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Residues important for Ca^{2+} ion transport in the neuronal K^+-dependent Na^+-Ca^{2+} exchanger (NCKX2)

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ABSTRACT

K^+-dependent Na^+-Ca^{2+} exchangers (NCKXs) belong to Ca^{2+}/cation antiporter gene superfamily. NCKX proteins play an important role in Ca^{2+} homeostasis and are bi-directional plasma membrane Ca^{2+}-transporters which utilize the inward Na^+ and outward K^+ gradients to move Ca^{2+} ions into and out of the cytosol ($4Na^+$:$1Ca^{2+}$ + $1K^+$). In this study, we examined residues in the two regions with the highest degree of homology between the different NCKX isoforms (α-1 and α-2 repeats) to determine which residues are important for Ca^{2+} coordination. Using fluorescent intracellular Ca^{2+}-indicating dyes, we measured NCKX-mediated Ca^{2+} transport in HEK293 cells expressing wildtype or mutant NCKX2 and analyzed shifts in the apparent binding affinity (K_m) of mutant proteins when compared to the wildtype exchanger. Of the 93 residue substitutions tested, 31 were found to show a significant shift in the external Ca^{2+} ion dependence of which 18 showed an increased affinity to Ca^{2+} ions and 13 showed a decreased affinity, and, hence, are believed to be important for Ca^{2+} ion binding and transport. When compared to the crystal structure of the archaeal Na^+-Ca^{2+} exchanger NCX_Mj and the NCKX2 homology model based on this crystal structure, our biochemical data reveal that these 13 residues are either in direct contact with the Ca^{2+} ion or lining a Ca^{2+} transport pathway through the exchanger. *Supported by CIHR MOP-81327.*

1. Introduction

K^+-dependent Na^+-Ca^{2+} Exchangers (NCKX) are bi-directional plasma membrane Ca^{2+} transporters which belong to the Solute Carrier Family 24 A (*SLC24 A*) of membrane transporters. NCKXs operate via the alternating access model and mediate the extrusion of one Ca^{2+} ion coupled with one K^+ ion in exchange for four Na^+ ions ($4Na^+ \leftrightarrow 1Ca^{2+} + 1K^+$) [1]. Five different human NCKX isoforms exist (NCKX1-5) which play key roles in the regulation of many biological processes such as: vision in retinal rod (NCKX1) and cone (NCKX2, NCKX4) photoreceptors [2–6], enamel maturation in ameloblasts (NCKX4) [7–11], olfaction in olfactory neurons (NCKX4) [12], satiety in neurons of the paraventricular nucleus (PVN)(NCKX4) [13], synaptic plasticity in hippocampal neurons (NCKX2) [14] and pigmentation in epidermal melanocytes (NCKX5) [15]. NCKX1-4 are all thought to operate in the cell surface membrane, whereas NCKX5 is not found in the surface membrane but located within the cell in the TGN [16].

Topological studies on the human NCKX2 protein resulted in a model consisting of two membrane spanning domains, each consisting of 5 α-helical trans-membrane segments (TMS), separated by a large hydrophilic cytoplasmic loop and preceded by an N-terminal hydrophilic loop located in the extracellular space [17,18] (see also Fig. 5). Sequence conservation between NCKX1-5 is largely restricted to the two sets of TMS. It is most pronounced in two central clusters within the two sets of TMS known as the α-1 and α-2 repeats and thought to contain the cation binding sites (reviewed in [19]). NCKX proteins are bidirectional and can carry out both Ca^{2+} influx (reverse exchange) and Ca^{2+} efflux (forward exchange) dependent on the transmembrane Na^+ and K^+ ionic gradients. With the use of reverse exchange measurements, the kinetic parameters of cation transport (turnover number, K_m values for Na^+, K^+ and Ca^{2+}, resp.) were found to be very similar for NCKX1-4 [20]. This study concerns the outward facing Ca^{2+}-binding site, which, in the absence of competing cations such as Na^+ and Mg^{2+}, has a surprisingly low K_m value in the 1–8 μM range [19,20].

