Trends in Cell & Molecular Biology

Original Communication

New insights on Na^+/Ca^{2+} exchangers and protein glycosylation in human cells

María Beatriz Bistué Millón[a,b], Cyntia Amorosi[b,c], Magalí Papazoglu[a,b], Myriam Siravegna[b,c], Graciela Elso-Berberian[b,c] and Carla Gabriela Asteggiano[a,b,d,*]

[a]Centro de Estudio de las Metabolopatías Congénitas (CEMECO), Hospital de Niños de la Sma. Trinidad, Universidad Nacional de Córdoba, Ferroviarios 1250, Córdoba;
[b]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET);
[c]Laboratorio de Biofísica, Instituto de Investigación Médica M. y M. Ferreyra, INIMEC CONICET, Universidad Nacional de Córdoba, Friuli 2434, Córdoba;
[d]Cátedra de Farmacología, Facultad de Medicina, Universidad Católica de Córdoba, Jacinto Ríos 571, (X5004ASK), Bº General Paz, Córdoba, Argentina.

ABSTRACT

Na^+/Ca^{2+} exchangers (NCX and NCKX proteins) contribute to Ca^{2+} homeostasis and recent studies have demonstrated the expression of NCX1 (*SLC8*) and NCKX1 (*SLC24*) proteins in human platelets. A tight ($[Ca^{2+}]_i$) is necessary for platelets to prevent inappropriate thrombus formation or bleeding due to altered platelet aggregation, a severe clinical manifestation in congenital disorders of glycosylation (CDG) PMM2-CDG patients. Very little is known about glycosylation and Ca^{2+} uptake. In this study, we propose to examine Na^+/Ca^{2+} activity ($^{45}Ca^{2+}$ uptake) and protein glycosylation in human cells. Immunopurified NCX1 and NCKX1 proteins from microsomal fractions of human platelets were detected with anti-SLC8 antibody and anti-SLC24 antibody, respectively, and lectin staining (concanavalin A (Con A) and wheat germ agglutinin (WGA)). Additionally, enzymatic *N*- and *O*-deglycosylation strategies (PNGase F and *O*-glycosidase digestion) were assayed. In healthy control subjects, we observed *N*-linked glycans attached to NCX1 and NCKX1 proteins and *O*-linked sialo oligosaccharides attached to NCKX1. To better understand the clinical relevance of altered protein *N*-glycosylation, we analyzed the $^{45}Ca^{2+}$ uptake in PMM2-CDG platelets and transfected NCX1 cDNA in tunicamycin-treated HEK293 cells. Western blot showed that $^{45}Ca^{2+}$ uptake and NCX1 protein were greatly diminished. We present evidence that suggests for the first time that *N*-hypoglycosylation alters Na^+/Ca^{2+} exchange in platelets from CDG patients or tunicamycin-treated HEK293 cells. Additional studies will be necessary to further elucidate the structure of glycans bound to these proteins and the pathological aspects of cell hypoglycosylation.

KEYWORDS: glycosylation, Ca^{2+} homeostasis, platelets, Na^+/Ca^{2+} exchange, CDG disease

1. INTRODUCTION

Calcium (Ca^{+2}) is one of the most ubiquitous and versatile intracellular signaling molecules. The Ca^{2+} signaling process is triggered by an increase in intracellular Ca^{2+}concentration ($[Ca^{2+}]_i$), which gives rise to a wide variety of events, depending on the different sensitivity of the cells. Specialized Ca^{2+} transport systems are responsible for controlling intracellular Ca^{2+} concentrations. Sarco-endoplasmic reticulum Ca^{+2}ATPase (SERCA) and plasma membrane Ca^{+2}ATPase (PMCA) utilize the energy from ATP hydrolysis to pump Ca^{2+} into the

*Corresponding author: asteggianocarla@hotmail.com

endoplasmic reticulum and outside the cell, respectively. Together with the plasma membrane Na^+/Ca^{2+} exchangers, these are all key players in intracellular Ca^{2+} homeostasis [1, 2].

The plasma membrane Na^+/Ca^{2+} exchangers are a family of bidirectional antiporters whose main function is the extrusion of Ca^{2+} from the cytosol. In many cells, including platelets, the Na^+/Ca^{2+} exchanger is capable of exchanging 3 or 4 extracellular Na^+ ions for 1 intracellular Ca^{2+} ion into or out of the platelet cytosol [3]. In the resting state, it removes Ca^{2+} from the platelet cytosol [4, 5]. Stimulation of platelets by different agonists results in increased $[Ca^{+2}]_i$, which appears to be due to both a release from intracellular storage organelles and an influx across the plasma membrane [6, 7, 8-10].

Mammalian Na^+/Ca^{2+} exchangers can be divided into two different protein families based upon the ion transport specificities of the molecules, Na^+/Ca^{2+} (NCX) (*SLC8*) (GenBank™ accession number NM_021097.1) and Na^+/Ca^{2+}- K^+ (NCKX) (*SLC24*) (GenBank™ accession number AF062922) [11, 12]. Three members of the NCX family have been cloned and are products of three different genes [13]. For the NCKX gene family, five members have been identified so far. NCKX activity is distinguished from that of NCX proteins by their absolute requirement for K^+. NCKX proteins transport 4 Na^+ in exchange for one Ca^{2+} and one K^+. The five NCKX proteins exhibit relatively high sequence similarity [14] and the distribution of NCKX1 has been described in different tissues [12, 15]. Functional studies have demonstrated the existence of the Na^+/Ca^{2+} exchanger in human platelets [3, 4]. The isoform NCX1 has been shown to be expressed in human platelets, together with NCKX1, the K^+-dependent Na^+/Ca^{2+} exchanger [9].

Platelet activation is a dynamic process that must be tightly regulated to prevent inappropriate or excessive thrombus formation [10, 16]. Calcium, as an essential second messenger, regulates platelet integrin activation, granule secretion and procoagulant activity [17, 18]. In human platelets, D. Roberts *et al*. (2012) reported that collagen activation led the NCX1 exchanger to transiently reverse thus promoting Ca^{2+} influx, while NCKX1 continued to operate in Ca^{2+} efflux mode to reduce $[Ca^{2+}]_i$. The Ca^{2+} influx mode was blocked by the NCX inhibitors, a isothiourea derivative (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methane sulfonate (KB-R7943) and (2-[4-[(2,5-difluorophenyl)methoxy]phenoxy]-5-ethoxyaniline) (SEA0-400) [1]. Disruptions of Ca^{2+} signaling may contribute to a broad spectrum of pathologies [7, 19]. Very little is known about glycosylation and Ca^{2+} uptake in human cells. Only an *in silico* report from Kim *et al*. (1998) has shown that the retinal rod Na^+/K^+- Ca^{+2}exchanger (NCKX1) contains a long *N*-terminal extracellular hydrophilic segment that is heavily glycosylated [20].

In this study, we focused on the analysis of glycans bound to NCX1 and NCKX1 exchangers, in order to understand the contribution of glycosylation to Na^+/Ca^{2+} activity. We studied human cells with an altered *N*-glycosylation pathway by transfection with NCX1 cDNA in tunicamycin-treated HEK293 (Human Embryonic Kidney 293 cells) or platelets from patients presenting protein *N*-hypoglycosylation due to congenital disorders of glycosylation (CDG). The CDG diseases are metabolic hereditary pathologies and the most frequent type of CDG is an enzymatic deficiency called PMM2-CDG (Phenotype MIM number 212065) [21, 22]. PMM2-CDG is caused by mutations in the *PMM2* gene (Gene/Locus MIM number 601785) that affect the enzyme phosphomannomutase (PMM; EC 5.4.2.28) altering the *N*-glycosylation pathway. This enzyme catalyzes the conversion of mannose-6-phosphate to mannose-1-phosphate in the synthesis of the lipid-linked oligosaccharides (LLO) in endoplasmic reticulum (ER) [22-24]. Different thrombohemorrhagic events due to abnormal platelet aggregation have been described in PMM2-CDG patients [23-25]. A recent report suggests a potential abnormal glycosylation of platelet membrane proteins in PMM2-CDG patients that could enhance spontaneous aggregation and agglutination [26].

