Modulation of Kv4-encoded K\(^+\) Currents in the Mammalian Myocardium by Neuronal Calcium Sensor-1*

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Voltage-gated K\(^+\) channels are multimeric proteins, consisting of four pore-forming \(\alpha\)-subunits alone or in association with accessory subunits. Recently, for example, it was shown that the accessory Kv channel interacting proteins form complexes with Kv4 \(\alpha\)-subunits and modulate Kv4 channel activity. The experiments reported here demonstrate that the neuronal calcium sensor protein-1 (NCS-1), another member of the recoverin-neuronal calcium sensor superfamily, is expressed in adult mouse ventricles and that NCS-1 co-immunoprecipitates with Kv4.3 from (adult mouse) ventricular extracts. In addition, co-expression studies in HEK-293 cells reveal that NCS-1 increases membrane expression of Kv4-\(\alpha\)-subunits and functional Kv4-encoded K\(^+\) current densities. Co-expression of NCS-1 also decreases the rate of inactivation of Kv4 \(\alpha\)-subunit-encoded K\(^+\) currents. In contrast to the pronounced effects of Kv channel interacting proteins on Kv4 channel gating, however, NCS-1 co-expression does not measurably affect the voltage dependence of steady-state inactivation or the rate of recovery from inactivation of Kv4-encoded K\(^+\) currents. Taken together, these results suggest that NCS-1 is an accessory subunit of Kv4-encoded I\(_{\text{to,f}}\) channels that functions to regulate I\(_{\text{to,f}}\) density in the mammalian myocardium.

The rapidly activating and inactivating depolarization-activated fast transient outward K\(^+\) current, I\(_{\text{to,f}}\), underlies the rapid initial phase of action potential repolarization in mammalian cardiac cells, and heterogeneities in I\(_{\text{to,f}}\) expression contribute to observed variations in action potentials waveforms in different regions of the heart (1, 2). In addition, there are changes in the expression and the properties of I\(_{\text{to,f}}\) during normal cardiac development (3) and in a variety of myocardial disease states (2, 4, 5). Changes in I\(_{\text{to,f}}\) density can result in marked alterations in action potential waveforms, influence the normal propagation of activity in the myocardium, and increase the susceptibility to lethal cardiac arrhythmias (2, 4, 5). Thus, there is considerable interest in defining the molecular correlates of functional I\(_{\text{to,f}}\) channels and in delineating the molecular mechanisms controlling I\(_{\text{to,f}}\) expression in the mammalian myocardium. Importantly, considerable evidence has accumulated demonstrating that pore-forming \(\alpha\)-subunits of the Kv4 subfamily encode cardiac I\(_{\text{to,f}}\) channels (6–10). In contrast, the role of accessory subunits in the generation of functional I\(_{\text{to,f}}\) channels in the mammalian heart is poorly understood (1).

In addition to the diversity of voltage-gated (Kv) pore-forming \(\alpha\)-subunits (1, 11), a number of Kv channel accessory subunits, including Kv\(\beta\), MinK, MiRP\(s\), KChAP, and KChIPs, have been identified (1, 11–14). Interestingly, Kv\(\beta\)1.1, Kv\(\beta\)1.2, and Kv\(\beta\)2.1 reportedly form complexes with Kv4 \(\alpha\)-subunits in (rat) brain (15, 16), and experiments in heterologous expression systems have demonstrated that accessory subunits can modulate the properties and the functional expression of K\(v\)4 \(\alpha\)-subunit-encoded K\(^+\) currents (14, 17–20). Co-expression of Kv\(\beta\)1.2 (in HEK-293 cells), for example, alters the oxygen sensitivity of Kv4.2-encoded K\(^+\) currents (17). Recently, it was also reported that co-expression of MiRP1 with Kv4.2 (in Xenopus oocytes) affects the time- and the voltage-dependent properties of the currents (18). The Kv channel interacting proteins (KChIPs), which are calcium-binding proteins in the recoverin-neuronal calcium sensor superfamily, have been shown to bind specifically to the N-terminal intracellular domain of Kv4 \(\alpha\)-subunits and to affect Kv4 channel gating (14, 19, 20). Heterologous co-expression of KChIP1, –2, or –3 with Kv4.2 or Kv4.3, for example, increases functional (cell surface) K\(^+\) current densities, slows inactivation, accelerates the rate of recovery from inactivation, and shifts the voltage dependence of steady-state inactivation (14, 19, 20). Importantly, KChIP2 is expressed in the mammalian heart (14, 20), and recent biochemical studies (20) reveal that KChIP2 co-immunoprecipitates with Kv4 \(\alpha\)-subunits from adult mouse ventricles.