NCKX proteins belong to the CaCA superfamily of Ca^{2+}/cation antiporters, and, within this superfamily, they are most closely related to

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Abbreviations: NCKX, K^+- dependent Na^+- Ca^{2+} exchangers; NCX, Na^+- Ca^{2+} exchangers; CaCA, Ca^{2+}/ cation antiporter gene family; *SLC24*, solute carrier 24 gene family; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; EDTA, ethylenediamine triacetic acid; HEDTA, hydroxyethyl ethylenediamine triacetic acid; NTA, nitrilotriacetic acid; TMS, transmembrane segment; TBST, tris buffered saline and tween; K_m, Michaelis-Menten constant; Mj, *Methanococcus jannaschii*

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the *SLC8* gene family of K^+-independent Na^+-Ca^{2+} Exchangers (NCX) and to several putative Na^+-Ca^{2+} Exchangers found in lower organisms. However, sequence similarities between NCKX and NCX proteins are confined to the two α-repeat areas mentioned above. No crystal structures have been obtained to date for eukaryotic NCX and NCKX proteins, but the crystal structure of a distantly related archaeal NCX protein from *Methanococcus jannaschii* (NCX_Mj) has been published [21]. NCX_Mj is a considerably smaller protein compared with eukaryotic NCX and NCKX proteins and mostly consist of the two sets of 5 TMS in a topological arrangement identical to that described before for NCKX2 [17,18]. Even though sequence conservation between NCKX and NCX_Mj is very limited, the NCX_Mj residues involved in cation coordination are almost completely conserved and allow for cation coordination in a homology model of human NCKX2 based on the NCX_Mj structure [22]. Consistent with their importance for cation coordination in NCKX2, substitutions in ten of the twelve residues thought to coordinate cations in NCKX2 were previously shown to result in a large (> 80%) loss of transport activity when expressed and tested in cell lines [23,24]. Despite the high degree of conservation between the NCKX2 and NCX_Mj residues thought to make up the cation binding sites, the reported K_m values for external Ca^{2+} transport differ greatly, i.e. low micromolar values for NCKX proteins versus a value of 0.25 mM observed for NCX_Mj [25], and values between 0.1 and 0.3 mM reported for NCX1 [26]. We previously reported for a small number of α-repeat residues that substitutions to other amino acids resulted in a decrease in Ca^{2+} affinity although the resolution did not permit proper measurements of K_m values [27]. This study identified two acidic residues, one in each of the two α-repeats of NCKX2 (E188 and D548), that showed the largest shifts in Ca^{2+} affinity for the charge-conserving E188D and D548E substitutions, while substitutions that removed the charge (e.g. E188Q and 548 N) resulted in mutant NCKX2 proteins without any detectable transport activity although protein expression was not affected. Significantly, the homologous residues in NCX_Mj, E54 and E213, resp., are the two main Ca^{2+} coordinating residues in the Ca^{2+} binding site [21].

We have now improved our analytical procedures using sodium-loaded HEK293 cells and better suited Ca^{2+}-indicating dyes (Fluo4-AM and Fluo4FF-AM) to permit accurate measurements of K_m values for external Ca^{2+}, even in NCKX2 mutants that have as little as 1% of WT NCKX2 maximal transport activity [20]. Here, we report on shifts in Ca^{2+} ion K_m values in NCKX2 mutant proteins representing substitutions of all α-1 and α-2 repeat residues (ninety-three residues in total). Surprisingly, a rather large number of the mutant NCKX2 proteins showed statistically significant shifts in Ca^{2+} ion K_m values, both representing mutants with lower K_m values and mutants with higher K_m values. We will discuss these results in the context of the cation binding sites in the occluded outward facing NCX_Mj structure and in our homology model of NCKX2 derived from this structure.

2. Methods

All tissue culture reagents were purchased from Invitrogen and all other chemicals were purchased from Sigma-Aldrich unless stated otherwise.

2.1. Human embryonic kidney 293 (HEK293) cells transfection and loading with Ca^{2+} indicating dyes

We used mutant NCKX2 cDNAs representing 93 residue substitutions in the α-repeat regions of the human NCKX2 splice variant 2 (Accession # NM_001193288) which were described previously [23,24]. Details of the HEK293 cell transfection protocol, the Na^+ loading and the Fluo-4/4FF-AM loading protocol as well have been described in details previously [20].

