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Calbindin-D in peripheral nerve cells is vitamin D and calcium dependent

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ABSTRACT **The vitamin D-induced calcium-binding protein calbindin-D (CaBP) was localized immunohistochemically in some but not all of the cell bodies and axons within the intestinalis nerve of the chicken. Unlike other nerve tissue thus far examined, the CaBP content of the intestinalis nerve was decreased in vitamin D deficiency and increased in chicken adapted to a calcium-deficient diet. These changes are qualitatively similar to the pattern of response of enterocytes. The inclusion of calcium-containing solutions within the duodenal lumen caused, directly or indirectly, a decrease in the amount of CaBP in this nerve in a dose-dependent manner. The exact role of CaBP in intestinalis nerve cells is unknown but may be in the regulation of intracellular ionic Ca^{2+} concentrations during excitation, although other functions of CaBP cannot be excluded.**

The induction of the synthesis of the calcium-binding protein calbindin-D (CaBP) in intestine, kidney, and certain other tissues has been shown to be dependent on the seco-steroid vitamin D or its biologically active metabolites (1, 2). In severe vitamin D deficiency, the CaBP content of intestinal mucosa is virtually absent and is considerably decreased in renal tissue (2). However, brain CaBP does not change in vitamin D deficiency and remains essentially the same as in the normal vitamin D-replete animal (3). Based on such observations, a relationship between the rate of cell turnover and the rate of decrease of CaBP content with the onset of vitamin D deficiency in a particular tissue was suggested.

CaBP occurs in two general forms, the avian type of M_r ≈28,000 and with four high-affinity calcium-binding sites (K_a, ≈2×10^6 M^{-1}) and the mammalian type of M_r ≈9,000 and with two high-affinity binding sites (2). These proteins are homologous with calmodulin and other members of the troponin C superfamily of calcium-binding proteins.

The concentration of CaBP in intestinal mucosa is well-correlated with the transport of calcium across the epithelial cell barrier and is considered to increase the transfer of calcium from the microvillar region to the basal–lateral membrane (4, 5), as well as possibly having other functions in the transport process (6). CaBP has been localized immunohistochemically in certain cells of the brain and central nervous system, such as the Purkinje cells of the cerebellum, the hippocampus, and cells of sensory systems (7–15). Although its function therein is unknown, CaBP may play a role in the management of intracellular calcium ion concentrations. Calcium ions are known to subserve various functions of the nervous system, including the release of neurotransmitter, the stimulation of calcium–calmodulin-dependent protein kinases, the operation of calcium-dependent potassium channels, and, from studies with invertebrates, the possible involvement in such processes as habituation and sensitization (16, 17).

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Studies reported herein had their origin from the investigation of vitamin D dependency of contractility of smooth muscle of the vascular system. Preliminary experiments suggested that the contraction–relaxation cycle of the smooth muscle present in the caudal mesenteric vein and artery of the chicken, in response to norepinephrine and K^+ depolarization, differed as a function of vitamin D status. Analysis of the preparation by radioimmunoassay disclosed the presence of CaBP, subsequently localized in some of the neurons of the intestinalis nerve, a nerve closely associated with these blood vessels. Further experimentation showed that CaBP in intestinalis nerve cells is decreased in vitamin D deficiency and increased in dietary calcium deficiency, responding qualitatively the same as CaBP in intestine. These changes in peripheral nerve were unexpected, based on the prior reports of the insensitivity of brain CaBP to vitamin D deficiency. It was also observed that the presence of Ca^{2+} solutions in the duodenal lumen markedly affected the concentration of CaBP in the peripheral nerve.

MATERIALS AND METHODS

Tissue (Mesenteric Tissue) for Assay. Blood vessels and nerves plus immediately adjacent mesentery were dissected together, starting from the region of the distal intestine in chickens and progressing in a rostral direction for 4–6 cm. Specifically, these tissues (here and after referred to as mesenteric tissue) consisted of the mesothelium and loose connective tissue of the mesentery, the caudal mesenteric artery and vein, and closely associated intestinalis nerve (nerve of Remak). The intestinalis nerve, which is unique to birds, arises from the central nervous system near the caudal pole of the kidneys and courses rostralward within the mesentery.

Immunohistochemical Localization of CaBP. The mesenteric tissue, taken for immunohistochemical analysis, was removed from 4-week-old White Leghorn chickens, quick frozen, fixed by freeze substitution for 2 weeks, imbedded, sectioned and reacted with goat anti-CaBP antiserum, and the presence of CaBP was determined by the peroxidase-antiperoxidase technique as described (18). Immunocontrols underwent reaction with nonimmune goat serum in place of anti-CaBP.