Here we describe for the first time glycans attached to the Na^+/Ca^{2+} exchanger's protein. Lectins (WGA and Con A) revealed the presence of *N*-acetylglucosamine, sialic acid, mannose and/or glucose residues that co-localize with NCX1 and NCKX1 detected proteins. These results indicated that the exchanger's proteins in human platelets are glycosylated, most probably with *N*-glycans

because of the presence of mannose residues. We present evidence that suggests that *N*-hypoglycosylation affects NCX1 protein and Ca^{2+} uptake, and that protein glycosylation plays a key role in Na^+/Ca^{2+} exchange activity, maintaining intracellular Ca^{2+} homeostasis mainly in human platelets. The characterization of glycoproteins attached to platelet plasma membranes could provide new targets for regulating Ca^{2+} homeostasis in health and disease.

2. MATERIALS AND METHODS

2.1. Samples

Blood was collected *via* peripheral blood extraction to obtain serum, DNA and platelet-rich plasma (PRP). Samples were obtained from healthy volunteers (normal platelets) and PMM2-CDG patients, after parental informed consent. Genomic DNA was isolated from peripheral blood leukocytes obtained from ethylenediaminetetraacetic acid (EDTA) blood samples, using a commercially available purification kit and according to the manufacturer's instructions (Wizard Genomic Purification Kit, Promega, Madison, WI).

2.2. Platelet extraction

Blood was mixed with 1/10 volume citrate anticoagulants (3.2%, 0.109 M sodium citrate), and platelet-rich plasma (PRP) was prepared according to a protocol [27]. Blood was centrifuged at 600 x g for 20 min and then platelets were isolated from the PRP by centrifugation at 800 x g for 15 min. Platelet homogenates were subsequently fractionated by differential centrifugation to obtain the following fractions: cell homogenate; 600 x g fractions, 10000 x g fractions and microsomal fraction enriched in plasma membranes. Protein concentrations were determined by Lowry's method using bovine serum albumin as standard.

2.3. HEK293 cell culture and transient cDNA NCX1 transfections

Dog heart cNCX1.1 was cloned into pcDNA3.1 expression vector (kindly loaned by Dr. L. Annunziato) [28]. Human embryonic kidney (HEK293) cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Transient expression of wild-type NCX1 cDNAs was carried out using the calcium phosphate precipitation method [29]. Briefly, 38 µl of transfection solution with calcium phosphate-cDNA precipitates was prepared by adding 2.1 µl of DNA solution (2.1 µg of plasmid DNA in 2.5 M $CaCl_2$ solution) to 36 µl of phosphate-containing solution (274 mM NaCl, 1.8 mM Na_2HPO_4, and 50 mM of 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.07; the mixture was applied to 12-well dishes containing 50% confluent HEK293 cells for 16-20h. Next day, the medium containing the transfection solution was replaced with fresh DMEM. Tunicamycin at a final concentration of 1.5 µg/ml was added to the medium for 8 hours. Two days after transfection, the cells were collected for Western blot analysis or $^{45}Ca^{2+}$ uptake.

2.4. Western blot analysis

Western blotting was performed according to standard protocols. Microsomal fractions, enriched with platelet plasma membrane or HEK293 homogenate, were suspended in RIPA buffer (Tris-HCl 10 mM PH:7.4, NaCl 150 mM, SDS 0.1%, sodium deoxycholate 0.5%, Nonidet P-40 1% and protease inhibitor cocktail (Roche)). After sonication (10 cycles), proteins (~80 ug) were loaded and separated on 7.5% SDS-polyacrylamide gels for 2 h (20-40V). Proteins were transferred onto PVDF membranes (GE Healthcare) (Immobilon-Polyvinylidene Difluoride) for 1 h (240 mA). The filters were blocked overnight at 4 °C with 5% nonfat dry milk (Bio-Rad) in Tris-buffered saline (2 mM Tris-HCl, 50 mM NaCl (pH7.5) plus 0.1% Tween 20. The appropriate primary antibody was incubated in 5% nonfat dry milk. After washing the membranes in tris-buffered saline (TBS 1X), the blots were incubated for 2 hours at room temperature (RT) with primary monoclonal mouse anti-human NCX1 (ACNB300-127 Anti SLC8A1) (1/1000) or primary polyclonal rabbit anti-human NCKX1 (H00009187-A01 Anti SLC24A1) (1/500) antibodies from Abcam antibodies. After 3 washes (5 min each) with T-TBS (0.1% Tween 20), blots were incubated for 1 hour in horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse 1/5000 or anti-rabbit 1/5000, InmunoPure) and detected with the enhanced chemiluminescence assay (ECL Plus, GE) [30]. For lectin detection, the blots were incubated first with *Concanavalin A* (1/500 Sigma) or *Wheat Germ Agglutinin*, (1/500 Sigma) for 2 hours, followed by 3 washes (5 min each)

with TBS 1X, and then were incubated with an anti-IgG streptoavidin-horseradish peroxidase-conjugated secondary antibody (1/1000 Sigma). Transferrin was detected with rabbit anti-human transferrin (1/1500), Dako). The images were revealed by chemiluminescence (ECL Plus Enhanced chemiluminescence assay, GE) [4]. As a loading control, we evaluated the expression of GAPDH protein using a mouse monoclonal antibody (A5441, Sigma-Aldrich).

The HEK293 cells extract was denatured by adding 0.5% SDS and 1% *β*-mercaptoethanol and incubated at 37 °C for 10 min. NP-40 and Na_2-HPO_4 were added to each sample to a final concentration of 1% and 50 mM, respectively and Western blot was performed according to standard protocols.

2.5. NCX1 and NCKX1 protein immunoprecipitation

All steps were performed at 4 °C. Microsomal fractions of platelets (200 µg of protein) were lysed for 60 min on ice with 500 µl of lysis buffer (50 mM Tris-HCl, pH 7.2, 1.0% Triton X-100, 300 mM NaCl, with the complete protease inhibitor cocktail tablets from Roche). Lysates were preabsorbed with protein G-Sepharose beads (Amersham, Pharmacia, 75% suspension washed with lysis buffer before use) for 60 min at 4 °C. In parallel, 1 µg IgG anti-NCKX1 (1/200) or anti-NCX1 antibody (1/50) (AbNova) was incubated with 100 µl protein G-Sepharose beads for 60 min at 4 °C. Preabsorbed microsomal fractions were incubated with 100 µl anti-NCKX1 antibody-protein G-Sepharose beads or anti-NCX1 antibody-protein G-Sepharose beads (50% slurry) at 4 °C for 4 h. Immunocomplexes were pelleted by centrifugation at 2,500 x g for 10 s at 4 °C and then washed four times at 4 °C with PBS. Pellet was resuspended in sample buffer (1.25 M Tris-Cl, SDS 25%, DTT 0.5% pH:6.8, glycerol 5%, bromophenol blue 0.5% and PMSF 100 mM, leupeptin 10 mM, pepstatin 10 mM and aprotinin 10 mM) (Roche). The samples were boiled for 2 min at 100 °C and centrifuged for 10 min (10.000 x g). The supernatant was collected for immunoblotting [31].

2.6. Enzymatic deglycosylation

To release the *N*-glycans, 200 µg of microsomal fractions from human platelets were incubated overnight at 37 °C with 2 µl PNGase F enzyme (500 u/ml) (SIGMA) or 2 µl H_2O (control) solubilized in a reaction mixture containing 250 mM sodium phosphate (pH 7.5), 2% SDS, 1 M β-mercaptoethanol, and 15% (v/v) Triton X-100.

For enzymatic *O*-deglycosylation assays, microsomal fractions were resuspended in 200 mM sodium phosphate (pH 7.2), containing SDS up to 0.1% and Triton X-100 1% and were first treated with 10 milliunits of neuraminidase (Roche) for 1 h at 37 °C and then treated with 2.5 mU of *O*-glycosidase (BioRad) at 37 °C overnight. The reactions were stopped by adding Laemmli sample buffer, followed by SDS-PAGE. Controls for *N*- and *O*-deglycosylation assays were incubated only with the respective buffer.

