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Calcium-Binding Protein: Its Cellular Localization in Jejunum, Kidney and Pancreas (38742)

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(Introduced by J. P. Hannon)

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A vitamin D-dependent calcium binding protein (CaBP) has been demonstrated in homogenates of gut mucosa from chick, dog, cow and rat and in kidney homogenates from chick (1–4). A calcium binding protein has also been reported in homogenates of human kidney and gut mucosa (5–9), but its vitamin D dependency has not been established. Localization of CaBP in the goblet cells and in association with the surface coat microvillar region of chick intestine has been reported (10). More recently, a paracellular cation absorption process has been suggested to account for at least part of the calcium transport across intestinal mucosa (11), based on the affinity of CaBP for La^{3+} and the paracellular distribution of La^{3+} demonstrated by electron microscopy. The present study was designed to localize CaBP more precisely using enzyme immunohistochemistry and thus determine whether CaBP could be a component of a paracellular transport mechanism or not.

Methods. Reagent preparation. CaBP was purified from postmortem human kidney by a combination of DEAE cellulose and Sephadex G75 chromatography and acrylamide gel electrophoresis as reported earlier (6). Antiserum (Anti CaBP) for tissue localization of CaBP was prepared by injecting a homogenate of equal volumes of Freund's complete adjuvant and CaBP in normal saline into the footpads of a New Zealand White rabbit.[2] The initial protein dose was 500 μg, followed by 100 μg after 31 days. The rabbit was exsanguinated after 56 days. Serum gamma globulin was precipitated by adding an equal volume of saturated ammonium sulfate solution to the serum. The precipitate was removed by centrifugation and dissolved in pH 7.4 Tris buffer (6). The ammonium sulfate was removed by chromatography on a 5 × 100 cm Sephadex G75 column which had been equilibrated with pH 7.4 Tris buffer. Gamma globulin from a normal untreated rabbit (NRG) was prepared in the same manner.

Specificity of the Anti CaBP was assessed by immunoelectrophoresis of renal CaBP-containing material (DEAE + G75 purified) in the presence of 2.5 mM EDTA or 0.3 m*M* calcium chloride. As reported by Hitchman and Harrison (9), CaBP migrates much faster relative to other proteins when Ca^{2+} is excluded from the buffer and/or complexed with EDTA. When electrophoresis was performed in the presence of Ca^{2+}, migration of CaBP was retarded as indicated by both amido black staining and immunoprecipitate. There was neither amido black staining nor immunoprecipitate in the area where CaBP would be expected in the absence of Ca^{2+}. Thus, we are confident that the antibody is specific for CaBP or at least for proteins whose rate of electrophoretic migration is affected by Ca^{2+}.

Localization technique. CaBP was localized in tissues with the indirect peroxidase-labeled antibody method of Nakane and Pierce (12), employing 3-3′ diaminobenzidine (DAB) and H_2O_2 as peroxidase substrate. We followed the procedure of Kawaoi and Nakane (13) to prepare peroxidase-conjugated goat anti-rabbit gamma globulin (Anti RGG-HPO).

[1] The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

[2] In conducting the research described in this report, the investigators adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care, of the Institute of Laboratory Animal Resources, National Academy of Sciences—National Research Council.

CaBP was demonstrated in tissues fixed three hours in pH 7.3 phosphate-buffered 2% paraformaldehyde with 4% sucrose and washed overnight in pH 7.3 phosphate-buffered saline. It was also localized in tissues quick-frozen in ethanol cooled with dry ice. The paraformaldehyde-fixed tissues were sectioned after either freezing or embedding in paraffin.

Four types of controls were used on serial tissue sections to establish the specificity of the staining reaction. On some, NRG was applied in lieu of the specific Anti CaBP, while others were incubated with Anti CaBP which had first been mixed with purified CaBP. To determine the presence of endogenous peroxidatic activity, the Anti RGG-HPO was omitted on some sections and to assess the effect of the DAB, still others were incubated in substrate lacking H_2O_2. All control sections were negative except that erythrocytes contained endogenous peroxidatic activity, and goblet cells of the jejunum bound Anti RGG-HPO nonspecifically (Fig. 1A).

Results. Intestine. Biopsy specimens of normal human jejunum[3] were obtained with a Crosby–Kugeler capsule and fixed in paraformaldehyde. Upon staining, the presence of CaBP was indicated by brown reaction product in the intercellular space around absorptive cells of the villus tips (Fig. 1B, C). It was associated with the lateral and basal plasma membranes, but there was little in the microvillar region of the epithelial cells. Reaction product was also present in the basement membrane region beneath absorptive cells. There was no reaction product within the cytoplasm of absorptive cells. Because of nonspecific binding of Anti RGG-HPO, we were unable to determine whether CaBP was present in goblet cells in our human material. This pattern of CaBP localization was consistent in jejunal biopsies from three humans, but we were not able to obtain specific localization in biopsies of human duodenum or in any level of intestine from several species of animals.

