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Nickel reduces calcium dependent dimerization in neural cadherin†

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Cadherins are the transmembrane component in adherens junctions, structures that link the actin cytoskeletons in adjacent cells within solid tissues including neurological synapses, epithelium and endothelium. Cell–cell adhesion by cadherins requires the binding of calcium ions to specific sites in the extracellular region. Given the complexity of the cell adhesion microenvironment, we are investigating whether other divalent cations might affect calcium-dependent dimerization of neural (N) cadherin. The studies reported herein characterize the impact of binding physiological magnesium(II) or neurotoxic nickel(II) on calcium-dependent N-cadherin function. Physiological levels of magnesium have only a small effect on the calcium-binding affinity and calcium-induced dimerization of N-cadherin. However, a tenfold lower concentration of nickel decreases the apparent calcium-binding affinity and calcium-induced dimerization of N-cadherin. Competitive binding studies indicate that the apparent dissociation constants for nickel and magnesium are 0.2 mM and 2.5 mM, respectively. These K_d values are consistent with concentrations observed for a range of divalent cations in the extracellular space. Results from these studies indicate that calcium-induced dimerization by N-cadherin is attenuated by natural and non-physiological divalent cations in the extracellular microenvironment.

Significance to metallomics

In order to understand signal transduction and long term potentiation, it is critical to determine the effect of the complex ionic milieu of neurological tissue upon neural cadherin, the major cell adhesion molecule at excitatory synapses. The studies reported here are basic protein chemistry studies that monitor the direct effect of alternative divalent ions on adhesive dimer formation by neural cadherin. Results indicate that nickel attenuates calcium-induced adhesive dimerization, particularly at the low calcium levels characteristic of potentiated synapses.

Introduction

Due to the role of cations in the structure and function of neurological tissue, the ionic complexity of the neural extracellular environment has significant impact on brain physiology.[1] A special symposium entitled "Cell Adhesion Molecules as Potential Targets in Metal Toxicity" at the Society of Toxicology in 2001 addressed this issue directly. Since that time, significant effort has been focused on understanding the complexity of the extracellular matrix and its apparent role in sequestering cations (*cf.* Morawski *et al.*[2] and Nicholson *et al.*[3]), including computational modeling of ionic currents.[4] Cadherins are calcium-dependent cell–cell adhesion molecules that are the synaptic-tag for potentiated synapses.[5,6] Calcium binds to the extracellular region at sites that comprise clusters of negatively charged residues at the interface between each of the five tandem modular extracellular domains. Given the itinerant nature of the calcium-binding sites in cadherins in comparison to EF-hand calcium-binding sites[7] or Zn-finger sites,[8] we might expect that other divalent cations would bind to cadherin and compete with calcium for occupancy of the calcium-binding sites. For our purposes here, we have undertaken the measurement of the direct impact of two alternative divalent metals on calcium-dependent adhesive dimer formation.

This paper addresses three related issues: Do alternative divalent cations compete with calcium binding? Can their binding induce dimer formation? And does competition with calcium binding affect adhesive dimer formation by N-cadherin (NCAD)? Our studies focus on the effect of two divalent cations, a naturally occurring ion (Mg^{2+}) and a neurotoxic, environmental hazard (Ni^{2+}). To our knowledge, these are the first studies to determine the direct effect of alternative divalent ions on adhesive dimer formation in NCAD *in vitro* and highlight the effect on the binding affinity of calcium and the formation of dimers as separate, but linked, phenomena.

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N-Cadherin is responsible for mediating a variety of specific cell–cell adhesive events through calcium-dependent dimerization of identical proteins on adjoining synaptic membranes.[9] It is involved in a variety of morphological and developmental processes such as synapse formation and maintenance, neurulation, and neurite outgrowth.[10–12] Because these morphological processes have been shown to occur concurrently with changes in cadherin expression, regulated modifications of the adhesive strength and selectivity of these cell adhesion molecules are critical for proper embryonic development.[13] Cell adhesion molecules, including N-cadherin, are involved in the dynamics and regulation of synaptic structure and function that directly impact induction of long-term potentiation.[14–16] Given the role of N-cadherin in these physiological processes, it is important to understand the microenvironmental factors that influence its adhesive behavior.