2.7. Clinical and genetic studies

Biochemical and genetic studies were carried out in patients enrolled under the clinical criteria for CDG at CEMECO, Children's Hospital, Córdoba, Argentina. Two patients were included in this study and diagnosed as PMM2-CDG. Patient 1 (P1) was a 4-year old boy, and patient 2 (P2) a 1-year old boy at diagnosis. Patient 1 presented clinical features such as psychomotor global retardation, cerebellar hypoplasia, microcephaly, strabismus, hypothyroidism, abnormal fat distribution and inverted nipples. The neurological presentation included developmental delay, generalized hypotonia, bilateral ophthalmoparesis, optic nerve hypoplasia, strabismus and macrocephaly. He had a first tonic-clonic seizure at 2 months of age. Patient 2 had a multisystem clinical presentation with a severe neonatal period complicated by pericardial effusion and a severe thrombohemorrhagic event. He presented high platelet levels with abnormal aggregation.

Studies for stimulation of platelet aggregation were performed with different agonists (ADP, epinephrine, arachidonic acid, collagen and ristocetin). Platelet aggregation was simultaneously measured in the Jasco CAF-102 analyzer. A stirrer speed of 1,000 rpm was used, and the temperature was set at 37 °C. Platelet aggregation was measured as a change in optical density. For the initiation of aggregation, ADP (2.5 µM), adrenalin (50 µM) and collagen (1 µg/ml) were used [32]. The maximum rate of aggregation was determined during the initial 3 min

after the addition of agonists. These studies were conducted at the Institute of Hematology of the National Academy of Medicine, Buenos Aires, Argentina.

2.7.1. Biochemical studies

Isoelectric focusing: Serum transferrin from P1 and P2 samples (15 µl) was saturated for 30 min with Fe^{2+} at room temperature with a solution of NaCl 0.9% (35 ul) and 10 mM ferric citrate (7.5 ul) and centrifuged at 3,500 g. Two µl of each sample was transferred to a hydrated immobiline gel acrylamide 0.2 % (PhastGel pH 4-6.5; GE Healthcare) and separated by PhastSystem isoelectric focusing (GE Healthcare). Transferrin isoforms were detected after immunofixation with rabbit anti-human transferrin antibody (Sigma) and Coomassie blue staining [33]. The relative amounts of the transferrin isoforms were determined and quantified using the ImageJ 1.42q software (http://imagej.nih.gov; Wayne Rasband, National Institutes of Health, USA).

High-performance liquid chromatography (HPLC): Separation of the transferrin glycoforms was performed by liquid chromatography on a HPLC Agilent 1100 Series system. For the anion-exchange chromatography, a SOURCE 15Q 4.6/100 PE column (GE Healthcare) was used at 25 °C with a linear salt gradient elution at a flow rate of 1.0 ml/min. Quantification of the transferrin glycoforms relied on the selective absorbance of the iron-transferrin complex at 470 nm. The relative amount of each transferrin isoform was expressed as a percentage of the area under the curve (%AUC) [34, 35].

2.7.2. Genetic studies

Genomic DNA was amplified using the primers for the *PMM2* gene (MIM number 601785) and analyzed by direct Sanger sequencing in an AB3130 system (Applied Biosystems) according to previous reports ([36] with modification). The DNA sample (200 ng) was incubated in 50 µL of buffer containing 10 µl reaction commercial buffer 5X, 0.5 µl *Go Taq DNA polymerase* (Invitrogen), 100 nM of each primer and 10 mM dNTPs. Polymerase chain reaction (PCR) was performed by an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, of 40 s at different temperatures and of 45 s at 72 °C. PCR products were purified with a kit (GFX^{TM} PCR DNA or Gel Band Purification Kit, Amersham Pharmacia Biotech, Piscataway, USA).

2.8. Measurement of Na^+/Ca^{2+} exchange

2.8.1. Ca^{2+} uptake in platelets

In this work, the Na^+-dependent Ca^{2+} uptake in the absence of K^+ represents Na^+/Ca^{2+} exchange activity by NCX1 and in the presence of [K^+] represents NCKX1 activity. Ca^{2+} uptake was measured in healthy control platelets (from a group of healthy individuals who are representative of the control population) and PMM2-CDG patient platelets. Using the original techniques described by Smith *et al*., 1987 and Urbanczyk *et al*., 2006 with modification [37, 38], the platelets were washed with a solution containing 150 mM NaCl and 30 mM Bis-Tris propane-HCl, 180 mM Bis-Tris propane-HCl (NCX1 in activity) or 150 mM KCl and 30 mM Bis-Tris propane-HCl (NCKX1 in activity and NCX1 not activate). NCKX1 is not active under the conditions used to measure NCX1 (in the absence of K^+). The pH was adjusted to 7.4 and the osmolarity to 330 mosmol/kg of water (Wescor osmometer). To initiate the $^{45}Ca^{2+}$ influx through reverse-mode NCX, about 1.10^6 platelets were pre-incubated with the ionophore gramicidin (2 µg/µl) at room temperature for 4 min, 0.2 mM of $CaCl_2$ ($^{45}Ca^{+2}$) was added and, after gentle mixing, the incubation for $^{45}Ca^{2+}$uptake started at 37 °C ($^{45}Ca^{2+}$specific activity was 0.01 µCi/nmol). The transport reaction was stopped by the addition of 500 µl of ice-cold stop solution containing 100 mM $MgCl_2$, 2 mM ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA) and 20 mM Bis-tris propane-HCl, pH 7.4 and 330 mosmol/Kg. Afterwards, it was filtered, washed with 10 ml of medium containing EGTA to remove the extracellular bound radioactivity and counted by the liquid scintillation method. The contribution of Ca^{2+} influx due to the NCKX1 exchanger was calculated as the difference between the $^{45}Ca^{2+}$ uptake determined with vehicle control (DMSO) (corresponding to 100%) and that obtained in the presence of the inhibitor SEA 0400 (% given by NCKX1) [39].

2.8.2. Ca^{2+} uptake in HEK293 cells

Na^+-dependent Ca^{+2}-uptake was measured in dog NCX1 cDNA HEK293 transfected cells, using a previously described protocol. Briefly, transient transfected cells were grown in 12-well plates to

80-90% confluence, and were loaded with Na^+ by incubating the cells for 20 min in 10 mM HEPES-Tris (pH 7.4), 146 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 10mM glucose, and 0.1% bovine serum albumin (BSA) containing 1mM ouabain and 10 µM monensin. $^{45}Ca^{2+}$ uptake was then initiated by simultaneously adding standard uptake medium [above solution modified containing 146 mM LiCl, 0.1 mM $^{45}CaCl^{2+}$ (1.5 µCi/ml) and 1 mM ouabain in each well]. After 30 s, $^{45}Ca^{2+}$ uptake was stopped by washing the cells three times with an ice-cold solution containing 10 mM HEPES-Tris (pH 7.4), 150 mM KCl and 1 mM EGTA. Cells were then solubilized with 0.1 N NaOH and their $^{45}Ca^{2+}$ content was determined in a liquid scintillation counter. To determine the contribution of the NCX1 exchanger in transfected HEK293 cell Ca^{2+} uptake, the effect of a selective inhibitor, KB-R7943 (Sigma, 3µM), on calcium influx was measured (data not shown).

2.9. Statistical analysis

The Student's t-test for unpaired data was used for comparisons between control and PMM2-CDG patient platelets, with $p < 0.05$ considered a significant difference. Results from $Na^+/Ca^{2+}$and Na^+/K^+Ca^{2+} exchange were expressed as mean ± SEM. The GraphPad Prism v5.01 software (La Jolla, CA, USA) was used for statistical analysis. Data normality was verified using the D'Agostino-Pearson omnibus K2 normality test.

3. RESULTS

3.1. NCX1 and NCKX1 proteins

We detected the expression of NCX1 and NCKX1 proteins in microsomal fractions from human platelet homogenate. Bovine cardiac vesicles express only the NCX1 protein and this was analyzed by Western blot to corroborate the specificity of the NCX antibody that did not appear to cross-react with the NCKX protein that is detected by the specific antibody [40] (data not shown). Different reports described the NCX1 protein as composed of 970 aa with a predicted molecular mass of 108 kDa. By SDS-PAGE, NCX1 migrates at MW of 70 kDa and 120 kDa, with some tissues showing an additional MW of 160 kDa, believed to reflect the presence of intramolecular disulfide bonds or aggregation [13, 41]. However, we analyzed the 120 kDa band in our samples (Figure 1).

