Retinoic Acid Increased Expression of the Na^+/Ca^{2+} Exchanger in the Heart and Brain

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Abstract. The Na^+/Ca^{2+} exchanger (NCX) is an important calcium transport system, which regulates intracellular calcium homeostasis. In particular, NCX is highly expressed in the plasma membrane of excitable neuronal and cardiac cells. We report here that binding of retinoic acid enhances expression of the NCX1 in the rat cardiac atria, brain stem and hypothalamus, but not in cerebellum. Differences in the regulation of the NCX1 by retinoic acid might suggest that $GATA_4$-retinoic acid inducible transcription factor is activated in the hypothalamus and brain stem, but not in the cerebellum. Our results support the idea that inducible transcription factors play an important role in the fine-tuning of local tissue calcium homeostasis.

Key words: Retinoic acid — Na^+/Ca^{2+} exchanger — Heart — Brain

Introduction

Calcium movement is one of the most important and powerful mechanisms controlling a variety of processes in the cells. Complexity and accuracy of calcium handling is secured by diverse calcium transport systems and also calcium binding proteins. The Na^+/Ca^{2+} exchanger (NCX) is an important calcium transport system, which regulates calcium levels by exchange of three ions of Na^+ for one Ca^{2+} and can act in either directions (Blaustein and Lederer 1999). Three mammalian genes are encoding NCX1, NCX2 and NCX3 type of the Na^+/Ca^{2+} exchanger (Gabeliny et al. 2003) and all three types are expressed in the neuronal tissue. Alternative splicing of six exons is also responsible for numerous variants of the NCX. The major variant of this transporter present in neurons can be upregulated by protein kinase A (PKA), whereas the predominant isoform in astrocytes is not modulated by PKA (He et al. 1998). Thus, different modulation of NCX isoforms may selectively regulate different physiological processes. The NCX1 isoform gene contains three types

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of alternatively used promoters: H1, K1 and Br1. Br1 promoter regulates expression in the brain and low level ubiquitous expression (Muller et al. 2002). Several studies have shown that different transcription factors affect the promoter activity, which can modulate expression of the NCX. Nicholas and Philipson (1999) have found that the rat cardiac NCX1 promoter is dependent on $GATA_4$ transcription factor. E-box and potentially MEF2 elements are probably other regulatory elements of the NCX1 (Cheng et al. 1999). Retinoic acid (RA) regulates expression of the IP_3R1 and 2 receptors in several brain areas, probably *via* RA-responsive element (RARE) (Stefanik et al. 2004, submitted). In our previous work we have shown that activation of the adrenergic system can potentiate gene expression of the cardiac NCX1 (Hudecova et al. 2004). In this work we tested whether retinoic acid can modulate the gene expression of the NCX1. We focused on regulation of the NCX1 by RA in selected rat brain areas – cerebellum, hypothalamus and brain stem and also in the rat cardiac atria and ventricles, where the NCX1 is known to play a crucial role in the process of excitation-contraction coupling.

Materials and Methods

Animals

Male Wistar rats (cca 160 g, Dobrá Voda, Slovakia) 3 months old were used. Prior to experiments, animals were housed for 1 week, four animals *per* cage in a controlled environment (22±2 ℃, 12 h light/dark cycle, light on at 6.00 a.m.). Food and water were available *ad libitum*. The Ethic Committee of the Institute of Experimental Endocrinology SAS approved all presented experiments. One experimental group of rats was treated by administration of Roaccutane (13-cis retinoic acid in tylose) 1 mg *per* 1 kg of body weight by gavage, during a period of 5 days with 1 day of rest. Control rats were treated in the exactly same way with tylose only. Each group was composed of 6 animals. Animals were sacrificed by decapitation 24 h after the last Roaccutane administration; cerebellum, hypothalamus, brain stem, cardiac atria and ventricles were frozen immediately in the liquid nitrogen.