N-Cadherin is a classical or type I cadherin comprising five tandem extracellular (EC) domains, a single-pass transmembrane region, and a conserved C-terminal cytoplasmic region. Formation of adhesive dimers between cadherins on apposed cell surfaces occurs through the formation of a strand-swapped structure. This end state forms *via* the exchange of the N-terminal β-strand between juxtaposed EC1 domains, resulting in the mutual insertion of the side chain of a conserved tryptophan (Trp2) into the conserved hydrophobic pocket of the neighboring molecule.[17] Because the critical information necessary for formation and dynamics of cadherin-based adhesion resides in the primary structure of the EC region, the studies in this paper utilize a highly studied construct possessing only the first two domains of the extracellular region of N-cadherin, NCAD12.

The interface between tandem EC domains contains clusters of carboxylates that constitute three calcium-binding sites. Binding of three calcium ions at these interdomain sites causes a conformational change resulting in a rigidified EC region with an elongated, curved structure.[18,19] Because the residues comprising the calcium-binding sites are anionic, we hypothesized that the presence of other divalent cations in the extracellular microenvironment will compete with calcium for site occupancy, resulting in a reduction in calcium-binding affinity with a concomitant decrease in dimer formation. Studies, herein, report the effect that Ni^{2+} and Mg^{2+} have on calcium-dependent dimerization of NCAD12.

Cations reside in varying concentrations within the neural extracellular matrix and are thought to play important roles in modulating neuronal structure and function.[20–22] Previous work from our lab demonstrated that adhesive dimer formation by cadherin is systematically increased as the concentration of NaCl increases from 60 mM to 1 M, indicating that there is an attenuation of dimerization affinity due to electrostatic repulsion.[23] While the origin of this electrostatic effect is unknown, it does indicate the critical role of ionic interactions between protomers at the dimerization interface.

Magnesium is an essential nutrient in every cell type in the human body in concentrations of up to 1.5 mM within the extracellular matrix.[24] It participates in a number of physiological processes including signaling, enzyme activation, and catalysis.[25] Magnesium is also the most abundant free divalent cation within cells, further demonstrating its ubiquity in physiological processes.[26]

Nickel possesses an essential role in many physiological processes,[27,28] and its concentration in normal tissues is in the submicromolar range.[29] Environmental pollution by nickel has resulted from the rising consumption of nickel-containing products and from the by-products of their production, recycling and disposal.[30,31] Human exposure to nickel primarily occurs by inhalation and ingestion, and excessive amounts of nickel in different forms may be deposited in the human body over time, by means of both occupational and dietary exposure.[32,33] Bioaccumulation can cause disruption of normal function, particularly in redox regulation.[34,35] Nickel accumulates in fetal brain and, at only 10 μM concentration, can disrupt many cellular functions in a cell-culture model of fetal brain.[36] Recent studies highlight deficits in 3–5 month old infants who were exposed to cigarettes during their gestation.[37] While this is clearly a multifaceted effect, including nicotine exposure, we argue that alternative divalent cations could play a pivotal role at even low concentrations. Further, concentrations of nickel in tissues from workers exposed to nickel containing particles were increased from 30 μM to 2 mM,[38] thereby increasing their risk for cancer.[39]

Because the chemistry of the extracellular matrix has numerous implications in the fields of brain physiology and pathophysiology,[40–42] we explored the effects of Ni^{2+} and Mg^{2+} on the biophysical properties of NCAD12. While these studies show that the function of NCAD12 is weakly affected by the presence of Mg^{2+}, the presence of Ni^{2+} strongly affected both calcium-binding and dimerization affinities. These results suggest that the presence of alternative divalent cations within the neural extracellular matrix may modulate synaptic dynamics and efficacy.

