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Developmental Expression of NCS-1 (Frequenin), a Regulator of Kv4 K^+ Channels, in Mouse Heart

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ABSTRACT

The channel proteins responsible for the cardiac transient outward K^+ current (I_{to}) of human and rodent heart are composed, in part, of pore-forming Kv4.3 or Kv4.2 principal subunits. Recent reports implicate K^+ channel interacting proteins (members of the neuronal Ca^{2+}-binding protein family) as subunits of the I_{to} channel complex. We reported that another Ca^{2+}-binding protein, frequenin [or neuronal calcium center protein-1 (NCS-1)], also functions as a Kv4 auxiliary subunit in the brain. By examining cardiac expression of NCS-1, the aim of this study was to examine the potential physiologic relevance of this protein as an additional regulator of cardiac I_{to}. Immunoblot analysis demonstrates NCS-1 protein to be expressed in adult mouse ventricle at levels comparable to that found in some brain regions. Cardiac NCS-1 protein expression levels are much higher in fetal and neonatal mouse hearts when compared with the adult. Immunocytochemical analysis of isolated neonatal mouse ventricular myocytes demonstrates co-localization of NCS-1 and Kv4.2 proteins at the sarcolemma. Given its high levels of expression in the heart, NCS-1 should be considered an important potential Kv4 regulatory subunit, particularly in the immature heart. **(*Pediatr Res* 53: 554–557, 2003)**

Abbreviations

I_{to}, transient outward K^+ current
KChIPs, K^+ channel interacting proteins
NCS-1, neuronal calcium center protein-1 (also known as frequenin)

I_{to} contributes to the repolarization process of the cardiac action potential and hence plays an important role in determining excitability of the heart. At the molecular level, it is generally believed that Kv4.3 or Kv4.2 subunits are components of channels responsible for human and rodent I_{to} (1). The expression and localization of Kv4 channels can be regulated by various channel-interacting proteins, including Kvβ-subunits (2), the chaperon-like proteins (KChAP) (3), MiRP1 (4) and filamin (5), which is a cytoskeleton-associated protein. More recently, a group of Ca^{2+}-binding proteins (KChIPs) has been described as physiologically important auxiliary subunits for Kv4 channels (6). The expression of KChIP2 mRNA in the heart (6, 7) and the fact that KChIP2 knock-out mice lack I_{to} (7) suggest that KChIP2 assembles with Kv4 α subunits to produce the cardiac I_{to}. The neuronal Ca^{2+} sensor protein-1 (NCS-1; the mammalian homologue of frequenin) belongs to the same family of neuronal Ca^{2+}-binding proteins that contains the KChIPs. We found NCS-1 to have all of the characteristics that identify it as a *bona fide* regulatory subunit of Kv4 currents (8). In heterologous expression systems, NCS-1 increases the Kv4 current amplitude, slows its inactivation time course, and accelerates recovery from inactivation. It physically associates with and co-localizes with Kv4.2 subunit in mouse brain. NCS-1 was originally described to have neuronal-specific expression. However, if expressed in the heart, then NCS-1 may, together with KChIPs (6, 7), be considered as an additional regulatory subunit of native cardiac I_{to}. The aim of this study was therefore to examine, using complimentary approaches, whether NCS-1 is expressed in ventricular myocytes of the mouse heart and to determine its expression level during development.

METHODS

All experimental protocols were performed in accordance with institutional animal guidelines.

Antibodies. The primary antibodies used were monoclonal anti-myc (clone 9E10), polyclonal chicken anti-NCS-1 (Research Diagnostics, Flanders, NJ, U.S.A.), rabbit anti-NCS-1

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(obtained from Dr. Bai Lu, National Institutes of Health, Bethesda, MD, U.S.A.) and rabbit anti-Kv4.2 antibodies (Sigma Chemical, St. Louis, MO, U.S.A., or Chemicon International, Temecula, CA, U.S.A.). The secondary antibodies used for immunoblotting were horse radish peroxidase-conjugated anti-mouse, anti-rabbit IgG (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.) or anti-chicken antibodies (Jackson Immunoresearch, Bar Harbor, ME, U.S.A.). Secondary antibodies for immunocytochemistry included Cy2- or Cy3-conjugated anti-chicken or anti-rabbit IgG (Jackson Immunoresearch).

Western blotting. Myc-tagged NCS-1 was constructed by subcloning the NCS-1 coding region into the pCS2+MT vector, which contains six myc epitope copies (9). Tissue homogenates were obtained from the hearts or brains of CO_2-anesthetized 13 d postconception (dpc) fetal, or from 1-wk-old (neonatal) Swiss Webster mice. A panel of tissues was also obtained from pentobarbital-anesthetized adult mice. Western analysis was performed as described previously (8).

