 15 min at 37°C. The cells were harvested by centrifugation and lysed with 0.1 M HCl with boiling. Intracellular cAMP was measured using a direct cAMP enzyme immunoassay kit (Assay Designs, Ann Arbor, MI, USA) following the manufacturer's instructions. The cAMP concentrations were calculated by the displacement curve obtained from the standard cAMPs.

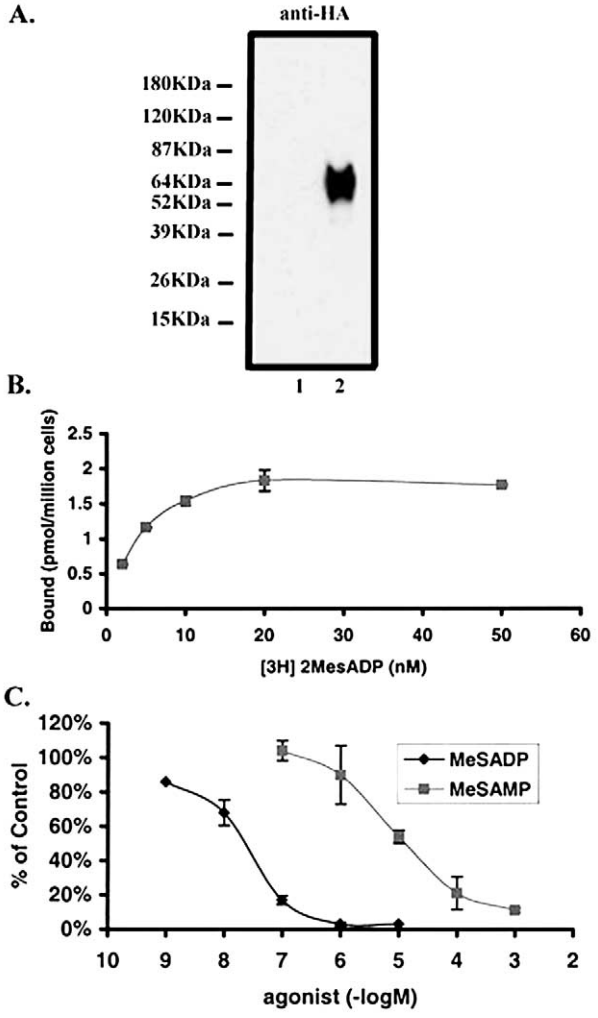

Fig. 1. Stable expression of the P_2Y_{12} receptor in CHO cells. A: Immunodetection of the P_2Y_{12} receptor with anti-HA antibody. The whole cell extracts of the control CHO cells (lane 1) and the P_2Y_{12}-expressing CHO cells (100-17) (lane 2) were separated by 4–20% SDS–PAGE and immunoblotted with rat monoclonal anti-HA 3F10 antibody. B: Saturation binding of [^{3}H]2MeSADP to the P_2Y_{12}-expressing cells. The radioligand binding assay was performed as described in Section 2. Cells were incubated with 1–50 nM [^{3}H]2MeSADP in the absence or presence of 150 μM unlabeled 2MeSADP. C: Competition for [^{3}H]2MeSADP binding to the P_2Y_{12}-expressing cells. 10 nM [^{3}H]2MeSADP was added with cell aliquots in the presence of 2MeSADP or 2MeSAMP. The data are the means ± S.D. ($n = 4$).