The reported NCKX1 protein has 1199 aa and migrates in SDS-polyacrylamide gels with a predicted molecular mass of 130 kDa and 220 kDa [20, 40]. In our assays, the protein detected with anti-NCKX1 migrated at 220 kDa and a band of 130 kDa [40] (Figure 2).

3.2. Glycosylation studies

Bio-informatics analysis using the NetNGlyc and NetOGlyc CBS prediction Servers (www.cbs.dtu.dk/services) predicted 2 *N*-glycosylation sites (Asn 44 and 136) for NCX1 protein and different *N*-glycosylation (Asn 290, 552 and 664) and *O*-glycosylation (Thr 329, 340, 412 and 413) sites for NCKX1 protein. To study the glycosylation sites predicted by bio-informatics analysis, total homogenates from human platelets were immunoprecipitated with anti-NCX1 (Figure 1) or NCKX1 (Figure 2) antibodies and detected with lectin staining using wheat germ agglutinin (WGA) and concanavalin A (Con A), two of the most commonly used lectins in cell biology. Equally loaded immunoprecipitated material resolved by Western blot was probed with either WGA or Con A, which showed the presence of reactivity in the immunoprecipitated samples and undetectable levels in the controls (albumin-IP with anti-NCX1 or anti-NCKX1, beads and albumin). For NCX1, a 120 kDa band was detected with WGA (Figure 1 A) and Con A (Figure 1 B) that colocalized with the immunoprecipitated NCX1-Ab complex (Figure 1C). Similarly, for the NCKX1 immunocomplex, both lectins, WGA (Figure 2 A) and Con A (Figure 2 B), revealed a band at 130 kDa that colocalized with the NCKX1-Ab immunoprecipitated complex (Figure 2 C). Taken together, these lectins (WGA and Con A), revealed the presence of *N*-acetylglucosamine, sialic acid, mannose and/or glucose residues bound to NCX1 and NCKX1 proteins. These results indicated that the exchanger's proteins in human platelets are glycosylated, most probably with *N*-glycans because of the presence of mannose residues.

3.3. *N*-deglycosylation of NCX1 and NCKX1 proteins

To investigate the presence of *N*-glycans attached to the Asn residues in these proteins, entire glycan chains were removed by enzymatic deglycosylation with PNGase F enzyme. This enzyme cleaves the

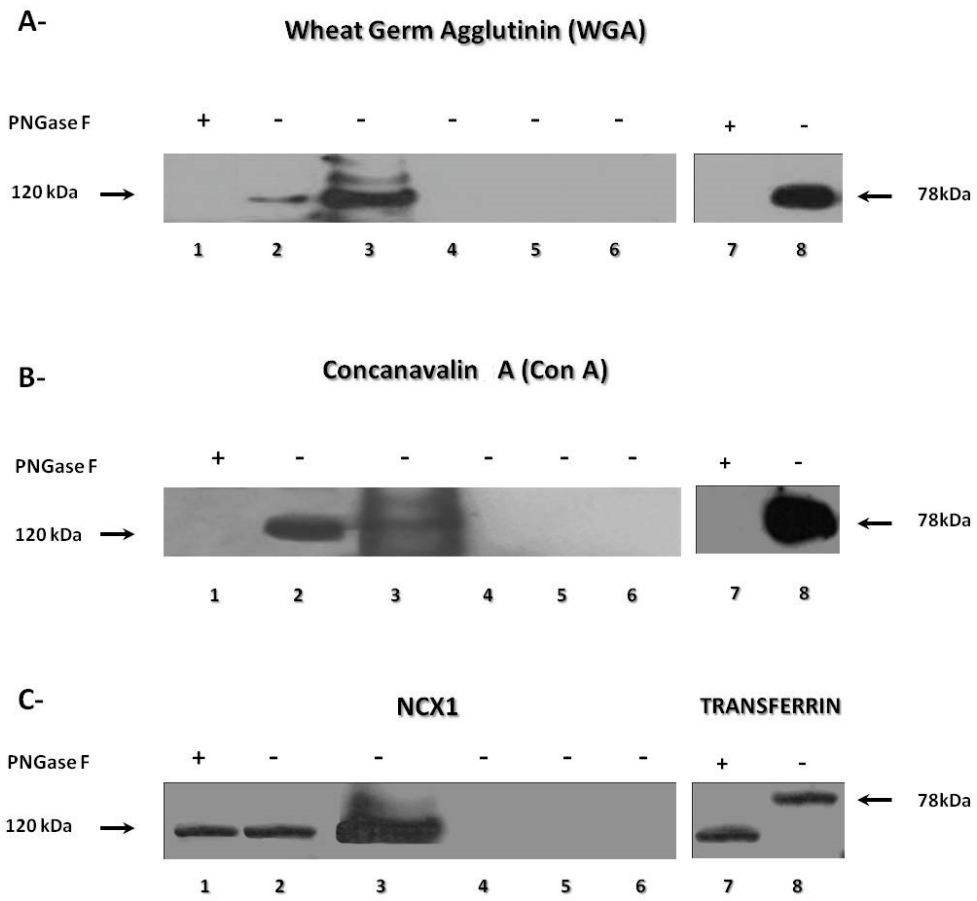

Figure 1. Western blot and lectin staining of NCX1 protein. *N*-deglycosylation of NCX1 protein. Two hundred micrograms (200 ug) of total homogenates from human platelets were immunoprecipitated with NCX1 antibody, resolved by Western blot and further blotted with WGA lectin (A), Con A lectin (B) and anti-NCX1 antibody (C). The NCX1-Ab immunoprecipitated complex treated with (+) or without (-) PNGase F and the 120 kDa band was not observed with lectin staining and PNGase F (+): (A) WGA (lane 1) or (B) Con A (lane 1) compared with non-enzymatic PNGase F treatment (-) (lane 2A and B). Total homogenate non-immunoprecipitated (lane 3 A and B). A strong NCX1 band appeared when it was detected with NCX1 antibody (C) (lanes 1 to 6). NCX1-Ab immunoprecipitated albumin (lane 4), albumin (lane 5) and protein G-Sepharose beads (lane 6) are proved as non-glycosylated protein-negative controls for lectin detection. Transferrin (78 kDa) is a very well-known *N*-glycoprotein used as a control for PNGase F treatment (+) (lane 7) or (-) (lane 8).

glycan *N*-acetylglucosamine (GlcNAc) bond to the Asn. The deglycosylation treatment was performed after the proteins were immunoprecipitated with NCX1 (Figure 1) or anti NCKX1 antibodies (Figure 2).

In the NCX1-Ab immunoprecipitated complex treated with PNGase F (+), the 120 kDa band was not observed to be stained with WGA (Figure 1 A) or Con A (Figure 1 B) lectins. A strong NCX1 protein band colocalized when it was detected with NCX1 antibody (Figure 1 C). In contrast, when the NCKX1-Ab immunoprecipitated complex was incubated in the presence of PNGase F (+), the treatment did not show any effect (Figure 2 A). Under our experimental conditions, the *N*-deglycosylation treatment produced no significant effect on the molecular weight of the NCKX1 exchanger, but abolished lectin detection in the immunopurified NCX1 complex. Transferrin is a very well-known *N*-glycoprotein of 78 kDa, used as a positive deglycosylation control. The mobility of the serum transferrin shifted down after PNGase F treatment (Figure 1).

