Characterization of the Cation-Binding Properties of Porcine Neurofilaments†

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Received March 10, 1988; Revised Manuscript Received June 21, 1988

ABSTRACT: In the presence of physiological levels of Na^+ (10 mM), K^+ (150 mM), and Mg^{2+} (2 mM), dephosphorylated neurofilaments contained two Ca^{2+} specific binding sites with K_d = 11 μM per unit consisting of eight low, three middle, and three high molecular subunits, as well as 46 sites with K_d = 620 μM. Only one class of 126 sites with K_d = 740 μM was detected per unit of untreated neurofilaments. A chymotryptic fraction enriched in the α-helical domains of neurofilament subunits contained one high-affinity Ca^{2+}-binding site (K_d = 3.6 μM) per domain fragment of ~32 kDa. This site may correspond to a region in coil 2b of the α-helical domain, which resembles the I–II Ca^{2+}-binding site in intestinal Ca^{2+}-binding protein. Homopolymeric filaments composed of the low or middle molecular weight subunits contained low-affinity Ca^{2+}-binding sites with K_d = 37 μM and 24 μM, respectively, while the K_d values for the low-affinity sites in heteropolymeric filaments were 8–10-fold higher. Competitive binding studies, using the chymotryptic fraction to assay the high-affinity Ca^{2+}-binding sites and $^{22}Na^+$ to monitor binding to the phosphate-containing low-affinity sites, yielded K_d values for Al^{3+} of 0.01 μM and 4 μM, respectively. This suggests that the accumulation of Al^{3+} in neurons may be due in part to its binding to neurofilaments.

Neurofilaments (NFs),[1] the intermediate filaments found in neurons, are made up of subunits that contain multiple phosphorylation sites (Julien & Mushynski, 1982). The midsized (NF-M) and high molecular weight (NF-H) subunits of porcine axonal NFs contain about 8 and 13 phosphate moieties, respectively, while the low molecular weight subunit (NF-L) contains 3 (Georges et al., 1986). The phosphorylation sites are located in the carboxy-terminal tail domain of the subunits (Julien & Mushynski, 1983), which also contains glutamate-rich tracts (Geisler et al., 1983; Myers et al., 1987; Julien et al., 1987; Lewis & Cowan, 1987; Levy et al., 1987; Napolitano et al., 1987). The abundance of negatively charged groups in NF proteins prompted a study of their Ca^{2+}-binding properties, which demonstrated the presence of both high- and low-affinity binding sites (Lefebvre & Mushynski, 1987).

It is not known at present whether the binding of Ca^{2+} by NFs has any physiological relevance. NFs are closely associated with intracellular membrane systems known to sequester and release Ca^{2+} (Burton & Laveri, 1985) and may be one of the components involved in maintenance of Ca^{2+} homeostasis in the neuron. The neurofibrillary tangles that accumulate in certain neurological disorders have been shown to contain Ca^{2+} and Al^{3+} deposits as well as NF antigens (Dahl et al., 1982; Gambetti et al., 1983; Garruto et al., 1984). Since Ca^{2+} affects the physical state of many proteins (Williams, 1986), the binding of Ca^{2+} or other cations to NFs may induce conformational changes resulting in phase transitions of the neurofilamentous network (Metuzals et al., 1981). The more highly phosphorylated state of axonal NFs are compared to NFs in the perikaryon and dendrites (Sternberger & Sternberger, 1983) may reflect regional differences in the functional properties of NFs as untreated and dephosphorylated NFs differ in their Ca^{2+}-binding properties (Lefebvre & Mushynski, 1987).

In the present study we have evaluated the effects of Na^+, K^+, Mg^{2+}, and other cations on Ca^{2+} binding to untreated and in vitro dephosphorylated NFs and have characterized the binding of Na^+ to the low-affinity Ca^{2+}-binding sites. The high-affinity Ca^{2+}-binding sites were localized in a particulate fraction enriched in α-helical domains that was obtained by treating NFs with chymotrypsin (Julien & Mushynski, 1983; Geisler et al., 1983). Binding studies using NFs with different subunit compositions indicated that intersubunit interactions as well as peripheral domains affected the Ca^{2+}-binding properties. It was also shown that Al^{3+} binds to both the high- and low-affinity Ca^{2+}-binding sites on NFs.