Radioimmunoassay of CaBP. For the quantification of CaBP in the mesenteric tissue, the following procedure was used. The tissue was rinsed in cold 0.9% NaCl solution, blotted with absorbent paper, minced in 1 ml of ice-cold Tris buffer mixture (13.7 mM Tris·HCl/0.12 M NaCl/4.7 mM KCl, pH 7.4), finely dispersed by sonication (Heat Systems/

Abbreviation: CaBP, calbindin-D, the vitamin D-induced calcium-binding protein.

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Ultrasonics, Plainview, NY), and centrifuged at 30,000 × *g* for 1 hr at 4°C. The supernate was recovered and analyzed for CaBP by a radioimmunoassay using ^{125}I-labeled CaBP, essentially as described by McCann *et al.* (19). Purified standard CaBP was isolated from chicken duodenum as described (20).

Electrophoretic Mobility of CaBP from Duodenal and Nervous Tissue. The mesenteric tissue and cerebellar tissue from 4-week-old vitamin D-repleted chicken were prepared as described above in Tris buffer mixture and centrifuged at 30,000 × *g* for 30 min. Aliquots of the supernate were disassociated by incubating at 37°C for 30 min in 1% $NaDodSO_4$/2% 2-mercaptoethanol/0.2% Tris; purified duodenal CaBP was similarly treated. These samples, singly or in combination, were subjected to electrophoresis on $NaDodSO_4$/polyacrylamide slab gels (21). The resolved proteins were electrophoretically transferred to nitrocellulose sheets (pore size, 0.45 μm; Millipore), essentially as described by Towbin *et al.* (22). The electrophoretic blots were incubated in a solution composed of 10% fetal bovine serum/3% bovine serum albumin in the Tris buffer mixture for 2 hr at 37°C. The membrane was rinsed in Tris-buffered saline (20 mM Tris·HCl/0.5 M NaCl, pH 7.5). After incubating with rabbit anti-chicken CaBP antiserum diluted in Tris-buffered saline overnight at 20°C–22°C, the blot was thoroughly rinsed. The blot was then incubated for 2 hr at 20°C–22°C with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) diluted 1:100 in the Tris buffer mixture containing 10% fetal bovine serum and 3% bovine serum albumin and was then thoroughly rinsed again in Tris-buffered saline. For color development, the Bio-Rad HRP Color Development Kit was used.

Radial Diffusion Immunoassay for CaBP in Duodenal Mucosa. CaBP in the mucosa of the duodenum was determined by a radial immunodiffusion assay as described (3).

Protein Analysis. The protein concentrations of the mucosal and mesenteric tissue were determined by the procedure of Lowry *et al.* (23), using bovine serum albumin as the standard protein.

Effect of Vitamin D Deficiency. One-day-old chicks were fed a rachitogenic diet for 4 weeks. One half of the chicks were given 500 international units (IU) of vitamin D_3 in 0.2 ml of propylene glycol intramuscularly 72 hr before the experiment. The other half received propylene glycol only. Concentrations of CaBP in the duodenal mucosa and the intestinalis nerve associated with caudal mesentery vessels were determined as indicated above.

Effect of Adaptation to Calcium-Deficient Diet. One-day-old chicks were fed a commercial chick starter diet (Agway, Ithaca, NY) for 3 weeks and subsequently divided into three groups of five or six chickens per group. The groups were then fed complete diets that varied only in calcium content and contained either 0.1%, 1.0%, or 2.0% calcium. Each diet contained 0.67% phosphorus and 1200 IU of vitamin D_3 per kg of diet. After 8 days on these diets and an overnight fast, the chickens were killed and the duodenum and mesenteric tissue were excised for CaBP analysis.

Effect of Intraluminal Calcium and Calcium Absorption. Vitamin D-repleted chickens were anesthetized with ether and the duodenum was exposed. Solutions containing NaCl (150 mM) and $^{45}CaCl_2$ (1 μCi/ml; 1 Ci = 37 GBq) (pH 7.2), with the total calcium concentration ranging from 1 to 40 mM, were injected into the duodenal lumen (1 ml per chicken), using the *in situ* ligated loop procedure as described (24, 25). Chickens were anesthetized 15 min later, blood was withdrawn from the mesenteric vein draining the duodenum, and ^{45}Ca in the venous serum was quantified using a liquid scintillation counter (Beckman). The mesenteric tissue was then excised and analyzed for CaBP by radioimmunoassay as noted above.