The experiments reported here were focused on examining the expression and the functioning of neuronal calcium sensor protein-1 (NCS-1), another member of the recoverin-neuronal calcium sensor superfamily (21), in the mammalian myocardium. Biochemical data are presented demonstrating that NCS-1 is highly and uniformly expressed in adult mouse ventricles and that NCS-1 co-immunoprecipitates with Kv4.3 from mouse ventricular extracts. In addition, co-expression studies revealed that NCS-1 increases functional Kv4-encoded K\(^+\) curr-

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1 The abbreviations used are: KChIPs, Kv channel interacting proteins; PFUs, plaque-forming units; ASODNs, antisense oligodeoxynucleotides; NCS-1, neuronal calcium sensor protein-1; GDNF, glial derived neurotrophic factor.
rent densities and decreases the rate of current inactivation. Taken together, these observations suggest that NCS-1 is an integral component of cardiac I_{slow} channels. A preliminary account of some of the findings presented here has appeared previously in abstract form (22).

**EXPERIMENTAL PROCEDURES**

*Western Blots—*Lysates and membrane preparations were prepared from adult (6–8 week) C57BL6 mouse brains, ventricles, and isolated ventricular myocytes as well as from Kv4.3-expressing HEK-293 cells, using methods described previously (25, 24). The protein content of each of the solubilized samples was determined by using a Bio-Rad protein assay kit with bovine serum albumin as a standard. Proteins were fractionated on 8–15% SDS-PAGE gels and transferred to PVDF membranes. The PVDF membrane strips were then incubated in 0.2% 1-Block (Tropix) in PBS containing 0.1% Tween 20 (blocking buffer) for 1 h at room temperature, followed by overnight incubations at 4 °C with a specific polyclonal anti-Kv4.3 antibody, anti-Kv4.3a (20), or a polyclonal anti-NCS-1 antibody (25) at 5 μg/ml. After washing, the membrane strips were incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (Tropix) diluted 1:5000 in the blocking buffer, and bound antibodies were detected using the CFPSD chemiluminescent alkaline phosphate substrate (Tropix).

*Immunoprecipitations—*Immunoprecipitations were performed with protein A-Sepharose beads (50 μl) (Sigma) prior to the addition of aliquots (100 μl) of mouse ventricular lysates. After mixing (by inversion) overnight at 4 °C, precipitating material was collected by centrifugation and extracted by heating in PBS containing 5% normal goat serum, 0.02% Triton X-100 and 0.1% NaNH3 for 1 h. HEK-293 cells were placed in blocking buffer (PBS containing 5% normal goat serum, 0.02% Triton X-100 and 0.1% NaNH3) for 1 h. HEK-293 cells were placed in blocking buffer (PBS containing 5% normal goat serum, 0.02% Triton X-100 and 0.1% NaNH3) for 1 h. HEK-293 cells were then incubated with an anti-Kv4.3 antibody (Abcam), anti-Kv4.3b, as described previously (20). The anti-Kv4.3b antibody (0.5 μg) was incubated with equilibrated protein A-Sepharose beads (50 μl) (Sigma) prior to the addition of aliquots (100 μl) of mouse ventricular lysates. After mixing (by inversion) overnight at 4 °C, precipitating material was collected by centrifugation and extracted by heating in PBS containing 1% SDS. The resulting protein samples were fractionated on SDS-PAGE gels and subjected to immunoblot analysis with either the polyclonal anti-Kv4.3a (20) or the anti-NCS-1 (25) antibody. Following exposure to secondary antibody, protein bands were visualized by enhanced chemiluminescence as described above.

*Immunohistochemistry—*For immunohistochemistry, HEK-293 cells were fixed in 4% paraformaldehyde for 30 min. Following washing with PBS, cells were placed in blocking buffer (PBS containing 5% normal goat serum, 0.02% Triton X-100 and 0.1% NaH2PO4) for 1 h. HEK-293 cells were then incubated with an anti-Kv4.2 antibody (20) at 1:50 dilution in blocking buffer at 4 °C overnight. After washing, cells were incubated with a Cy3-labeled goat anti-rabbit IgG secondary antibody (Chemicon), and labeled cells were visualized under epifluorescence illumination.

