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Rapid turnover of the "functional" Na^+–Ca^{2+} exchanger in cardiac myocytes revealed by an antisense oligodeoxynucleotide approach

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Abstract

Antisense oligodeoxynucleotides (AS-ODNs) were used in combination with transient functional expression of the cardiac Na^+–Ca^{2+} exchanger (NCX1) to correlate suppression of the Na^+–Ca^{2+} exchange function with down-regulation of NCX1 protein expression. In a de-novo expression system (Sf9 cells), a decrease in both, NCX1 mRNA and protein after AS-ODN application was paralleled by diminished NCX1 activity, a typical hallmark of a true "antisense effect". Although AS-ODN uptake was also efficient in rat neonatal cardiac myocytes, in whole-cell extracts of these cells treated with AS-ODNs, the amount of NCX1 protein determined in a quantitative binding assay remained almost unchanged, despite a prompt loss of NCX1 function. Immunocytochemical staining of myocytes revealed that most of the immunoreactivity was not localized in the plasma membrane, but in intracellular compartments and was barely affected by AS-ODN treatment. These results indicate that the "*functional half-life*" of the NCX1 protein in the plasma membrane of neonatal cardiac myocytes is surprisingly short, much shorter than reported *half-lifes* of about 30 h for other membrane proteins.

Keywords: Sodium–calcium exchange; NCX; Antisense oligodeoxynucleotides; Cardiac myocyte; Heart; Intracellular calcium; Calcium transport; Caged calcium; Sf9 cells

1. Introduction

The Na^+–Ca^{2+} exchanger (NCX) represents a transport protein that is expressed in the cell membrane of almost any cell type. In cardiac muscle cells, the activity of the NCX is particularly pronounced and the transporter is primarily responsible for the extrusion of Ca^{2+} from the cytosol during the relaxation of muscle force (for review, see [1–3]). The cardiac subtype NCX1 mediates countertransport of Na^+- and Ca^{2+} ions with a stoichiometry of 3:1, thus generating a membrane current (I_{NCX}) [4–6] (but see [7,8]). To date there are still considerable gaps in the understanding of the cellular and molecular function of the NCX1. This is, at least in part, due to the lack of a completely specific pharmacological inhibitor. The emerging knowledge about the cDNA encoding the cardiac NCX1 protein which has been cloned and sequenced in 1990 enabled several studies using molecular biology techniques in order to overcome this limitation [9]. This includes site-directed mutagenesis [10–13], transgenic animals overexpressing NCX1 [14,15], the generation of knock-out mice [16,17], and in addition, antisense oligodeoxynucleotide (AS-ODN) strategies to block the de-novo synthesis of NCX in cell cultures [18–21]. The AS-ODN approach led to an almost complete functional suppression of NCX activity assessed by measuring I_{NCX} and by examining the Ca^{2+} transport using laser-scanning confocal imaging [18]. However, some discrepancies with regard to the protein stability and turnover of NCX1 in the cell membrane remain unsolved. The relatively quick decrease in I_{NCX}, which was substantial at 24 h and almost complete at 48 h in AS-ODN-treated neonatal cardiac cells indicated a compara-

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bly short half-life time ($t_{1/2}$) of less than 24 h [18]. In contrast, results from metabolic protein labeling studies suggested a $t_{1/2}$ of 33 h for NCX1 [22]. In principle, the action mediated by the AS-ODNs could be due to several mechanisms. The most obvious and expected one is the specific blocking and degradation of newly synthesized NCX1 mRNA. In addition, the antisense oligonucleotides may have either a direct pharmacological effect on the NCX1 protein or unspecific toxic effects as previously shown in other systems [23–25].

To address the question of specificity of the "antisense" approach, two models (neonatal rat cardiac myocytes and insect Sf9 cells) and three methods (reverse-transcriptase polymerase chain reaction (RT-PCR), immunohistochemistry and a quantitative binding assay) were used, all of which are independent of the NCX1 function. The advantage of Sf9 cells is the lack of endogenous NCX1 protein and the ease at which these cells can be infected with baculovirus containing the cDNA for NCX1. In Sf9 cells expressing NCX1, the application of AS-ODNs resulted in a significant reduction of NCX1 mRNA, of the de-novo synthesis of the exchanger as evidenced by a quantitative binding assay and also by measuring the exchanger function. In contrast, in neonatal cardiac myocytes, we observed that in whole-cell homogenates the total amount of NCX1 epitopes recognized by the antibody was essentially unaltered after 48 h of AS-ODNs treatment, while the functionally active fraction of NCX1 in the sarcolemma had virtually disappeared. These observations provide a likely explanation for some of the discrepancies in previous reports on the functional or biochemical half-life of NCX1.

