N-Linked Protein Glycosylation Is a Major Determinant for Basal TRPC3 and TRPC6 Channel Activity*

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The TRPC family of receptor-activated cation channels (TRPC channels) can be subdivided into four subfamilies based on sequence homology as well as functional similarities. Members of the TRPC3/6/7 subfamily share common biophysical characteristics and are activated by diacylglycerol in a membrane-delimited manner. At present, it is only poorly understood whether members of the TRPC3/6/7 subfamily are functionally redundant or whether they serve distinct cellular roles. By electrophysiological and fluorescence imaging strategies we show that TRPC3 displays considerable constitutive activity, while TRPC6 is a tightly regulated channel. To identify potential molecular correlates accounting for the functional difference, we analyzed the glycosylation pattern of TRPC6 compared with TRPC3. Two N-linked motifs in TRPC6 were mutated (Asn to Gln) by in vitro mutagenesis to delete one or both extracellular N-linked glycosylation sites. Immunoblotting HEK293 cells expressing TRPC6 wild type and mutants favors a model of TRPC6 that is dually glycosylated within the first (e1) and second extracellular loop (e2) as opposed to the monoglycosylated TRPC3 channel (Vannier, B., Zhu, X., Brown, D., and Birnbaumer, L. (1998) J. Biol. Chem. 273, 8675–8679). Elimination of the e2 glycosylation site, missing in the monoglycosylated TRPC3, was sufficient to convert the tightly receptor-regulated TRPC6 into a constitutively active channel, displaying functional characteristics of TRPC3. Reciprocally, engineering of an additional second glycosylation site in TRPC3 to mimic the glycosylation status in TRPC6 markedly reduced TRPC3 basal activity. We conclude that the glycosylation pattern plays a pivotal role for the tight regulation of TRPC6 through phospholipase C-activating receptors.

Following binding to cognate receptors on the cell membrane, many hormones, neurotransmitters, and growth factors elicit increases in the intracellular free calcium concentration ([Ca^{2+}]) subsequent to phospholipase C activation. In addition to inositol 1,4,5-trisphosphate (InsP_3)-mediated calcium release from intracellular stores, Ca^{2+}-permeable plasma membrane ion channels are activated in a PLC-dependent manner in most cells either by second messenger-mediated pathways or by store depletion (1). To date, seven mammalian homologues of the Drosophila melanogaster transient receptor potential (TRP) visual transduction channels (2), the classical TRPC channel proteins (TRPC), have been identified. They were characterized as subunits of receptor-activated (ROCs) and store-operated (SOCS) cation channels (see Refs. 3–6 for recent reviews). TRPC proteins are thought to span the plasma membrane 6 times with a pore loop inserted between transmembrane segments 5 and 6 (7), and functional channel complexes are believed to be homo- and heterotetrameric. In principle, all TRPC proteins combine into multimeric complexes within the confines of TRPC subfamilies as assessed in living cells (8).

Based on structural and functional criteria, TRPC3, -6, and -7 form a distinct subgroup within the TRPC family. The primary amino acid sequences of TRPC3, -6, and -7 are ~75% identical, and the latter proteins represent non-selective cation channels characterized by both inward and outward rectification. A functional hallmark of the TRPC3, -6, -7 subfamily is their membrane-delimited activation by diacylglycerols (DAGs) independent of protein kinase C (9, 10). Furthermore, in a heterologous expression system (8) as well as in rat brain synaptosomes (11) TRPC3, -6, and -7 interact with each other, but not with other members of the TRPC family (8). Interestingly, both TRPC3 and TRPC6 are expressed in brain (12, 13). TRPC3 expression in the brain is most prominent around the time of birth, and in pontine neurons channel activation occurs through a signaling pathway initiated by binding of brain-derived growth factor to its cognate receptor, the transmembrane receptor tyrosine kinase TrkB (14). The role of TRPC6 or putative TRPC3/6 heteromultimers in brain still remain elusive. TRPC3 was originally cloned from human embryonic kidney cells (HEK 293 cells) (12) and functionally contributes to calcium permeable ion channels gated by store depletion in these cells (15). Low level stable expression of TRPC3 in HEK 293 cells gave rise to ion currents only when IP_3 is bound to its receptor and internal calcium stores are depleted (16). At the molecular level, interacting domains of the IP_3 receptor and TRPC3 have been characterized in great detail, thus entertaining the notion of store-dependent activation by a conformational coupling mechanism (Ref. 17 and see Ref. 18 for a recent review) and inhibition by Ca^{2+}/calmodulin (19). Although TRPC3 expressed in DT40 avian B-lymphocytes show activation by store depletion (20), the latter concept is not undisputed, and a predominant store-operated activation mecha-


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The abbreviations used are: InsP_3, inositol 1,4,5-trisphosphate; TRPC, classical transient receptor potential channel; DAG, diacylglycerol; HeLa, human embryonic kidney cells; eGFP, enhanced green fluorescent protein; YFP, yellow fluorescent protein; CHO, Chinese hamster ovary; ANOVA, analysis of variance; Me_2SO, dimethyl sulfoxide; e1/2, first/second extracellular loop.
nism could not be confirmed in transiently transfected HEK 293 cells (20), Chinese hamster ovary (CHO) cells (21) and in a HEK 293 cell line stably transfected with TRPC3 (22). Most notably, the latter two studies and a report by Hurst et al. (23) describe a high basal activity of TRPC3 channels.