RESULTS

Immunohistochemical Localization. After the presence of immunologically reactive CaBP had been demonstrated in the mesenteric tissue, the localization of CaBP was determined immunohistochemically (26). CaBP was not present in the endothelial cells or in the smooth muscle cells of the mesenteric vein and artery, but was found in nerve cell bodies and axons of the intestinalis nerve, a nerve anatomically associated with these vessels (Fig. 1). It can be seen that only some of the nerve cells in the ganglion contained CaBP. This is reminiscent of other nerve tissues—e.g., the brain—in which only specific neuronal cells were shown to contain CaBP (7–15). CaBP was also visualized in axons and specifically associated with axonal varicosities.

Radioimmunoassay and Immunoblot. In validating the radioimmunoassay procedure, it was shown that serial dilu-

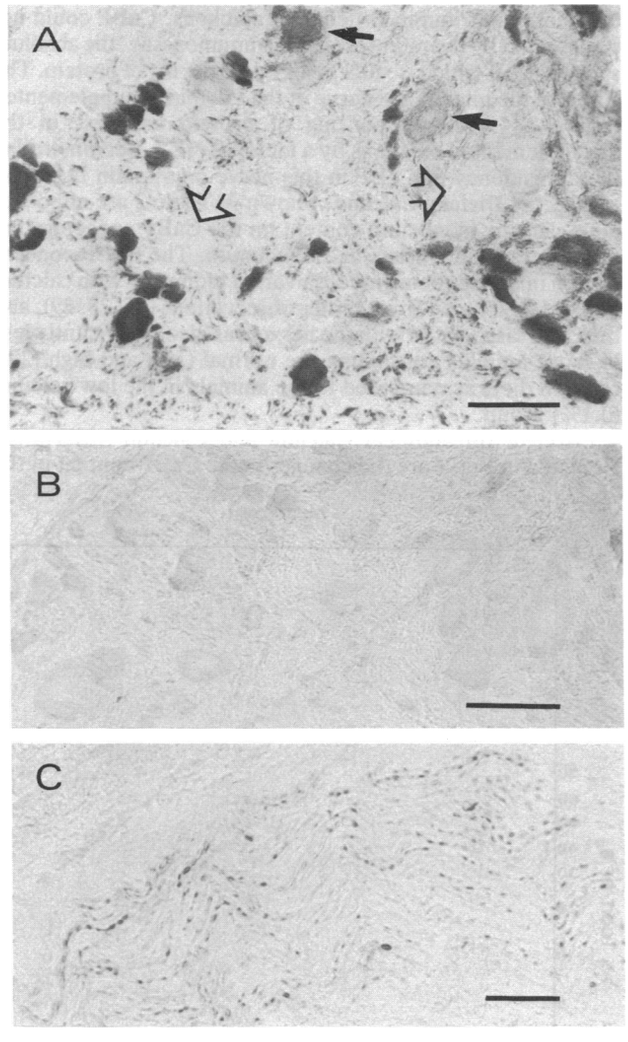

FIG. 1. Localization of CaBP in the intestinalis nerve present in the mesenteric tissue. (*A*) Heavy deposits of immunomarker for CaBP are present over several neuronal cell bodies in the ganglion. Some cell bodies have immunomarker at a much lower concentration (arrows), while others have none (open arrowheads). Axons also contain CaBP within the ganglion but are better visualized outside the ganglion (*C*). (*B*) The immunocontrol is from an adjacent section reacted with nonimmune serum. (*C*) Immunomarker for CaBP in peripheral nerve is present over the axonal varicosities of some axons. (*A* and *B*, ×250, bar = 50 μm; *C*, ×400, bar = 25 μm.)

tions of the extract from mesenteric tissue inhibited ^{125}I-labeled CaBP binding to antibody in parallel to the inhibition produced by purified CaBP standard solutions (Fig. 2). The slopes of these two curves were not different, indicating that the reactive compound in the intestinalis nerve was immunologically identical to CaBP purified from the duodenum. Furthermore, the immunoblot (Fig. 3) showed that the immunoreactive proteins in the nerve and cerebellar preparations have essentially the same electrophoretic mobility as purified chicken intestinal CaBP. The protein in cerebellum appears as a doublet, suggesting heterogeneity of CaBP in this tissue. Previously, Pochet *et al.* (27) reported the presence of two CaBPs in rat brain, with apparent molecular weights of 27,000 and 29,000. A very faint protein band above the denser band in the intestinalis nerve extract also suggests the possible presence of two CaBPs in this peripheral nerve.