Kidney. The pattern of localization of CaBP in the kidney was strikingly similar in man (autopsy), monkey, dog, cat, rat, mouse, and chick, and is illustrated with a section from mouse kidney (Fig. 1D, E). The same pattern was observed with both methods of fixation. In the outer cortex, only certain regularly-spaced proximal[4] and distal tubules contained CaBP, suggesting a non-random distribution. CaBP was also present inconstantly in cells of straight segments, collecting ducts, and in thin loops deep in the papilla.

Sections of positive tubules contained cells with reaction product in cytoplasm or nucleus, or both. Cell membranes and brush borders were consistently stained in positive cells. Completely unstained cells were present immediately adjacent to positive ones. No reaction product was present in blood vessel walls, in cells of the glomerulus, or in basement membrane of glomerulus or tubules.

Pancreas. CaBP was present in the pancreatic islets of the cat, dog, rat, mouse and chick (Fig. 1F). Human pancreas was not examined. In the islets, the reaction product was distributed intracellularly in a majority of the islet cells and the nuclei were unstained. The distribution of the labeled cells and the coarse granular pattern of the reaction product within cells suggest that the CaBP is associated with Beta cells. Exocrine tissue of the pancreas did not contain CaBP.

Discussion. If CaBP is a component of an intestinal calcium transport system, then its cellular localization is of critical importance to an understanding of the transport process. As mentioned above, Taylor and Wasserman (10) reported localization of CaBP in goblet cells and on the brush border of intestinal epithelial cells. However, interpretation of unidirectional calcium flux data

[3] The normal subjects volunteered for the study under the provision of Contract No. DA-49-193-MD-2596 between the University of Colorado and the U. S. Army Research and Development Command as a part of the conscientious objector program. The provisions of the contract make it obligatory to obtain the informed consent, in general, for the subjects' services, and specifically for each definitive study, and precluded the *in vivo* use of any radioactive substance.

[4] Sense submission of the manuscript special staining procedures suggest that it may be the thick segment of the ascending loop of Henle rather than proximal tubule.

