Neuronal Ca\textsuperscript{2+} sensor protein-1 (NCS-1) is a member of the Ca\textsuperscript{2+} binding protein family, with three functional Ca\textsuperscript{2+} binding EF-hands and an N-terminal myristoylation site. NCS-1 is expressed in brain and heart during embryonic and postnatal development. In neurons, NCS-1 facilitates neurotransmitter release, but both inhibition and facilitation of the Ca\textsuperscript{2+} current amplitude have been reported. In heart, NCS-1 co-immunoprecipitates with K\textsuperscript{+} channels and modulates their activity, but the potential effects of NCS-1 on cardiac Ca\textsuperscript{2+} channels have not been investigated. To directly assess the effect of NCS-1 on the various types of Ca\textsuperscript{2+} channels we have co-expressed NCS-1 in Xenopus oocytes, with Ca\textsubscript{v}1.2, Ca\textsubscript{v}2.1, and Ca\textsubscript{v}2.2 Ca\textsuperscript{2+} channels, using various subunit combinations. The major effect of NCS-1 was to decrease Ca\textsuperscript{2+} current amplitude, recorded with the three different types of \( \alpha \) subunit. When expressed with Ca\textsubscript{v}2.1, the depression of Ca\textsuperscript{2+} current amplitude induced by NCS-1 was dependent upon the identity of the \( \beta \) subunit, expressed with no block recorded without \( \beta \) subunit or with the \( \beta \) subunit. Current-voltage and inactivation curves were also modified and displayed a different specificity toward the \( \beta \) subunits. Taken together, these data suggest that NCS-1 is able to modulate cardiac and neuronal voltage-gated Ca\textsuperscript{2+} channels in a \( \beta \) subunit specific manner.

Ca\textsuperscript{2+} entry through voltage-gated Ca\textsuperscript{2+} channels is essential for various cellular processes that include muscle contraction, pacemaker activity, synaptic transmission, or gene expression. Several types of Ca\textsuperscript{2+} channels have been characterized (T, L, N, P/Q, and R) that appear to play a specific role in each of these functions. These channels share a common architecture composed of a major \( \alpha \) subunit (for which ten genes are known) tightly associated with regulatory subunits \( \alpha_{2-6} \) (four different genes), \( \beta \) (four genes), and possibly \( \gamma \) (eight genes) in a functional multimeric complex (1–4). This molecular diversity, further expanded by the existence of several splice variants for each of these genes, produces a large number of possible Ca\textsuperscript{2+} channel subunit combinations with different pharmacological and biophysical properties and specific cellular and subcellular localization (5). The precise regulation of the Ca\textsuperscript{2+} influx in response to various physiological situations is further controlled by several regulatory mechanisms, working at different levels, including channel expression, localization, or activity, via additional interactions with modulatory proteins. Several Ca\textsuperscript{2+}-dependent feedback mechanisms sense incoming Ca\textsuperscript{2+} ions to finely tune channel activity to the cellular Ca\textsuperscript{2+} demands and prevent cytotoxic Ca\textsuperscript{2+} overload (6). These mechanisms use Ca\textsuperscript{2+}-sensing proteins and are specific of a given type of Ca\textsuperscript{2+} channel. It has been shown, for example, that Ca\textsuperscript{2+}-dependent inactivation of the L-type Ca\textsuperscript{2+} channel (encoded by the Ca\textsubscript{v}1.2 \( \alpha \) subunit) is governed by a Ca\textsuperscript{2+}-driven interaction between calmodulin and the C-terminal tail of the channel \( \alpha \) subunit (7–10). A similar functional interaction also appears to exist on the P/Q-type Ca\textsuperscript{2+} channel (encoded by the Ca\textsubscript{v}2.1 subunit), the major channel type involved in synaptic transmission in the mammalian central nervous system (11–13).

The neuronal Ca\textsuperscript{2+} sensor protein-1 (NCS-1),\textsuperscript{1} the mammalian homologue of frequenin, belongs to a group of small Ca\textsuperscript{2+}-binding proteins comprising four EF-hand motifs, three of which are able to bind Ca\textsuperscript{2+} in the micromolar (EF-2) or submicromolar (EF-3,4) range (14–16). NCS-1 also contains an N-terminal myristoylation site (17). NCS-1 has been shown to facilitate synapse formation, spontaneous and/or evoked neurotransmitter release, paired-pulse facilitation, and exocytosis in several cell types (18–22). NCS-1 interacts directly with phosphatidylinositol 4-kinase in yeast (23), COS-7 (24), and chromaffin cells (25), leading to the hypothesis that part of the effects mediated by NCS-1 could involve modifications in cellular trafficking through regulation of the phosphatidylinositol signaling pathway, thereby affecting vesicular transport and recycling, as well as inositol 1,4,5-trisphosphate-sensitive Ca\textsuperscript{2+} stores (24, 25).