Effect of Vitamin D Deficiency. The CaBP content of the intestinalis nerve varied significantly with various treatments, as did duodenal CaBP. As shown in Table 1, the CaBP content of the duodenal mucosa of the vitamin D-replete chickens was ≈40 μg per mg of protein, whereas, in the duodenum of vitamin D-deficient chickens, CaBP could not be detected by the radial diffusion immunoassay, the absolute sensitivity of which is <0.3 μg of CaBP per mg of protein. The CaBP in the intestinalis nerve of the vitamin D-supplemented birds was ≈480 ng per mg of protein and less in the unsupplemented chickens by a factor of ≈2.4, demonstrating the dependency of CaBP in this nerve on vitamin D status.

Effect of Dietary Calcium. Shown in Table 2 are effects of diets varying in calcium content on the CaBP concentration of the intestinalis nerve and duodenum. The CaBP concentration in the duodenal mucosa varied indirectly with calcium intake as expected from earlier observations (25, 28, 29), and the CaBP associated with the nerve was also significantly less in those animals consuming the normal (1%) and high (2%) calcium diets as compared to the animals in the low calcium (0.1%) group.

Effect of Intraluminal Calcium and Calcium Absorption. Depicted in Fig. 4 are the changes in the CaBP content of the intestinalis nerve in response to intraluminal calcium. When the calcium content of the solution injected into the duodenal lumen was 10 mM or greater, the CaBP content of the intestinalis nerve decreased significantly. The absorption of calcium from the various calcium-containing solutions was calculated from the specific activity of ^{45}Ca in precursor compartment (duodenal lumen) and the ^{45}Ca in venous blood, and these data are also shown in Fig. 4.

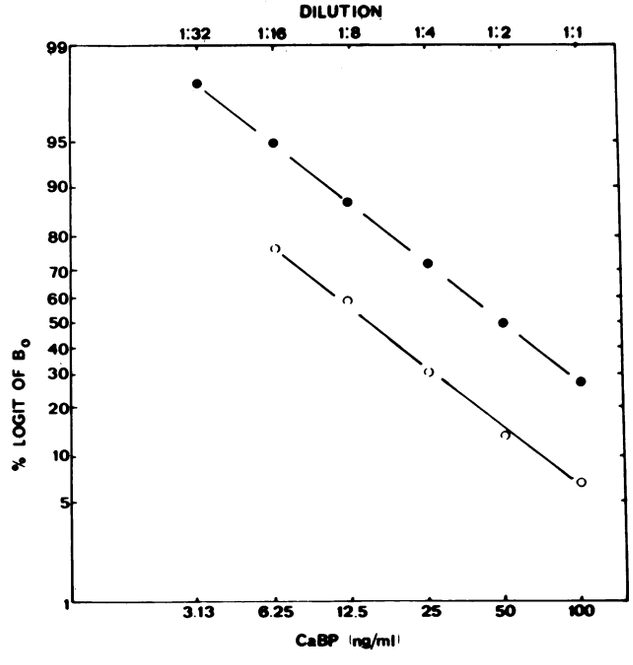

FIG. 2. Effect of unlabeled CaBP and mesenteric tissue extract on binding (B_0) of ^{125}I-labeled CaBP to specific rabbit anti-CaBP antiserum. Note the parallel decrease in binding by various dilutions of unlabeled CaBP and the tissue extract. Each point represents the mean of three determinations. ●, CaBP; ○, mesenteric tissue extract.

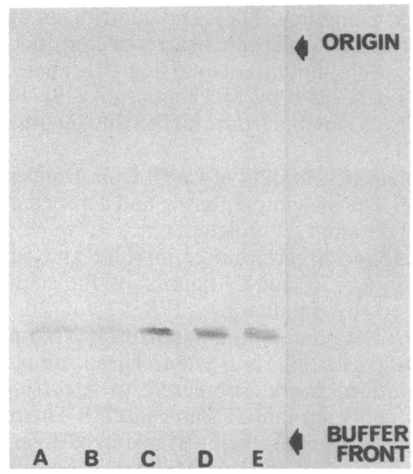

FIG. 3. Immunoblot of extracts of mesenteric tissue and brain (cerebellum) and purified CaBP. After $NaDodSO_4$/PAGE, proteins were electrophoretically transferred to the blots and CaBP was visualized immunologically (see text). Lanes: A, mesenteric tissue; B, same as lane A + CaBP; C, CaBP; D, cerebellum + CaBP; E, cerebellum. The immunoreactive material in each preparation migrated on the $NaDodSO_4$ gels essentially the same as purified CaBP. A doublet was apparent in the cerebellar extract, which suggests multiple forms of the brain protein.