2. Methods

2.1. Oligodeoxynucleotide uptake by rat cardiac myocytes and SF9 cells

Antisense oligodeoxynucleotides (AS-ODNs), nonsense (NS) and mismatch (MM) ODNs were identical to the ones described before [18]. The targeted sequence is part of the 3′ untranslated region of the cardiac Na^+–Ca^{2+} exchanger (position 3065–3083, numbering according to the rat sequence) [26]. Phosphorothioate AS-ODNs (high purity salt free) were purchased from MWG Biotech (Ebersberg, Germany). In these ODNs, phosphorothioate groups replace four of the internucleotide phosphate groups at each end (5′ and 3′), while all other linkages are normal phosphoester bonds. The modified "backbone" of such a mixed phosphorothioate oligo is resistant to the action of most exonucleases, but has a natural DNA center. Stock solutions of purified ODNs (100–300 μM) were stored at 4 °C or for longer periods at −70 °C. ODNs were added to the culture medium (3–5 μM final concentration for myocytes) for 24–48 h. Cultivated myocytes were then either fixed for immunohistochemistry or harvested for the binding assay experiments. To check for ODNs uptake, we used either AS-ODNs against the calcium-binding protein calretinin as described before or AS-ODNs against the NCX1, additionally labeled with FITC at the 3′ end [27]. The calretinin AS-ODNs have the same length and approximately the same guanine/cytosine content as the AS-ODNs directed against the NCX1 used for the experiments. For SF9 cells higher concentrations of ODNs (20–30 μM) were used to obtain comparable loading.

2.2. Neonatal rat cardiac myocytes

Primary cultures of neonatal rat cardiac myocytes from 2- to 3-day-old Wistar rats were prepared using established methods [28]. After enzymatic dissociation, myocytes were resuspended in medium M199 (Gibco) supplemented with 20 U/ml penicillin, 20 μg/ml streptomycin and 10% fetal calf serum, then ODNs were added after 2 h at an initial concentration of 1 or 3 μM. Cultures were maintained in an incubator (1.5% CO_2) at 37 °C for 24–48 h for experimental purposes. For binding experiments, myocytes were seeded in 25 cm^2 flasks at a density of (1–2) × 10^6 cells/ml and the AS-ODNs or NS-ODNs were added at an initial concentration of 1 or 3 μM after 2 h. After 48 h, cells were washed with phosphate buffered saline (PBS), scraped off the flask, transferred into a 10 ml tube and briefly centrifuged (3 min, 1000 rpm). The pelleted cells were resuspended in 5 ml of Dulbecco's Minimal Essential Medium supplemented with 5% fetal calf serum (to block unspecific binding sites) and 0.1% saponin (to permeabilize spontaneously forming membrane vesicles). This stock suspension was finally homogenized in a Potter-type glass-teflon homogenizer and used for the binding assay.

2.3. Quantification of NCX mRNA by RT-PCR

To quantify NCX1 mRNA levels in ODNs-treated cells (Sf9 or cultured neonatal myocytes), semi-quantitative RT-PCR with isolated total RNA was carried out. For first strand synthesis, 5 μg of total RNA was reverse-transcribed using random primers and reagents (RevertAid^{TM} H^- First Strand cDNA Synthesis Kit, MBI Fermentas, Lab Force, Nunningen, Switzerland). Three different PCR reactions were carried out from the same batch (20 μl) of RT product isolated from Sf9 cells (2 μl RT product per PCR). The first reaction was aimed at amplifying part of the coding region (446–716) of the human NCX1 mRNA yielding a fragment of 271 base pairs. The second PCR reaction used primers that amplify a fragment of 232 base pair centered around the annealing site of the AS-ODN in the 3′UTR region of the NCX1 mRNA. Forward primer: ATCCTGCCTCTTTGTGCTCCTATGGCTC; reverse primer: TACTTCGGCCCTAGTACAGAGTATGCTC. For the normalization of the RT-PCR reaction and serving as an internal standard, amplification of Sf960S acidic ribosomal protein P2 (60S RP) mRNA with primers 60SP2-5′ (AGACGTAGAGAAGATCCTCAGCTCTGTTGG) and 60SP2-3′ (TTGGTCTCCTTCTCTTCCTTCTTCTCCTCG) resulting in a band of