However, a possible involvement of NCS-1 in expression and regulation of voltage-gated Ca\textsuperscript{2+} channels has also been proposed. Overexpression of a dominant-negative mutant of NCS-1, which displays impaired Ca\textsuperscript{2+}-dependent conformational changes (19), or direct loading of presynaptic nerve terminals with NCS-1 suggested that voltage-independent inhibition, as well as activity-dependent facilitation of P/Q-type Ca\textsuperscript{2+} channels (Ca\textsubscript{v}2.1), could be controlled by NCS-1, possibly via direct protein-protein interactions (18). Effects on N-type Ca\textsuperscript{2+} channel (Ca\textsubscript{v}2.2) properties have also been reported (20), and the expression of NCS-1 in mammalian cardiac myocytes and subsequent effect on K\textsuperscript{+} channel expression (26) gave rise to the possibility that NCS-1 may regulate multiple types of Ca\textsuperscript{2+} channels and other voltage-dependent ion channels, not only in neurons (27).

\textsuperscript{1} The abbreviation used is: NCS-1, neuronal Ca\textsuperscript{2+} sensor protein-1.
In a first step to explore this possibility, we have co-expressed NCS-1 with three different types of Ca\textsuperscript{2+} channel; Cav\textsubscript{1.2}, Cav\textsubscript{2.1}, and Cav\textsubscript{2.2}, associated with different combinations of auxiliary \(\beta\) subunit, and measured the resulting Ba\textsuperscript{2+} and Ca\textsuperscript{2+} currents. These combinations are likely to be expressed in different cell types where they represent potential targets for NCS-1 effects. Our goal was to explore the effect of NCS-1 on both Ca\textsuperscript{2+} channel expression and properties and to provide a first description of the molecular requirements necessary for NCS-1 effects on Ca\textsuperscript{2+} channels that may help in the understanding of the precise mode of action of this Ca\textsuperscript{2+}-bind-}

The protein we have, however, focused this study on the P/Q-type (Cav\textsubscript{2.1}) Ca\textsuperscript{2+} channels, which seem to be a primary target in various cell types (28). Our results show that NCS-1 down-regulates expression of L-, N-, and P/Q-type Ca\textsuperscript{2+} channels in a \(\beta\) subunit-specific manner and induces minor modifications of the electrophysiological properties of the channel. We provide evidence of direct functional effects of NCS-1, in addition to modifications in the expression level and/or trafficking of the channels to the membrane.

**EXPERIMENTAL PROCEDURES**

Materials and Oocyte Preparation—The following cDNA were used, and the GenBank\textsuperscript{TM} accession number is provided: Cav\textsubscript{1.2} (\(\alpha_{1C}\)), M67515; Cav\textsubscript{2.1} (\(\alpha_{2D}\)), M64373; Cav\textsubscript{2.2} (\(\alpha_{2D}\)), D14157; NCS-1, L27421; \(\beta_{1C}\), X61394; \(\beta_{2C}\), M80545; \(\beta_{3}\), M85781; \(\beta_{4}\), L02315; and \(\alpha_{2D}\), M86621. Mutations NCS-1\textsubscript{R186C} and NCS-1\textsubscript{L208F} have been described previously (29). Ca\textsuperscript{2+} channel subunits were subcloned into the pm2 vector, whereas NCS-1 and its mutants were subcloned into pcDNA3 (Invitrogen).

*Xenopus laevis* oocyte preparation and injection were performed as described previously (30). Each oocyte was injected with 5–10 nl of a cDNA mixture containing the cDNA at a cDNA concentration of 0.3 ng/ml with a ratio of 1:2.3:1. When one or more of these cDNAs was omitted, cDNA concentrations were kept constant by addition of the appropriate volume of deionized water. Oocytes were kept for 2 to 4 days before recordings at 18 °C and under gentle agitation.