Experimental Procedures

Preparation of Neurofilaments. The reassembled NFs were prepared from subunits purified by hydroxylapatite chromatography of an NF-enriched fraction from porcine spinal cord (Georges et al., 1986). To prepare filaments with different subunit compositions, NF proteins were separated by preparative electrophoresis on 6 mm thick sodium dodecyl sulfate (SDS)–6.0% polyacrylamide slab gels, and polypeptide bands were visualized and electroeluted as described previously (Julien & Mushynski, 1982). SDS was removed from the polypeptides by an ion-pair extraction method (Henderson et al., 1979), and the precipitate was dried under a stream of nitrogen and dissolved in 0.05 M 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.8, containing 8 M urea and 0.1% β-mercaptoethanol. The homopolymers were reassembled by dialysis against 0.1 M Mes, 0.2 M NaCl, and 0.001 M dithiothreitol, pH 6.5, at 37 °C for 24 h (Gardner et al., 1984). The copolymers were reassembled by overnight dialysis at room temperature against 0.025 M imidazole, 0.15 M KCl,

† This work was supported by Medical Research Council of Canada Grant MT-5159. S.L. was supported by a studentship from the Fonds de la Recherche en Santé du Québec and an internal award from the Faculty of Medicine, McGill University.

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[1] Abbreviations: binding buffer, 0.02 M Hepes and 0.004 M diethanolamine, pH 6.8; class I and class II binding sites, high- and low-affinity Ca^{2+}-binding sites, respectively; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; ICaBP, vitamin D dependent intestinal Ca^{2+}-binding protein; Mes, 2-(*N*-morpholino)ethanesulfonic acid; NF, neurofilament; NF-L, NF-M, and NF-H, low, middle, and high molecular weight neurofilament subunits, respectively; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate.

0006-2960/88/0427-8503$01.50/0

0.005 M $MgSO_4$, 0.002 M dithiothreitol, and 0.001 M phenylmethanesulfonyl fluoride (PMSF), pH 7.1 (Zackroff et al., 1982).

Enzymatic Treatment. NFs reassembled from purified subunits (Lefebvre & Mushynski, 1987) were suspended in 0.05 M Mes, pH 6.5, at a concentration of 3 mg/mL and incubated with α-chymotrypsin (Boehringer Mannheim) at an enzyme:NF ratio of 1:3000 for 15 min at 30 °C (Julien & Mushynski, 1983). Digestion was stopped by addition of PMSF to 0.002 M, and the suspension was centrifuged for 30 min at 4 °C in an Eppendorf microfuge. The pellet was resuspended and dialyzed against 0.02 M *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonic acid (Hepes) and 0.004 M diethanolamine, pH 6.8 (binding buffer), containing 2 g/L Chelex 100 (Bio-Rad Laboratories, Inc.), and the protein concentration was adjusted to 0.25 mg/mL for Ca^{2+}-binding studies. NFs were dephosphorylated with *Escherichia coli* alkaline phosphatase as previously described (Georges et al., 1986).

Binding Assay. Ion binding was measured by partition centrifugation as described previously (Lefebvre & Mushynski, 1987). Stock solutions of Ca^{2+} were prepared in Chelex 100 treated binding buffer. EGTA was not used to regulate Ca^{2+} concentrations because of its reported interference with some Ca^{2+}-binding proteins (Kronman & Bratcher, 1983). Consequently, the Ca^{2+} concentrations in the range of 2–10 μM may not be as precise as can be obtained with a buffering system. Prior to their use in binding studies, all proteins were dialyzed against binding buffer containing 2 g/L Chelex 100. Different protein concentrations were used depending on the nature of the filaments. NF samples of 0.5 mg/mL were incubated for 10 min at room temperature with the appropriate concentrations of cations. Ca^{2+} binding was tested over a range of 2 μM–2 mM $CaCl_2$ and Na^+ binding over a range of 50 μM–10 mM NaCl. In each case the binding was monitored by the use of the corresponding radioactive cation, $^{45}Ca^{2+}$ or $^{22}Na^+$. The binding properties of the copolymer composed of NF-L and NF-H, and of the homopolymers containing NF-L or NF-M, were tested at protein concentrations of 0.25, 0.215, and 0.11 mg/mL, respectively. Scatchard plots were fitted by the ligand binding protein program converted for the Apple II computer (Biomedical Computing Technology Information Center, Nashville, TN).

Materials. Hydroxylapatite was obtained from Bio-Rad Laboratories. All competitor cations were Aristar-grade chloride salts from BDH. $^{45}CaCl_2$ and $^{22}NaCl$ were obtained from Du Pont–New England Nuclear (Canada) Ltd. All other chemicals used were of reagent grade or the best quality available.