2.2. Fluorescence-based assay to measure the external Ca^{2+} dependence of Wild Type (WT) and mutant human NCKX2 proteins expressed in HEK293 cells

50 µl aliquots of the Fluo-loaded HEK293 cells expressing the WT or mutant NCKX2 proteins were added to a cuvette containing 1950 µl of a solution of 150 mM LiCl, 20 mM HEPES pH7.4, 50µM EDTA and 1 mM HEDTA (or 1 mM NTA) under constant stirring. The Ca^{2+} ion chelators (Hydroxyethyl)-ethylene-diaminetriacetic acid (HEDTA) and Nitrilotriacetic acid (NTA) were used to control the free $[Ca2^+]_{ext}$. concentration as shown in Supp. Fig. 1. Various amounts of a 200 mM CaHEDTA or 200 mM CaNTA stock solutions were added separately to different cuvettes containing 2 ml of either 1 mM HEDTA or 1 mM NTA respectively. The different CaHEDTA/HEDTA or CaNTA/NTA ratios yielded different free $[Ca^{2+}]_{ext}$ that ranged between 60 nM and 53 µM based on a calibration curve (Supp. Fig. 1). Time–dependent rises in fluorescence were recorded for 30 s after the addition of different concentrations of $[Ca2^+]_{ext}$ and 50 mM $[K^+]_{ext}$ to initiate Ca^{2+} influx via NCKX-mediated reverse exchange; after which the cells were permeabilized with a cocktail of 0.1% saponin and 7.5 mM $CaCl_2$ (final concentrations) to determine Fluo4/Fluo4FF fluorescence at saturating Ca^{2+}. Eight different $[Ca^{2+}]_{free}$ concentrations were used for each mutant NCKX2 protein (Supp. Fig. 2).

2.3. Data and statistical analysis

The fluorescence signals recorded from the Ca^{2+} dependence experiments for all the WT and mutant NCKX2 proteins were normalized to the maximum fluorescence signal and then graphed using SigmaPlot 12.5 software. Each single residue substitution was replicated 3 to 10 times. The rates of linear rises in fluorescence rates, between 2 and 30 s, depending on the activity of the mutant protein, were fitted using the single-site saturation equation to calculate the Michaelis-Menten constant (K_m) using GraphPad Prism 5 software. Statistical analysis was also performed using GraphPad Prism 5 where the two-tailed *t*-test was performed to calculate p-values and compare the difference in the means of K_m values between the WT group and each of the mutant proteins.

3. Results

3.1. Single residue substitutions in the α-repeat regions of the human NCKX2: changes in external Ca^{2+} dependence measured via reverse exchange

The topological model of the human NCKX2 protein is comprised of two sets of five trans-membrane segments (TMS) separated by a large hydrophilic cytoplasmic loop reported not to be important for exchange [28]. The α-1 and α-2 repeat regions are the most conserved sequence elements among the five human NCKX isoforms (Fig. 1A) and NCKXs from different species [29]. Here, we tested single residue substitutions of all residues in the α-repeat regions, i.e. from D172 to I220 and from E532 to D575 (highlighted in bold in Fig. 1A). The residue numbering used here represents residues of the full-length human cone NCKX2 sequence (Accession #. AAF21810) and these mutants have been described in previous studies [23,24]. As described in detail in an earlier study [20], we used a fluorescence-based assay to measure shifts in the external Ca^{2+} dependence of mutant NCKX2 proteins, expressed in Na^+-loaded HEK293 cells, by the rise in intracellular free Ca^{2+} concentration resulting from reverse exchange at high external K^+ concentration. Our external Ca^{2+} dependence assay (and K^+ dependence in the companion paper) measured reverse Na^+-Ca^{2+}-K^+ exchange because this transport mode permits high resolution measurements of these dependencies. Although forward exchange is the physiologically more relevant mode of NCKX proteins, our study aimed to examine the cation binding sites in NCKX proteins where our biochemical data