Electrophysiology—Whole-cell Ba\textsuperscript{2+} currents were recorded under two-electrode voltage clamp using the GeneClamp 500 amplifier (Axon Instruments, Union City, CA). Current and voltage electrodes (less than 1 meq/ml) were filled with 3 M KCl, pH 7.2, with KOH. Ba\textsuperscript{2+} and Ca\textsuperscript{2+} current recordings were performed after injection of BAPTA (~50 nl of the following in mM): BAPTA-free acid (Sigma), 100; CsOH, 10; HEPES, 10; pH 7.2 with CsOH; using the following bath solution (in mM): Ba\textsuperscript{2+}/CsOH, 10; TEAOH, 20; NMDG, 50; CsOH, 2; HEPES, 10; pH 7.2, with methanesulfonic acid. Currents were filtered and digitized using a DMS-Teclam Labmaster and subsequently stored on a Pentium-based personal computer using the pClamp software (version 6.02, Axon Instruments). Ba\textsuperscript{2+} or Ca\textsuperscript{2+} currents were recorded during a 400-ms test pulse from -80 to +10 mV. Current amplitudes were measured at the peak of the current. Comparisons of averaged amplitudes between batches were always made with amplitudes measured the same day after injection. Comparisons between similar experiments were made by normalizing all averaged amplitudes with respect to the control current amplitude set as 100%. Isochronal steady-state inactivation curves (2.5 s of conditioning voltage followed by a 400-ms test pulse to +10 mV) were fitted using the equation, \(I(t) = I_{\infty}A_1e^{-\frac{t}{\tau_1}} + A_2e^{-\frac{t}{\tau_2}} + C\), where \(I(t)\) is the current amplitude after a conditioning pulse, \(I_{\infty}\) is the current amplitude measured during the test pulse at +10 mV for conditioning voltages varying from -80 to +50 mV, \(I_{\text{max}}\) is the current amplitude measured during the test pulse for a conditioning voltage of -80 mV, \(V_{\text{act}}\) is the potential for half-inactivation, \(V\) is the voltage, \(k\) is the slope factor, and \(R_{\infty}\) is the proportion of non-inactivating current. Current to voltage curves were fitted using the equation, \(I(V) = G(V - V_{\text{act}})/[(1 + \exp((V - V_{\text{act}})/k)]\), where \(I(V)\) is the current amplitude measured during the voltage steps varying from -80 to +50 mV, \(V_{\text{max}}\) is the peak current amplitude measured at the minimum of the current-voltage curve, \(G\) is the normalized macroscopic conductance, \(R_{\infty}\) is the apparent reversal potential, \(V_{\text{act}}\) is the potential for full activation, \(V\) is the value of the voltage step, and \(k\) is a slope factor.

Inactivation kinetics were estimated by fitting Ba\textsuperscript{2+} current decay with two exponential components using the equation, \(I(t) = A_1e^{-\frac{t}{\tau_1}} + A_2e^{-\frac{t}{\tau_2}} + C\), where \(I(t)\) is the current amplitude, \(t\) is the time, \(\tau_1\), \(\tau_2\), \(A_1\), and \(A_2\) represent the time constants and amplitudes of the two compo-

**RESULTS**

Expression of NCS-1 in *Xenopus* Oocytes—Previous works on chromaffin, human embryonic kidney 293, and COS-7 cells have shown that NCS-1 was endogenously expressed at non-negligible levels (24, 28). In non-injected *X. laevis* oocytes, the level of expression of the endogenous NCS-1 was barely detectable in Western-blots and much lower than in human embryonic kidney 293 and COS-7 cells (see Fig. 1A). Thus *X. laevis* oocytes are a system of choice to study the functional effect of NCS-1 on voltage-gated Ca\textsuperscript{2+} channels. In these oocytes, injection of the cDNA coding for rat NCS-1 led, as expected, to a massive expression of a protein of a molecular mass of ~23 kDa, in accordance with the theoretical molecular mass of

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\(^2\) A. Joromin, unpublished observations.
co-expression of NCS-1 with the CaV2.1 and control). A similar result was also found upon co-expression of NCS-1 (see Fig. 2A). This effect was most pronounced in oocytes expressing the CaV2.2 Ca2+ channel, where the averaged Ba2+ current amplitude recorded in the batch of oocytes co-injected with NCS-1 cDNA (N = 1 experiment, n = 38 oocytes) was only 10% of the control current amplitude recorded from oocytes co-injected with H2O instead of the NCS-1 cDNA (N = 1, n = 35). However, a similar effect was also found when NCS-1 was co-injected with CaV1.2 (N = 3, n = 71; 52% of the control amplitude n = 70) or CaV2.1 Ca2+ channel subunits (N = 7, n = 206; 46% of the control amplitude n = 162).

The CaV2.1 channel β subunit is an important determinant of the final Ca2+ current amplitude observed at the oocyte surface membrane and regulates many of its electrophysiological properties (4, 31, 32). Hence, we tested the role of the β subunit on the effects of NCS-1. Using the same experimental approach, we co-expressed NCS-1 with the CaV2.1 Ca2+ channel either with the α2-δ subunit alone or with the α2-δ and one of the four β subunits (β1, β2, β3 or β4). Again, in each case, the resulting current amplitude was compared with the current amplitude recorded in oocytes injected with the same combination of Ca2+ channel subunits but without NCS-1. Interestingly, when NCS-1 was co-expressed with CaV2.1 without β subunit, almost no effect on current amplitude was observed (N = 2, n = 32 and 26 for control). A similar result was also found upon co-expression of the β subunit (N = 12, n = 198, with control n = 198), whereas co-expression of NCS-1 with the CaV2.1 and β1, β2, β3 or β4 subunit decreased the expressed Ba2+ current amplitude to between 25 and 45% of their respective control values (see Fig. 2B, N = 3, 7, 2 and n = 100, 206, 71 and n = 65, 162, 84 for controls, respectively). The lack of effect of NCS-1 on the CaV2.1 Ca2+ channel subunit, expressed alone or with the β subunit, could not be attributed to a deficit of NCS-1 protein in these oocytes, because a robust expression of NCS-1 was also detected in these oocytes by Western blot (see bottom of Fig. 2B). On the other hand, to ensure that the decrease of the Ba2+ current amplitude obtained upon co-expression of NCS-1 with the β1, β2, β3 or β4 subunit was not because of a deleterious effect on the expression of any cloned protein, we analyzed the level of expression of the CaV2.1 protein in these conditions by Western blot. Fig. 2C demonstrates that a clear band of an approximate molecular mass of 250 kDa, matching of the theoretical molecular mass of the CaV2.1 subunit (251 kDa), could be specifically detected only in oocytes injected with the CaV2.1 subunit cDNA (+α2-δ+β2 subunits; see Fig. 2C, left lane). In oocytes co-injected with NCS-1 cDNA and the same combinations of Ca2+ channel subunits, this CaV2.1 immunoreactive band was also present at similar intensity (Fig. 2C, middle lane, each lane approximately loaded with three oocytes), whereas it was absent in non-injected oocytes (Fig. 2C, right lane). These results therefore revealed a true, β subunit-specific effect of NCS-1 on the Ba2+ current amplitude that cannot be attributed to nonspecific down-regulation of protein expression.