Results

Our previous studies of Ca^{2+} binding by NFs were carried out in binding buffer only (Lefebvre & Mushynski, 1987). To determine whether Ca^{2+} binding could take place under more physiological conditions, binding studies were conducted in the presence of Na^+ (10 mM), K^+ (150 mM), and Mg^{2+} (2 mM) at their approximate intracellular concentrations. Scatchard plot analysis (not shown) by a best-fit program yielded the values for the dissociation constant (K_d) and number of class I and class II binding sites (n) listed in Table I. The number of binding sites was calculated per unit consisting of eight NF-L, three NF-M, and three NF-H, with a combined molecular mass of 1200 kDa, representing the relative amounts of the three subunits in chromatographically purified, reassembled NFs (Lefebvre & Mushynski, 1987). Under conditions where all three cations were present, only one class of Ca^{2+}-binding sites, having a K_d of 740 μM, was detected in untreated NFs (Table I). The high-affinity Ca^{2+}-binding sites were either totally blocked or else the K_d was shifted upward so that the two classes of binding sites could no longer be resolved. On the other hand, two high-affinity Ca^{2+}-binding sites per unit of dephosphorylated NFs could still be detected in the presence of Na^+, K^+, and Mg^{2+}. Dephosphorylation with *E. coli* alkaline phosphatase had removed about 42 of the 85 phosphate moieties from each unit consisting of eight NF-L, three NF-M, and three NF-H (data not shown). It is interesting to note that the latter treatment resulted in a corresponding reduction in the number of low-affinity Ca^{2+}-binding sites.

Table I: Effect of Na^+, K^+, and Mg^{2+} on the Dissociation Constant (K_d) and Number (n) of Ca^{2+}-Binding Sites in Untreated and Dephosphorylated Neurofilaments[a]

addition to buffer	class I		class II	
	n (mol/mol of unit)	K_d (μM)	n (mol/mol of unit)	K_d (μM)
no addition				
untreated NF	4.0 ± 0.9	4.1 ± 1.3	126 ± 4	293 ± 26
dephos NF	8.0 ± 2.0	15.0 ± 3.0	54 ± 3	332 ± 61
Na^+, K^+, Mg^{2+}				
untreated NF	nd[b]	nd	126 ± 12	740 ± 86
dephos NF	2.0 ± 0.7	11.0 ± 2.9	46 ± 9	620 ± 136

[a] The unit used for calculating n consists of eight NF-L, three NF-M, and three NF-H. About half (42 of 85) of the phosphates were removed per unit by *E. coli* alkaline phosphatase, and the term dephos NF refers to the dephosphorylated NFs. The values are the means of 15 experiments performed on seven different preparations. Each value is given with its SE. Na^+, K^+, and Mg^{2+} were added at their approximate intracellular concentrations of 10, 150, and 2 mM, respectively. [b] nd = not detectable.

The selectivity of the Ca^{2+}-binding sites was tested at Ca^{2+} concentrations between 2 and 10 μM and multivalent cation concentrations of 5 and 10 μM. The competitive effects of the bivalent and trivalent cations on Ca^{2+} binding to untreated NFs could be arranged in the decreasing order $Co^{2+} \simeq Mn^{2+} > La^{3+} \simeq Zn^{2+} > Fe^{3+} > Mg^{2+}$. The apparent K_d of the Ca^{2+}-binding sites on untreated NFs was increased by all of these cations, while the number of binding sites was unchanged. In the case of dephosphorylated NFs, the number of binding sites also remained unchanged in the presence of the different cations, but the apparent K_d was decreased in all cases. The latter finding suggested that the cations exerted an allosteric effect rather than binding directly to the high-affinity Ca^{2+}-binding sites (not shown).

Using the same binding assay as for $^{45}Ca^{2+}$, we demonstrated that $^{22}Na^+$ binds to untreated and in vitro dephosphorylated NFs (Figure 1). In each case only one class of Na^+-binding sites was detected, with a K_d of 3 mM for 131 sites per unit and a K_d of 2 mM for 65 sites per unit for untreated and in vitro dephosphorylated NFs, respectively. It is noteworthy that the ratio 3:2 Na:PO_4 remained unchanged after dephosphorylation. Since dephosphorylation similarly reduced the number of low-affinity Ca^{2+}-binding sites (Table I), we propose that Na^+ also binds to these class II sites. When the binding of K^+, Ca^{2+}, or Mg^{2+} to the class II sites in untreated or dephosphorylated NFs was monitored with $^{22}Na^+$, the major effect of dephosphorylation was a 7-fold increase in the K_d value for K^+ (from 64 to 450 mM) (not shown).