Reverse transcription PCR. Total RNA, obtained from adult and 13 dpc fetal mouse hearts and brain, was used for reverse transcription. PCR was performed to amplify full-length NCS-1 using the forward primer 5'-GCCATGGGGAAATCCAACAGC-3' and the reverse primer 5'-CTATACCAGCCCGTCGTAGAGG-3'.

Immunofluorescence microscopy. Ventricular myocytes were enzymatically isolated from 7- to 10-d-old neonatal mice. Myocytes were plated on poly L-lysine coated glass coverslips and immunocytochemistry was performed as described previously (8). Images were acquired on an Olympus AX-70 using a MagnaFire cooled charge-coupled device camera, or a confocal microscope (LSM 510, Carl Zeiss Inc., Thornwood, NY, U.S.A.).

RESULTS

NCS-1 is expressed in mouse heart. We first characterized the specificity of the chicken anti-NCS-1 antibody by Western blotting (Fig. 1*A*). Anti-NCS-1 antibody detected a band of the expected molecular size (30 kD) only in lysates of HEK293 cells that have been transfected with myc-tagged NCS-1 cDNA, but not in untransfected or myc-tagged KChIP1 transfected cells (the presence of the proteins is demonstrated using anti-myc antibodies).

Proteins prepared from a panel of tissues from adult mice were subjected to immunoblotting (Fig. 1*B*). In the brain, NCS-1 is expressed at highest levels in the forebrain (which includes the olfactory bulb), with lower levels in the midbrain and the cerebellum. Although NCS-1 expression was originally described to be neuron-specific, recent evidence demonstrates that NCS-1 is also expressed in other cell types (10). Our immunoblot data show that NCS-1 is expressed in the mouse heart at levels corresponding to those found in the midbrain and cerebellum. NCS-1 expression is undetectable in liver, skeletal muscle, spleen, and thymus.

When examining cardiac expression of NCS-1 during development (Fig. 1*C*), we found NCS-1 to be expressed at high levels in the fetal heart with declining protein levels after birth. This expression pattern was mirrored when measuring NCS-1 mRNA levels (Fig. 1*D*), suggesting that the developmental regulation of NCS-1 expression is transcriptional in nature. In contrast to the declining expression with age in the heart, we found NCS-1 expression in brain tissue to peak after birth (with lower expression levels at the fetal or adult stages; Fig. 1*C*).

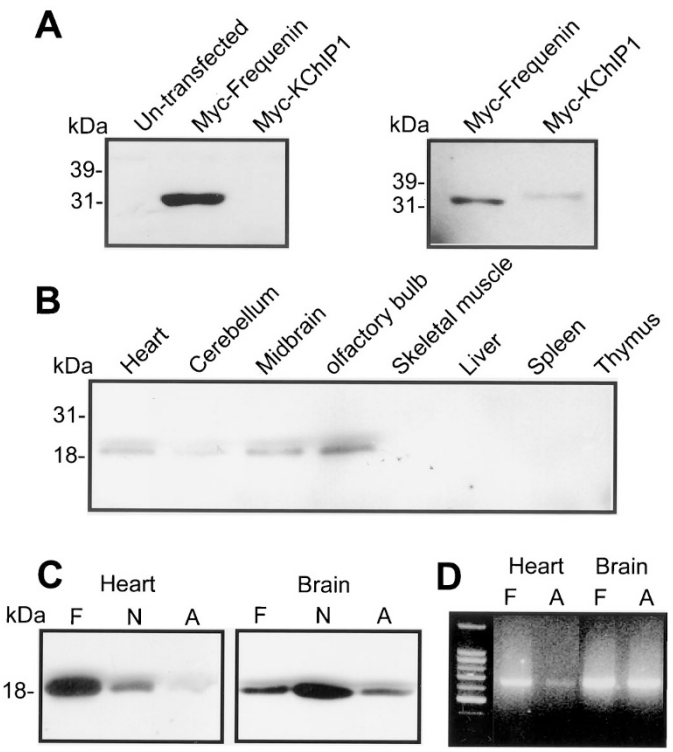

Figure 1. NCS-1 expression in mouse tissues. (*A*) Characterization of the chicken anti-NCS-1 antibody. HEK293 cells were transfected with myc-tagged NCS-1 or myc-tagged KChIP1 cDNA and Western blotting was performed using anti NCS-1 antibodies (*left panel*) or anti-myc antibodies (*right panel*). (*B*) Tissue distribution of NCS-1 protein expression in adult mouse (2 μg of proteins per lane). Immunoblotting was performed using chicken anti- NCS-1 antibodies (1/5000). (*C*) Expression of NCS-1 protein in mouse heart and brain during development [5 μg protein per lane from 13 dpc fetal (*F*), 7-d neonatal (*N*), or adult (*A*) mouse heart or brain]. Immunoblotting was performed using rabbit anti-NCS-1 antibodies (1/300). Similar results were obtained using chicken anti-NCS-1 antibodies. (*D*) Expression of NCS-1 mRNA in mouse heart and brain during development. Reverse transcriptase PCR was performed using total RNA from adult (*A*) or 13 dpc fetal (*F*) mouse heart or brain.