3. Results

3.1. Stable expression of P_2Y_{12} receptor in CHO cells

In order to study structure and function of the P_2Y_{12} receptor, stably expressing P_2Y_{12} receptor with an amino-terminal HA tag and a carboxy-terminal 1D4 tag was achieved by transfecting CHO-DHFR$^-$ cells with the receptor-expressing plasmid and by selecting clones resistant to 100 nM methotrexate. The stable clones were screened by immunoblot detection with rat monoclonal anti-HA 3F10 antibody. Out of 80 clones screened, one of the high expressing clones (100-17) was chosen for further studies. As shown in Fig. 1A, the anti-HA antibody 3F10 recognized a predominant band with an approximate molecular weight of 64 kDa (estimated 41.7 kDa), which is consistent with the notion that P_2Y_{12} is a glycoprotein. To confirm whether the HA tagged P_2Y_{12} receptor can bind ADP, the stable cell line was also measured for the whole cell binding to [^{3}H]2MeSADP (Fig. 1B). The clone displays a K_d of approximately 5 nM and a B_{max} of 2–3 pmol/10^6 cells. To determine the binding specificity of the HA tagged receptor, competition binding experiments were carried out in the stable cell line (Fig. 1C). The IC_{50} value of 2MeSADP is around 25 nM, whereas IC_{50} of 2MeSAMP is around 16 μM. These values are consistent with the previously reported values [16]. Surface expression of the wild-type P_2Y_{12} in the stable cell line was confirmed by flow cytometry anal-

ysis using rat monoclonal antibody directed against the HA tag at the amino-terminus of the receptor (see Fig. 2A).

3.2. Treatment with tunicamycin has no effect on the surface expression and ligand binding of the P_2Y_{12} receptor

Analysis of its amino acid sequence [9,10] predicts that the human P_2Y_{12} receptor contains two consensus sequences for N-linked glycosylation at residues 6 and 13 within its extracellular amino-terminus. To study the role of N-linked glycosylation on cell surface expression, we used tunicamycin, an inhibitor that specifically inhibits the first step in the formation of the core oligosaccharide in N-linked glycosylation. The P_2Y_{12} stable cell line 100-17 was treated with 5 μg/ml of tunicamycin or with Me_2SO solvent for 48 h. Cells were then labeled with anti-HA antibody for the flow cytometry analysis. As shown in Fig. 2A, the P_2Y_{12} receptors in tunicamycin-treated and mock-treated cells were both detected at the cell surfaces. When cell extracts from these samples were analyzed on SDS–PAGE and immunoblotted with anti-HA antibody, the apparent molecular weight of P_2Y_{12} decreased to approximately 42 kDa in the tunicamycin-treated cells (Fig. 2B, lane 2), a size consistent with the estimated molecular weight of P_2Y_{12} with tags (41.7 kDa). Moreover, the size of the protein expressed in tunicamycin-treated cells was identical to that of a mutant P_2Y_{12} receptor lacking two glycosylation sites (see below), indicating that the tunicamycin treatment successfully prevented the N-linked glycosylation of the receptor. These results suggest that N-linked glycosylation is not required for surface expression of the P_2Y_{12} receptor.

To assess the specific binding of [^{3}H]2MeSADP to the non-glycosylated P_2Y_{12} receptors in the tunicamycin-treated cells, saturation whole cell binding studies were performed (Fig. 2C). Analysis of the [^{3}H]2MeSADP binding data revealed that the mock-treated cells had a K_d value of 4.6 nM and a B_{max} value of 3.6 pmol/10^6 cells, whereas the tunicamycin-treated cells had a K_d value of 9.6 nM and a B_{max} value of 3.89 pmol/10^6 cells. These results indicate that the ligand binding properties of non-glycosylated P_2Y_{12} receptors in tunicamycin-treated cells are similar to that of the wild-type receptors. Since [^{3}H]2MeSADP is membrane impermeable, this result also supports the flow cytometry analysis data showing that the non-glycosylated P_2Y_{12} receptor is present on the cell surface.

3.3. Site-directed mutagenesis further confirms that the glycans carried at Asn^6 and Asn^{13} of the P_2Y_{12} receptor are not required for its surface expression and ligand binding activity

To further confirm the roles of N-linked glycosylation, Asn^6 and Asn^{13} of the glycosylation sites were mutated to Gln by site-directed mutagenesis either individually or simultaneously to prevent glycosylation. The resulting mutant P_2Y_{12} receptors were transiently expressed in COS-7 cells and the protein expression was examined by immunoblotting