Since the low-affinity Ca^{2+}-binding sites appeared to contain phosphate moieties, which are located in the carboxy-terminal domain of NF subunits (Julien & Mushynski, 1983), it was of interest to localize the high-affinity sites. The treatment of NFs with chymotrypsin releases carboxy-terminal domains

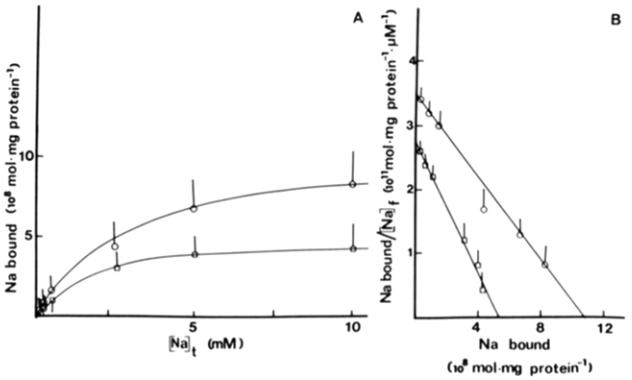

FIGURE 1: Na^+ binding to untreated (○) and dephosphorylated (□) neurofilaments as determined by partition centrifugation (A) and Scatchard plot analysis (B). The amount of bound Na^+ per milligram of protein was plotted as a function of total Na^+ concentration. The curves show the mean and standard deviation for each point. The lines in the Scatchard plots were generated visually. Each mean is the average of 10 experiments performed on six different preparations.

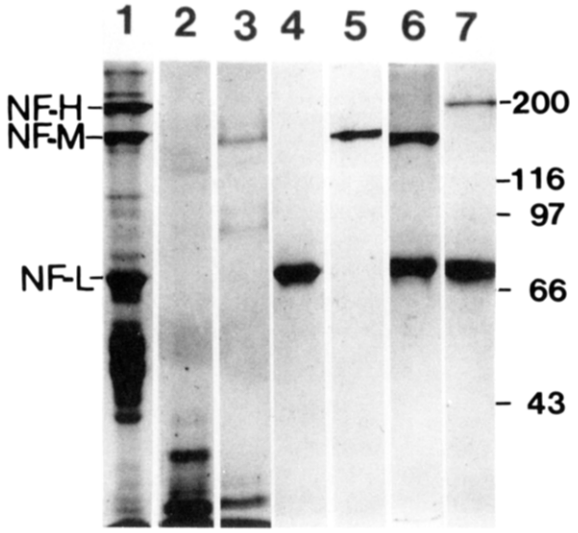

FIGURE 2: Analysis of different NF preparations by SDS–polyacrylamide gel electrophoresis. Lane 1 contains the crude porcine NF preparation. Lanes 2 and 3 show the pellet and supernatant fractions, respectively, after centrifugation of chymotrypsin-treated NFs. Lanes 4 and 5 represent the respective homopolymers of NF-L and NF-M. Lanes 6 and 7 show the respective heteropolymers composed of NF-M/NF-L and NF-H/NF-L. The numbers at the right indicate the positions of molecular weight standards ($\times 10^{-3}$). The gel contained 7.5% acrylamide.

as soluble fragments, leaving an insoluble fraction enriched in coiled-coil α-helical domains (Geisler et al., 1983; Julien & Mushynski, 1983). Gel electrophoretic analysis of this core fraction from porcine NFs indicated the presence of major polypeptides at ~32–35 kDa (Figure 2, lane 2). Ca^{2+} binding to the latter fraction was assayed in binding buffer by partition centrifugation. At saturation, 4.2×10^{-8} mol of Ca^{2+} was bound per milligram of NF core fraction, and the K_d was 3.6 μM (Figure 3). In the presence of physiological concentrations of Na^+ (10 mM), K^+ (150 mM), and Mg^{2+} (2 mM) combined, one-fifth of the high-affinity binding sites was still available, and the global effect of the added cations was noncompetitive in nature (not shown).

Attempts were also made to study Ca^{2+} binding by the soluble carboxy-terminal domains of NF-H and NF-M (Figure 2, lane 3) released by chymotryptic cleavage (Julien & Mushynski, 1983) using equilibrium dialysis and millipore filtration methods. No binding was detected under these conditions, perhaps due to the low-binding affinity of the phosphate-containing class II sites.