Results and discussion

The experiments reported here address three related questions: Does Mg^{2+} or Ni^{2+} compete with Ca^{2+} for occupancy of the calcium-binding sites? Does Mg^{2+} or Ni^{2+} binding induce dimer formation? And does their competition with calcium binding affect adhesive dimer formation by N-cadherin? These studies show that neither Mg^{2+} nor Ni^{2+} can promote dimerization by NCAD12; however, both compete with Ca^{2+} for the binding sites and attenuate calcium-induced dimerization. Thus, the presence of divalent cations within the extracellular matrix will impact the stability and dynamics of adherens junctions.

Binding of divalent cations

Changes in the CD spectra were monitored during calcium titrations of NCAD12 in the absence and presence of fixed concentrations of Ni^{2+} and Mg^{2+}. Representative calcium titrations are shown in Fig. 1. As the concentration of Ni^{2+} and Mg^{2+} increased, the midpoint of the titration curve shifted to a higher calcium concentration, especially in the presence of Ni^{2+}, indicating a lower apparent calcium-binding affinity as a function of increasing divalent cation concentration. These results imply that there is

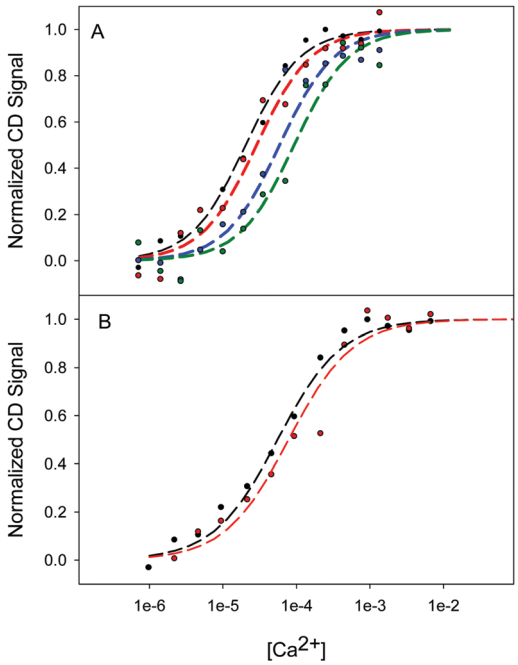

Fig. 1 (A) Calcium titrations of NCAD12 as a function of Ni^{2+} concentration. NCAD12 was titrated with Ca^{2+} only (black), and in the presence of 100 μM (red), 500 μM (blue), and 1 mM Ni^{2+} (green). (B) Calcium titrations of NCAD12 as a function of Mg^{2+} concentration. NCAD12 was titrated with Ca^{2+} only (black) and in the presence of 1.5 mM Mg^{2+} (red). In both figures, the CD signal is plotted against total calcium concentration. Dashed lines are simulated based on parameters resolved from global analysis of at least two separate experiments at each experimental condition. Data are normalized for clarity. Example CD spectra are shown in ESI,†Fig. S2.

Table 1 Association constants for calcium binding monitored by circular dichroism

Independent calcium titration	$K_a(Ca^{2+})$	$18 \pm 4 \times 10^3$
Competitive titrations[a]	$K_a(Ca^{2+})$	$20 \pm 4 \times 10^3$
	$K_a(Ni^{2+})$	$5 \pm 2 \times 10^3$
	$K_a(Mg^{2+})$	$4 \pm 3 \times 10^2$

[a] Calcium titrations with added Ni^{2+} or Mg^{2+} (eqn (1)).

competition between calcium and other divalent cations in the neural extracellular matrix for the same binding sites in N-cadherin.

In order to determine the apparent binding constants for Ni^{2+} and Mg^{2+}, competitive binding data were fit to eqn (1). Resolved association constants for Ca^{2+}, Ni^{2+} and Mg^{2+} are shown in Table 1. The resolved association constant for calcium binding in the absence of Ni^{2+} and Mg^{2+} is also shown for comparison, and agrees with the value resolved from the competitive titrations. Ni^{2+} binds with an apparent dissociation constant of 200 μM, fourfold weaker binding affinity than for Ca^{2+}. Mg^{2+} binds with an apparent dissociation constant of 2.5 mM, significantly weaker than either of the other two cations. A direct titration of NCAD12 by Ni^{2+} is shown in ESI,†Fig. S1.