Subcellular localization of NCS-1 and co-localization with Kv4.2 proteins. To determine the subcellular localization of NCS-1 in immature ventricular myocytes, we performed immunocytochemistry using myocytes isolated from neonatal mouse hearts. Strong immunostaining was detected at sarcolemmal regions in all myocytes examined (Fig. 2*A*), with clustering occurring at regular intervals (*arrows in inset*). NCS-1 immunostaining was not observed in nonpermeabilized myocytes (Fig. 2*B*).

We performed double-staining experiments to examine the possibility of co-localization of Kv4.2 and NCS-1 proteins in immature myocytes. Although all cells were stained with Kv4.2 antibody, a subpopulation of myocytes (~30%) exhibited stronger staining [this is most likely due to the known regional heterogeneity of Kv4.2 expression in the rodent heart (11)]. Consistent with a previous report (12), we found strong

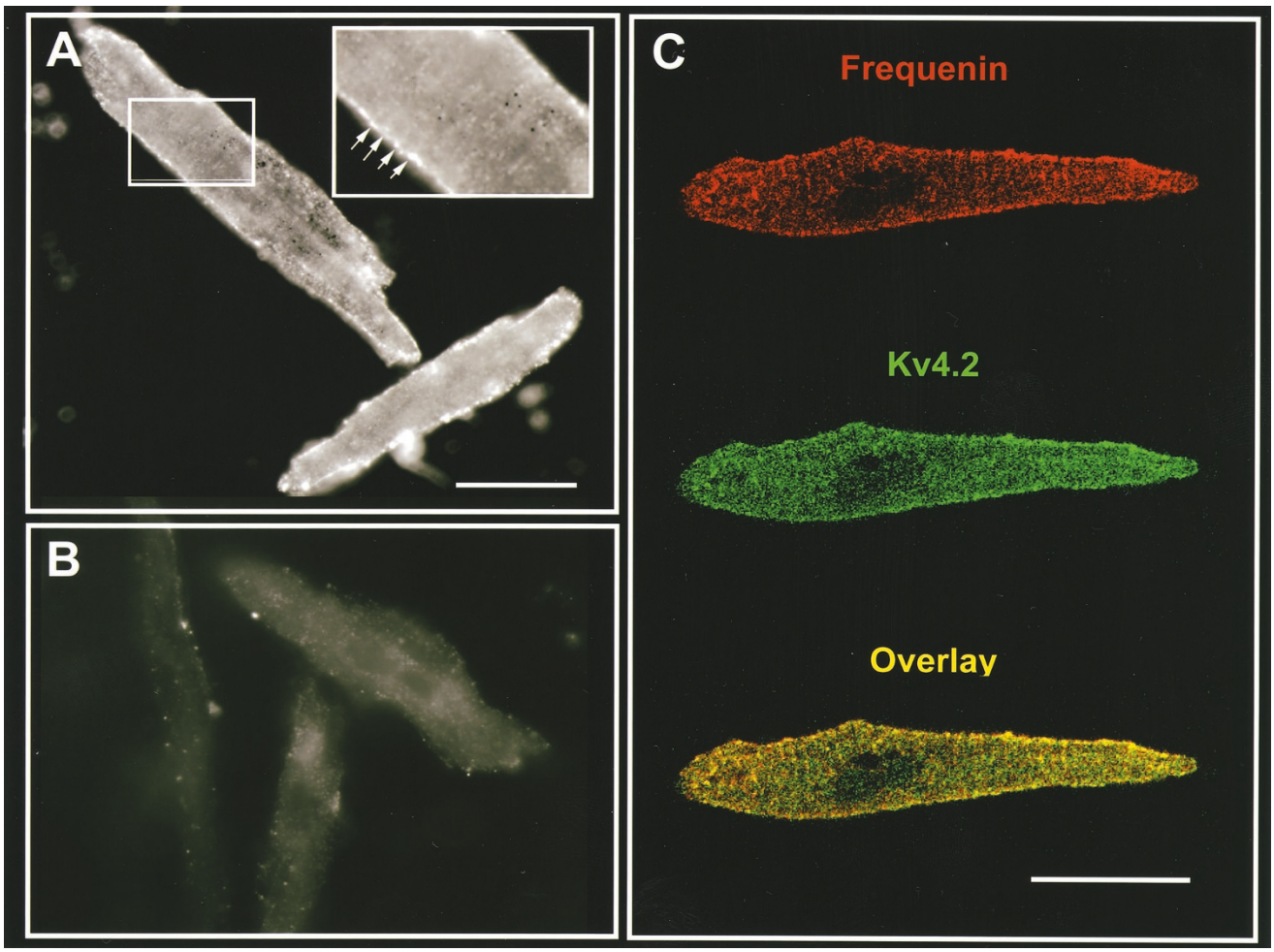

Figure 2. NCS-1 is expressed at the sarcolemma and co-localizes with Kv4.2 channel protein in isolated neonatal (7- to 10-d-old) mouse ventricular myocytes. Immunocytochemistry was performed. Strong immunostaining was detected at sarcolemmal regions in all myocytes examined (*A*), with clustering occurring at regular intervals (*arrows in inset*). NCS-1 immunostaining was not observed in nonpermeabilized myocytes (*B*) Double staining of a permeabilized myocyte using chicken anti-NCS-1 and rabbit anti-Kv4.2 antibodies (the secondary antibodies used were, respectively, CY3-conjugated anti-chicken and CY2-conjugated anti-rabbit IgG) (*C*). The scale bar depicts 20 μm.

Kv4.2 immunostaining at the t-tubular system (Fig. 2*C*). Co-localization of Kv4.2 and NCS-1 at sarcolemmal regions and the t-tubular system is illustrated by the overlay image (yellow punctate staining in Fig. 2*C*). These data extend our previous finding of co-localization of Kv4.2 and NCS-1 protein in mouse brain and further demonstrate that, at a subcellular level, NCS-1 is localized in heart cells in a manner that interaction with Kv4 proteins is likely to occur.

DISCUSSION

KChIP proteins modulate Kv4 current amplitude and gating kinetics when expressed in heterologous expression systems. KChIPs also co-localize with, and co-immunoprecipitate with Kv4.2 proteins in mouse brain (6). Because KChIP2 mRNA is expressed in the heart (6, 13, 7), and deletion of KChIP2 gene leads to a complete loss of I_{to} in ventricular myocytes (7), KChIP2 appears to be an essential component of Kv4 channels in the heart. We recently demonstrated that NCS-1/frequenin exhibit many of these properties, making it a candidate