DISCUSSION

CaBP, discovered in the intestinal mucosa, has been localized in a number of tissues and organs of chickens, including the central nervous system and sensory nerves (7–15). Unlike the intestine, the CaBP content of the brain is not diminished in the vitamin D-deficient state and remains essentially the same as in brains of vitamin D-replete chickens (3). However, Taylor (30) demonstrated an increase in the CaBP concentration in brain tissue when vitamin D was chronically administered to rachitic chickens. The only other manipulation shown to affect brain CaBP was the production of epileptic rats by way of the "kindling" procedure, a procedure in which electrical impulses are delivered to the brain at periodic intervals over a number of days (31); in this situation, the depletion of CaBP-like immunoreactivity was localized to the dentate granule cell–mossy fiber system of the hippocampus. Siegel *et al.* (32) also reported that the direct injection of pharmacological doses of 1,25-dihydroxyvitamin

Table 1. Effect of vitamin D_3 on the CaBP content of the intestinal mucosa and intestinalis nerve of 4-week-old rachitic chickens

Treatment	Mucosal CaBP, μg per mg of protein	Neuronal CaBP, ng per mg of protein
Vehicle	Undetectable	197 ± 27
Vitamin D_3 (500 IU)*	40 ± 1	477 ± 47†

Values represent mean ± SEM of five or six chickens per group.
*Administered intramuscularly 72 hr before experiment.
†The +vitamin D values are significantly greater than −vitamin D values at $P < 0.001$.

Table 2. Effect of dietary calcium on CaBP content of intestinal mucosa and intestinalis nerve of chickens

Dietary calcium	Mucosal CaBP, μg per mg of protein	Neuronal CaBP, ng per mg of protein
0.1% Ca	34 ± 1*	188 ± 9*
1.0% Ca	13 ± 0.4**	116 ± 11**
2.0% Ca	10 ± 1**	67 ± 6***

Values represent the mean ± SEM of five or six chickens per group. Dietary calcium contained 0.67% P and 1200 IU of vitamin D_3 per kg. Levels of dietary Ca varied as indicated.
*Values designated with different numbers of asterisks in a column are significantly different at $P < 0.001$.

D_3 into the brain increased the resistance of "kindled" rats to epileptic-like episodes. This effect of 1,25-dihydroxyvitamin D_3 on seizure activity occurred rapidly (within 5–10 min), was transient, and is probably too rapid in onset to be manifested by new protein synthesis. Large doses of vitamin D were also shown to decrease the number of seizures in epileptic patients without concomitant changes in serum calcium and magnesium concentrations (33).

In the present study, it was demonstrated that the concentration of CaBP in the intestinalis nerve associated with the caudal mesentery vein and artery was significantly lower in vitamin D-deficient chickens than in the vitamin D-replete chickens. However, in contrast to the rachitic duodenal mucosa, which was virtually devoid of CaBP in the severely vitamin D-deficient chicken, the intestinalis nerve still contained CaBP. The difference in the response of the two tissues to vitamin D deficiency could be due to differences in the rate of cell turnover and/or the longevity of the CaBP mRNA and/or the rate of release of CaBP.

Restriction of the dietary intake of calcium is a known stimulus for increased production of 1,25-dihydroxyvitamin D_3 by the renal 25-hydroxyvitamin D-1α-hydroxylase enzyme system (34, 35). As was reported by us (25) and others (28, 29), the concentration of CaBP in the mucosa varies indirectly with calcium intake. The data shown herein indicate that CaBP in the intestinalis nerve varies indirectly with dietary calcium levels—i.e., in the same direction as does the change in duodenal CaBP concentration. Duodenal CaBP in the group receiving the 0.1% calcium diet was greater by ≈340% when compared to the 2% dietary calcium group and the increase in nerve CaBP was ≈280%.