RNA isolation and relative quantification of mRNA levels by RT-PCR

Population of total RNAs was isolated by TRI Reagent method (MRC Ltd.). Briefly, tissue samples were homogenized by tissue homogenizer (Biospec Products Inc.) in TRI Reagent and stored for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes. The homogenate was extracted by chloroform and RNAs in the aqueous phase were precipitated by isopropanol. RNA pellet was washed with 75% ethanol and stored in 96% ethanol at −70 ℃. The purity and integrity of isolated RNAs was checked on GeneQuant Pro spectrophotometer (Amersham Biosciences). Reverse transcription was performed using 1.5 μg of total RNAs and Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences) with $pd(N_6)$ primer. PCR specific for Na^+/Ca^{2+} exchanger isoform 1 NCX was carried out afterwards using primers NCX1: 5′-AGG CGG CTT TTT

TAC-3′ (position 1127–1145) and NCX2: 5′-CGA CTT CCA GAG-3′ (position 1286–1304, *Rattus norvegicus* GI 451571), giving a 177 bp fragment. After the initial denaturation (94 ℃ for 7 min), 30 cycles of denaturation at 94 ℃ for 1 min annealing at 48 ℃ for 1 min and polymerization at 72 ℃ for 1 min were performed. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as a housekeeper gene control for semi-quantitative evaluation of PCR. Primers GA1: 5′-AGA TCC ACA ACG GAT ACA TT-3′ and GA2: 5′-TCC CTC AAG ATT GTC AGC AA-3′ (Terada et al. 1993) were used to amplify 309 bp fragment from each first strand sample. After the initial denaturation (94 ℃ for 7 min), 30 cycles of denaturation at 94 ℃ for 1 min annealing at 60 ℃ for 1 min and polymerization at 72 ℃ for 1 min were performed. Both products, NCX and GAPDH were analyzed in 2 % agarose gels.

Statistical analysis

Each value represents the average for 6 animals. Results are presented as a means ± S.E.M. Statistical differences among groups were determined by one-way analysis of variance (ANOVA). Statistical significance was defined as *** $p < 0.001$. For multiple comparisons, an adjusted *t*-test with p values corrected by the Bonferroni method was used (Instat, GraphPad Software, USA).

Results

Expression of the NCX1 mRNAs in the rat brain stem tissue was almost two times higher compared to hypothalamus (54.58 ± 4.52 a.u. *versus* 27.57 ± 4.62 a.u.; Figure 1, empty columns), but similar to that in cerebellum (61.28 ± 5.49 a.u.). Treatment of rats with RA resulted in increase in the NCX mRNA in both brain

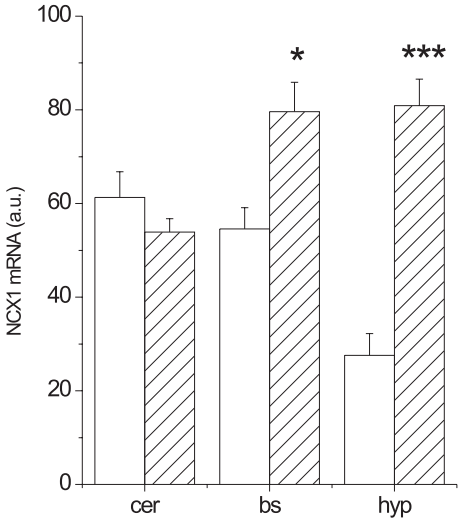

Figure 1. Effect of the RA on levels of the NCX1 mRNA in cerebellum (cer), brain stem (bs) and hypothalamus (hyp). Empty columns represent the gene expression of the NCX1 in control, untreated rats and slashed columns in rats treated with RA. Each column represents an average of at least 5 animals and is displayed as mean ± S.E.M. Statistical significance is * $p < 0.05$ and *** $p < 0.0001$.