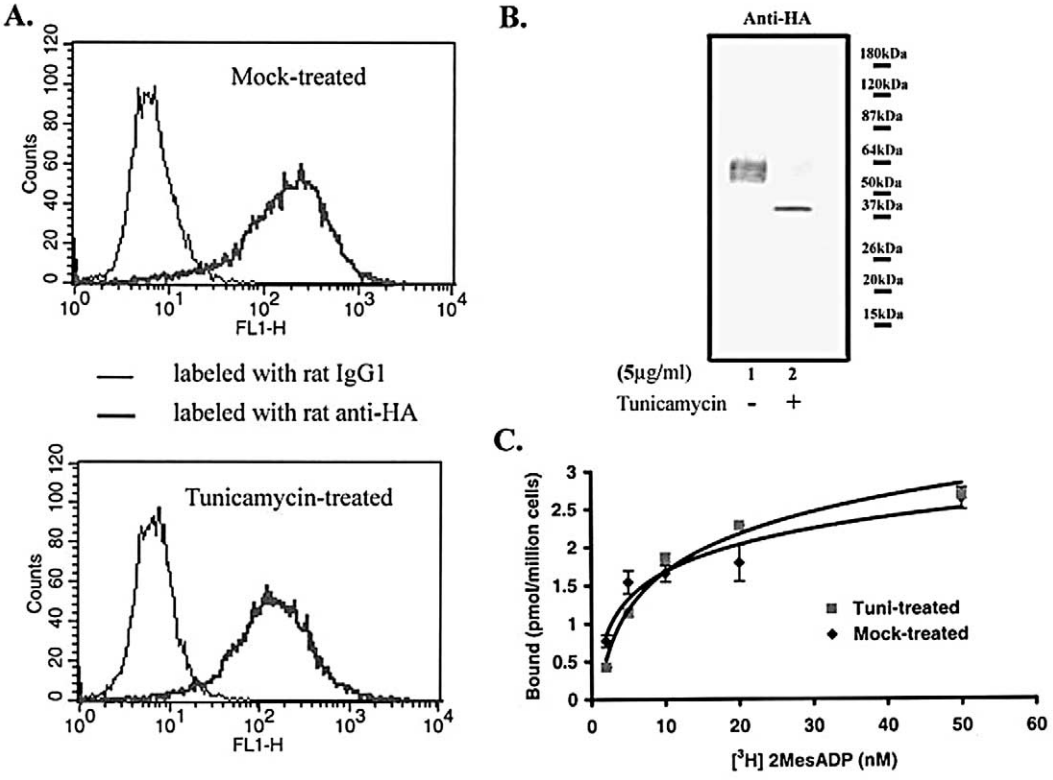

Fig. 2. Treatments with tunicamycin have no effect on the surface expression and ligand binding of the P_2Y_{12} receptor. A: The P_2Y_{12}-expressing cells, treated with tunicamycin or with Me_2SO alone, were stained with anti-HA monoclonal antibody 3F10 or isotypic rat IgG_1, and subjected to FACS analysis, as described in Section 2. B: The P_2Y_{12}-expressing cells were incubated for 48 h in the presence of Me_2SO (lane 1) or tunicamycin (lane 2). The whole cell extracts were resolved in 4–20% SDS–PAGE and immunoblotted with rat anti-HA 3F10 antibody. C: The [^{3}H]2MeSADP binding of the tunicamycin-treated or the mock-treated cells was measured as described in Section 2. The data are the means ± S.D. ($n = 2$).

with anti-HA. As shown in Fig. 3A, substituting either Asn^6 (lane 3) or Asn^{13} (lane 2) with Gln caused a faster migration of P_2Y_{12} in SDS–PAGE, suggesting the mutant receptors receive fewer N-glycan modifications than the wild-type receptor. When both Asn^6 and Asn^{13} were mutated to Gln (lane 1), one single 42-kDa band of P_2Y_{12} was detected by the anti-HA antibody, which is identical to the migration of the deglycosylated band shown in Fig. 2C. These results indicate that both Asn^6 and Asn^{13} were utilized for N-linked glycosylation in vivo. To determine the cell surface expression of the mutant receptors, COS-7 cells transiently transfected with the mutant constructs were labeled with anti-HA antibody and subjected to FACS analysis. As shown in Fig. 3B, all three mutant receptors (N6Q, N13Q, N6Q/N13Q) exhibit almost identical surface expression patterns to the wild-type receptor in the flow cytometry analysis data, which is consistent with the results of tunicamycin treatments (Fig. 2A). All these data indicate that N-linked glycosylation is not essential for the surface expression of the P_2Y_{12} receptor.