**Effect of NCS-1 on CaV2.1 Properties**—We next investigated whether co-expression of NCS-1 with the CaV2.1 subunit might have other functional consequences on Ca2+ channel proper-
ties. Current-voltage curves and isochronal inactivation curves were constructed from currents recorded in oocytes expressing the Ca\textsubscript{v}2.1 subunit with the \(\alpha_2\)-\(\delta\) alone or with the \(\beta_1\), \(\beta_2\), or \(\beta_3\) subunits, in the presence or absence of NCS-1. These record-ings were performed using either Ba\textsuperscript{2+} or Ca\textsuperscript{2+} ions as extracellular permeant cations to track any Ca\textsuperscript{2+}-specific modulation.

As presented in Table I, in the presence of Ca\textsuperscript{2+} ions and in the absence of \(\beta\) subunit (\(\alpha_1\)\(\alpha_2\)\(\alpha_\delta\) subunits), no modifications in the activation and inactivation parameters were observed upon co-expression of NCS-1. This lack of effect was also noted upon co-expression of the \(\beta_1\) or \(\beta_2\) subunit (see Fig. 3 and Table I).

Interestingly, when co-expressed with Ca\textsubscript{v}2.1 and the \(\beta_{2a}\) subunit, NCS-1 significantly depolarized the current-voltage curve \((V_{\text{act}} = -5.0 \text{ and } -0.8 \text{ mV without and with NCS-1 respectively, } p < 0.05)\) and reduced inactivation \((R_{\text{in}} = 69 \text{ and } 56\% \text{ respectively, } p < 0.05)\). Similar effects were also found in the presence of extracellular Ba\textsuperscript{2+} (Table II) and thus were not Ca\textsuperscript{2+}-dependent.

We then analyzed the effects of NCS-1 on Ba\textsuperscript{2+} current kinetics. Ca\textsubscript{v}2.1 current inactivation could be approximated by an exponential decaying phase, best described using a fast \((\tau_1)\) and a slow \((\tau_2)\) component. None of these components appeared to be significantly affected by expression of the Ca\textsuperscript{2+}-binding protein NCS-1 (Fig. 4). This lack of effect was found for channels co-expressed with the \(\beta_1\) or the \(\beta_2\) subunit and in the presence of either extracellular Ba\textsuperscript{2+} or Ca\textsuperscript{2+}. Neither the time constants \((\tau_1 \text{ and } \tau_2)\) nor their respective amplitude \((\%\tau_1 \text{ and } \%\tau_2)\) were changed at all voltages examined (see Fig. 4). Moreover, no effect on channel activation and reactivation were observed upon co-expression of NCS-1, whether the Ca\textsubscript{v}2.1 subunit was expressed with the \(\alpha_2\)-\(\delta\) alone or with any of the four \(\beta\) subunits (not shown). Therefore, although both channel expression and channel properties seemed to be regulated by NCS-1 in a \(\beta\) subunit-specific manner, they appear to require different subunit arrangements, i.e. expression was modified when \(\beta_1\), \(\beta_2\), or \(\beta_3\) subunits were expressed, whereas modifications in channel properties were only recorded in the presence of the \(\beta_2\) subunit.

To get some insight into the possible molecular determinants involved in NCS-1 effects, the same experiments, with the \(\beta_{2a}\) subunit, were conducted using two mutants of NCS-1. NCS-1\textsubscript{E120Q}, with its third EF-hand disrupted, showed impaired Ca\textsuperscript{2+}-dependent conformational changes (19) but was still able to bind cellular proteins. NCS-1\textsubscript{G2A}, a myristoylation-deficient mutant of NCS-1, relocalized NCS-1 from the perinuclear region to the cytosol (29).