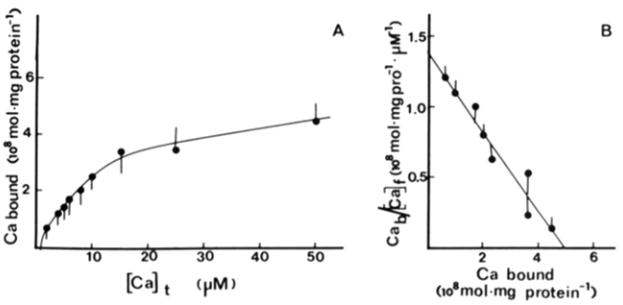

FIGURE 3: Ca^{2+} binding to the insoluble, α-helix-enriched core fraction from chymotrypsin-treated NFs. (A) The amount of bound Ca^{2+} per milligram of protein was plotted as a function of total Ca^{2+} concentration. The protein concentration was 0.25 mg/mL. The curve shows the mean and standard deviation for each point. Each mean is the average of 12 experiments performed on eight different preparations. (B) Scatchard plot of the means of the Ca^{2+}-binding data obtained in (A). The straight line was generated visually.

Table II: Dissociation Constants and Maxima of Bound Calcium for Neurofilaments of Different Subunit Composition[a]

	class I		class II	
unit	n (mol/mol of unit)	K_d (μM)	n (mol/mol of unit)	K_d (μM)
NF-L (3)	1.3 ± 0.4	4.6 ± 1.4	5.9 ± 1.7	37 ± 5
NF-M (8)	0.8 ± 0.2	2.1 ± 0.8	14.0 ± 4.0	24 ± 3
NF-L, NF-M (19)	6.0 ± 2.4	18.0 ± 5.4	40.0 ● 16.0	340 ± 70
NF-L, NF-H (20)	6.3 ± 2.0	20.0 ± 4.5	51.0 ● 17.0	243 ● 40

[a] The bound Ca^{2+} (n ± SE) and dissociation constants (K_d ± SE) were determined by computer analysis of Scatchard plots. NF-L and NF-M were reassembled alone, and n was calculated for each reference unit of 62 and 107 kDa, respectively. In the other cases, n was calculated for reference units of one NF-L/two NF-M and three NF-L/one NF-H with molecular masses of 276 and 326 kDa, respectively. The numbers in parentheses represent the number of phosphate moeties per unit (Georges et al., 1986). The values are the means of 10 experiments performed on five different preparations.

The contributions of the different NF subunits to Ca^{2+} binding were determined by preparing filaments with different subunit compositions. NF-L and NF-M were reassembled alone, and Ca^{2+} binding was calculated for each reference unit of 62 or 107 kDa, respectively (Kaufmann et al., 1984). In the case of filaments composed of NF-L and NF-M, NF-L and NF-H, or dephosphorylated NF-L and NF-M, their compositions were determined by densitometric scanning of SDS–polyacrylamide gels (Figure 2), and the values one NF-L, two NF-M and three NF-L, one NF-H were used to calculate molecular masses of 276 and 326 kDa, respectively, for each reference unit. The molecular masses of porcine NF proteins were determined by hydrodynamic methods (Kaufmann et al., 1984), which yield more precise size estimates than those obtained by SDS–polyacrylamide gel electrophoresis.

Analysis of Scatchard plots (not shown) indicated that all filament preparations made up of untreated subunits contained two classes of Ca^{2+}-binding sites, the numerical values being summarized in Table II. Both NF-L and NF-M homopolymers had a single high-affinity Ca^{2+}-binding site per polypeptide, and the K_d values for the class II Ca^{2+}-binding sites were lower than those determined for the heteropolymers. When NF-M or NF-H were assembled with NF-L the K_d values for the high-affinity Ca^{2+}-binding sites increased to a similar extent, being greater than the K_d of 4.1 μM obtained with NFs containing all three subunits (Lefebvre & Mushynski, 1987). The K_d of the low-affinity Ca^{2+}-binding sites increased to the same extent for both heteropolymers and was comparable to the value obtained for NFs composed of three subunits (Lefebvre & Mushynski, 1987). Filaments containing

dephosphorylated NF-L alone or dephosphorylated NF-L and NF-M had no detectable high-affinity Ca^{2+}-binding sites, and the binding affinity of class II sites was in both cases similar to that observed for the heteropolymers composed of untreated subunits (not shown).