To characterize the ligand binding of the N-linked mutants

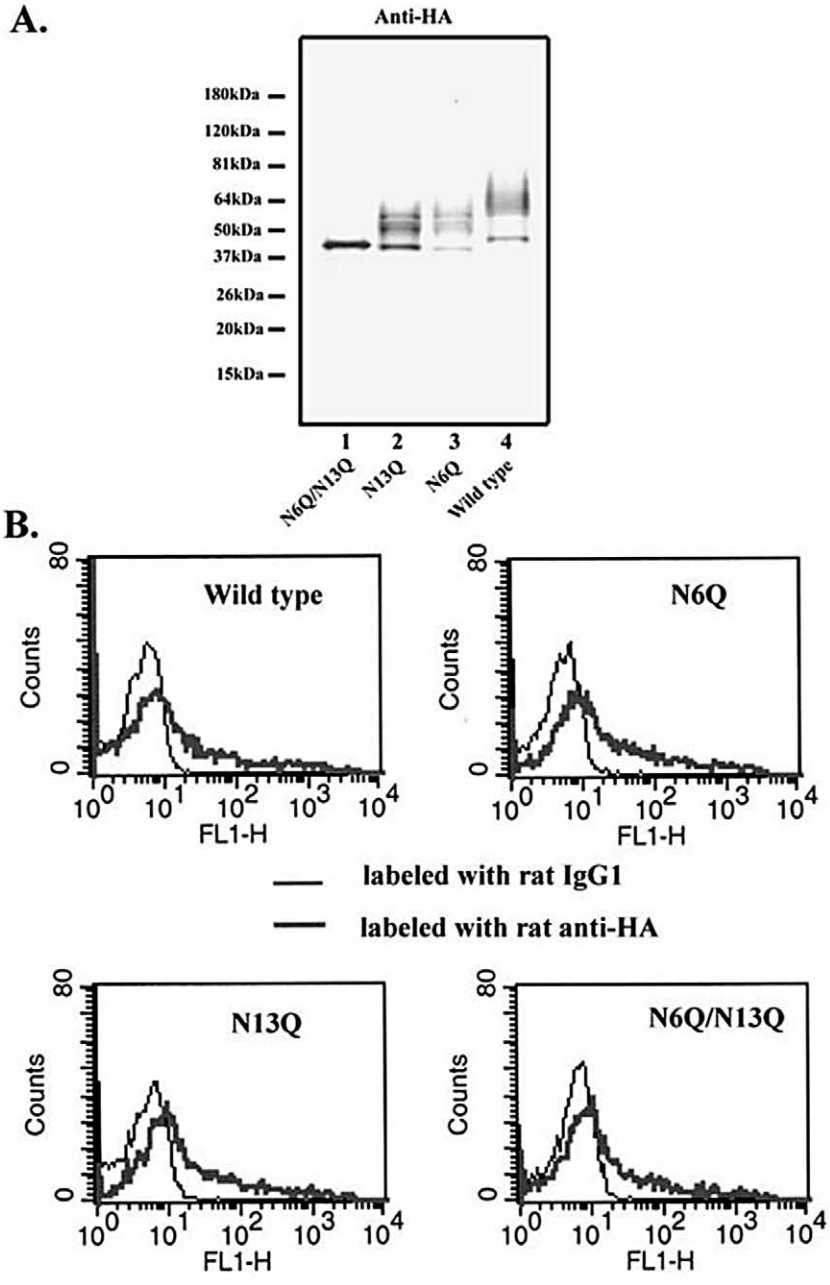

Fig. 3. N-linked glycans at Asn^6 and Asn^{13} of the P_2Y_{12} receptor are not required for its surface expression. A: Plasmid DNA of pWZ1002 (wild-type receptor), pWZ1006 (N6Q mutant), pWZ1007 (N13Q mutant), and pWZ1008 (N6Q/N13Q mutant), were transiently transfected into COS-7 cells as described in Section 2. 50 μg/ml of crude membranes isolated from these cells were subjected to 4–20% SDS–PAGE and immunoblotted with rat monoclonal anti-HA 3F10 antibody. B: Flow cytometry analysis indicates that the P_2Y_{12} receptors missing either or both N-linked glycosylation sites have a normal cell surface expression. COS-7 cells, transiently transfected with wild-type or mutant P_2Y_{12}-expressing plasmids, were stained with anti-HA 3F10 antibody or isotypic rat IgG_1, and subjected to FACS analysis, as described in Section 2.

(N6Q, N13Q, N6Q/N13Q), the COS-7 cells transiently expressing the mutant and wild-type receptors were measured for the saturation whole cell binding of [^{3}H]2MeSADP (Fig. 4A). The results showed that B_{max} of mutants N6Q (1.82 pmol/10^6 cells), N13Q (1.50 pmol/10^6 cells), N6Q/N13Q (0.97 pmol/10^6 cells), and wild-type (1.47 pmol/10^6 cells) are similar, whereas the K_d of the three mutants (11.3, 9.6, 14 nM, respectively) slightly increased as compared to wild-type (5.6 nM). To find out whether the binding specificity of the non-glycosylated mutant receptor is affected, competition experiments were carried out for the COS-7 cells transiently expressing wild-type and N6Q/N13Q mutant receptors. As shown in Fig. 4B, both wild-type and N6Q/N13Q mutant receptors display similar competition curves for 2MeSADP and 2MeSAMP. The N6Q/N13Q mutant has a slightly decreased IC_{50} of 2MeSADP (6.3 nM) in comparison with wild-type (20 nM), while the IC_{50} of 2MeSAMP for wild-type (4 μM) and N6Q/N13Q (2.5 μM) were similar. Taken together, these data indicate that the elimination of either or both N-linked glycosylation sites does not appear to have a significant effect on affinity or specificity of ligand binding to P_2Y_{12} receptor.