Co-expression of either NCS-1\textsubscript{E120Q} or NCS-1\textsubscript{G2A} had the same effect as wild-type NCS-1 on the current-voltage curve of the Ca\textsubscript{v}2.1+\(\alpha_2\)-\(\delta\)\(\beta_2\) Ca\textsuperscript{2+} channel (i.e. a small but significant positive shift of \(-5 \text{ mV}; \text{ see Fig. 5B and Table III}). They differed, however, in their effects on the inactivation curve. Although the NCS-1\textsubscript{E120Q} completely suppressed the effect of NCS-1 on the residual current \((R_{\text{in}}:\text{ see Fig. 5 and Table III}), \) co-expression of NCS-1\textsubscript{G2A} left this parameter unchanged but suppressed the shift in \(V_{\text{in}}\) induced by wild-type NCS-1. These two mutants also had different actions on current amplitudes. Indeed, whereas NCS-1\textsubscript{E120Q} reduced the Ba\textsuperscript{2+} current amplitude when compared with control currents, recorded the same day in oocytes not injected with NCS-1, NCS-1\textsubscript{G2A} had no consistent effect, although a small, albeit not significant, re-
duction could be noted (see Fig. 5A). In the case of NCS-1\textsubscript{E120Q}, however, this effect was less marked than when recorded in oocytes co-injected with the wild-type NCS-1, suggesting that both Ca\textsuperscript{2+}-dependent conformational changes, and possibly myristoylation, participated to the observed effects. It should be noted that none of these mutants had any effect on the functional properties of the Ca\textsubscript{v}2.1+\(\alpha_2\)-\(\delta\) Ca\textsuperscript{2+} channels, expressed alone or with the \(\beta_1\) or \(\beta_3\) subunits, whereas the effects

### Table I

**Effects of NCS-1 on Ca\textsubscript{v}2.1 Ca\textsuperscript{2+} currents**

Current-voltage and inactivation curve parameters calculated from oocytes expressing the indicated combination of subunits, in the presence of 10 mM external Ca\textsuperscript{2+}. See “Experimental Procedures” for details.

| \(\alpha_1\) + \(\alpha_2\) + \(\alpha_\delta\) (n = 3) | \(\alpha_1\) + \(\alpha_2\) + \(\alpha_\delta\) + \(\beta_1\) (n = 4) | \(\alpha_1\) + \(\alpha_2\) + \(\alpha_\delta\) + \(\beta_2\) (n = 8) | \(\alpha_1\) + \(\alpha_2\) + \(\alpha_\delta\) + \(\beta_3\) + NCS-1 (n = 8) | \(\alpha_1\) + \(\alpha_2\) + \(\alpha_\delta\) + \(\beta_1\) + NCS-1 (n = 8) | \(\alpha_1\) + \(\alpha_2\) + \(\alpha_\delta\) + \(\beta_2\) + NCS-1 (n = 10) | \(\alpha_1\) + \(\alpha_2\) + \(\alpha_\delta\) + \(\beta_3\) + NCS-1 (n = 5) |
|---|---|---|---|---|---|---|
| \(V_{\text{act}}\) | \(-8.2 \pm 1.7\) | \(-13.3 \pm 2.5\) | \(-4.9 \pm 0.5\) | \(-17.3 \pm 0.7\) | \(-17.3 \pm 0.6\) | \(-17.6 \pm 1.1\) |
| \(K_{\text{act}}\) | \(-7.1 \pm 1.3\) | \(-4.3 \pm 1.7\) | \(-17.3 \pm 0.7\) | \(-6.1 \pm 0.5\) | \(-17.3 \pm 0.6\) | \(-17.6 \pm 1.1\) |
| \(V_{\text{m}}\) | \(9.5 \pm 2.0\) | \(10.7 \pm 1.7\) | \(5.7 \pm 0.3\) | \(4.3 \pm 1.1\) | \(5.4 \pm 0.3\) | \(5.6 \pm 0.3\) |
| \(K_{\text{m}}\) | \(0 \pm 14\) | \(0 \pm 3.5\) | \(15 \pm 3.2\) | \(69 \pm 2.9\) | \(56 \pm 3.9\) | \(6 \pm 1.7\) |
| \(R_{\text{m}}\) | \(-1.3 5.5\) | \(-0.8 10.4\) | \(-4.6 0.4\) | \(-17.3 6.0\) | \(-17.3 0.6\) | \(-17.6 1.1\) |

\* Statistically different from control without NCS-1 expressed (0.05 level).