as an additional Kv4 regulatory subunit (8). We found NCS-1 to increase Kv4.2 and Kv4.3 current amplitude, inactivation time course, and channel availability without affecting other rapidly inactivating K^+ channels (including Kv1.4 and Kv3.4). As is the case for KChIPs, we found overlapping expression patterns of NCS-1 and Kv4.2 in mouse brain and direct interaction occurring between NCS-1 and Kv4.2 proteins isolated from mouse brain.

In our present study, we demonstrate NCS-1 to be expressed strongly in mouse heart. Our observation is supported by the recent finding that NCS-1 co-immunoprecipitates with Kv4.3 proteins in mouse heart (14). We also found NCS-1 to be co-localized with Kv4.2 protein at the sarcolemma of immature cardiac myocytes. This unique subcellular localization of NCS-1 (together with its known interaction with Kv4.2 proteins) and physical association between NCS-1 and Kv4 channels make it an additional candidate as a regulatory subunit of native cardiac I_{to} channels.

We found the expression of NCS-1 in the mouse heart is highest in the fetus and declines after birth. Previous reports demonstrate that Kv4.2 mRNA and protein levels (as well as I_{to}

density) increase during postnatal development in the rat (15). However, the increase in I_{to} density between postnatal d 5 and 30 is larger than that expected based on Kv4.2 protein expression patterns (15). It is therefore possible that NCS-1, with an elevated expression level in the fetal and neonatal heart, may play an important role in increasing the density of I_{to} at this early developmental stage. In contrast, it has been reported that KChIP2 expression is higher in adult heart than in fetal heart (7). Thus, KChIP2 may be the predominant regulator of Kv4 channels in the adult heart, whereas NCS-1 may be more physiologically relevant in the immature heart (or perhaps also during heart failure, when reversal to fetal gene expression patterns often occurs (16). Given its high Ca^{2+} sensitivity relative to KChIPs (17, 10) and the higher cytosolic Ca^{2+} levels in immature heart (18), our data suggest that NCS-1 is poised as a potentially important regulatory subunit of cardiac I_{to} channels, especially so in the immature heart. However, NCS-1 is known to be a multifunctional protein in neurons and other tissues, with roles ranging from regulation of neurotransmission, phosphatidylinositol 4-kinase activity and ion channel activity (10, 19–21). It is therefore possible that NCS-1 may have additional functions in the immature heart that remain to be elucidated.

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