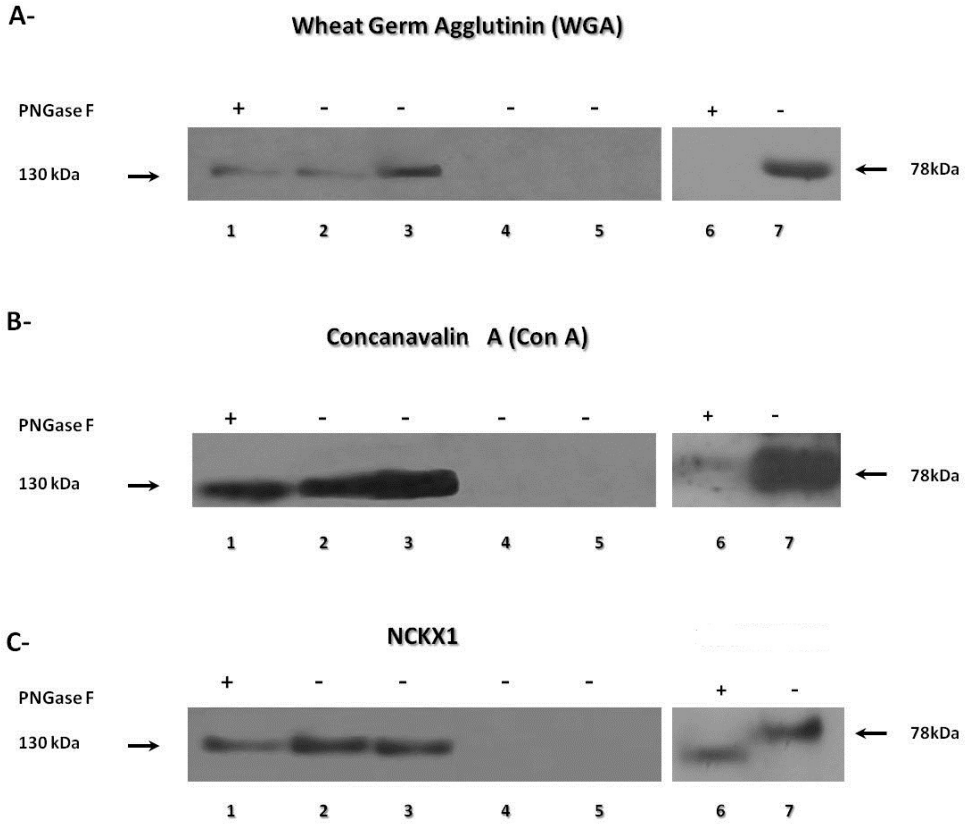

Figure 2. Western blot and lectin staining of NCKX1 protein. *N*-deglycosylation of NCKX1 protein. Total homogenates (200 ug) from human platelets were immunoprecipitated with anti-NCKX1 antibody, resolved by Western blot and further blotted with WGA lectin (A), Con A lectin (B) and anti-NCKX1 antibody (C). The NCKX1-Ab immunoprecipitated complex was treated with (+) or without (-) PNGase F and the 120 kDa band was not observed with lectin staining and PNGase F (+): (A) WGA (lane 1) or (B) Con A (lane 1) compared with non-enzymatic PNGase F treatment (-) (lane 2A and B). Total homogenate non-immunoprecipitated (lane 3 A and B). We observed a 130 kDa band detected with WGA (Figure 2 A), Con A (Figure 2 B) as well as with anti NCKX1 antibody (Figure 2C). Under our experimental conditions, the *N*-deglycosylation treatment produced no significant effect on the molecular weight of the NCKX1 protein. Controls: NCKX1-Ab immunoprecipitated albumin (lane 4) and albumin (lane 5) are proved as non-glycosylated protein-negative controls for lectin detection. Transferrin (78 kDa) is a very well-known *N*-glycoprotein used as a control for PNGase F treatment (+) (lane 6) or (-) (lane 7).

3.4. *O*-deglycosylation of NCKX1 protein

To further investigate the presence of *O*-glycans, microsomal fractions of human platelets were immunoprecipitated with anti-NCKX1 antibody and deglycosylated with neuraminidase and *O*-glycosidase. The enzyme *O*-glycosidase DS (BioRad) is a recombinant endoglycosidase that cleaves all non-sialylated Ser/Thr-linked Gal(ß1-3)-GalNAc(a1) disaccharides from glycoproteins and glycopeptides after the neuraminidase treatment required for *O*-glycosidase activity. Following deglycosylation, the immunocomplexes were analyzed by Western blot with WGA lectin staining. After neuraminidase and *O*-glycosylation treatment, we could not detect any band with lectin WGA staining (Figure 3A), but a strong band appeared in the same immunopurified proteins detected with anti-NCKX1 antibody (Figure 3B). These results suggest that the 130 kDa band of the NCKX1 exchanger contains *O*-linked sialo-carbohydrate chains.

3.5. $^{45}Ca^{2+}$ uptake in platelets

To analyze Na^{+}/Ca^{2+} exchange, aliquots of 1.10^{6} platelets were washed in a Ca^{2+}-free Bis-Tris propane

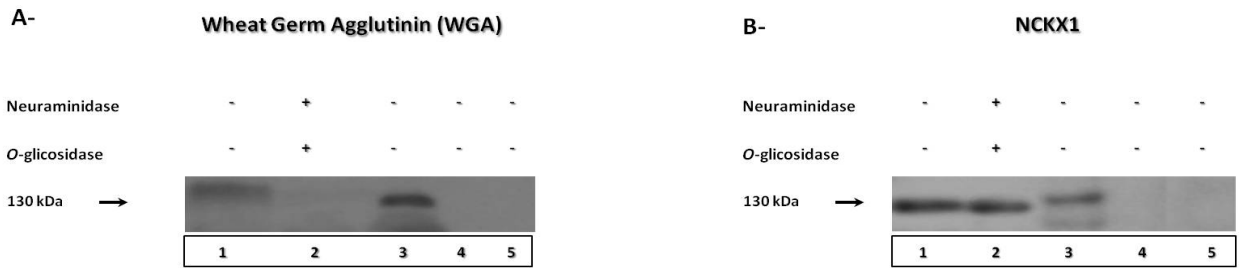

Figure 3. *O*-deglycosylation of NCKX1 protein. Microsomal fractions of human platelets were immunoprecipitated with anti-NCKX1 antibody, deglycosylated with neuraminidase and *O*-glycosidase and resolved by Western blot with WGA lectin staining (A) and with anti-NCKX1 antibody (B). After neuraminidase and *O*-glycosylation treatment (+) (lane 2), we could not detect any band with the lectin staining (A). A strong band appeared in the same immunopurified proteins detected with anti-NCKX1 antibody (B). Controls: Total homogenate non-immunoprecipitated (lane 3). NCKX1-Ab immunoprecipitated albumin (lane 4) and free albumin (lane 5) are proved as non-glycosylated protein-negative controls for lectin detection.

solution, containing 150 mM NaCl and 30 mM Bis-Tris propane-HCl, and were pre-incubated with gramicidin (2 µg/µl) for 4 min. When required, the concentration of extracellular potassium [K^+_e] was adjusted to 5 or 150 mM before each experiment. Samples were obtained from healthy volunteers (normal control platelets) (n = 20). To initiate the $^{45}Ca^{2+}$ influx, 0,2 mM $^{45}CaCl_2$ was applied to the cells, incubated at 37 °C at the indicated times, and the aliquots were harvested by filtration (see section 2.7). As shown in figure 4, we observed a typical time course curve of Ca^{2+} influx in the absence or in the presence of different concentrations of extracellular K^+ (K^+_e). In the absence of K^+_e, we observed a Ca^{2+} influx increase to a maximum Ca^{2+} uptake of 0.047 ± 0.004 nmol/1.10^6 cells at 50 seconds (n = 5) (Figure 4 A). Additionally, in the presence of K^+_e, an initial increase of $^{45}Ca^{2+}$ influx was observed until a maximum uptake at 60 seconds, which remained constant over time (Figure 4 B). It was also observed that the influx of Ca^{2+} is dependent on K^+ concentration. With a concentration of 5 mM K^+, it was 0.051 ± 0.007 nmol/1.10^6 cells and, at higher concentrations of K^+ (150 mM), it significantly increased to 0.081 ± 0.007 nmol/ 1.10^6 cells (Figure 4 B). To further determine the contribution of each exchanger to platelet calcium uptake, we measured the effect of a selective NCX1 inhibitor, SEA 0400 (2 µM) on calcium influx with a concentration of 150 mM K^+_e. The activity of NCKX1 corresponds to 63 ± 2% compared to DMSO control cells (t-test $p < 0.05$) (Figure 4 C). Taken together, our results demonstrate that both K^+-independent (NCX1) and K^+-dependent (NCKX1) exchangers are functional in platelets from a group of healthy individuals that are representative of control population.