**Fig. 3.** Effect of NCS-1 on the functional properties of Ca\textsubscript{v}2.1. Normalized current-voltage and inactivation curves recorded in oocytes injected with the Ca\textsubscript{v}2.1 \(\alpha_1\)\(\alpha_2\)\(\alpha_\delta\)\(\beta_1\) (A) or \(\alpha_1\)\(\alpha_2\)\(\alpha_\delta\)\(\beta_2\) (B) Ca\textsuperscript{2+} channel subunits with (open circle) or without (open square) NCS-1 are shown. Recordings were made using 10 mM extracellular Ca\textsuperscript{2+}. NCS-1 induced a significant depolarization of the voltage for activation (marked by an asterisk on the bottom of the current-voltage curve; see “Experimental Procedures” and Table I) and decreased the residual current (marked by an asterisk on the inactivation curve). This effect was only seen with the \(\beta_2\) subunit.
Current-voltage and inactivation curve parameters calculated from oocytes expressing the indicated combination of subunits, in the presence of 10 mM external Ba²⁺. See “Experimental Procedures” for details.

| Ba²⁺ currents | Vₐ₅ | Kₐ₅ | Vₘ | Kₘ | Rₘ |
|---------------|-----|-----|----|----|----|
| a₁A + a₂ – δ (n = 4) | 4.6 ± 1.1 | -5.8 ± 0.2 | -13.8 ± 1.4 | 5.9 ± 0.9 | 0 ± 2.7 |
| a₁A + a₂ – δ + β₁ (n = 7) | 6.1 ± 1.2 | -6.3 ± 0.2 | -14.2 ± 0.8 | 6.5 ± 0.4 | 0 ± 2.7 |
| a₁A + a₂ – δ + β₁ + NCS-1 (n = 7) | -6.0 ± 0.7 | -4.9 ± 0.3 | -25.3 ± 0.8 | 5.5 ± 0.1 | 16 ± 2.1 |
| a₁A + a₂ – δ + β₂ (n = 7) | -5.0 ± 0.6 | -5.0 ± 0.2 | -25.0 ± 0.9 | 5.7 ± 0.2 | 16 ± 1.9 |
| a₁A + a₂ – δ + β₂ + NCS-1 (n = 7) | -5.0 ± 0.9 | -3.1 ± 0.2 | -10.2 ± 1.3 | 5.4 ± 0.6 | 67 ± 1.7 |
| a₁A + a₂ – δ + β₂ (n = 5) | -6.9 ± 1.2 | -4.1 ± 0.2 | -5.7 ± 0.8 | 6.4 ± 0.5 | 62 ± 1.7 |
| a₁A + a₂ – δ + β₂ + NCS-1 (n = 5) | -3.8 ± 0.7 | -4.6 ± 0.3 | -27.4 ± 0.6 | 5.6 ± 0.2 | 7 ± 0.8 |
| a₁A + a₂ – δ + β₂ + NCS-1 (n = 8) | -5.2 ± 0.6 | -4.5 ± 0.3 | -30.8 ± 1.5 | 6.1 ± 0.2 | 6 ± 1.0 |

* Statistically different from control without NCS-1 expressed (0.05 level).

**DISCUSSION**

Recent studies have extended the area of expression of NCS-1 from the nervous system to neuroendocrine cells and even cardiac myocytes (21, 26, 33). In these cell types, NCS-1 modulates synaptic transmission (14, 22, 34), secretion (21, 25), or cellular excitability (18, 20) via complex processes that include regulation of key enzymes for membrane transport (15, 17, 23, 24, 35) and specific regulation of various ion channels (16, 18–20, 28). K⁺ channels were the first ion channel target to be characterized in expression systems (26, 36). Co-expression studies described an up-regulation of channel expression and activity, specifically recorded with the Kᵥ4 K⁺ channel family (26, 36). Modifications in the expression and properties of other ion channels have also been reported. In endocrine cells and neurons P/Q-type and N-type Ca²⁺ channels are clearly affected (18–20, 28), but no effect on L-type channels has been reported so far (20). These results suggest the existence of specific effects among the different voltage-activated Ca²⁺ channel types or among different tissues. However, the molecular basis of this specificity, and in particular the role of...
the subunit composition of the channel, in the observed effects remains unknown.

In the present work, using heterologous expression of defined Ca\(^{2+}\) channel subunits, we show that NCS-1 has two major effects on high voltage-activated Ca\(^{2+}\) channels: (1) a decrease in the current amplitude that is \(\beta\) subunit-specific but observed with Ca\(_{V}1.2\), Ca\(_{V}2.1\), and Ca\(_{V}2.2\) Ca\(^{2+}\) channels; and (2) a small modification in the activation and inactivation parameters of the Ca\(_{V}2.1\), only seen when the \(\alpha_1\) subunit is expressed with the \(\beta_2\) subunit.