3.4. *N-linked glycosylation is important for the P_2Y_{12}-mediated inhibition of adenylyl cyclase*

It has been previously shown that P_2Y_{12} receptor is coupled to a G_i-mediated signaling pathway, which inhibits adenylyl cyclase [9,10]. To assess whether N-linked glycosylation plays a role in the signal transduction function of the receptor after ligand binding, stably expressed CHO cells of wild-type and N6Q/N13Q mutant HA-P_2Y_{12} receptors were constructed. As shown in the inset in Fig. 5, the cell lines of wild-type and N6Q/N13Q mutant receptors possess similar [^{3}H]2MeSADP binding levels and binding patterns, indicating that the expression levels of the receptors are similar. The effect of P_2Y_{12} activation on the adenylyl cyclase activity in these cells was then measured. As described in Section 2, the P_2Y_{12}-expressing CHO cells were challenged with 1 μM forskolin and the cellular cAMP contents were strongly increased. When the cells were co-stimulated with different concentrations of 2MeSADP, the intracellular levels of cAMP in the wild-type HA-P_2Y_{12}-expressing cells decreased in a concentration-dependent manner (Fig. 5). No such inhibition was observed when performed on non-transfected CHO cells (data not shown). The EC_{50} value of 2MeSADP for the wild-type HA-P_2Y_{12}-expressing cells is around 0.4 nM, which is within the previously reported values ranging from 0.078 to 14 nM [9,22–23]. However, when the N6Q/N13Q mutant HA-P_2Y_{12}-expressing cells were co-stimulated with 2MeSADP and forskolin, no inhibitory response to the forskolin-induced cAMP accumulation was observed (Fig. 5). 2MeSADP failed to trigger a concentration-dependent inhibition of the adenylyl cyclase activity in the cells expressing the non-glycosylated mutant receptor, even though the mutant receptor had a similar [^{3}H]2MeSADP binding activity to the wild-type receptor. Thus, these results indicate that although the non-glycosylated P_2Y_{12} receptor is functional in the ligand binding, it is defective in signal transduction.