The interaction of Al^{3+} with untreated NFs in the presence of low levels of Ca^{2+} could not be measured because the two cations caused NFs to aggregate at the high protein concentrations required to measure Ca^{2+} binding to the high-affinity sites. Since the NF core fraction obtained by chymotrypsin treatment did not undergo similar aggregation in the presence of low levels of Al^{3+} and Ca^{2+}, it was used to assay the binding of Al^{3+} to the high-affinity Ca^{2+}-binding sites. A K_d for Al^{3+} of about 0.01 μM was observed at the latter sites (not shown). Competition for the low-affinity Ca^{2+}-binding sites in untreated and dephosphorylated NFs was tested at 5, 10, and 15 μM Al^{3+}. $^{22}Na^+$ was used to monitor binding at the low-affinity sites in order to avoid aggregation, and a K_d for Al^{3+} of 4 μM was observed (not shown).

Discussion

The effects of various cations on the Ca^{2+}-binding properties of NFs were assessed in order to learn more about the nature of the binding sites and to determine whether binding occurs in the presence of physiological levels of the major intracellular cations. The untreated NFs, which are derived from myelinated axons (Georges et al., 1986) and are highly phosphorylated (Sternberger & Sternberger, 1983; Carden et al., 1987), did not display any high-affinity Ca^{2+}-binding sites in the presence of Na^+, K^+, and Mg^{2+}, and the K_d of the low-affinity sites increased. Under the same conditions, high-affinity Ca^{2+}-binding sites could still be detected in dephosphorylated NFs, which are analogous to perikaryal and dendritic NFs in their low phosphate content (Sternberger & Sternberger, 1983; Carden et al., 1987). The phosphate moieties thus appear to influence the accessibility of high-affinity Ca^{2+}-binding sites in addition to increasing the K_d value for these sites (Lefebvre & Mushynski, 1987).

The use of $^{22}Na^+$ to monitor competitive binding at the low-affinity (class II) sites provides a means of avoiding interference by the high-affinity Ca^{2+}-binding sites. This approach was particularly useful for studying the binding of Al^{3+} to class II sites because NFs aggregate in the presence of Ca^{2+} and Al^{3+}. The class II sites in highly phosphorylated, axonal NFs are likely to be occupied by either Na^+ or K^+ under resting conditions in situ. On the other hand, the binding of K^+ to these sites in hypophosphorylated NFs would be much lower, as indicated by the 7-fold higher K_d value for K^+ in dephosphorylated NFs as compared to that in untreated NFs. Following depolarization, when the intracellular Ca^{2+} concentration can be as high as 10 μM (Murase & Randic, 1983), some Ca^{2+} could bind to these low-affinity sites. Nuclear magnetic resonance studies have shown that Ca^{2+} bound to serine phosphate-containing sites is mobile. The diffusion of Ca^{2+} among phosphate moieties thus enables phosphoproteins to sequester large amounts of the ion (Cookson et al., 1980).

The presence of high-affinity Ca^{2+}-binding sites in an NF core fraction enriched in α-helical domains prompted a comparison of the amino acid sequences of porcine NF-L and NF-M (Geisler et al., 1983) with known Ca^{2+}-binding domains (Figure 4). The comparison revealed significant homologies between sites in NF-L and NF-M and the Ca^{2+}-binding I–II domain of vitamin D dependent intestinal Ca^{2+}-binding protein (ICaBP) (Szebenyi et al., 1981). The respective sites in NF-L and NF-M from four different mammalian species contain the same 12 amino acid sequence, in coil 2b of the rod domain

NF-L	^{337}(M)	N	(E)	A	(L)	E	K	(Q)	L	(O)	E	L	(L)
NF-M	^{337}(T)	K	(E)	S	(L)	E	R	(Q)	L	(S)	D	I	(E)
ICaBP	A	K	E	G	D	P	N	Q	L	S	K	E	E
EF-hand	*	_	*	_	*	G	1	*	I	*	_	_	*

FIGURE 4: Comparison of amino acid sequences of putative Ca^{2+}-binding domains in porcine NF-L and NF-M with the EF-hand domain of vitamin D dependent intestinal Ca^{2+}-binding protein. (*) indicates an oxygen-containing residue (D, E, N, Q, S, T); (I) indicates a hydrophobic residue (isoleucine preferred). The superscript 337 is the position in the amino acid sequence of each NF subunit of the first amino acid in the putative EF-hand sequence. The number 1 indicates an amino acid insertion compared to the classical EF-hand domain. Calcium ligands, predicted on the basis of sequence homology, are circled. The NF-L and NF-M sequences are from Geisler et al. (1983), while the ICaBP sequence is from Szebenyi et al. (1981).