In the present study we address the question of whether TRPC3 and TRPC6 are functionally redundant or whether they display distinct functional characteristics. Thus, we monitored the basal activity of TRPC3 and compared it to that of TRPC6 by analysing HEK293 and ITRPC6-expressing HEK 293 cells in patch-clamp experiments and electrophysiological recordings. In contrast to TRPC6, which turns out to be a tightly receptor- and phospholipase C-regulated channel, TRPC3 shows pronounced basal channel activity despite a high degree of overall amino acid identity between the two proteins (5). To identify the underlying cause for the different functional characteristics, we examined the glycosylation pattern of TRPC6 and identified two glycosylated asparagine residues as opposed to the monoglycosylated TRPC3 channel (7). Elimination of the distal glycosylation site in TRPC6 (N561Q) by in vitro mutagenesis was sufficient to convert the tightly regulated TRPC6 protein into a constitutively active channel, resembling TRPC3 in this regard. Considerably, engineering of a second distal glycosylation site in TRPC6 (N561Q) by in vitro mutagenesis kit (Stratagene Europe, Amsterdam, The Netherlands) was found to partially rescue channel activity, but not to completely reconstitute baseline activity as observed in TRPC3, thereby arguing against a simple all-or-nothing effect of the N-glycosylation sites. It appears likely that additional factors contribute to the activation of TRPC3 channels.

**EXPERIMENTAL PROCEDURES**

Materials—All cell culture media and supplements were purchased from PAA laboratories (Linz, Austria)

**Cell Culture and Transient Transfections**—HEK 293 cells were grown in Earle’s medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, and 50 units/ml streptomycin. For transient transfections, cells were seeded into 6-well dishes on glass coverslips and transfected the following day (80% confluency) with Metafectene reagent (Biontex, Munich, Germany). The concentration of transfected cDNA was kept constant by combining 0.2 μl of Metafectene with 1 μl of pcDNA3 (2 μg) downstream of the internal ribosome entry site (IRES) and corrected for by the Pulse software. Current-voltage relations were recorded at 330-ms intervals at 360 nm and normalized to 100% of the initial value. Basal decreases of fluorescence after adding Mn2+, which were calculated in percent of fluorescence at no Mn2+ addition, were subtracted. Each experiment was performed at least three times with similar results.

**Electrophysiological Techniques**—HEK 293 cells were transiently transfected with cDNAs coding for the indicated TRPC subunits in pcDNA3 (2 μg), guinea pig H1 histamine receptor in pcDNA3 (0.5 μg) and GFP reporter plasmid (0.1 μg) using FuGENE 6 reagent (7.8 μl) per 1 μl of pcDNA3 as described above (8). Whole cell patch clamp recordings were carried out at room temperature (23°C) 12–48 h after transfection, except for the TRPC6GM1.2 and TRPC6GM2 mutants, which were analyzed 48–120 h after transfection. Cells were superfused with bath solution (in mM): NaCl 140, CaCl2 1, MgCl2 1, glucose 10, HEPES 10, buffered to pH 7.4 with NaOH. Data of whole cell recordings were collected with an EPC9 patch clamp amplifier (HEKA, Lambrecht, Germany) using the Pulse software. Patch pipettes made of borosilicate glass (Science Products, Hofheim, Germany) had resistances of 2.5 to 3.5 MΩ when filled with intracellular solution (in mM): CsCl 120, NaCl 9.4, MgCl2 1, GTP 0.3, HEPES 15, adjusted to pH 7.2 with CsOH. The liquid junction potential was +4.3 mV and corrected for by the Pulse software. Current-voltage relations were obtained from triangular voltage ramps from −100 to +60 mV with a slope of 0.4 V/s applied at a frequency of 1 Hz. Data were acquired at a frequency of 5 kHz after filtering at 1.67 kHz. Inside-out single-channel measurements were carried out in 48–90 s. Data analysis was performed with an EPC9 and EPC10 patch clamp amplifier using the Pulse software. For the evaluation of channel activity, defined as NPo (the product of the number of channels and open probability) PC DAC 1.1.4 of Marburg University Software Team was