**Effect of NCS-1 on Ca\(^{2+}\) Current Amplitude**—Effects of NCS-1 on P/Q- and N-type Ca\(^{2+}\) channel current amplitudes have been documented over the past years but always based on experiments using either dominant negative mutants or overexpression of wild-type NCS-1 in native cells constitutively expressing the protein (18–20). Our study is therefore the first to analyze the effects of NCS-1 on multiple types of Ca\(^{2+}\) channel in the same environment, using heterologous expression of defined Ca\(^{2+}\) channel subunits. In neurons, NCS-1 has been shown to clearly increase N-type Ca\(^{2+}\) channel expression (20), with significant modifications of the electrophysiological parameters. The opposite effects have been found on P/Q-type Ca\(^{2+}\) channels in bovine chromaffin cells, where a dominant negative mutant of NCS-1 increased Ca\(^{2+}\) current amplitude (19) without effect on L-type Ca\(^{2+}\) channels. Using cDNA injection, we show that all three channel types are down-regulated by co-expression of NCS-1. Our experimental conditions and our analysis of the expression level of the Ca\(_{V}2.1\) subunit (Fig. 2C) suggest that this effect must take place after protein synthesis and could involve modifications in the correct folding and trafficking of the channel complex to the plasma membrane and/or regulation of the channel activity per se, taking place after insertion of the channel into the plasma membrane. Membrane expression of high voltage-activated Ca\(^{2+}\) channels is known to rely on a short sequence located within the intracellular loop connecting the homologous domains I and II of the \(\alpha_1\) subunit (37). This sequence, located close to the \(\beta\) subunit binding site (AID), acts as a retention signal in the endoplasmic reticulum and thus reduces trafficking of the \(\alpha_1\) subunit to the membrane, just like a surface expression brake. This brake is usually removed by the \(\alpha_1/\beta\) subunit association, which occurs in the reticulum. The fact that the decrease in current amplitude induced by NCS-1 was only observed when \(\beta_1\), \(\beta_2\), or \(\beta_4\) subunits were expressed suggests that NCS-1 may interfere with this mechanism. A similar effect on channel expression has been recently reported (38) for the small GTPase KirGq, which binds directly to the \(\beta\) subunit, thus preventing the \(\alpha_1/\beta\) association and restoring the expression brake imposed by the retention signal. Direct specific binding of NCS-1 to this site and/or to the \(\beta\) subunit is thus an attractive hypothesis to explain the reduction of the current amplitude but needs to be further explored by additional experiments designed to directly test the biochemical interactions between these subunits. Such a mechanism, however, may not be exclusive, and other pathways, acting directly or indirectly on channel activity, such as Src-dependent inhibition (28), or direct modulation of the G-protein-coupled receptor pathways (19), may also exist. Although in our recording conditions, the Src-dependent and G-protein pathways could be discarded (the Src kinase inhibitor, PP1, had no effect; data not shown), it is worth noting that G-protein \(\beta\gamma\) subunits and the Ca\(^{2+}\) channel \(\beta\) subunit possess very close binding sites on the main \(\alpha_1\) Ca\(^{2+}\) channel subunit (39–41), leaving open the possibility that interactions between the \(\alpha_1\) subunit on one hand, and G-protein, Ca\(^{2+}\) channel \(\beta\) subunit, and NCS-1 on the other hand, could be mutually exclusive and/or under the control of tissue-specific conditions. The I-II loop would therefore acts as a cross-road for cell-specific regulations.

Interestingly, NCS-1 had no effect on Ca\(_{V}2.1\) Ca\(^{2+}\) channel co-expressed with the \(\beta_3\) subunit, the \(\beta\) subunit that displays the lowest affinity for the AID (42), and which also shows the weakest potency to increase current amplitude in expression systems (43). In neurons, where the N-type Ca\(^{2+}\) channel is predominantly associated with the \(\beta_2\) subunit (44), the increase in vesicular transport and membrane trafficking induced by NCS-1, acting through the activation of the phosphatidylinositol 4-kinase \(\beta\) (45), may overcome the effect on the \(\alpha_1\) retention signal, poorly masked/removed by the \(\beta_2\) subunit, and lead to the observed overexpression of N-type Ca\(^{2+}\) channels (20). In this scenario, the tissue specificity of the effects of NCS-1 on Ca\(^{2+}\) channels should thus be critically dependent on the subunit composition of the channel. Whether NCS-1 acts directly or indirectly on the Ca\(^{2+}\) channel \(\alpha_1\) or \(\beta\) subunit requires further investigation.

The fact that mutants that prevented Ca\(^{2+}\) binding (NCS-1\(_{E120Q}\)) and protein myristoylation (NCS-1\(_{I20A}\)) both decreased the effects of NCS-1 on channel expression underlines the requirement for a fully functional NCS-1 protein to record these effects. Mutation of glutamate 120 to glutamine (NCS-1\(_{E120Q}\) mutant) disrupts a high affinity Ca\(^{2+}\) binding site and impairs Ca\(^{2+}\)-dependent conformational changes (19). This mutant acts as a dominant negative mutant for the regulation of the P/Q channel in chromaffin cells (19). In *Xenopus* oocytes the NCS-1\(_{E120Q}\) mutant appeared also less potent than wild-type NCS-1. However, a clear and significant decrease in current amplitude was nevertheless observed, suggesting that the mutation did not completely suppress NCS-1 activity. These differential effects constitute another argument in favor of the presence of different mechanisms working in chromaffin cells or *Xenopus* oocytes to regulate Ca\(^{2+}\) channels, i.e. removal of a voltage-independent inhibition versus down-regulation of channel trafficking, and suggest that Ca\(^{2+}\)-dependent changes are absolutely necessary only for the removal of the voltage-independent inhibition of the P/Q channels (19, 28). Preserved interactions of NCS-1\(_{E120Q}\) with cellular proteins can be an argument for the dominant negative effect of the mutant, as suggested (19), but can also constitute an interesting area of investigation to identify preserved interactions that may be still functional and involved in a Ca\(^{2+}\)-independent down-regulation of the channel activity.