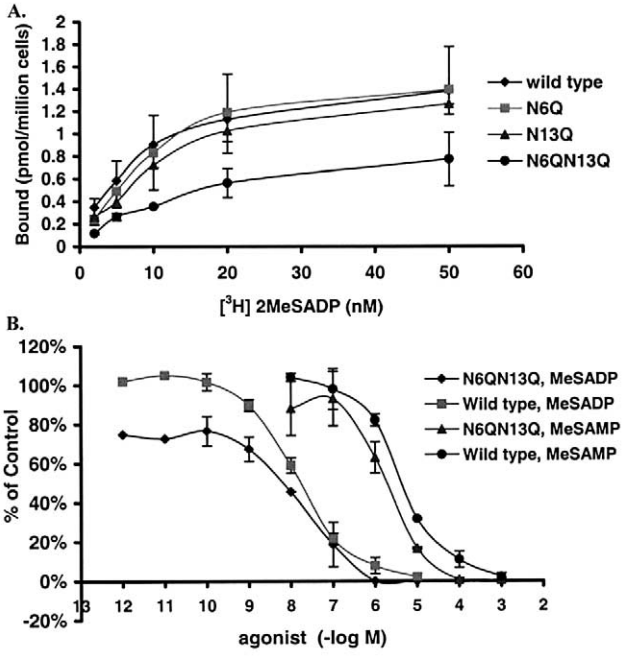

Fig. 4. N-linked glycosylation is not required for the ligand binding activity of the P_2Y_{12} receptor. A: Saturation binding of [^{3}H]2MeSADP to the COS-7 cells transiently transfected with P_2Y_{12}-expressing plasmids. The [^{3}H]2MeSADP binding assays, as described in Section 2, were performed in COS-7 cells transfected with wild-type or mutant P_2Y_{12} receptor cDNAs. B: Competition for [^{3}H]2MeSADP binding to the COS-7 cells expressing wild-type and non-glycosylated mutant P_2Y_{12} receptors. COS-7 cells, transiently transfected with plasmid DNAs expressing wild-type and N6Q/N13Q mutant receptors, were incubated with 10 nM [^{3}H]2MeSADP and different concentrations of 2MeSADP or 2MeSAMP. All data represent the means ± S.D. ($n = 4$).

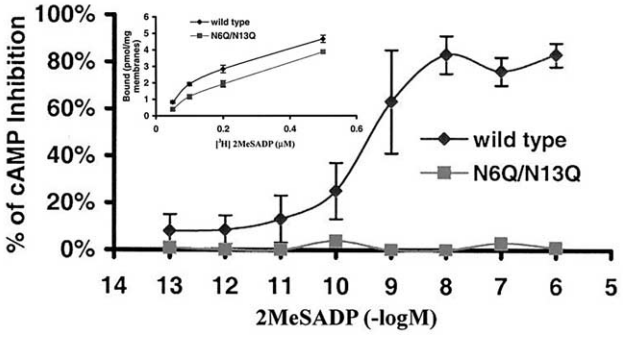

Fig. 5. N-linked glycosylation is important for the P_2Y_{12}-mediated inhibition of adenylyl cyclase. The concentration-dependent inhibition by 2MeSADP of the forskolin-induced cAMP accumulation in the HA-P_2Y_{12} stably expressed CHO cells were performed as described in Section 2. Both wild-type and N6Q/N13Q mutant receptor-expressing CHO cells were treated with IBMX (1 mM) and challenged with forskolin (1 μM) and 2MeSADP. The cAMP concentrations in the presence of IBMX alone, and in the presence of forskolin and IBMX, were normalized to 0 and 100%, respectively. The data represent the means ± S.D. ($n = 4$). The graph in the inset compares the [^{3}H]2MeSADP binding on crude membrane fractions isolated from the same cells. The data represent the means ± S.D. ($n = 2$).