**Tunicamycin Treatment and Innublotting**—HEK 293 cells were transiently transfected with cDNAs coding for the indicated TRPC subunit in pcDNA3 (3 μg) using 9 μl of the FuGENE 6 reagent (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s recommendations. 14 h after transfection one-fifth of the medium was replaced with medium containing 0.1% Me2SO or tunicamycin (10 μg/ml, Calbiochem, San Diego) in 0.1% Me2SO giving rise to a final tunicamycin concentration of 2 μg in 0.02% Me2SO. Cells were washed in phosphate-buffered saline 36 h after transfection and resuspended in a buffer containing 20 mM Tris/HCl (pH 7.5) and protease inhibitors provided through MiniProtein tablets (Roche Applied Science). After repetitive aspiration through a 26-gauge needle, one volume of 2× Laemmli buffer was added. The cell lysate was heated (65 °C for 5 min), and proteins were resolved by 7% SDS-PAGE. After electrophoretic transfer to nitrocellulose (Hybond N, Amersham Biosciences, Freiburg, Germany), membranes were blocked in phosphate-buffered saline supplemented with 5% bovine serum albumin and incubated with an anti-TRPC3 antibody (14) (1:1000 in phosphate-buffered saline supplemented with 0.05% Tween 20). After washing with phosphate-buffered saline/0.05% Tween 20 (3 × 15 min), membranes were incubated with a secondary peroxidase-coupled goat anti-rabbit Ig-G (1: 2000 in blocking medium; Sigma, Taufkirchen, Germany) coupled to an in vitro produced Odyssey IX70 microscope. Cells expressing GFP were selected at 470 nm and analyzed in Mn2+ quench experiments at the isosbestic wavelength of fusion-2 (380 nm in our system) and at 340 and 380 nm for measuring [Ca2+]. Cells were superfused with 2 or 0.2 mM MnCl2 in HBS and 100 μM histamine in HBS/Mn2+ at the time points indicated. Fluorescence signals of cells expressing GFP and the histamine receptor were recorded at 330-ms intervals at 360 nm and normalized to 100% of the initial value. Basal decreases of fluorescence after adding Mn2+, which were calculated in percent of fluorescence at no Mn2+ addition, were subtracted. Each experiment was performed at least three times with similar results.
FIG. 1. Basal and receptor-mediated activity of TRPC3 and TRPC6. A, electrophysiological recordings. HEK 293 cells were transiently cotransfected with plasmids encoding eGFP, the H1 histamine receptor and human TRPC3 or human TRPC6. Current-voltage relationships were analyzed by patch clamp recordings in the whole cell mode in non-transfected unstimulated cells and in transfected cells before (basal) and after application of receptor agonist (histamine). Insets, continuous recordings of TRPC3- and TRPC6-expressing cells before and after application of histamine. Duration of histamine applications is indicated by bars. B, current-voltage relationships (n = 11) of TRPC3-expressing HEK 293 cells were analyzed by patch clamp recordings in the whole cell mode after application of receptor agonist (100 μM histamine). Reversal potentials were...
used. NPo values were calculated for voltage ladders from −60 to +60 mV, recorded in 1-s steps. 1 μM 1-stearoly-2-arachidonoyl-sn-glycerol (SAG), a potent non membrane-permeable diacylglycerol derivative, was added to stimulate the channels in inside-out patches. The sampling rate was 15 kHz and the data were filtered with a −3dB cut-off frequency of 5 kHz. Only patches with baseline noise of <560 fA r.m.s. were used.

Statistical Analysis—Data are presented as means ± S.E. of the mean (S.E.). In the case of Mn2+ quench measurements, one representative experiment out of three is shown. Unless stated otherwise, data were compared by an unpaired Student’s t test, if a Gaussian distribution was confirmed by applying a Kolmogorov-Smirnov (normality) test, and significance was accepted at p < 0.05. For multiple comparisons a one-way ANOVA was applied.

RESULTS

Different Basal Activities of TRPC3 and TRPC6—To address the issue as to whether TRPC3 and TRPC6 are functionally redundant or whether they have discernible characteristics, we set out to compare basal channel activities. To this end, human TRPC3 or TRPC6 were transiently coexpressed with the H1 histamine receptor in HEK 293 cells. Current-voltage relationships before and after application of histamine to the bath solution were recorded in whole cell patch clamp experiments (Fig. 1A). In both TRPC3- and TRPC6-expressing cells H1 receptor activation gave rise to rapidly developing transient outward and inward currents at holding potentials of +60 and −60 mV (Fig. 1A, insets), respectively. The current-voltage relations revealed rectification in the inward and outward direction, a property which is consistently encountered with members of the TRPC3/6 subfamily (25). Most notably, basal TRPC3 activity in the absence of histamine was higher than that of TRPC6 when recorded both at negative and at positive potentials (Fig. 1A). Reversal potentials for TRPC3-expressing cells were −2.1 ± 0.8 and −1.7 ± 1.1 mV before and after histamine application, respectively, and −1.5 ± 1.0 and −2.6 ± 1.0 mV for TRPC6-expressing cells and were not significantly different (p > 0.05 One-way ANOVA). By plotting reversal potentials of TRPC3-expressing cells against current densities at +60 mV and at −60 mV no correlation between both values was detected (Fig. 1B). When basal TRPC3 and TRPC6 activities at −60 and +60 mV were normalized to maximal currents in response to histamine challenge monitored at the same holding potentials, TRPC3-expressing cells displayed significantly (p < 0.002) increased constitutive channel activities (−46.0 ± 5.0% and 48.7 ± 4.3% at −60 and +60 mV, respectively) as compared with HEK 293 cells studied after transfection of TRPC6 cDNA (−10.6 ± 2.3% and 20.9 ± 4.3% at −60 and +60 mV, respectively) (Fig. 1C). The functional differences between the two TRPC proteins did not depend on the type of Gq/11-coupled receptor coexpressed, because TRPC3 and TRPC6 could likewise be distinguished by means of their discrepant basal activities in HEK 293 cells expressing m5 muscarinic instead of H1 histamine receptors (data not shown).