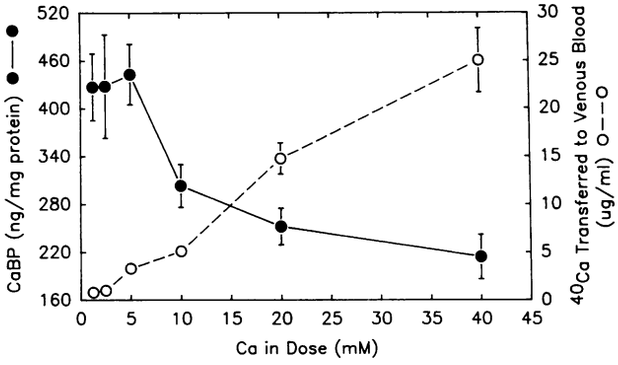

FIG. 4. Effect of the duodenal absorption of calcium on the concentration of CaBP in the mesenteric tissue. Vitamin D-repleted chickens were anesthetized and solutions containing various concentrations of $CaCl_2$ (labeled with ^{45}Ca) were injected into the ligated duodenal loops (see text). After 15 min, mesenteric venous blood draining the duodenum and the mesenteric tissue were obtained. Serum from the venous blood was assayed for ^{45}Ca content and the depicted ^{40}Ca values were calculated from the quantity of ^{45}Ca per ml of serum divided by the specific activity ($^{45}Ca/^{40}Ca$) of the injected solution. CaBP was determined by radioimmunoassay. Note that the CaBP content of the intestinalis nerve decreased significantly ($P < 0.001$) when the intraluminal concentration of ^{40}Ca was 10 mM or greater. ●, CaBP; ○, serum ^{40}Ca.

Of interest and indeed unexpected was the response of CaBP in the intestinalis nerve to a pulse of calcium placed in the duodenal lumen. The CaBP content of the nerve tissue decreased substantially 15 min after solutions containing 10 mM or more of calcium were deposited in the duodenal lumen. The precise stimulus for the decrease of CaBP under these circumstances is unknown but might be due to a transient hypercalcemia or, indirectly, the secretion of intestinal hormones, such as cholecystokinin and pentagastrin; the latter hormones are known affectors of calcitonin release in some species (36). Perhaps related to these observations is the report that adrenocorticotropic hormone and insulin (via the resultant hypoglycemia) caused an increase in CaBP in plasma of pigs (37), indicating an effect of certain hormones on the release of CaBP from intracellular sites. Although the primary source of blood CaBP seems to be the intestinal mucosa (38, 39), other CaBP-containing tissues, such as nerve cells, could constitute another source.

The calcium ion plays a significant role in physiological and biochemical processes in nerve tissue. As is known, calcium is required for neurotransmitter release and activates, with calmodulin, such processes as ATP-dependent calcium transport, phosphorylation of neuronal proteins, glycogenolysis, and neurotransmitter synthesis. The calcium that participates in such reactions could be derived from extracellular sources, entering neuronal tissue through potential and/or receptor-operated calcium channels, or from internal stores. The presence of CaBP in specific neurons might exert important effects on the control of neuronal function through its ability to bind calcium with high affinity and thereby control intracellular Ca^{2+} levels by essentially serving as a calcium buffer, as suggested by Baimbridge *et al.* (40). The resting free Ca^{2+} concentration in most cells is considered to be $\approx 10^{-7}$ M and CaBP, with a dissociation constant of $\approx 0.5 \times 10^{-7}$ M, could influence intracellular free Ca^{2+} levels in this capacity. The buffer function of CaBP could place a constraint on the excessive increase in intracellular Ca^{2+} upon agonist stimulation or depolarization, or it could maintain intracellular Ca^{2+} levels at stimulatory concentrations over a longer period of time than in its absence. It is also possible that neuronal CaBP has other functions since there is evidence that the M_r 28,000 CaBP from chicken intestine and the M_r 9000 CaBP from bovine intestine can stimulate the activity of the intestinal basolateral ATP-dependent calcium pump (6, 41, 43).

The physiological and pathological implications of the dependency of CaBP in the intestinalis nerve on calcium and vitamin D status cannot be defined from current data. However, a relationship between calcium, vitamin D intake, and hypertension in certain individuals has been reported (42). The possibility exists that CaBP in peripheral nerve might modulate smooth muscle contractility, thereby determining the degree of vascular resistance and, as a consequence, regional blood flow and blood pressure. It would be of interest, therefore, to determine whether CaBP is present in peripheral nerves of humans and, if so, its dependency on vitamin D and calcium status.

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