**Construction of Adenoviral Vectors**—The recombinant adenoviral vectors, AdEGI-Kv4.2 and AdEGI-Kv4.3, were generated by Cre-lox recombination, as reported previously (26, 27). The coding sequence for mKv4.2 or mKv4.3 was cloned into the adenovirus shuttle vector pXCRSV bicistronic shuttle vector that also expresses EGFP. The recombinant adenoviral vector, pXCRSV-NCS-1 (or pXCRSV-KChIP2) was then co-transfected into 293 cells, yielding NCS-1 (and KChIP2) adenoviruses were constructed using methylation interference (20). The adenoviral vector AdVgRXR, encoding the ecdysone receptor virus (at 50 PFU/cell), and 2% fetal calf serum. After 2 h, the infection medium was replaced with growth medium. Approximately 24 h later, cells were secondarily infected with either the NCS-1 virus (or the KChIP2 virus) at 500 PFU/cell. Kv4.3 expression was then detected by using a biotinylated primary antibody specific for Kv4.3. After fixation with 4% paraformaldehyde, the membranes were incubated at room temperature for 15 min prior to addition of the LipofectAMINE/AdSOON mixture to the cells. After 12 h, the AdSOON-containing medium was removed and replaced with selection medium, and the cells were harvested 24–36 h later.

*Electrophysiological Recordings—*Whole-cell voltage-gated outward K+ currents were recorded at 25 °C from adenovirus infected EGFP-positive HEK-293 cells. The bath solution contained (in mM) 136 NaCl, 4 KCl, 1 CaCl2, 2 MgCl2, 10 HEPES, and 10 glucose; pH 7.4. Recording pipettes contained (in mM) 135 KCl, 1 MgCl2, 10 EGTA, and 10 HEPES, and 5 glucose; pH 7.2. Experiments were conducted using a Dagan 3900A amplifier (Dagan Corp.), interfaced to a Gateway microcomputer equipped with a Digidata 1200 Series analog/digital interface (Axon). The pClamp software package was employed to deliver voltage steps and to record electrophysiological data. From a holding potential of −70 mV, voltage-gated outward K+ currents were evoked during the 400-ms depolarizing voltage steps to potentials between −40 and +60 mV; voltage steps were applied in 10-mV increments at 15-s intervals.

To determine the rates of recovery from steady-state inactivation, cells were first depolarized to +50 mV for 400 ms to inactivate the current by prepulse conditioning to potentials of −110, −20, or −50 mV for varying times ranging from 5 ms to 2 s, and finally stepped to +50 mV for 400 ms (test pulses) to activate the currents and assess the extent of recovery. The voltage dependence of steady-state inactivation were also examined using a two-pulse protocol; 1-s prepulses to potentials between −110 and +50 mV followed by a 400-ms test pulse to +50 mV were used. Experimental data were acquired at variable sampling frequencies, and current traces were filtered on-line at 5 kHz prior to digitization and storage.

*Data Analysis—*Analysis of electrophysiological data was conducted using Clampfit 6.0.5 (Axon). The decay phases of the voltage-activated, Kv4.x-encoded outward K+ currents in HEK-293 cells were well described by single exponentials at all test potentials. In addition, as reported previously (14, 20), the inactivation time constants for the Kv4.x-encoded K+ currents do not display any appreciable voltage dependence. The mean ± S.E. decay time constants (τdecay) reported here (Table I) were determined from fits of the decay phases of the currents evoked at +40 mV. To determine the kinetics of recovery (from steady-state inactivation) of the Kv4-encoded K+ currents, the pulse protocol described above was used, and the amplitudes of peak outward K+ currents evoked during the test pulses (in each cell) were measured and normalized to the amplitude of the peak current determined during the conditioning pulse (in the same cell). Data obtained from several cells were pooled, and mean ± S.E. normalized recovery data were plotted against the interpulse interval and fitted with single exponential functions.

The voltage dependence of steady-state inactivation of the Kv4.x-encoded K+ currents were obtained by normalizing the peak current amplitudes determined for the currents evoked at +50 mV from each conditioning potential to the maximal amplitude of current evoked from the conditioning prepulse of −110 mV (determined in the same cell).

* Alternate References—

1. S. A. Malin and J. M. Nerbonne, unpublished data.

2. S. A. Malin and J. M. Nerbonne, unpublished data.
Data from several cells were pooled, and the mean ± S.E. normalized data were plotted as a function of the prepulse potential and fitted with a Boltzmann equation: \( V_{1/2} = \frac{1}{1 + \exp(V - V_{b}/k)} \), where \( V_{b} \) is the voltage \( (V_{m}) \) at which 50% of the channels are inactivated, and \( k \) is a slope factor. All data are presented as means ± S.E. The statistical significance of observed differences between groups of cells was evaluated using a one-way analysis of variance followed by Student-Newman-Keuls test; \( p \) values are presented in the text, and statistical significance was set at the \( p < 0.05 \) level.