By adding Mn2+ to the bath solution, the influx of extracellular cations into single TRPC protein-expressing cells can be monitored by the quenching of fura-2 fluorescence by Mn2+ ions recorded at the isosbestic wavelength. Subsequent to the addition of 2 mM Mn2+ to the extracellular medium, a profound acceleration of the basal decrease of fura-2 fluorescence (−0.13 ± 0.01%/s) was observed only in TRPC3-expressing cells (Fig. 1D). On the contrary, the presence of TRPC6 did not give rise to increased fura-2 quenching by Mn2+ ions (−0.034 ± 0.008%/s, similar to untransfected cells, data not shown), thus revealing a significantly reduced (p < 0.001) basal activity. These data lend further credence to the concept of high basal TRPC3 activity as opposed to the quiescence of TRPC6 in the absence of phospholipase C activation.

Glycosylation Status of TRPC3 and TRPC6—TRPC3 and TRPC6 are closely related members of the same subfamily of TRPC proteins. We hypothesized that in addition to differences in the primary structure post-translational modifications like protein glycosylation may also contribute to the distinctive functional properties of the two cation channels. Therefore, we searched for potential glycosylation consensus sites (NX(S/T)) in the TRPC6 primary amino acid sequence. We detected seven potential glycosylation sites, only two of which are predicted to face the extracellular space as deduced from the TRPC3 transmembrane topology proposed by Vannier et al. (7). Thus, the N-terminal site, Asn-473, appears to be located in the first extracellular loop (e1) and corresponds to the single glycosylated asparagine residue in TRPC3 (7), while Asn-561 in the second extracellular loop (e2) has no counterpart in the TRPC3 primary amino acid sequence. To test whether these putative glycosylation sites were indeed post-translationally modified in TRPC6 and whether such modifications would entail any functional consequences, we initially created three glycosylation mutants (TRPC6GM1[N473Q], TRPC6GM2[N561Q], TRPC6GM1.2[N473/561Q]) as illustrated in Fig. 2A. In order to minimize the possibility of conformational disruption and to preserve the local charge distribution within the ion channel proteins, we exchanged Asn for structurally very similar Gln residues differing only by one methylene group in the amino acid side chains. TRPC6 and its three mutated versions C-terminally fused to GFP were expressed in HEK 293 cells and analyzed by immunoblotting (Fig. 2B) and confocal laser-scanning microscopy (Fig. 2C).

When lysates of wild-type TRPC6GFP-expressing cells were resolved by SDS-PAGE and probed with an anti-TRPC6 antibody, two specific bands of ~120 and 160 kDa were detected (Fig. 2B). The diffuse band migrating with a higher molecular mass disappeared upon preincubation of cells with the glycosylation inhibitor tunicamycin, commensurate with the assumption that it represented a glycosylated form of TRPC6, while the 120-kDa band conforms to the non-glycosylated ion channel protein. Bands with apparent molecular masses around 80 kDa were also detected in non transfected control cells (left panel of Fig. 2B). Elimination of the N-terminal glycosylation site in TRPC6GM1 did not prevent the appearance of a diffuse slowly migrating, tunicamycin-sensitive band of ~140 kDa indicating that protein glycosylation still occurred at the C-terminal site in e2. Reciprocally, removal of the C-terminal Asn-561 in TRPC6GM2 did not abolish protein glycosylation, because the relevant Asn residue in e1 could still be post-translationally modified. Only lysates of cells expressing TRPC6GM1.2 in which both possible glycosylation sites were eliminated, did no longer show slowly migrating tunicamycin-
and analysis of their glycosylation status. Lysates (50 μg) of HEK 293 cells transfected with TRPC6 and three TRPC6 mutants (TRPC6GM1, 2, and 1.2) with altered potential glycosylation sites in TRPC3 (according to Ref. 6), potential glycosylation sites in TRPC6 and its glycosylation mutants. A, schematic representation of glycosylated sites in TRPC6 (according to Ref. 6), potential glycosylation sites in TRPC6 and three TRPC6 mutants (TRPC6GM1, 2, and 1.2) expressed in HEK 293 cells. TRPC6 and three TRPC6 mutants (TRPC6GM1, 2, and 1.2) fused to GFP were analyzed with the same antibody (TRPC6-Ab). Band corresponding to the glycosylated form of TRPC6GFP are indicated by black triangles. Numbers indicate the molecular masses of the marker proteins in kDa.