Dimerization studies

The assay for calcium-dependent dimerization of NCAD12 depends upon a unique property of N-cadherin that has not been observed in other members of the classical cadherin family. NCAD12 forms a kinetically trapped dimer upon rapid decalcification of the saturated dimer (D_{sat}). This trapped apo dimer (D_{apo}*) is not in exchange with monomer and, therefore, appears as a distinct dimer peak in size exclusion chromatography (SEC). As reported here, we can exploit this unique property to determine the effect of alternative metals on dimerization of NCAD12.

Assembly studies. Initial studies tested the ability of Mg^{2+} and Ni^{2+} to promote the formation of dimer as assessed by analytical SEC. In order to perform this study, we created an apo protein stock that had low intrinsic levels of the kinetically trapped dimer. Data presented in Fig. 2A indicate that the level of dimer formed upon addition of 1 mM Ca^{2+} is consistent with expectations based on the protein concentration (25 μM) and the K_d for dimerization (25 μM).[43] Further, neither Mg^{2+} nor Ni^{2+} promoted the assembly of the D_{sat} dimer as indicated by the similarity of the dimer levels in the Ni^{2+}- and Mg^{2+}-added samples to the apo-stock.

Disassembly studies. To determine whether Mg^{2+} or Ni^{2+} will disassemble D_{apo}*, the cations were added to an apo stock that was enriched in D_{apo}*. While 1 mM Mg^{2+} did promote some dimer disassembly, 1 mM Ni^{2+} had a greater effect. As expected, Ca^{2+} disassembled D_{apo}* to create a dimer peak that was expected based on the final concentration of NCAD12 injected on the analytical SEC column (Fig. 2B). The ability of Mg^{2+} and Ni^{2+} to disassemble D_{apo}* suggests that these cations bind to the same binding sites as calcium.

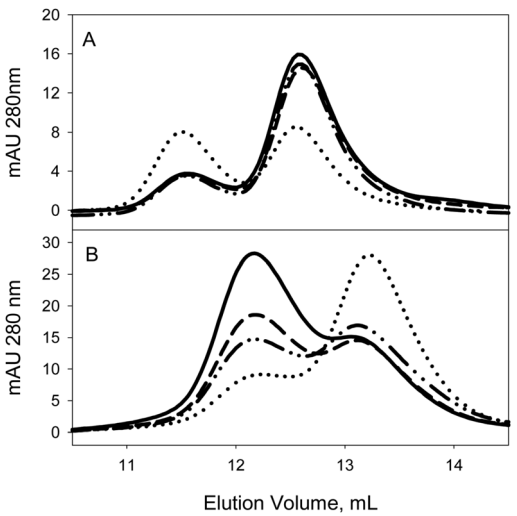

Fig. 2 (A) Assembly of dimer assayed by analytical SEC. Ca^{2+} (1mM; ···), Mg^{2+} (1.5 mM; - - -), or Ni^{2+} (1 mM; -..-..-) was added to an apo solution of NCAD12 (25 μM; —) that had a low level of kinetically trapped dimer. Chromatograms illustrate that Ca^{2+} promotes the formation of D_{sat}, while Mg^{2+} and Ni^{2+} do not. (B) Disassembly of kinetically trapped dimer assayed by analytical SEC. An apo NCAD12 stock was prepared (80 μM) with enriched levels of kinetically trapped dimer (—). The apo stock (—) was disassembled with the addition of 1 mM Ca^{2+} (···), 1 mM Mg^{2+} (- - -) or 1 mM Ni^{2+} (-..-..-). After addition of cation, the level of dimer decreased, indicating that cation binding "unlocks" the trapped dimer that was in the apo stock.