RESULTS

Expression of NCS-1 in Adult Mouse Ventricular Myocytes—Western blot analysis of fractionated adult mouse brain and ventricular lysates probed with the anti-NCS-1 antibody revealed the presence of a single ~25-kDa band in both the brain (B) and the ventricular (V) samples (Fig. 1A). Importantly, robust expression of NCS-1 was also evident in Western blots of lysates from isolated mouse ventricular myocytes (M) (Fig. 1A), indicating that the expression of NCS-1 evident in ventricular

lysates (V) arises predominantly, if not exclusively, from cardiac myocytes, rather than from non-myocytes, such as intra-cardiac neurons. In addition, Western blots of lysates prepared from the right ventricle (RV), the left ventricular apex (A), and the ventricular septum (S) reveal that NCS-1 is expressed at similar levels throughout the ventricles of the adult mouse myocardium (Fig. 1B).

 Association of NCS-1 with Kv4.3 in Adult Mouse Ventricle—The KChIPs are members of the recoverin-neuronal calcium sensor superfamily, which also includes frequenin, NCS-1, recoverin, calmodulin, neurocalcin, and hippocalcin (21). Recent studies (14, 20) have demonstrated that the KChIPs associate with Kv4.x α-subunits and modify the gating of Kv4.x-encoded K⁺ channels in heterologous expression systems. It has also been shown that the KChIPs co-immunoprecipitate with Kv4.x α-subunits from adult rat and mouse brain (14, 20) and from adult mouse ventricles (20). To explore the possibility that NCS-1 is also associated with Kv4.x α-subunits in (mouse) cardiomyocytes in situ, adult mouse ventricular proteins were immunoprecipitated with a polyclonal anti-Kv4.3 antibody, Kv4.3b, shown previously (20) to be specific for Kv4.3. The resulting immunoprecipitates were then fractionated and probed with another anti-Kv4.3-specific antibody anti-Kv4.3a (20) (Fig. 1C) or with an anti-NCS-1 antibody (Fig. 1D). As reported previously (20), the anti-Kv4.3b antibody reliably precipitates Kv4.3 (IP, Fig. 1C) or Kv4.2 (20) but not Kv α-subunits (such as Kv2.1 or Kv1.4) in other subfamilies. When the fractionated immunoprecipitated proteins were immunoblotted with the polyclonal anti-NCS-1 antibody, a single band at ~25 kDa was detected (IP, Fig. 1D), demonstrating that NCS-1 and Kv4.3 are associated in the adult mouse ventricle. The lanes labeled IN (for input) in C and D of Fig. 1 are the lysates prior to immunoprecipitation. The difference in the intensities of the ~25-kDa bands detected with the anti-NCS-1 antibody in the original tissue lysates (IN) and in the samples following immunoprecipitation (IP) with the anti-Kv4.3b antibody (Fig. 1D) suggests that only a fraction of the total NCS-1 protein expressed in adult mouse ventricles is associated with Kv4.3-encoded K⁺ channels in vivo (see “Discussion”).

Functional Consequences of NCS-1 Co-expression on Kv4-encoded K⁺ Currents—The functional consequences of co-expression of NCS-1 on Kv4.2- and Kv4.3-encoded K⁺ currents expressed in HEK-293 cells (see “Experimental Procedures”) were also examined. Parallel experiments were conducted on cells co-expressing KChIP2 to allow direct comparison of the modulatory effects of NCS-1 and KChIP2 on Kv4.x-encoded K⁺ currents. Representative whole-cell voltage clamp recordings from HEK-293 cells expressing Kv4.2 or Kv4.3 alone (top) or in combination with NCS-1 (middle) or KChIP2 (bottom) are presented in the left and right panels, respectively, of Fig. 2A. As is evident, rapidly activating and inactivating outward K⁺ currents were evident in all cells. Comparison of the top and middle panels of Fig. 2A reveals that co-expression of NCS-1 increased peak Kv4.2- (Fig. 2A, left) and Kv4.3-encoded (Fig. 2A, right) outward K⁺ current densities. Mean ± S.E. peak outward K⁺ current densities (at +40 mV), for example, were significantly

\( p < 0.001 \) higher in Kv4.2+NCs-1- (89 ± 6 pA/pF, \( n = 12 \)) and Kv4.3+NCs-1-expressing cells (114 ± 15 pA/pF, \( n = 13 \)) compared with cells expressing Kv4.2 (53 ± 4 pA/pF, \( n = 16 \)) or Kv4.3 (64 ± 6 pA/pF, \( n = 10 \)) alone (Table I). Similar to previous reports (14), co-expression of KChIP2 with either Kv4.2 (Fig. 2A, left) or Kv4.3 (Fig. 2A, right) also significantly (\( p < 0.001 \)) increased peak outward K⁺ current densities (Fig. 2A, lower panel). Mean ± S.E. peak current densities (at +40 mV) in cells expressing Kv4.2 and KChIP2 or Kv4.3 and KChIP2 were 184 ± 23 pA/pF (\( n = 8 \)) and 128 ± 17 pA/pF (\( n = 6 \)), respectively.
Analysis of peak outward K⁺ current versus voltage relations for both Kv4.2 and Kv4.3 revealed that current densities were increased at all test potentials in the presence of NCS-1 or KChIP2 (Fig. 2B). For Kv4.2, however, the average increases in peak outward K⁺ current densities at all test potentials were larger in cells co-expressing KChIP2 than in cells co-expressing NCS-1 (Fig. 2B, left panel). In contrast to the marked effects of NCS-1 (and KChIP2) co-expression on Kv4 α-subunit-encoded
Kv channels, were evident in HEK-293 cells co-expressing NCS-1 or KChIP2 (not illustrated; see “Discussion”).