**Fig. 2.** Glycosylation status of TRPC3, TRPC6, and TRPC6 glycosylation mutants. A, schematic representation of glycosylated sites in TRPC6 (according to Ref. 6), potential glycosylation sites in TRPC6 and three TRPC6 mutants (TRPC6GM1, 2, and 1.2) expressed in HEK 293 cells and analysis of their glycosylation status. Lysates (50 μg) of HEK 293 cells transfected with pcDNA3 or pcDNA3 with an inserted TRPC6GFP- or TRPC3YFP-cDNA (left panel) were analyzed with an anti-TRPC6 antibody (TRPC6-Ab). Cell lysates (10 μg) expressing human TRPC6 or each of the three glycosylation mutants (TRPC6GM1, 2, 1.2) fused to GFP were analyzed with the same antibody (right panel).

responsive protein bands. Thus, in contrast to TRPC3 modified only at one site in e1, our results strengthen the notion of a dually glycosylated TRPC6 channel. As protein glycosylation is known to have a profound impact on proper folding and plasma membrane targeting, we asked whether the glycosylation mutants would be correctly inserted into the cell membrane and assessed the cellular localization of TRPC6 proteins C-terminally fused to GFP by confocal laser microscopy. As shown in Fig. 2C, the cellular distribution of the three TRPC6 mutants was indistinguishable from the wild-type channel, and the overlay of fluorescence and differential interference contrast images demonstrated that all TRPC6 proteins were correctly targeted to the plasma membrane.

**Increased TRPC6 Basal Activity of TRPC6 Glycosylation Mutants**—To evaluate the effect of protein glycosylation on TRPC6 basal activity, TRPC6GM2, devoid of N-linked glycosylation in e2, thereby mimicking the glycosylation status of TRPC3, and TRPC6GM1.2 lacking any oligosaccharide modifications (Fig. 2, A and B) were transiently expressed in HEK 293 cells in conjunction with the G_{o11} coupled H_{3} histamine receptor. Icnic currents before and after addition of histamine were examined with the whole cell patch clamp technique. When compared with wild-type TRPC6 (Fig. 1A, lower panel), the current-voltage relationships of HEK 293 cells expressing either glycosylation mutant revealed increased basal channel activity, which was most conspicuous at negative membrane potentials (Fig. 3A). Histamine stimulation further increased inward and outward whole cell currents.

When basal activities at −60 and +60 mV were normalized to maximal agonist-induced currents, TRPC6GM2-expressing cells displayed significantly (p < 0.002) increased constitutive channel activity (−44.9 ± 4.3% and 47.4 ± 4.1% at −60 and +60 mV, respectively) as opposed to HEK 293 cells transfected with TRPC6 cDNA (Fig. 3B; see Fig. 1C). Likewise, normalized TRPC6GM1.2 basal activity (−47.6 ± 5.3% and 59.4 ± 4.9% at −60 and +60 mV, respectively) was significantly higher than that observed upon TRPC6 expression, but almost identical to basal activity levels recorded for TRPC3 (Fig. 3B; see Fig. 1C).

When comparing the current-voltage relationships characteristic for TRPC6 (Fig. 1A) with those obtained with the two glycosylation mutants (Fig. 3A), we noted that apart from differences in basal channel activity outward rectification upon histamine stimulation was reduced in the case of the mutant TRPC6 proteins. Therefore, it appeared deemed legitimate to ask whether our normalization procedure might systematically overestimate basal channel activity at positive potentials. To address this issue, current densities were determined under basal conditions immediately after establishing the whole cell configuration in cells expressing the wild-type or mutant TRPC6 proteins. The synopsis of cells expressing wild-type TRPC6 or one of the glycosylation mutants (Fig. 3C) illustrates that progressive ablation of protein glycosylation (wild-type TRPC6 → TRPC6GM2 → TRPC6GM1.2) resulted in an increase in current densities at negative and positive membrane potentials.

**Experimental Procedures.** Proteins were detected by the TRPC6-specific antibody. Bands corresponding to the glycosylated form of TRPC6GFP are indicated by black triangles. Numbers indicate the molecular masses of the marker proteins in kDa. C, subcellular localization TRPC6 and TRPC6GM1, 2, 1.2. Human TRPC6 and TRPC6GM1, 2, 1.2 were C-terminally fused to GFP, transiently transfected into HEK 293 cells and analyzed by confocal laser scanning microscopy. GFP fluorescence, differential interference contrast (DIC) images, and overlays of GFP fluorescence and corresponding DIC images are depicted.
FIG. 3. Basal activity of TRPC6 glycosylation mutants (TRPC6GM1, 2, 1.2). A, electrophysiological recordings. HEK 293 cells were transiently cotransfected with a pcDNA3 plasmid encoding the H1 histamine receptor and a pIRES plasmid encoding the TRPC6 glycosylation mutant TRPC6GM2 or TRPC6GM1.2 downstream and the cDNA of eGFP upstream of the internal ribosomal entry site (IRES). Current-voltage relationships were analyzed by patch clamp recordings in the whole cell mode before (basal) and after application of the receptor agonist (histamine) to the pipette solution. B, summary of basal activities of TRPC6GM2- (n = 22) and TRPC6GM1.2- (n = 16) expressing cells analyzed at −60 and +60 mV. Values were normalized to histamine-induced increases at the respective holding potentials. C, analysis of current densities of TRPC6- (n = 12), TRPC6GM2- (n = 18), and TRPC6GM1.2- (n = 18) expressing cells. Transfected cells were analyzed by patch clamp recordings.
Fig. 4. Glycosylation status of TRPC3 and TRPC6Gly2. A, schematic representation of the glycosylated site in TRPC3 and a mutant (TRPC3Gly2) with an additional potential glycosylation site (E512XS → N512XS). B, immunoblot analysis of TRPC3 and TRPC3Gly2 expressed in HEK 293 cells and analysis of their glycosylation status. Lysates (50 μg) of HEK 293 cells transfected with pcDNA3 or pcDNA3 with an inserted TRPC6GFp- or TRPC3YFP-cDNA (left panel) were analyzed with an anti-TRPC6 antibody (TRPC6-Ab). Cell lysates (10 μg) expressing human TRPC3 or TRPC3Gly2 were analyzed by the same antibody (right panel). MeSO (0.02% [−]) or tunicamycin (2 μg) as a glycosylation inhibitor dissolved in 0.02% MeSO (+)) was added to the cells as described under “Experimental Procedures.” Proteins were detected by the TRPC3-specific antibody. Bands corresponding to the glycosylated forms of TRPC3 are indicated by black triangles. C, subcellular localization TRPC3 and TRPC3Gly2. Human TRPC3 and TRPC3Gly2 were C terminally fused to YFP, transiently transfected into HEK 293 cells and analyzed by confocal laser scanning microscopy. YFP fluorescence, differential interference contrast (DIC) images, and overlays of YFP fluorescence and corresponding DIC images are depicted.