3.6. PMM2-CDG patients

Patients enrolled according to the clinical criteria for CDG were studied in our center. The analysis of serum transferrin isoforms was carried out by at least two different methodologies (IEF, HPLC and/or CE) to detect altered protein glycosylation. We observed abnormal Tf-IEF type I patterns in P1 and P2 with increased asialo- and disialotransferrin isoforms and decreased tetrasialotransferrin (Figure 5 A). The %AUC obtained by HPLC were: disialotransferrin 11.80% (P1) and 46.9% (P2) (NV: 0.5-1.6%); and asialotransferrin 5.6% (P1) and 35.70% (P2) (NV: 0%), indicating altered sialylation in transferrin isoforms observed by both methodologies. The genotypes for the *PMM2* gene were analyzed and P1 presented two mutations in exon 5 (c.481G>A; p.E139K and c.488G>A; p.R141H), and P2 presented the same exon 5 mutation (c.488G>A; p.R141H) and a second change in exon 8 (c.756G>A; p.V231M).

In both patients, we studied the agonist response to platelet aggregation. This study was conducted in collaboration with the Progress of Medicine Foundation, Córdoba, Argentina (P1) and Institute of Hematology, National Academy of Medicine, Buenos Aires, Argentina (P2). We observed a decreased primary aggregation (P1 and P2) and an absent secondary aggregation (P2) when the platelets

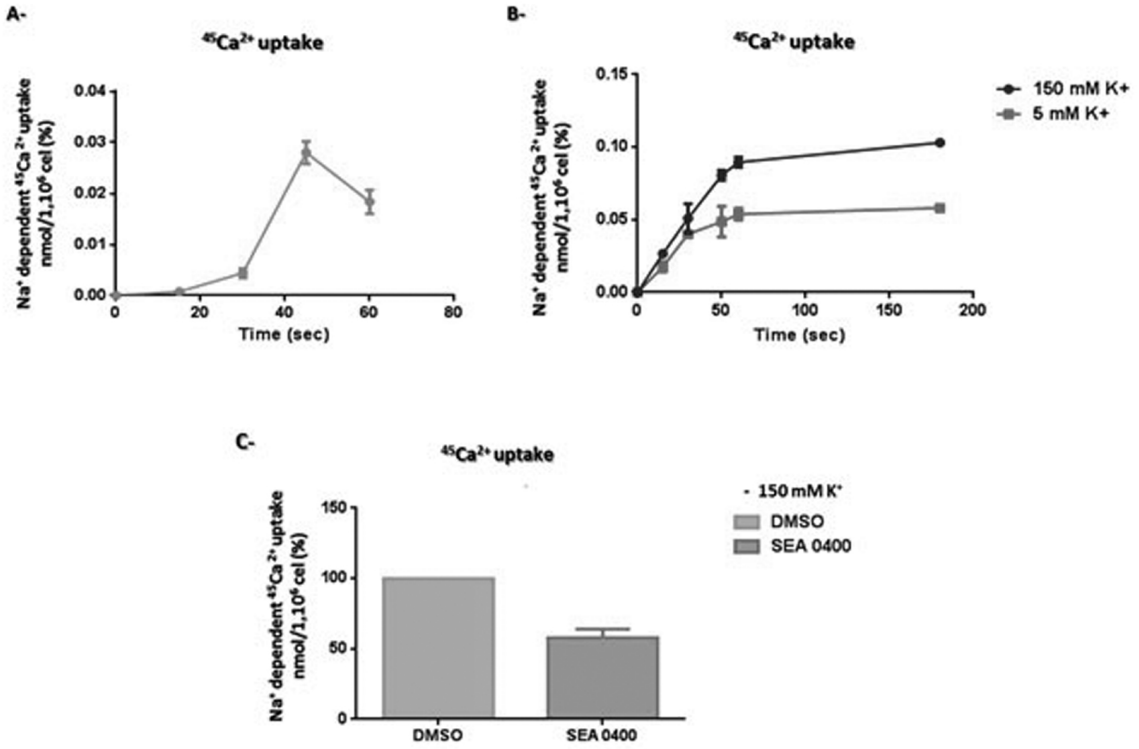

Figure 4. Ca^{2+}uptake in healthy human platelets. Cells (1.10^6 cells) were washed in a Ca^{2+}-free Bis-Tris propane solution containing 150 mM NaCl and 30 mM Bis-Tris propane-HCl and pre-incubated with gramicidin (2 µg/µl) for 4 min. When required, the concentration of extracellular potassium ($[K^+]_e$) was adjusted to 5 or 150 mM. To initiate the $^{45}Ca^{2+}$ influx, 0.2 mM $^{45}CaCl_2$ was applied to the cells and incubated at 37 °C at the indicated times (see section 2.7). (A) The graphic shows a typical time course curve of $^{45}Ca^{2+}$ influx in the absence of extracellular K^+ (n = 20). We observed an increase in $^{45}Ca^{2+}$ influx to a maximum Ca^{2+} uptake of 0.047 ± 0.004 nmol/1.10^6 cells at 50 seconds. (B) In the presence of K^+_e, an initial increase of $^{45}Ca^{2+}$ influx was observed until a maximum uptake at 60 s, which remained constant over time. The influx of $^{45}Ca^{2+}$ is dependent on K^+ concentration, which was 0.051 ± 0.007 nmol/1.10^6 cells with a concentration of 5 mM K^+ (squares) and was significantly increased at higher concentrations of K^+ (150 mM) to 0.081 ± 0.007 nmol/1.10^6 cell (circles). (C) $^{45}Ca^{2+}$ influx in the presence of the NCX1 selective inhibitor, SEA0-400, compared to control vehicle (DMSO). The contribution of NCKX in $^{45}Ca^{2+}$ uptake was 63 ± 2%. The data represent the mean values (±S.E.) of $^{45}Ca^{2+}$ uptakes from 5 independent experiments in triplicate.

were stimulated with different agonists (ADP for P1 and ADP, adrenalin and collagen for P2). Only patient 2 presented a severe bleeding event during the neonatal period.

3.6.1. Detection of NCX1 and NCKX1 proteins in PMM2-CDG platelets

The platelet membrane of PMM2-CDG patients were analyzed by Western blot according to standard protocols (Section 2.2). The levels of NCX1 and NCKX1 proteins detected with both antibodies were normal in P1 (NS) (Figure 5 A and B), whereas P2 showed a dramatically reduced expression of NCX1 (n = 3; P < 0.01) (Figure 5 A and B).

3.6.2. Ca^{2+} uptake in PMM2-CDG platelets

It was possible to perform Na^+-dependent and independent $^{45}Ca^{2+}$ uptake only in the platelets of P2, who presented a severe bleeding event during the neonatal period. In this patient, the Na^+-dependent $^{45}Ca^{2+}$ uptake was 0.015 ± 0.003 nmol/ 1.10^6 cells, in the absence of K^+_e, while in the control (normal platelets) the uptake was 0.058 ± 0.003 nmol/1.10^6 cells at 45 seconds (Figure 5 C). This observation seems to indicate a dramatically reduced NCX1 activity in P2 platelets compared with control platelets (t-test, p < 0.01). No difference was observed in NCKX1 activity between control