Analysis of the decay phases of the currents in records such as those presented in Fig. 2A revealed that the time constants of inactivation (τdecay) of the Kv4.2-encoded K+ currents were significantly (p < 0.01) altered by NCS-1 co-expression. At +40 mV, for example, the mean ± S.E. τdecay for the Kv4.2-encoded K+ currents in the absence and in the presence of NCS-1 are 46 ± 4 and 64 ± 3 ms, respectively (Table I). Co-expression of NCS-1 with Kv4.3 also resulted in marked slowing of the time course of current decay (Table I). In addition, and as reported previously (14), co-expression of KChIP2 also significantly (p < 0.05) slowed the rates of inactivation of the Kv4.2- and the Kv4.3-encoded K+ currents (Table I).

By using a double pulse protocol (see “Experimental Procedures”), the effects of NCS-1 and KChIP2 co-expression on the kinetics of recovery from steady-state inactivation of the Kv4.2- and Kv4.3-encoded K+ currents were examined. Recordings from representative Kv4.2- (left) and Kv4.3-expressing cells (right) are shown in Fig. 3, and similar results were obtained on many cells (Fig. 4A and Table I). Analysis of the normalized current amplitudes as a function of the recovery time (interpulse interval) revealed that the time courses of recovery of both the Kv4.2- (see also Fig. 4A) and Kv4.3-encoded K+ currents at −70 mV are well described by single exponentials. The mean ± S.E. time constants of recovery (τrecovery) of the Kv4.2- and Kv4.3-encoded K+ currents are 260 ± 21 ms (n = 16) and 153 ± 14 ms (n = 16), respectively (Table I). In addition, for both Kv4.2 and Kv4.3, the rates of recovery of the currents were accelerated at −90 mV (Table I).

Co-expression of NCS-1 did not appear to affect the rate of recovery from inactivation of either the Kv4.2- or Kv4.3-encoded K+ currents (Fig. 3, middle panels). Analysis of the mean normalized recovery data confirmed that there was no significant difference in the kinetics of recovery of the Kv4.2- (Fig. 4A) or the Kv4.3-encoded K+ currents in the presence of NCS-1 (Table I). The mean ± S.E. recovery time constants for the Kv4.2- and Kv4.3-encoded K+ currents at both −70 and −90 mV in the presence and absence of NCS-1 were indistinguishable (Table I). In contrast, and as reported previously (14), co-expression of KChIP2 dramatically accelerated the time course of recovery from inactivation of the Kv4.2- and the Kv4.3-encoded K+ currents (Fig. 3, bottom panels). The mean ± S.E. time constant of recovery from inactivation of the Kv4.2-encoded K+ currents at −70 mV (Fig. 4A) in the presence of KChIP2 (69 ± 5 ms) was significantly (p < 0.01) faster than in cells expressing Kv4.2 alone (260 ± 21 ms) (Table I). Co-expression of KChIP2 also markedly accelerated the rate of recovery of the Kv4.2-encoded K+ currents at −90 mV (Table I). The rates of recovery of the Kv4.3-encoded K+ currents expressed in HEK-293 cells were also increased by KChIP2 co-expression (Table I).

As reported previously (14), co-expression of KChIP2 also affected the voltage dependence of steady-state inactivation of heterologously expressed Kv4.x-encoded K+ currents. Co-expression of KChIP2 with Kv4.2, for example, resulted in a significant (p < 0.01) depolarizing shift in the V1/2 value and increased the steepness (k value) of the normalized current versus conditioning voltage plot (Fig. 4B). Similar results were obtained with Kv4.3 (Table I). Co-expression of NCS-1 also resulted in a (6 mV) depolarizing shift in the voltage dependence of steady-state inactivation of Kv4.3-encoded K+ currents, although the slope factor was unaffected (Table I). In contrast, NCS-1 co-expression did not measurably affect the V1/2 or the k value for the voltage dependence of steady-state inactivation of Kv4.3-encoded K+ currents, although these effects are less pronounced than seen with KChIP2 (see “Discussion”).