43.3% at +60 mV were observed in TRPC6GM2-containing isolated patches. Thus, basal channel activities determined by two different electrophysiological approaches were very similar in isolated inside-out patches and in the whole cell configuration of TRPC6 and TRPC6GM2-expressing cells (see Figs. 1C and 3B) and strengthen the concept that the protein glycosylation pattern has a profound impact on basal channel activity.

The notion of increased basal activity of TRPC6 glycosylation mutants was additionally supported by measuring basal Mn2+ entry into fura-2-loaded cells expressing TRPC6 mutants (Fig. 3D). In the case of all three glycosylation mutants tested, the addition of 2 mM Mn2+ to the extracellular medium was followed by a pronounced decline of fura-2 fluorescence (TRPC6GM1: −0.16 ± 0.02%/s; TRPC6GM2: −0.15 ± 0.02%/s; TRPC6GM1.2: −0.17 ± 0.02%/s), reminiscent of genuine TRPC3 properties, but in contrast to the quiescence of wild-type TRPC6 (p < 0.001 for all three glycosylation mutants compared with TRPC6) under these experimental conditions (cf. Fig. 1D, lower panel).
Engineering of a Dually Glycosylated TRPC3 Mutant (TRPC3GM2)—After having learned that a reduction of the TRPC6 glycosylation pattern resulted in increased basal channel activity, we embarked on a reciprocal approach and introduced an additional glycosylation consensus site into e2 of TRPC3 to generate TRPC3Gly2 [E512N], hypothesizing that such a measure should reduce receptor-independent activity. TRPC3Gly2 was hence assumed to be subject to protein glycosylation at two sites corresponding to those in the dually modified TRPC6 (Fig. 4A). TRPC3 and its glycosylation mutant were C-terminally fused with the yellow fluorescent protein (YFP), expressed in HEK 293 cells and examined by immunoblotting (Fig. 4B) and confocal laser scanning microscopy (Fig. 4C).

Lysates of TRPC3-expressing cells were subjected to SDS-PAGE and probed with an anti-TRPC3 antibody. An incompletely resolved diffuse TRPC3 doublet was detected, the higher molecular mass component of which disappeared after
pretreatment of cells with tunicamycin indicating that it represented the mono-glycosylated version of wild-type TRPC3 (Fig. 4B), while the faster migrating band of the doublet indicated the non-glycosylated protein. Two to three faint bands migrating with an apparent molecular mass smaller than 85 kDa were only detected in TRPC3-expressing HEK 293 cells (Fig. 4B, left panel) and were most likely proteolytic cleavage products of TRPC3. The antibody showed some minor cross-reactivity toward overexpressed recombinant TRPC6GFP (50 μg of lysate protein loaded; see: Fig. 4B, left panel). Introduction of a second glycosylation site in e2 allowed for an improved separation of two bands, most likely because of an increase in the molecular mass of the upper, tunicamycin-sensitive band due to dual glycosylation (Fig. 4B, right panel). To assess cell membrane targeting of TRPC3 and its glycosylation mutant TRPC3Gly2, we performed laser scanning microscopy with GFP-tagged proteins. Both wild-type as well as mutant TRPC3 were inserted into the plasma membrane as illustrated by the overlay of fluorescence and differential interference contrast images (Fig. 4C). In line with a previous observation (26) both C-terminally GFP-tagged TRPC3 proteins yielded a punctuated fluorescence staining of the cell membrane, clearly set apart from the uniform distribution of the fluorescence signal in the case of TRPC6 (Fig. 2C).