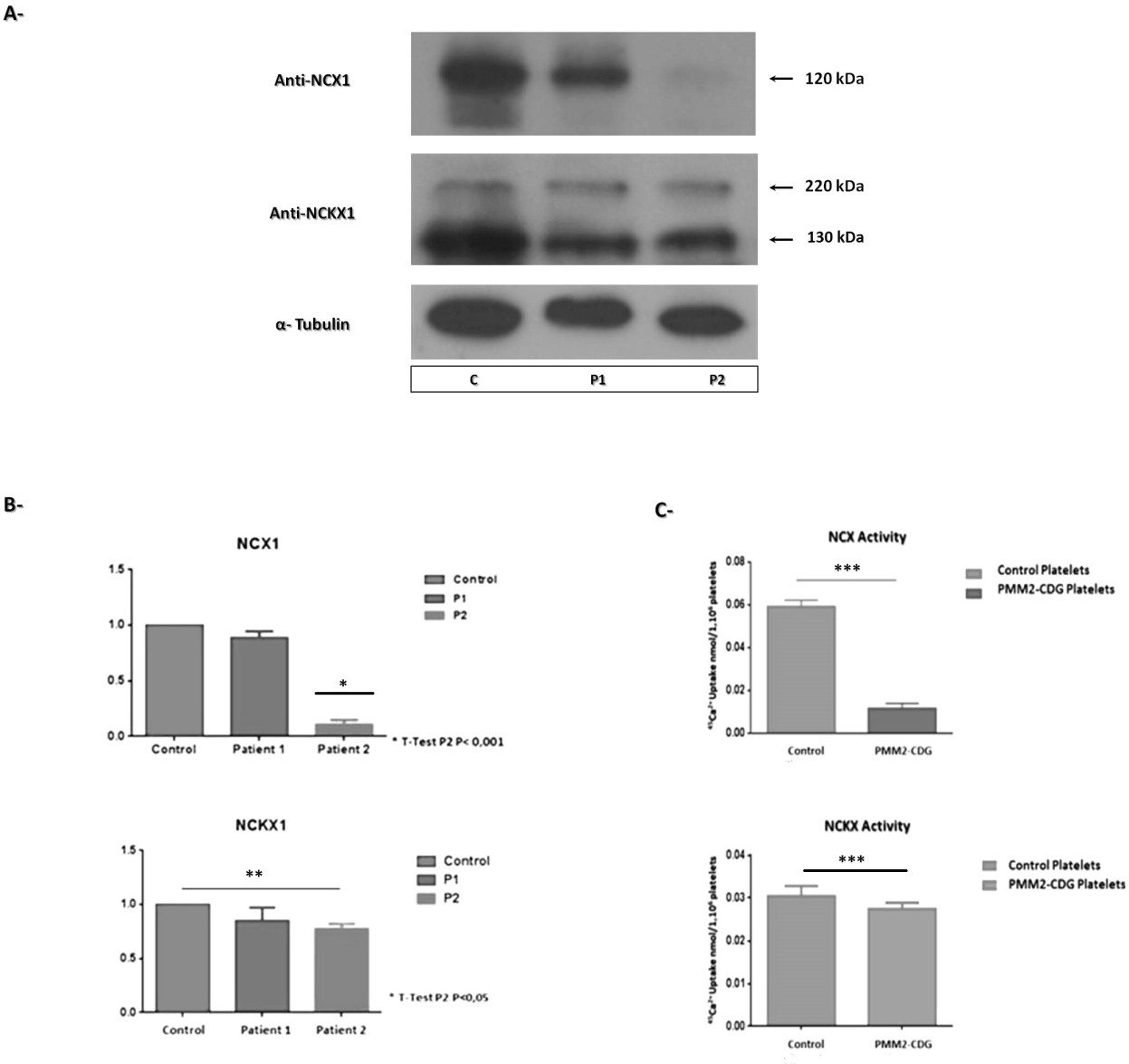

Figure 5. NCX1 and NCKX1 protein expression and Ca^{2+}uptake in PMM2-CDG patients. Microsomal fractions of PMM2-CDG platelets were probed with monoclonal anti-NCX1 (Anti SLC8A1) and polyclonal anti-NCKX1 (Anti SLC24A1). (A) Western blot analysis and (B) arbitrary densitometry. Statistically significant difference between means of control subjects (n = 10) and platelets of PMM2-CDG was observed using the Student's t-test in NCX1 expression in patient 2 with a reduced expression of NCX1 protein (n = 3; $P < 0.001$) (*). In patient 1, no difference was observed in NCX1 or NCKX1 (**) proteins compared to control human platelets (n: 3, $P > 0.05$). Graphics represent the mean ± SE values for 3 independent experiments in triplicate of the major band of 120 kDa of NCX1 and NCKX1. (C) Na^+-dependent $^{45}Ca^{2+}$ uptake measurements in PMM2-CDG platelets. In the absence of K^+_e, the PMM2-CDG platelets had an uptake of 0.015 ± 0.003 nmol/1.10^6 cells and, in the control, it was 0.058 ± 0.003 nmol/1.10^6 cells. In the presence of K^+_e 150 mM, PMM2-CDG platelets showed a Ca^{2+} influx of 0.042 ± 0.003 nmol/1.10^6 cells and, in the control, it was 0.081 ± 0.008 nmol/1.10^6 cells. NCX1 activity was reduced in PMM2-CDG platelets compared with control platelets (t-test, $p < 0.01$) (***). There was no statistically significant difference in NCKX1 activity between control and PMM2-CDG platelets, (n) number of independent samples analyzed.

and PMM2-CDG platelets in P2 (activity at 45 seconds was 0.030 ± 0.002 nmol/1.10^6 cells and 0.027 ± 0.001 nmol/1.10^6 cells, respectively) (Figure 5 C).

3.7. Analysis of NCX1 protein in HEK293 culture cells

The $^{45}Ca^{2+}$ uptake was analyzed in over-expressed NCX1 protein from transiently transfected full-length dog NCX1 cDNA in tunicamycin-treated HEK293 cells. The HEK293 cells were transiently transfected and exposed for 8 h to 1.5 μg/ml tunicamycin added to the medium. All samples were collected 48 h post-transfection and subjected to Western blot analysis (see section 2.1.3). Figure 6 illustrates the detection of a 120 kDa band corresponding to the over-expressed NCX1 protein (Figure 6 A). When tunicamycin is added to the medium, the molecular mass of NCX1 protein becomes lower than the corresponding immunoreactive protein synthesized in the absence of tunicamycin. A shift in the 120 kDa band seems to correspond to the lack of glycans due to the tunicamycin effect that blocks *N*-linked glycosylation (Figure 6 A).

The Na^+-dependent $^{45}Ca^{2+}$uptake was measured in HEK293 culture cells (see section 2.7.1). The NCX1 activity was indistinguishable in untransformed cells (not shown), indicating the absence of any endogenous Na^+/Ca^{2+} exchange in HEK293. When the cells were transiently transfected with NCX1 cDNA, we observed a $^{45}Ca^{2+}$ uptake of 0.012 nmol/ 0.5.10^6 cells and, when the culture cells were treated with tunicamycin, we observed a decrease

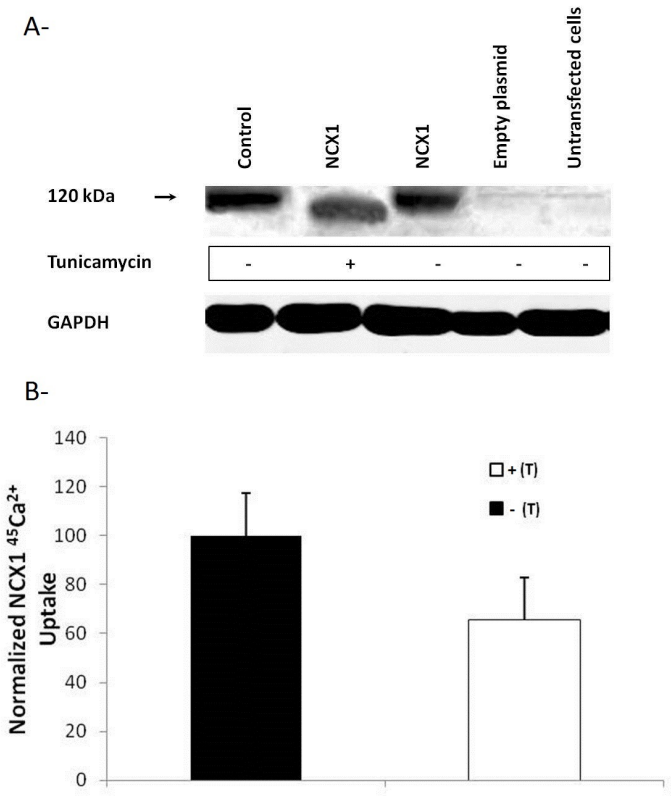

Figure 6. Transiently transfected full-length dog NCX1 cDNA in HEK293 culture cells. (A) Western blot of NCX1 over-expressed protein. Cell lysate (100 μg) from HEK293 over-expressing NCX1 protein was grown in the presence (+) or without (-) 1.5 μg/ml tunicamycin for 8 h. Blots were probed with anti-NCX1 monoclonal antibody. (A) Lane 1: Bovine cardiac vesicles express only the NCX1 protein and it was analyzed to corroborate the NCX-detected protein. Lane 2 and 3: treated (+) and untreated (-) cells with tunicamycin. Lane 4: cells transfected with empty plasmid. Lane 5: untransfected cells. (B) $^{45}Ca^{2+}$ uptake measurements in HEK293 cells transiently transfected with NCX1 cDNA. Treatment with tunicamycin decreases the activity of Na^+/Ca^{2+} exchange by around 35%. This effect is statistically significant and values are expressed as an arbitrary densitometry (normalized NCX1 Ca^{2+} uptake values).

in NCX1 activity (0.0113 nmol/0.5.10^6 cells) (Figure 6 B).