### Table I. Effects of NCS-1 and KChIP2 on Kv4-encoded K+ currents in HEK-293 cells

| Kv4.2 | Control | +NCS-1 | +KChIP2 |
|-------|---------|--------|---------|
| τdecay (ms) | 46 ± 4a | 64 ± 3a | 85 ± 5a |
| τrecovery (ms) | 260 ± 21b | 244 ± 27b | 69 ± 5b |
| V1/2 (mV) | −39.1 ± 0.2a | −40.2 ± 1.3a | −32.6 ± 1.0a |
| k (mV) | 10.2 ± 0.5 | 11.1 ± 0.7 | 6.6 ± 0.8 |

| n | 16 | 12 | 8 |

| Kv4.3 | Control | +NCS-1 | +KChIP2 |
|-------|---------|--------|---------|
| τdecay (ms) | 102 ± 6 | 141 ± 13b | 135 ± 10b |
| τrecovery (ms) | 190 ± 12c | 168 ± 19c | 63 ± 6c |
| V1/2 (mV) | −33.2 ± 1.4d | −27.3 ± 0.5d | −28.1 ± 0.8d |
| k (mV) | 9.0 ± 0.5 | 8.2 ± 0.6 | 4.8 ± 0.4 |

| n | 10 | 13 | 6 |

* Value is significantly different from control at the p < 0.01 level.
* Value is significantly different from control at the p < 0.05 level.
* Determined at a holding potential of −70 mV.
* Determined at a holding potential of −90 mV.

### NCS-1 Increased Membrane Expression of Kv4 α-Subunits—

Subsequent experiments were designed to test the hypothesis that NCS-1 augments Kv4.x-encoded K+ current densities by increasing the cell surface membrane expression of Kv4.x α-subunit-encoded K+ channels. In initial experiments aimed at testing this hypothesis, the effect of NCS-1 on the membrane expression of Kv4.3 was examined in HEK-293 cells stably expressing Kv4.3. Interestingly, Western blots of lysates of Kv4.3-expressing HEK-293 cells probed with the polyclonal antibody revealed endogenous expression of NCS-1 in these cells (Fig. 5A, left panel, −lanes). Nevertheless, infection of these cells with Ad-NCS-1 (see “Experimental Procedures”) revealed marked increases in NCS-1 expression (Fig. 5A, left panel, + lanes). In Western blots of fractionated membrane proteins from Kv4.3-expressing HEK-293 cells probed with the anti-Kv4.3 antibody, it was evident that infection with Ad-NCS-1 increased the membrane expression of Kv4.3 (Fig. 5A, right panel, + lanes) compared with cells expressing endogenous levels of NCS-1 (Fig. 5A, right panel, −lanes). Taken together, these results suggested that NCS-1 modifies the time- and voltage-dependent properties of Kv4.x-encoded K+ currents, although the effects are less pronounced than seen with KChIP2 (see “Discussion”).

In parallel experiments, the effects of decreased NCS-1 expression on the distribution of Kv4.3 in HEK-293 cells were examined. In these experiments, NCS-1 levels in Kv4.3-expressing HEK-293 cells were reduced using an antisense oligonucleotide (AsODN; 2 μM) targeted against human NCS-1. As
### DISCUSSION

**NCS-1 Is Expressed in Adult Mouse Ventricles and Is Associated with Kv4 α-Subunits**—The results of these experiments suggest that NCS-1 plays a functional role in the generation of cardiac $I_{to,f}$ channels. Western blot analyses revealed that the anti-NCS-1 antibody identified a single protein of ~25 kDa in adult mouse ventricles as well as in adult mouse brain. The apparent molecular mass (~25 kDa) of the NCS-1 protein detected here was consistent with predictions based on sequence analysis (21, 31) and another recent report (32) demonstrating robust expression of (22 kDa) NCS-1 in mouse brain. Importantly, although NCS-1 and other members of the recoverin-neuronal calcium sensor superfamily are highly expressed in neurons and often considered nervous system-specific proteins (21, 31), the results here demonstrated that NCS-1 was also readily detected in extracts of isolated adult mouse ventricular cells. This finding suggested that the NCS-1 evident in the Western blots of lysates of whole adult mouse ventricular tissue reflects predominantly, if not exclusively, expression in myocytes. Therefore, NCS-1 is similar to another distantly related member of the recoverin-neuronal calcium sensor superfamily, KChIP2 (14), which is also highly expressed in adult mouse ventricles (20).