Decreased Basal Activity of the TRPC3Gly2 Mutant—In order to test the hypothesis that the high basal activity of TRPC3 could be reduced by introducing an additional glycosylation site in e2, thus imitating the situation in TRPC6, we functionally analyzed the TRPC3Gly2 mutant after transient expression in HEK 293 cells. The current-voltage relationship of TRPC3Gly2 after application of histamine to the bath solution (Fig. 5A) resembles that obtained with wild-type TRPC3 (Fig. 1A, upper panel) and shows identical rectifying properties. However, Fig. 5A illustrates that additional glycosylation in e2 diminishes basal activity, a phenomenon which was evident when comparing basal outward currents between TRPC3 and TRPC3Gly2 (cf. Fig. 1A, upper panel, and Fig. 5A). Constitutive activities normalized to maximal histamine-induced currents at −60 or +60 mV (−29.4 ± 6.7% and 43.3 ± 8.9%, respectively) (Fig. 5B) in TRPC3Gly2-expressing cells showing outwardly rectifying currents were found to be reduced when compared with TRPC3 (Fig. 1C, upper panel) with differences attaining statistical significance at −60 mV (p = 0.046). A comparison of current densities in TRPC3- and TRPC3Gly2-expressing cells recorded immediately after patch rupture at holding potentials of −60 or +60 mV revealed a significantly (p = 0.027) lower current density for TRPC3Gly2 at +60 mV (Fig. 5C). As emphasized earlier, maximal current densities measured in response to histamine stimulation were not significantly different between TRPC3 and TRPC3Gly2 (data not shown), thus ruling out discrepant expression levels of the channel proteins in the cell membrane as the underlying cause of the divergent basal activities noted. Finally, we compared TRPC6, TRPC3, and TRPC3Gly2 by measuring Mn2+ entry in fura-2-loaded cells. (Fig. 5D). In this set of experiments, the extracellular Mn2+ concentration was reduced from 2 mM to 200 μM, thus diminishing fura-2 quenching due to basal channel activity, yet allowing for detection of receptor-stimulated Mn2+ influx. In contrast to TRPC3, TRPC6 expression did not give rise to fura-2 quenching upon Mn2+ addition to the bath. Of note, basal Mn2+ entry characteristic for wild-type TRPC3 (−0.061 ± 0.005%/s) was nearly abolished in the case of TRPC3Gly2 (−0.026 ± 0.008%/s; p < 0.001; Fig. 5D, lower panel). Histamine challenge resulted in rapid and massive Mn2+ influx in TRPC6-expressing cells. TRPC3-expressing cells consistently reacted to agonist-stimulation with a transient de-quenching effect followed by an increased rate of Mn2+ entry. The transient de-quenching effect is due to a higher transient rise in the cytoplasmatic Ca2+ concentration after emptying the internal stores of TRPC3-expressing cells and is completely abolished by preincubation of cells with thapsigargin (data not shown). Even though basal activity was no longer observed with TRPC3Gly2, H2 receptor stimulation initiated cation influx with a rate comparable to that seen with agonist-activated TRPC3.

DISCUSSION

In this study, we characterized the impact of different N-glycosylation patterns on functional differences between TRPC3 and TRPC6. Together with TRPC7 these channels constitute a TRPC subfamily, whose members are activated by diacylglycerol (DAG) in a membrane-delimited fashion (9). Recently, it was demonstrated that proteins of the TRPC3/6/7 subfamily can form heterotetramers by combining with each other, but not with other members of the TRPC family (8), giving rise to a select array of distinct channel tetramers, which may differ in terms of biophysical properties and regulation. To clarify the issue as to whether members of the TRPC3/6/7 subfamily are functionally redundant or whether they serve distinct physiological roles, a thorough characterization of their functional properties and regulation is an indispensable first step.

By applying whole cell patch clamp recordings as well as fluorescence imaging techniques we characterized TRPC6 as a tightly receptor-activated channel with very low basal activity. On the contrary, pronounced channel activation even in the absence of receptor agonist was invariably noted upon TRPC3 expression. To uncover potential molecular determinants for the different functional behavior of two closely related cation channels, we analyzed the glycosylation pattern of TRPC6 as compared with TRPC3. Our findings demonstrate that the TRPC6 protein is dually glycosylated as opposed to the mono-glycosylated TRPC3 channel (7). TRPC6 glycosylation not only occurs in heterologous expression systems, but has recently also been demonstrated in pulmonary vascular smooth muscle cells (PASMC) where TRPC6 is involved in PASMC proliferation (27). By deleting glycosylation site(s) in TRPC6 we were able to create mono- and non-glycosylated TRPC6 variants, which were found to be correctly inserted into the plasma membrane as assessed by confocal microscopy. N-linked glycosylation is implicated in correct protein folding and the assembly of functional ion channel complexes as demonstrated for Shaker potassium channels structurally related to the TRPC family (28). Thus, it is worthwhile mentioning that TRPC glycosylation mutants could be expressed as functional ion channels at a cell membrane density comparable to that of wild-type proteins as inferred from peak current densities after agonist challenge. Consistent with the notion that the N-glycosylation pattern is responsible for functional differences TRPC6 glycosylation mutants showed a similarly high basal activity when compared with TRPC3 in Mn2+ quench experiments. Electrophysiological recordings in the whole cell mode confirmed these observations, but revealed differences in the outwardly rectifying properties compared with wild-type TRPC6 indicating an additional as yet unappreciated contribution of e2-glycosylation to TRPC6 function distinct from the control of basal channel activity. The analysis of NPo values derived from isolated inside-out patches support our conclusion based on whole cell experiments and demonstrate that also under cell-free conditions basal channel activity is regulated by the protein glycosylation status.