4. Discussion

This study demonstrates that two potential N-linked glycosylation sites of the P_2Y_{12} receptor are both glycosylated in vivo, and that N-linked glycosylation is essential for signal transduction but not for the receptor's expression on the cell surface and ligand binding activity. This conclusion is

supported by the evidence obtained from two different approaches. First, in stably expressing P_2Y_{12} CHO cells, tunicamycin, an inhibitor of N-linked glycosylation, causes no effect on the surface expression and ligand binding of the receptor. The inhibition of N-linked glycosylation by tunicamycin is confirmed by a shift in the molecular weight of the protein in the tunicamycin-treated cells. Second, because tunicamycin has a general action on glycoprotein synthesis, the consensus N-linked glycosylation sequences have been removed by site-directed mutagenesis. The mutated non-glycosylated receptor retains cell surface expression and ligand binding activity. Furthermore, the study also revealed that the N-linked carbohydrate addition is instead required for the signal transducing step of the receptor, as the unglycosylated mutant receptor is defective in the P_2Y_{12}-mediated inhibition of the adenylyl cyclase activity despite a normal binding activity. This study is the first report to describe the roles of N-linked glycosylation in the normal activity of purinergic receptors of the P2Y subclass.

The data from this study suggest that the folding and the delivery of P_2Y_{12} receptor to the cell surface are apparently independent of the oligosaccharide addition process. This is clearly in contrast to those of the P2X receptors where N-linked glycans have been shown to play an essential role for their surface expression [17–20]. It has been speculated that N-glycan modification affects the oligomer assembly or initial folding of the receptors for further intracellular trafficking [17,19,20]. P2X receptors have two transmembrane domains and a large extracellular loop, while P2Y receptors contain seven transmembrane domains. The difference in topological arrangement between P2X receptors and P2Y receptors may reflect the difference in membrane trafficking requirement between these two types of purinergic receptors. It will be interesting to find out the roles of N-linked glycans on the surface expression of other P2Y receptors.

One surprising finding of this study is that the N-glycans at the extracellular amino-terminus of the P_2Y_{12} receptor are involved in the signal transducing step after the ligand is bound. It may imply that a conformational change of the extracellular amino-terminus affected may be required for signal transfer through the membrane upon agonist ligation. This result is consistent with a recent report [24] that the third extracellular loop (EL3) of the P_2Y_{12} receptor appears to be crucial for postoccupancy signaling. Two autosomal P_2Y_{12} mutations (one within EL3 and the other at the boundaries of EL3 and TM6), identified from a patient with a congenital bleeding disorder, result in a normal 2MeSADP binding but a defective ADP-dependent inhibition of forskolin-stimulated adenylyl cyclase activity [24]. It seems that the extracellular portions of the P_2Y_{12} receptor including its amino-terminus and extracellular loops are directly involved in the signal transfer to the cytoplasm, through a conformational change triggered by the ligand binding at the transmembrane domain regions.

N-linked glycan structures of P_2Y_{12} expressed on platelets are still unknown. Even though P_2Y_{12} expressed on CHO cells that generally produce bi-, tri- and tetra-antennary sialylated glycans, is functional, yet the effect of different glycan structures on its surface expression, ligand binding or signalling remains unknown. It will be interesting to examine P_2Y_{12} expressed on various CHO glycosylation mutants [25] producing hybrid, high mannose and truncated complex carbohydrates or produced in presence of various N-linked glycosylation inhibitors. It will also be interesting to find out whether refurbishing either of N-linked glycans to P_2Y_{12} will restore the signal transduction function. Further detailed experiments will be needed to address all these questions.

In summary, the present study demonstrates that although N-linked glycosylation is not absolutely required for cell surface expression and ligand binding of the P_2Y_{12} receptor, it is important for the signal transducing step upon ligand binding. As P_2X_1, P_2X_2, and P_2X_4 have similar functional consequences of blocking N-linked glycosylation, it is possible that other members of the P2Y family might share the phenotype of the P_2Y_{12} receptor.

Acknowledgements: We thanks Dr. Joel Bard for comments on the manuscript, Drs. Mark Pausch and Chu-Lai Hsiao for plasmids. We thank Ella Presman for tissue culture support.

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