(Myers et al., 1987; Julien et al., 1987; Lewis & Cowan, 1986; Levy et al., 1987; Napolitano et al., 1987). The putative Ca^{2+}-binding sequences in NF-L and NF-M are like the EF-hand in the ICaBP I–II domain, which retains its Ca^{2+}-binding capacity despite being modified by the insertion of one amino acid residue. The ligand at position 5 of the putative EF-hand in NF-L and NF-M is a peptide bond carboxyl oxygen of a leucine residue, while the other ligands are from side-chain oxygens. The lower affinity of these sites for Ca^{2+} as compared to the ICaBP site may be due to the presence of basic side chains, but the K_d value is of the same order of magnitude as that of major intracellular Ca^{2+}-dependent regulators (Kilhoffer et al., 1983). However, the high-affinity Ca^{2+}-binding sites on untreated NFs behave anomalously. La^{3+}, which is often referred to as "super calcium" (Williams, 1970), has a lower competitive effect at these sites than do Co^{2+} and Mn^{2+}. This may reflect the presence of nitrogen ligands in the binding site, and its size may differ from that of more conventional Ca^{2+}-binding sites.

The putative EF-hand sequence in NF-L and NF-M is located within the α-helical domain, which is common to all intermediate filament proteins and is involved in filament assembly. The location of this sequence a few amino acids from the point where inversion of the heptad repeat pattern occurs (Geisler et al., 1983) may have functional significance. It may be possible to perturb the coiled-coil α-helix at this point to permit the formation of an EF-hand secondary structure. It has recently been reported that a divalent cation binding site was contained in the coil 1a subdomain of another intermediate filament protein, the glial fibrillary acidic protein (Yang & Babitch, 1987). While searching for sequence homologies with Ca^{2+}-binding domains, we did not detect an EF-hand sequence in this region of the NF subunits. However, this does not exclude the possibility that an unconventional Ca^{2+}-binding site (Stuart et al., 1986) is present in glial fibrillary acidic protein.

The detection of a lower number of high-affinity Ca^{2+}-binding sites than the number of EF-hand sequences per NF unit may be due to some of the sites being made inaccessible as a result of subunit interactions. By assigning an approximate molecular mass of 32 kDa to the chymotryptic fragments making up the NF coiled-coil domains, the number of high-affinity binding sites per α-helical domain was calculated to be about 1.3. The number was reduced to 0.2–0.3 after addition of Na^+, K^+, and Mg^{2+} at their physiological concentrations. The apparent noncompetitive effect may have been due to a compaction of the charged NF core in the presence of high concentrations of counterions. Since no high-affinity

Ca^{2+}-binding sites were detected on untreated NFs under these conditions (Table I), the interaction of cations with the carboxy-terminal domains reduced the number of sites, perhaps by further stabilizing the NF core structure or by direct steric hindrance.

Phosphate moieties located in the carboxy-terminal domains of NF subunits (Julien & Mushynski, 1983) appear to be involved in regulating the accessibility of high-affinity Ca^{2+}-binding sites as dephosphorylation of NFs restored a high-affinity class of Ca^{2+}-binding sites with a K_d of 15 μM (Lefebvre & Mushynski, 1987). The effect of adjacent protein domains on Ca^{2+} binding was determined by using homopolymeric NFs composed of NF-L or NF-M and heteropolymers composed of NF-M or NF-H combined with NF-L. It is noteworthy that the class II sites in homopolymeric NFs had much lower K_d values than the corresponding sites in heteropolymeric NFs (Table II), except in the case of homopolymeric NFs composed of dephosphorylated NF-L (not shown).

Levels of Al^{3+} as high as 4 mg/kg of brain have been detected in patients with Alzheimer's disease, while control brains have an average content of 1.9 mg/kg (Crapper-Mclachlan & Farnell, 1985). Among its possible effects on neurons, Al^{3+} would impair the Ca^{2+}-binding capacity of both high- and low-affinity Ca^{2+}-binding sites on NFs. Al^{3+} has already been shown to bind to and alter the properties of other proteins. For example, Al^{3+} binding induces a conformational change in calmodulin that blocks its normal functions (Siegel & Haug, 1983). Very low concentrations of Al^{3+} also promote tubulin polymerization more efficiently than does Mg^{2+} and reduce the sensitivity of microtubules to Ca^{2+}-induced depolymerization (MacDonald et al., 1987). The finding that nerve tissue represents a preferential site of Al^{3+} accumulation may therefore be explained by the presence of high-affinity Al^{3+}-binding sites on NFs as well as on tubulin and calmodulin, which are all abundant in neurons. The observed aggregation of NFs in the presence of low concentrations of Ca^{2+} and Al^{3+} may account for the abnormal accumulation of NFs in neuronal perikarya of animals injected with Al^{3+} (Bizzi et al., 1984; Bizzi & Gambetti, 1986). In these Al^{3+}-induced neuropathies the perikaryal NFs are in an abnormal, hyperphosphorylated state (Bizzi & Gambetti, 1986), which provides the tangles with additional cation-binding sites.