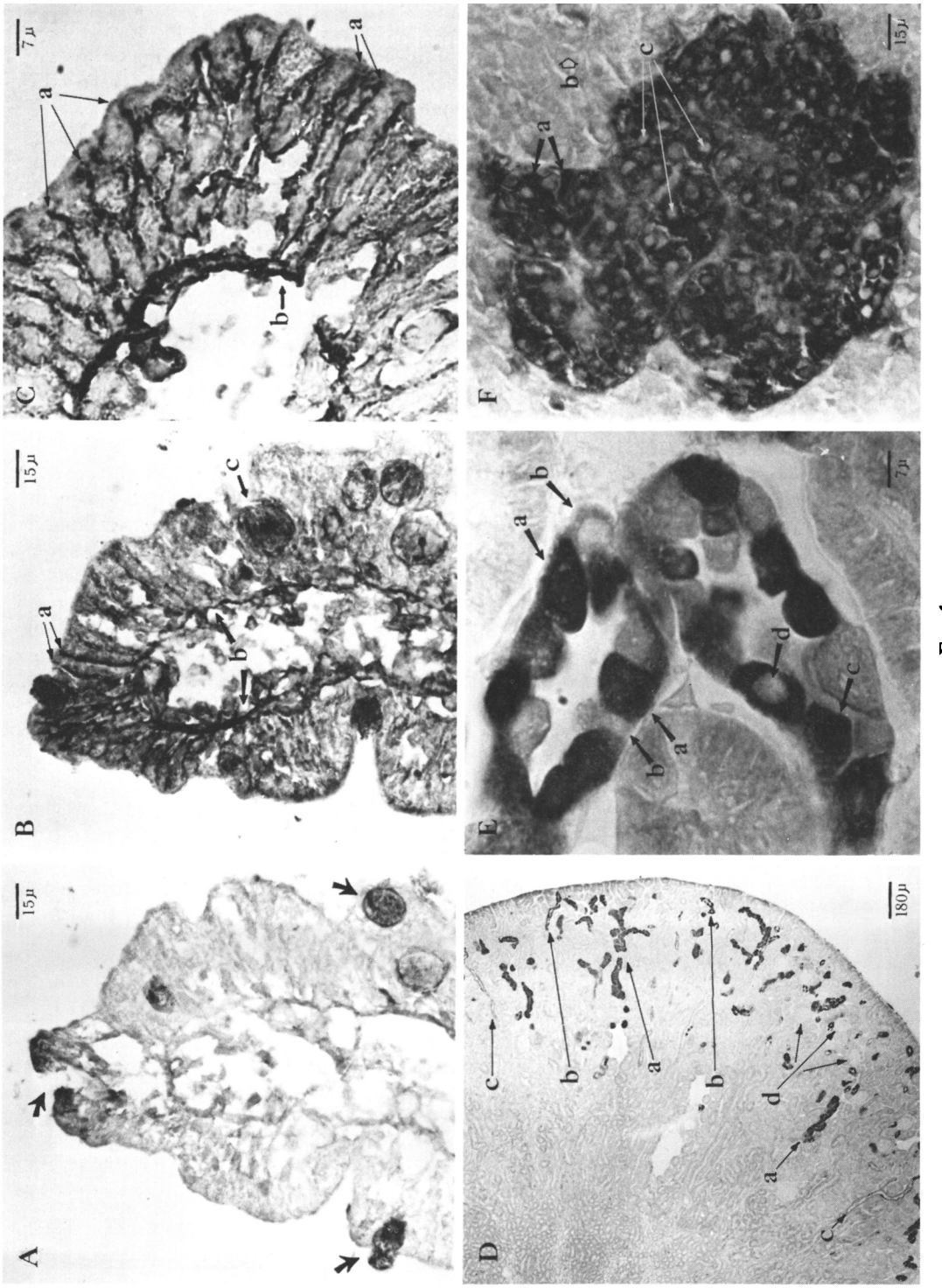

FIG. 1

suggests that the basal and/or lateral borders of the intestinal cell is the primary site of calcium permeability changes during adaptation of chicks to a low calcium diet (14). The localization reported herein is more consistent with the unidirectional flux data. However, it should be noted that Taylor and Wasserman used antibody against CaBP isolated from chick gut to localize CaBP in chick gut while we are using antibody against CaBP isolated from human kidney to localize CaBP in human gut. Thus, species, tissue and/or technique sensitivity differences could account for the different results.

The observation of CaBP in only selected renal tubules was an unexpected finding. Piazolo *et al.* (5) had reported a greater concentration of CaBP in renal cortex than that found in renal medulla, but to our knowledge the cellular localization of CaBP in kidney has not been previously reported. Structural heterogeneity among renal tubules has been reported (15), but we are not aware of other examples of such selective distribution of a specific protein in renal tissue. Whether the CaBP-positive regions are constant or shift with changing functional states remains to be shown. The presence of CaBP in renal cell nuclei and the variable nuclear staining in renal tubule cells raises the possibility that CaBP might function as a regulator of gene expression in those cells.

Previously reported methods had not demonstrated CaBP in pancreas (1), but a relatively high concentration of calcium in pancreatic islets has been reported (16). A link between CaBP and pancreas is also suggested by the observation of Schneider *et al.* (17) that the CaBP concentration in rat gut mucosa is markedly decreased by alloxan treatment. The functional significance of CaBP in pancreatic islets remains to be shown.

Another question raised by this study involves the immunologic cross-reactivity among the species and organs studied. With antibody prepared against human renal CaBP, we obtained good interspecies reactivity in kidney and pancreas, but we were able to stain only homologous intestine, suggesting an antigenic disparity in the heterologous intestinal CaBP. In man, intestinal CaBP is reported by Hitchman and Harrison (9) to have a mol wt of 13,700, while Morrissey and Rath (6) report that CaBP from kidney has a mol wt of 27,700. Both proteins bind Ca^{2+} avidly, and their sizes suggest the smaller could be a subunit of the larger. The amino acid sequence for human CaBP has not been reported. Our demonstration of immunologic cross-reactivity between these proteins is the first evidence that they share some degree of structural similarity. Antigenic similarity between human kidney CaBP and the smaller heterologous intestinal CaBP may be insufficient to permit interspecies reactions.

Summary. Calcium-binding protein (CaBP) was localized with peroxidase-labeled antibody in human jejunum and kidney, and in kidney and pancreas of

FIG. 1. CaBP Localization. A-C are human jejunal biopsy specimens fixed 3 hr in 2% paraformaldehyde with 4% sucrose, washed overnight in PBS at 4°, frozen in O.C.T. with ethanol–dry ice and sectioned with a freezing microtome. A is a control section which was incubated with normal rabbit serum in lieu of specific anti CaBP. Dark structures indicated by arrows are goblet cells, staining nonspecifically. B is an adjacent section from the same villus, incubated with anti CaBP. Dark lines between absorptive cells indicate reaction product and site of CaBP (a). Basement membrane region (b) is also stained specifically. Goblet cells contain reaction product (c). C is a higher magnification. The intercellular location of CaBP is evident as dark lines about absorptive cells (a). The basement region (b) is also stained. The microvillar region (surface, right) contains very little reaction product. Tissues depicted in D and E were embedded in paraffin after paraformaldehyde fixation and sectioned with a standard microtome. D is mouse kidney. CaBP is present in cells of particular proximal (a) and distal (b) tubules, and collecting ducts (c). Glomeruli are unstained (d). E is a distal tubule, mouse kidney. Cells containing CaBP (a) are immediately adjacent to negative cells (b). Nuclei in positive cells vary in staining reaction from strongly positive (c) to negative (d). F is rat pancreas. Cytoplasm of most islet cells (a) is heavily stained. Exocrine cells (b) and nuclei of islet cells (c) do not contain CaBP.

several animal species. The protein was associated with plasma membrane and intercellular space of jejunal absorptive cells. It was present in a specific population of renal tubule cells and in pancreatic islet cells. Immunohistochemical staining of intestinal CaBP with antibody to renal CaBP indicates structural similarity between the two proteins despite their different molecular weight.

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