As reported recently by Nakamura and colleagues (32), NCS-1 co-immunoprecipitates with Kv4 α-subunits from adult mouse brain. The experiments here revealed that NCS-1 also co-immunoprecipitates from adult mouse ventricles with a specific anti-Kv4.3 antibody, demonstrating directly that NCS-1 associates with Kv4 α-subunits in the (adult mouse) myocardium. It has been reported recently (20) that Kv4.2, Kv4.3, and KChIP2 co-immunoprecipitate from adult mouse ventricles, leading to the hypothesis that functional mouse ventricular $I_{to,f}$
This hypothesis is consistent with previous studies (21, 33, 36) and the subcellular localization of NCS-1 (33) suggest that the NCS-1 protein functions in the regulation of a variety of neuronal target proteins, including ion channels. In addition, the fact that NCS-1 is abundantly expressed in the Golgi apparatus and in neurofilament-rich structures has been interpreted as suggesting that NCS-1 likely functions in protein trafficking and cytoskeletal interactions (33). Although it has

 ventricular apex, and septum, i.e. there was no apparent “gradient” of NCS-1 protein expression in adult mouse ventricles. These observations are similar to those reported recently (20) for KChIP2 in adult mouse ventricles in that KChIP2 protein expression levels in right ventricles, left ventricular apex, and septum are also indistinguishable. It has also been demonstrated that the KChIP2 message is uniformly expressed in adult rat ventricles (37). In the ventricles of larger mammals, such as canine and human, however, KChIP2 mRNA expression reportedly varies through the thickness of the ventricular wall (37). These observations, together with the finding that Kv4.3 message levels are similar throughout the ventricles, have been interpreted as suggesting that the differential expression of KChIP2 underlies the transmural gradient in \( I_{\text{to,f}} \) density in human and canine ventricles (37). Nevertheless, it will be of interest to examine NCS-1 (message and protein) expression in canine and human myocardium. In mouse and rat ventricles, the uniform expression of KChIP2 (20, 37) and NCS-1 (present study) contrasts with the heterogeneous expression of Kv4.2 mRNA (6, 38) and protein (20, 38) and the regional differences in \( I_{\text{to,f}} \) density in these animals (38, 39). Indeed, in rats and mice, it appears that Kv4.2, not the Kv accessory subunits, is the primary determinant of the gradient in ventricular \( I_{\text{to,f}} \) expression (6, 20, 38).

**NCs-1 Regulates the Membrane Expression and the Properties of Kv4.x Subunits/Channels**—Similar to KChIP2 (14, 20), co-expression of NCS-1 with Kv4.x subunits in HEK-293 cells significantly increased K⁺ current densities and slowed inactivation. Similar results have been reported recently (32) for NCS-1 co-expression with Kv4.2 or Kv4.3 in Xenopus oocytes. In contrast to the effects of KChIP2 (14, 20), however, co-expression of NCS-1 had little or no effect on the voltage dependence of steady-state inactivation or the kinetics of recovery from inactivation of Kv4.x-encoded K⁺ currents in HEK-293 cells. Interestingly, co-expression of NCS-1 reportedly does result in measurable acceleration of the rates of recovery from inactivation of Kv4.2- and Kv4.3-encoded K⁺ currents in Xenopus oocytes (32).

The biochemical data presented here demonstrate that NCS-1 overexpression (in HEK-293 cells) increases the membrane expression of Kv4.3 and, conversely, that reducing NCS-1 attenuates the membrane expression of Kv4.3. In addition, the immunohistochemical experiments revealed that the overexpression of NCS-1 increased the cell surface expression of Kv4.3. Thus, it seems reasonable to suggest that NCS-1 forms complexes with Kv4.x α-subunits and functions to increase the cell surface expression of Kv4.x proteins and the densities of Kv4.x-encoded K⁺ channels. These results clearly highlight the complexity of the molecular basis of cardiac \( I_{\text{to,f}} \) channels, which appear to reflect heteromeric assembly of pore-forming Kv4.x α-subunits and diverse accessory subunits, including KChIP2 and NCS-1. Further studies aimed at defining the sites of interactions between Kv4.x α-subunits and NCS-1, determining the involvement of other accessory subunits and/or regulatory molecules in mediating these interactions, and delineating the underlying molecular mechanisms involved are warranted.

**Relationship to Previous Studies**—The widespread distribution of NCS-1 in the mammalian brain and spinal cord (21, 31, 33) and the subcellular localization of NCS-1 (33) suggest that the NCS-1 protein functions in the regulation of a variety of neuronal target proteins, including ion channels. In addition, the fact that NCS-1 is abundantly expressed in the Golgi apparatus and in neurofilament-rich structures has been interpreted as suggesting that NCS-1 likely functions in protein trafficking and cytoskeletal interactions (33). Although it has

channels are heteromeric consisting of both Kv4 α-subunits and KChIP2. The immunoprecipitation results here suggest that NCS-1 also contributes to the formation of heteromeric mouse ventricular \( I_{\text{to,f}} \) channels. Importantly, however, and in contrast to the findings with KChIP2 (20), only a fraction of the total ventricular NCS-1 protein co-immunoprecipitated with Kv4.3. The simplest interpretation of this finding is that NCS-1 likely subserves functional roles in mouse ventricular myocytes in addition to contributing to the formation of \( I_{\text{to,f}} \) channels. This hypothesis is consistent with previous studies (21, 33–36) in neurons suggesting that NCS-1 is a multifunctional protein. Clearly, additional experiments focused on testing this hypothesis and exploring the functional roles of NCS-1 in the mammalian myocardium are warranted.