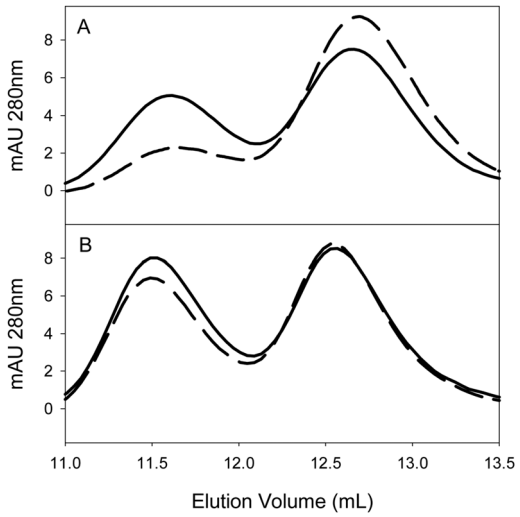

Fig. 3 (A) Effect of Ni^{2+} on calcium-induced dimer formation assayed by analytical SEC. The concentration of protein was 15 μM with 1 mM Ca^{2+} only (—) or with 1 mM Ca^{2+} and 1 mM Ni^{2+} (- - -). Additional Ni^{2+} concentrations were tested as described in the Experimental procedures. (B) Effect of Mg^{2+} on calcium-induced dimer formation assayed by analytical SEC. The concentration of protein was 25 μM with 1 mM Ca^{2+} only (—) or with 1 mM Ca^{2+} and 1 mM Mg^{2+} (- - -). Calculated values for the apparent dimer dissociation constants for each level of cation are shown in Table 1.

Competition studies. To understand the linked equilibria between calcium binding and dimer formation in NCAD12, we performed analytical SEC studies to determine the impact of competition for calcium-binding sites by Ni^{2+} or Mg^{2+} on dimer assembly. These chromatographic experiments provided the level of D_{sat} as a function of competing concentrations of Ni^{2+} or Mg^{2+} when the concentration of Ca^{2+} was at normal physiological levels (1 mM). As hypothesized, as the concentration of Ni^{2+} increased, we observed a reduction in dimerization affinity (increase in dissociation constant (K_d)) from 26 μM in the absence of Ni^{2+} to 107 μM in the presence of 1 mM Ni^{2+} (Fig. 3A). A smaller, but similar effect was observed for competition experiments with Mg^{2+} added as summarized in Table 2. Representative size exclusion chromatograms are shown in Fig. 3B, and the height of the D_{apo}* peak represents the level of calcium-saturated dimer in solution as a function of the concentration of protein and added Ni^{2+} or Mg^{2+}.

Simulation. We performed simulations to illustrate the effect of contaminating Ni^{2+} on calcium-induced dimerization in N-cadherin. Results are presented in Table 3. Given that the dissociation constant for calcium is 40 μM and for dimerization is 25 μM[43] in 1 mM calcium, we simulated the formation of adhesive dimer as a function of extracellular calcium concentration. The concentration of protein is 25 μM. Since this value is equivalent to K_d for dimerization in 1 mM Ca^{2+}, the level of dimer is close to its maximum value (0.5). As the nickel concentration increases to toxic levels, the fraction of dimer decreases to ~80% its expected value. The effect of nickel is relatively minor because the level of calcium is 20 times the K_d for calcium, and therefore, calcium occupies the calcium-binding sites leading to formation of dimer.

Table 2 Apparent K_d for Ca^{2+}-saturated dimer monitored by analytical SEC

	K_d (μM)
$[Ni^{2+}]$	
0	26 ± 7
100 μM	49 ± 6
500 μM	67 ± 5
1 mM	107 ± 8
$[Mg^{2+}]$	
0	32 ± 8
1.5 mM	45 ± 5

Table 3 Effect of calcium–nickel concentrations on fraction of dimer[a]

	$[Ca^{2+}]^c$, (M)	
$[Ni^{2+}]^b$, (M)	1×10^{-4}	1×10^{-3}
0.4×10^{-6}	0.28 (1.00)	0.48 (0.97)
200×10^{-6}	0.20 (0.70)	0.47 (0.94)
1×10^{-3}	0.07 (0.24)	0.41 (0.82)

[a] The numbers in parentheses are the fraction dimer values normalized for the total amount of dimer expected at 0 M Ni^{2+}. [b] Nickel concentrations under normal physiological conditions (0.4 μM), at the K_d for nickel binding to N-cadherin (0.2 mM), and at toxic levels (1 mM). [c] Calcium concentrations at excitatory synapses (0.1 mM) and in EC space, in general (1 mM).