### Experimental Procedures

Effects of mutated NCS-1 on Ca\(^{2+}\) channel currents: (1) a decrease in the current amplitude that is \(\beta\) subunit-specific but observed with Ca\(_{V}1.2\), Ca\(_{V}2.1\), and Ca\(_{V}2.2\) Ca\(^{2+}\) channels; and (2) a small modification in the activation and inactivation parameters of the Ca\(_{V}2.1\), only seen when the \(\alpha_1\) subunit is expressed with the \(\beta_2\) subunit.

**TABLE III**

Down-regulation of Neuronal Ca\(^{2+}\) Channels by NCS-1

| Ba\(^{2+}\) currents | \(V_{\text{act}}\) | \(K_{\text{act}}\) | \(V_{m}\) | \(K_{m}\) | \(R_{m}\) |
|----------------------|-----------------|-----------------|--------|--------|--------|
| \(\alpha_1 + \alpha_2 - \delta + \beta_2 (n = 8)\) | \(-10.7 \pm 0.9\) | \(-3.1 \pm 0.2\) | \(-10.2 \pm 1.3\) | \(5.4 \pm 0.4\) | \(67 \pm 1\) |
| \(\alpha_1 + \alpha_2 - \delta + \beta_2, NCS-1(n = 5)\) | \(-6.9 \pm 1.2^a\) | \(-4.0 \pm 0.2^a\) | \(-5.7 \pm 0.8^a\) | \(6.4 \pm 0.5\) | \(62 \pm 2\) |
| \(\alpha_1 + \alpha_2 - \delta + \beta_2, NCS-1_{E120Q} (n = 6)\) | \(-6.9 \pm 0.7^a\) | \(-4.1 \pm 0.3^a\) | \(-6.9 \pm 0.8\) | \(6.4 \pm 0.7\) | \(67 \pm 2\) |
| \(\alpha_1 + \alpha_2 - \delta + \beta_2, \ NCS-1_{I20A} (n = 8)\) | \(-6.6 \pm 4\) | \(-3.9 \pm 0.6\) | \(-10.2 \pm 0.3\) | \(5.1 \pm 0.6\) | \(58 \pm 10\) |

\(^a\) Statistically different from control without NCS-1 expressed (0.05 level).

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Current-voltage and inactivation curve parameters calculated from oocytes expressing the indicated combination of subunits, in the presence of 10 mM external Ba\(^{2+}\). See “Experimental Procedures” for details.
Mutation of the myristoylation site, NCS-1<sub>G2A</sub>, is known to affect subcellular localization of NCS-1 (29), with minor modifications in the protein structure (46). The tendency of the NCS-1<sub>G2A</sub> mutation to decrease Ca<sup>2+</sup> current amplitude was not found statistically significant. This may simply reflect a small decrease in the availability of the NCS-1<sub>G2A</sub> mutant at early stages of protein assembly, because of the loss of the perinuclear localization (29) or because of more indirect effects, related to mutation-induced modifications in the degree of cooperativity in Ca<sup>2+</sup> binding (46). The construction and testing of the double mutant NCS-1<sub>G2A,K120Q</sub> should help to solve this issue.

**Effect of NCS-1 on Ca<sup>2+</sup> Current Properties**—Beyond the decrease in current amplitude, we also noted that NCS-1 could specifically affect the electrophysiological properties of the Ca<sub>v</sub>2.1 channel, but only when co-expressed with the β<sub>2α</sub> subunit. Increased inactivation and a positive shift of the current-voltage curve were the most significant changes. These effects were seen in the presence of extracellular Ba<sup>2+</sup> or Ca<sup>2+</sup> and therefore, in our conditions, did not seem to be Ca<sup>2+</sup>-dependent. The fact that the decrease in current amplitude and the modifications in the channel properties did not have the same β subunit specificity suggested different underlying mechanisms. Direct interactions between Ca<sup>2+</sup> channels and NCS-1 at the plasma membrane have not been reported so far but, the presence of NCS-1 in synaptic-like microvesicles and its co-localization in presynaptic terminals with the proteins of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor complex, VAMP (45) and syntaxin (18), also known to interact with P/Q Ca<sup>2+</sup> channels (1), suggest that NCS-1 is appropriately located for these potential functional interactions. Indeed, a previously published work (18) reported an enhancement of the facilitation of the P/Q-type Ca<sup>2+</sup> channel in the Calyx of Held, occurring via an acceleration of current activation. A similar frequency-dependent facilitation was also observed in the Calyx of Held, occurring via an acceleration of current activation. A similar frequency-dependent facilitation was also observed in the Calyx of Held, occurring via an acceleration of current activation.

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