Although the data presented here and previously (Lefebvre & Mushynski, 1987; Krinks et al., 1987) permit only speculation about the possible role of Ca^{2+} binding by NFs, the evidence suggests that NFs may represent a Ca^{2+}-buffering system in regions of the neuron where they are abundant. In addition, the notion that all EF-hand proteins are involved in Ca^{2+}-binding regulation (Kretsinger, 1976) indicates that some aspects of NF structure or function may be under similar control. For example, the effect of Ca^{2+} on its binding proteins is mechanical in nature (Williams, 1986), and the location of an EF-hand at a point in the α-helical domain of NF proteins and other intermediate filament proteins where the heptad repeat pattern is reversed may indicate that the structure or assembly of NFs is influenced by Ca^{2+}.

ACKNOWLEDGMENTS

Porcine spinal cords were generously provided by Oliver Bienvenue Ltée, St. Valérien, Québec, Canada. The secretarial aid of Margaret Licorish is gratefully acknowledged.

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CORRECTIONS

Molecular Evolution of Serpins: Homologous Structure of the Human α_1-Antichymotrypsin and α_1-Antitrypsin Genes, by Jai-ju Bao, Richard N. Sifers, Vincent J. Kidd, Fred D. Ledley, and Savio L. C. Woo*, Volume 26, Number 24, December 1, 1987, pages 7755–7759.

Page 7758. In Table I, footnotes *a* and *b* should be replaced with the following: Optimal alignments were constructed pairwise between eight proteins in the serine proteinase inhibitor superfamily, and the percent difference and evolutionary distance in PAMs (accepted point mutations) between peptides were calculated with algorithms described (Dayhoff et al., 1972; Dayhoff, 1976). The percent difference between peptides is indicated in the table above the diagonal (top right), and the evolutionary distance (PAMs) is indicated below the diagonal (bottom left). Sequences were from the Protein Identification Resource (PIR).

The "γ Subunit" of Na,K-ATPase: A Small, Amphiphilic Protein with a Unique Amino Acid Sequence, by John H. Collins* and John Leszyk, Volume 26, Number 26, December 29, 1987, pages 8665–8668.

Page 8667. In column 2, the sentence beginning on line 7 should read as follows: In support of this idea, it was found that polyclonal antibodies raised to the isolated α and β subunits had some reactivity with purified γ [Ball, W. J., Jr., Collins, J. H., Land, L., & Schwartz, A. (1983) *Curr. Top. Membr. Transp. 19*, 781–785]. We are grateful to Dr. W. James Ball, Jr., for pointing out this error.

Identification and Sequence of a Binding Site Peptide of the β_2-Adrenergic Receptor, by Henrik G. Dohlman, Marc G. Caron, Catherine D. Strader, Nourdine Amlaiky, and Robert J. Lefkowitz*, Volume 27, Number 6, March 22, 1988, pages 1813–1817.

Page 1813. The author and address bylines should read as follows: Henrik G. Dohlman, Marc G. Caron, Catherine D. Strader, Nourdine Amlaiky, and Robert J. Lefkowitz*; Howard Hughes Medical Institute, Departments of Medicine, Biochemistry, and Physiology, Duke University Medical Center, Durham, North Carolina 27710, and Department of Biochemistry and Molecular Biology, Merck Sharp and Dohme Research Laboratories, Rahway, New Jersey 07065.

Orientation of Actin Monomer in the F-Actin Filament: Radial Coordinate of Glutamine-41 and Effect of Myosin Subfragment 1 Binding on the Monomer Orientation, by Andrzej A. Kasprzak,* Reiji Takashi, and Manuel F. Morales, Volume 27, Number 12, June 14, 1988, pages 4512–4522.

Page 4520. In column 2, the sentence beginning on line 2 should read as follows: The conventional position vector of N is $\hat{\imath}R + \hat{r}\overline{MN'} + \hat{u}\overline{N'N}$.... In column 2, three lines above eq A3, the distances should read $\overline{MN'}$, $\overline{N'N}$, $\overline{MP'}$, and $\overline{P'P}$.