The Western blot data presented reveal that NCS-1 is expressed at similar levels in adult mouse right ventricle, left

![Fig. 5. NCS-1 regulates membrane expression of Kv4.x α-subunits.](http://www.jbc.org/content/vol86/issue12/26442/F5)

**Fig. 5.** NCS-1 regulates membrane expression of Kv4.x α-subunits. HEK-293 cells stably expressing Kv4.3 (see “Experimental Procedures”) were infected with the adenoviral construct encoding NCS-1 (Ad-NCS-1; 500 PFU/cell) or treated (for 48 h) with an antisense oligodeoxynucleotide (AsODN-NCS-1; 2 μM) targeted against human NCS-1 (B). Whole-cell lysates and membrane proteins were then prepared (see “Experimental Procedures”), fractionated, and immunoblotted with either the anti-NCS-1 (left panels) or the anti-Kv4.3a (right panels) antibody. Equal amounts of proteins (20 μg) were loaded on each lane, and results obtained in two control (–) and two infected/AsODN-treated (+) experiments from each group are illustrated. A, although NCS-1 is detected in wild-type Kv4.3-HEK-293 cells (–), infection with Ad-NCS-1 (+) markedly increased NCS-1 expression (left panel). Immunoblots of fractionated membrane proteins from Kv4.3-expressing HEK-293 cells revealed that the membrane expression of Kv4.3 was increased in cells infected with NCS-1 (right panel). B, treatment of Kv4.3-expressing HEK-293 cells with the AsODN-NCS-1 (+) markedly reduced NCS-1 expression (left panel) and the membrane expression of Kv4.3 (right panel).

![Fig. 6. NCS-1 augmented cell surface expression of Kv4.x α-subunits.](http://www.jbc.org/content/vol86/issue12/26442/F6)

**Fig. 6.** NCS-1 augmented cell surface expression of Kv4.x α-subunits. Wild-type HEK-293 cells were transiently transfected with Kv4.2 (A) or Kv4.2 plus NCS-1 (1:1 ratio) (B). Approximately 48 h later, the cultures were fixed (see “Experimental Procedures”) and probed with a specific polyclonal anti-Kv4.2 antibody, followed by a Cy3-conjugated goat-anti-rabbit secondary antibody. In cells expressing Kv4.2 alone (A), the anti-Kv4.2 antibody labeling is diffuse, whereas in cells co-expressing NCS-1 (B), the labeling is punctate and appears predominantly at the cell surface (arrowheads).
long been recognized that NCS-1 plays a role in the regulation of synaptic transmission (34), direct support for a role for NCS-1 in the functioning of membrane ion channels was provided only recently (35) with the demonstration that NCS-1 regulates voltage-gated Ca\(^{2+}\) channel currents in adrenal chromaffin cells. In addition, it has been reported that the effects of glial derived neurotrophic factor (GDNF) on motoneurons are mediated by NCS-1 (36), suggesting an important role for NCS-1 in the functioning of neuronal Ca\(^{2+}\) channels. In support of this hypothesis, it was recently reported (40) that NCS-1 directly modulates the activity of P/Q-type voltage-gated Ca\(^{2+}\) currents in presynaptic nerve terminals.

The recent report by Nakamura and colleagues (32) and the results presented here clearly also reveal interactions between NCS-1 and the Kv4.x \(\alpha\)-subunit-encoded fast transient outward K\(^+\) currents, referred to as I\(_{K_{a}}\) and I\(_{K_{a,p}}\) in neurons (32) and myocardial cells (1, 20, 39), respectively. Interestingly, it has also been reported (41) that I\(_{K_{a}}\) in midbrain dopaminergic neurons is regulated by GDNF, through a mitogen-activated protein kinase-dependent pathway. Given the regulatory role of NCS-1 in the modulation of voltage-gated Ca\(^{2+}\) channels by GDNF (36), it seems reasonable to suggest that the effects of GDNF on I\(_{K_{a}}\) may also be mediated by NCS-1. Further experiments focused on testing this hypothesis directly, as well as on exploring the possibility that NCS-1 plays a role in the regulation/modulation of other voltage-gated K\(^+\) channels in neurons and cardiac cells, will clearly be of interest.

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