Conversely we asked if insertion of an additional glyco-
lational site in TRPC3 would lead to reduced basal activity and engineered a glycosylation site into the second extracellular loop (ε2) of the TRPC3 protein. Immunoblotting and confocal microscopy experiments showed that the new second consensus site was in fact glycosylated and that the mutant channel was inserted into the plasma membrane. In accordance with our initial hypothesis we noted that the basal activity of TRPC3 Gly2 was significantly reduced as opposed to wild-type TRPC3.

While TRPC3 was shown to be a receptor- and phospholipase C-controlled cation channel activated by intracellular DAG independent of store depletion (9), the situation with TRPC3 is considerably more complex. Heterologous expression of TRPC3 in HEK 293 and CHO cells revealed TRPC3 channel activity under resting conditions (21, 22, 23). A HEK 293 cell line stably expressing human TRPC3 also showed high basal Sr2+, Ba2+, and Mn2+-influx when compared with control cells (22). Using patch clamp recordings from inside-out patches, TRPC3 exhibited high intrinsic activity under resting conditions, which could be silenced by Ca2+/CaM (19). Furthermore, a tight physical and functional interaction of TRPC3 channels and InsP3 receptors was reported (16, 17). In a recent detailed analysis, however, Trebak et al. (29) provided compelling evidence to support the concept that in close analogy to TRPC6 (9) TRPC3 is activated in a receptor- and phospholipase C-dependent fashion via DAG, independently of InsP3 production and store depletion.

In light of similar biophysical pore properties (25) and channel activation mechanisms, the question arises as to whether TRPC3 and TRPC6 are functionally redundant and thus freely interchangeable in any given cellular setting. Systematic studies to describe principal rules of TRPC subunit combination in functional ion channel complexes revealed that in fact TRPC3 and TRPC6 can be incorporated into the same tetrameric complexes (8, 11). To assess the functional contribution of either channel subunit in such heterotetramers, the delineation of distinctive properties allowing us to differentiate between TRPC3 and TRPC6 is a necessary prerequisite. Apart from being activated by DAG all members of the TRPC3/6/7 subfamily display a potentiating effect of decreasing [Ca2+]i (30, 10). A first distinctive feature is the enhancement of TRPC6 currents by the frequently used cation channel blocker flufenamate which inhibits TRPC3 and TRPC7 (30, 31). In addition, we show by direct comparison that TRPC3 exhibits significant basal activity under resting conditions while TRPC6 is quiescent and under tight regulation by phospholipase C-activating receptors. Furthermore, we present evidence for a cardinal contribution of differences between the N-linked glycosylation pattern to the discrepant intrinsic activities observed. As yet, cell physiological implications of the constitutive activity of TRPC3 are unknown.

Usually N-glycosylation has a great impact on protein folding, intracellular trafficking or membrane targeting. We can exclude such effects for TRPC3 and TRPC6 by showing the insertion of all glycosylation mutants into the plasma membrane. The impact of protein glycosylation on functional characteristics of ion channels is considerably less understood. Our results show that in the case of TRPC3 and TRPC6 a dual glycosylation status is required for low basal and tight receptor-regulated channel activity. The molecular mechanism, however, by which TRPC protein glycosylation accomplishes low resting channel activity still remains elusive. Potential cues to a mechanistic understanding might be derived from studies on voltage-gated ion channels, some of which are heavily glycosylated with terminal sialic acid residues generating a cloud of negative charges at the external surface (see Ref. 32 for a recent review) thereby affecting the S4 voltage sensors of the channels. Obviously, such a mechanism cannot simply be extended to TRPC channels because they are devoid of S4 voltage sensors.

Recently, TRPC6 was reported as a receptor-activated cation channel in platelets (33), and in the aortic smooth muscle cell line A7r5 (31). TRPC6 may further be a likely molecular candidate for the vascular α1-adrenoreceptor-activated non-selective cation channel (30). Coexpression of TRPC3 and TRPC6 in smooth muscle cells of cerebral arteries (34), portal vein myocytes (30), but also in mouse spermatozoa (35) and in other tissues was demonstrated (36). At present it is unknown, however, whether TRPC3 and TRPC6 have distinct physiological roles in these cells and tissues or whether they are freely interchangeable without functional consequences. Here, we show in a heterologous cellular expression system that TRPC3 and TRPC6 are not functionally redundant, but display distinct properties: high basal activity of TRPC3 as opposed to tight regulation of TRPC6. Future studies with appropriate models like for other TRPC channels (37) will ultimately clarify whether these distinctions made at a cellular level also have a bearing on the living organism.

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