N-Cadherin occurs at excitatory synapses, where it is the "Synaptic tag" for long term potentiation. At such synapses, the concentration of calcium has been measured at 40 μM,[51] the K_d for calcium to N-cadherin. Simulations were performed at 0.1 mM Ca^{2+}, because this is the concentration for which we have accurate determination of K_d for dimerization (manuscript in preparation). First, notice that there is significantly less dimer predicted based on the lower calcium level ($f_{D(0.1\,mM\,Ca)} \approx 0.3$; $f_{D(1\,mM\,Ca)} \approx 0.5$). Second, the effect of nickel is more profound. When the concentration of nickel is equivalent to 200 μM, the K_d for Ni^{2+} binding to NCAD12, there is a 30% decrease in the simulated fraction of adhesive dimer in the presence of 0.1 mM Ca^{2+}. As the nickel concentration increases beyond this point, it successfully competes with calcium for the binding sites, effectively reducing the level of calcium-induced adhesive dimer.

Discussion. Natural and non-physiological divalent cations modulate the dimerization affinity of N-cadherin by decreasing calcium-binding affinity due to competition for the binding sites. While neither of the divalent metals we tested could directly induce formation of dimer, the observed decrease in calcium-binding affinity due to binding site competition directly decreased adhesive dimer formation.

In nature, ion binding sites in regulatory proteins often require a specific ion to execute their physiological function. This scenario is typified by the EF-hand sites in calmodulin. NMR studies have shown that the lower affinity N-terminal sites of calmodulin are occupied by Mg^{2+} under the typical concentrations of Mg^{2+} found *in vivo*. However, the occupancy of the site by Mg^{2+} does not cause the conformational change typical upon binding of calcium.[44,45] The selectivity for specific ions in EF-hand sites is due to the relatively subtle changes in the 6 amino acids that offer their side chain or backbone atoms to the ligand, thereby conferring specificity of binding.[46,47]

Calmodulin binds calcium ions to dynamically regulate the physiological response in a cell due to a stimulus. This scenario sharply contrasts the role of ion binding in cadherin.

For cadherin, ion binding is necessary for adhesive dimerization, a phenomenon that is not likely dynamically regulated by large transient fluxes in calcium concentration. In contrast to EF-hand sites, the calcium-binding sites in cadherin are located at the interface between adjacent modular domains and comprise clusters of negatively charged residues. The involvement of multiple loops from individual domains and the peptide linker between domains implies greater adaptability to ionic radii and coordination geometries. While we have no direct evidence that Ni^{2+} or Mg^{2+} binds to the calcium-binding sites, the fact that Ni^{2+} and Mg^{2+} disassemble the kinetically trapped dimer is consistent with their occupancy of the calcium-binding sites. The calcium-binding sites should have space for Ni^{2+} or Mg^{2+} given that the ionic radii are less than that for Ca^{2+} (Ca^{2+}– 0.99 Å, Ni^{2+}– 0.69 Å, and Mg^{2+}– 0.72 Å).[48]