4. DISCUSSION

Although the *SLC8* (NCX1 protein) and *SLC24* (NCKX1 protein) are related genes, there is very little sequence homology between them. In human platelets, it was reported that only collagen activation led the NCX1 exchanger to transiently reverse, promoting Ca^{2+} influx into the cytosol and subsequently to the platelet aggregation [4, 40]. The inhibition of the increase in $[Ca^{2+}]_i$ by NCXs inhibitors (KB-R7943 and/or SEA0-400) obstructs the collagen-induced aggregation of human platelets [4]. Previous reports studied glycosylation and its functional consequence in NCX1 and NCKX1 proteins in different cells and by other procedures but never in human platelet membranes [13]. Very little is known about the glycosylation status of Na^+/Ca^{2+}-exchangers in health or pathogenesis [20]. Glycosylation was described as a complex post-transcriptional protein modification in which the type of mature glycans bound to proteins depends on the genetic background, the regulation of the glycosylation machinery for each type of cell, and the abundance, affinity or location of the available donor and acceptor substrates [42]. Glycosylation of mammalian NCX and NCKX exchangers had not been assessed in platelets. We studied the glycans bound to these proteins based on bio-informatics analysis that predicted glycosylation and also analyzed Na^+/Ca^{2+} activity in cells from healthy subjects, in hypoglycosylated cells from PMM2-CDG patients, and in overexpressed NCX1 protein in transfected HEK293cells with an altered glycosylation pathway.

Following previous bioinformatic studies, potential *N*-glycosylation sites for NCX1 protein and particularly *O*-glycosylation sites for NCKX1 protein were detected by staining with lectins (Con A and WGA) bound to the platelet Na^+/Ca^{2+} exchangers. Additionally, *N*-deglycosylation analysis with PNGase F showed that NCX1 has *N*-linked glycans attached and, through *O*-deglycosylation strategies (neuraminidase and *O*-glycosidase digestion), we observed that NCKX1 contains mainly *O*-linked sialo-oligosaccharides. Removal of a *N*-linked oligosaccharide from NCX1 or several *O*-linked oligosaccharides from NCKX1 may change the electrophoretic mobility of the protein analyzed on gels; nevertheless, the data presented here (Figures 1 to 3) does not show changes in the apparent size of the bands for NCX1 and NCKX1. In platelets, under our experimental conditions, the *N*-deglycosylation treatment produced no significant effect on the molecular weight of the NCX1 exchanger, but abolished lectin detection in the immunopurified NCX1 complex. Lack of changes in the apparent size of NCKX1 protein by Western blot after deglycosylation treatments was explained by Kim *et al*. (1998), who postulate either that the predicted sites might not be glycosylated or that oligosaccharide chains are small and their removal does not affect the mobility of the exchanger on SDS-PAGE [20].

To understand the effect of hypoglycosylation in tissues and cells, van Geet *et al*. (2001) suggested a potential abnormal glycosylation on PMM2-CDG protein membrane platelets that could enhance non-specific platelet interactions [26]. We have observed reduced NCX1 protein detection and $^{45}Ca^{2+}$ uptake in platelets from a PMM2-CDG patient who presented a decreased primary aggregation and an absent secondary aggregation with collagen. According to a previous report, collagen activation led the NCX1 exchanger to transiently reverse, promoting Ca^{2+} influx [40]. The decrease in NCX1 protein expression and in $^{45}Ca^{2+}$ uptake results in overexpressed NCX1 is similar to that observed in HEK293 cells growing with tunicamycin. Our results suggest for the first time that the activity of Na^+/Ca^{2+} exchangers is affected by altered *N*-glycosylation in human cells. Future studies could provide new insights on specific glycans bound to Na^+/Ca^{2+} proteins and their contribution to exchanger activity in health or pathogenesis, for example the thrombohemorrhagic events described for CDG patients [23-25].

5. CONCLUSION

This work hopes to provide a significant advance in the knowledge of protein glycosylation and Na^+/Ca^{2+} exchange involved in platelet Ca^{2+} homeostasis in health and pathogenesis. It had been reported that human platelets express NCX and NCKX exchanger proteins but the role of glycans bound to these mammalian cells and the effect of protein hypoglycosylation on Ca^{2+} uptake had not been studied in platelets. We focused the study on

glycans attached to the Na^+/Ca^{2+} exchanger proteins, altered *N*-glycosylation due to congenital disorder of glycosylation (CDG) and transiently transfected full-length NCX1 cDNA HEK293 culture cells growing with tunicamycin. We observed *N*-linked glycans attached to NCX1 and particularly *O*-linked sialo oligosaccharides bound to NCKX1 protein in healthy control platelets. The clinical relevance of protein hypoglycosylation to Na^+/Ca^{2+} protein was analyzed in HEK293 tunicamycin-treated cells and in platelets from PMM2-CDG patients.

Additional studies will be necessary to further elucidate the role and structure of glycans attached to Na^+/Ca^{2+} exchangers. The key role of these exchangers in cardiovascular diseases [43-45] highlights new pharmacological molecules (neuronin-1) that were able to enhance NCX1 activity or show selective stimulation of its activity [46-48]. The development of new strategies capable of modulating Na^+/Ca^{2+} activity in platelets could provide new targets for regulating the Ca^{2+} homeostasis necessary to prevent inappropriate thrombus formation or bleeding, a severe clinical manifestation in congenital disorders of glycosylation (CDG) patients.

ACKNOWLEDGMENTS

The authors are grateful to the CDG patients and their parents. The authors would also like to thank Prof. Dr. Raquel Dodelson de Kremer (CEMECO, Córdoba Children's Hospital, Argentina); Dr. Norma Spécola (Neurometabolism Unit, Children's Hospital La Plata, Argentina) and Dr. Santiago Chacón (Neurology Unit, Gualeguaychu Children's Hospital, Entre Ríos, Argentina) for patient support and the acquisition of clinical history data. The NCX1.1 cDNA inserted into the pcDNA3.1 expression vector was kindly loaned by Prof. Lucio Annunziato and Dr. P. Molinaro (Division of Pharmacology, Department of Neuroscience, School of Medicine, University of Naples "Federico II"). We thank Dr. I. Martinez-Duncker for providing language help, writing assistance and technical assistance.

The authors would also like to thank the Progress of Medicine Foundation, Córdoba, Argentina for their collaboration in hemostatic studies, and the Institute of Hematology, National Academy of Medicine, Buenos Aires, Argentina. María Beatriz Bistué Millón holds a CONICET fellowship and Cyntia Amorosi and Magalí Papazoglu are recipients of fellowships from CONICET, Argentina. Myriam Siravegna is CPA of CONICET. Graciela Elso-Berberian and Carla Gabriela Asteggiano are CONICET researchers.

INFORMED CONSENT

This work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki 1975, as revised in 2000) for experiments involving human samples. Approval of Human Research was obtained from the institutional review boards of CIEIS (Human Research Ethics Committee), Children's Hospital of Córdoba, Argentina (CIEIS) Act Nº 95. Informed consent was obtained from all patients included in the study. Experiments involving recombinant DNA were reviewed and approved by applicable laws and regulations.

STATEMENT OF AUTHOR CONTRIBUTIONS

MBBM carried out the biochemical and molecular studies in platelet cells, measured the Ca^{2+} uptake and performed the statistical analysis. CA assayed the heterologous expression of NCX1 protein in HEK293 cells and the Ca^{2+} uptake and performed the statistical analysis. MP participated in analyzing the studies for CDG patients. MS gave technical support. GEB and ACG participated in the design and coordination of the study and drafted the manuscript. All authors made substantial contributions to all of the following: 1. the concept and design, acquisition of data or analysis and interpretation of data; 2. drafting the article or revising it critically for significant intellectual content; and 3. approving the version to be published.

FINANCIAL SUPPORT

This work was supported by grants from FONCYT (PICT2006-2350 and PICT2010-2824), Catholic University of Córdoba (UCC) (Grants 2010 to2015) and CONICET PID (11220090100063).

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interest.

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