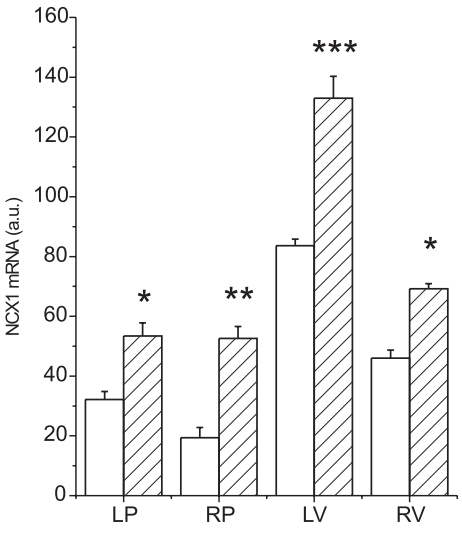

Figure 2. Effect of the RA on levels of the NCX1 mRNA in the left atrium (LP), right atrium (RP), left ventricle (LV) and right ventricle (RV). Empty columns represent the gene expression of the NCX1 in control, untreated rats and slashed columns in rats treated with RA. Each column represents an average of at least 5 animals and is displayed as mean ± S.E.M. Statistical significance is * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.0001$.

stem and hypothalamus (in brain stem to 79.61 ± 6.28 a.u. and in hypothalamus to 80.91 ± 5.67 a.u.; Figure 1, slashed columns), but not in cerebellum (53.91 ± 2.78 a.u.; Figure 1, slashed columns). Interestingly, increase in the NCX mRNA was much more pronounced in hypothalamus than in brain stem. In the cardiac atria and ventricles, RA also affects the NCX1 mRNA levels, which increased in the left atrium (LP) from 32.20 ± 2.69 to 55.76 ± 9.25 a.u., in the right atrium (RP) from 19.38 ± 4.83 to 52.61 ± 4.03 a.u., in the left ventricle (LV) from 83.6 ± 2.2 to 133.0 ± 7.3 a.u. and in the right ventricle (RV) from 46.0 ± 2.7 to 69.2 ± 1.7 a.u. (Figure 2).

Discussion

Our results clearly show that RA increases levels of the NCX1 mRNA in selected brain areas and also in the heart. We propose that increased levels of this transport system are due to augmented gene expression, since retinoids (acting through their receptors) operate as transcription factors. Increased levels of the Na^+/Ca^{2+} exchanger are commonly considered as evidence for some kind of misbalance in the cell metabolism. It was shown that higher amounts of the NCX are related with heart pressure overload (Muller et al. 2002), cardiac hypertrophy, ischemia and failure (Hasenfuss et al. 1994; Menick et al. 1996; Pogwizd et al. 1999). Also, stress as one of the most important risk factors in the development of variety civilization diseases increased gene expression of the NCX1 in the left ventricle of the rat heart (Zacikova et al. 1999). Thus, elevated levels of the NCX1 may serve as a marker of the development of pathophysiological condition.

Transcriptional regulation of the NCX1 is not completely described yet. How-

ever, three isoforms of the NCX are differently regulated due to different promoters. Sequence analysis of the NCX3 gene promoter region showed that there are binding sequences for transcription factors AP-1, $GATA_{2/3}$, AP-2, Sp-1 and ATF/CREB (Nicoll et al. 1996). Also, regional differences of the expression of NCX isoforms in the rat brain were reported by Yu et al. (1997). Retinoids comprise a family of polyisoprenoid compounds, which include vitamin A (retinol) and its natural metabolites as well as synthetic derivatives (for review see Brtko and Thalhamer 2003). RA induces its biological effects in target cells *via* binding to specific retinoic acid nuclear receptors. The resulting complexes bind to RARE in the promoters of RA-inducible genes to initiate gene transcription and to generate protein products that mediate the biological effects of RA.

In summary, we have shown that treatment of rats with RA *in vivo* increased gene expression of the NCX1 in the cardiac atria and ventricles and also in the brain stem and hypothalamus, but not in the cerebellum. Different transcriptional regulation of this important calcium transport system is probably responsible for different biological functions in diverse tissues.

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