Divalent nickel can adopt a variety of coordination geometries, including four-, five- and six-ligand coordination spheres, while divalent magnesium firmly prefers a six-ligand coordination sphere.[25] Notably, Ni^{2+} has a propensity to exist in equilibrium between multiple coordination geometries, explainable by the ease of reversible ligand coordination at the axial position of square-planar complexes, while Mg^{2+} exists in equilibrium between two hexa-coordinated isomeric states.[49] While the calcium-binding sites in NCAD12 might also be amenable to a variety of coordination geometries, explicit chelation geometry is likely necessary to create strain in the monomer required to promote dimer formation.[50] Furthermore, studies by Rodriguez-Cruz *et al.*[51] established that water dissociates more readily from Ca^{2+} than from Ni^{2+} or Mg^{2+}, suggesting that Ni^{2+} and Mg^{2+} may bind more weakly to potential chelation sites on NCAD12 due to the dehydration energy penalty. Calcium titration and dimer assembly data presented in Fig. 1 and 2 suggest that while Ni^{2+} and Mg^{2+} can occupy the calcium-binding sites, they are unable to cause the conformational change necessary to promote the formation of adhesive dimer.

Some known health effects nickel causes are skin allergies and lung fibrosis, along with causing toxicity to the kidneys and cardiovascular system.[52,53] Additionally, nickel exerts neurotoxic effects *via* the inhibition of acetylcholinesterase and Na^+, K^+-ATPase, as recently demonstrated in a rat model.[40] Nickel ions have also been shown to display differential effects on a variety of voltage-activated calcium channels, displaying binding affinities as high as several mM.[54] Because of this known disruption on calcium channels, it is logical to hypothesize that other divalent cations present in the extracellular matrix, such as Ni^{2+}, could also disrupt the binding of calcium to N-cadherin, thereby decreasing the formation of dimer, which is required for a panoply of physiological processes. Given the very high levels of nickel in workers with increased on-the-job exposure to nickel,[38] the nickel concentration can rise as high as 7× the K_d for nickel binding to NCAD12. In this extreme scenario, while one would expect other physiological effects,[55,56] we would expect calcium-induced dimerization at 80% of its normal level in most physiological compartments (1 mM calcium). At excitatory synapses where the extracellular calcium concentration has been measured as low as 40 μM,[57] then we would expect only 30% of its normal level of N-cadherin dimer.

The extracellular space in neural tissue is known to be a complex milieu. Monovalent and divalent cations are in high abundance relative to calcium, such that one might expect an effect on cell adhesion by calcium-dependent cell adhesion proteins. Recently published work from our laboratory illustrated this concept. Jungles *et al.* showed that a decrease in pH or an increase in NaCl concentration promotes the formation of adhesive dimer by N-cadherin, indicating that dimerization is sensitive to the charge state of the protein and the level of monovalent ions in solution.[23] Calcium levels have been shown to flux in neural tissue based on a number of different factors.[57,58] Thus, the impact of the ionic character of the extracellular microenvironment is a relevant and complex topic in cadherin-mediated cell adhesion.

Experimental

Protein expression and purification

The cloning of the gene for the first two extracellular domains (residues 1–221) of NCAD12 (EC1, linker1, EC2, and linker2) was described previously.[50] Briefly, recombinant pET30 Xa/LIC plasmids were amplified by KOD HiFi DNA Polymerase (Stratagene) and transformed into *Escherichia coli* BL21 (DE3) cells. Protein was overexpressed and purified. Protein purity was verified *via* SDS-PAGE in 17% Tris-glycine gels through standard protocols. The concentration of the protein stocks was determined spectrophotometrically (ε_{280} = 15 900 ± 400 $M^{-1}cm^{-1}$).

Divalent cation binding studies

The circular dichroism signal of NCAD12 was monitored during titrations with Ca^{2+}, Mg^{2+} and Ni^{2+} using an AVIV 202SF Circular Dichroism (CD) Spectrometer. For all titrations, solutions of 1 mM, 10 mM, 100 mM, and 700 mM of metal(II) chloride were each added sequentially in 2.5 μL, 5.0 μL, and 10.0 μL increments to a 5 μM solution of NCAD12. Titrations were performed in triplicate. CD signal was recorded in wavelength scans from 300 to 210 nm with an averaging time of 30 seconds. Titration data were considered at a fixed wavelength and then fit to an equation for equal and independent sites with linear apo and saturated baselines.[50] The Ni^{2+} and Mg^{2+} titrations did not yield clear transitions with saturated baselines precluding direct analysis for binding constants. In order to estimate the binding affinities of Mg^{2+} and Ni^{2+}, we performed competitive binding titrations, in which 5 μM NCAD12 solutions containing fixed concentrations of the Mg^{2+} and Ni^{2+} were incubated for five minutes at room temperature and then titrated with calcium as described above. Binding affinities were resolved from simultaneous analysis of all experiments by fitting to the following competitive binding model:

$$\frac{K_X \cdot X \cdot M_o \cdot \left(1 - \frac{K_Y \cdot Y}{1 + K_Y \cdot Y}\right)}{1 + K_X \cdot X \cdot \left(1 - \frac{K_Y \cdot Y}{1 + K_Y \cdot Y}\right)} = MX \qquad (1)$$

This model was derived based on the assumption that all 3 calcium-binding sites at the interface between EC1 and EC2 are of equal affinity and independent of each other. We recognize that the binding of calcium is likely to be cooperative, but our calcium binding data did not support analysis by a more complex binding model.

Disassembly and assembly studies: analytical size exclusion chromatography

The impact of Ca^{2+}, Mg^{2+} and Ni^{2+} binding on dimer formation was investigated using analytical size exclusion chromatography (SEC). A Superose-12 10/300 GL column (Amersham) was used on an ÄKTA Purifier HPLC system (Amersham) with UV absorbance detection at 280 nm, a 0.5 $mLmin^{-1}$ flow rate, and a 50 μL injection volume. The mobile phase consisted of 10 mM HEPES and 140 mM NaCl at pH 7.4. Two columns were used over the course of these experiments, leading to small differences in elution volume for the SEC data presented here.

Assembly studies. In order to assess the effects of divalent cation on the assembly of dimer, we exploited an analytically useful property of NCAD12. Rapid decalcification of the calcium-saturated NCAD12 dimer (D_{sat}) results in the formation of a kinetically trapped dimer (D_{apo}*); the concentration of D_{apo}* represents the amount of D_{sat} in the calcium-saturated solution prior to decalcification.[43] In order to test the ability of divalent cations to promote dimer formation, we added cations at a particular level to the apo sample to promote the formation of D_{sat}. In this case, the 25 μM apo protein samples were exposed to 1.5 mM $MgCl_2$ or 1 mM $NiCl_2$ to promote the formation of D_{sat}, and EDTA was added to a final concentration of at least fivefold the concentration of the cation before the sample was analyzed by SEC. Addition of EDTA depleted divalent cations in solution and converted D_{sat} to D_{apo}* for the purpose of analysis by SEC. The samples were subsequently injected on the SEC column, and the level of monomer and dimer was monitored as the height of the peaks detected at 280 nm.

Disassembly studies. To determine whether divalent cations can disassemble preformed D_{apo}*, we employed an apo protein stock that was previously prepared with a high level of D_{apo}*. Aliquots of the 80 μM apo stock were exposed to 1 mM Ca^{2+}, Mg^{2+} and Ni^{2+} for five minutes, and then the level of D_{apo}* remaining in the stock was determined using the SEC method described above.

Competition experiments. Finally, competitive binding studies were performed as follows. Fixed concentrations of $NiCl_2$ and $MgCl_2$ were added to aliquots of an apo NCAD12 stock (25 μM) and incubated at room temperature for five minutes. Next, 1 mM calcium was added and incubated for an additional five minutes, followed by 5 mM EDTA to form the D_{apo}* species for analysis by SEC.

Conclusions

We present data that show that Mg^{2+} and Ni^{2+} do not induce formation of adhesive dimer directly; however, they compete with calcium binding. Our data predict that Ni^{2+} competition with calcium binding strongly decreases calcium-induced dimerization at Ni^{2+} concentrations found at excitatory synapses, and the presence of Mg^{2+} would have only a small effect. Given the critical role of N-cadherin in synapse integrity and dynamics, it is essential to consider the metallic complexity of the extracellular milieu.

Conflicts of interest

There are no conflicts to declare.

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