Distribution of Protein Kinase C in the Villi of Duodenum of Developing Chick Embryos and Chicks: Immunohistochemical Studies at the Light and Electron Microscopic Levels

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Summary: We examined the immunohistochemical localization of protein kinase C (PKC) in chick embryonic and chick duodenal villi contained in cultured duodena using monoclonal antibodies that recognize types 1, 2 and 3 at the light and electron microscopic levels. In the light microscopic studies, staining with the type 2 and 3 PKC antibodies revealed immunoreactivities in the absorptive epithelial cells of the villi that approximated the time course after hatching, but was weak and partial just before the time of hatching. The epithelial cells of crypts seemed to lose their stain at all times. In the transmission electron microscopic studies, positive immunoreaction was found predominantly in the endoplasmic reticulum and nucleus, and also in the terminal web in the absorptive epithelial cells of villi.

In the cultured duodenal fragments, a change in the distribution of PKC was observed on the sheet-like duodenal fragments and its immunoreaction was mainly seen on the basal side of absorptive cells. In contrast, no change in the staining of PKC was revealed on the ring-like duodenal fragments.

These results suggested that PKC in the chick duodenal villi may be functionally related with the differentiation of absorptive epithelial cells and sensitive to changes in the cellular interactions of absorptive epithelial cells.

Protein kinase C (PKC) is a calcium-dependent, phospholipid-activated protein kinase first described by Inoue et al.⁹⁾. The enzyme is widely distributed in various tissues and organs, with the highest level found in brain. Among the physiological functions of PKC, the regulation of many cellular processes, including secretion, neurotransmitter release, differentiation, growth and gene activation, are widely accepted^{20–22)}. Cloning studies have demonstrated that PKC is a family of consisting of at least 7 subspecies (α , β_1 , β_2 , γ , δ , ϵ and ζ). Among them, α , β and γ gene products have been purified from a variety of different cells and tissues, and are referred to as types 3, 2 and 1 PKC, respectively^{5–7,22)}.

Immunocytochemical and biochemical studies have revealed differential localizations of some of these PKC enzymes in the mammalian brain mainly and in some other tissues, suggesting that each enzyme has a specialized function in different cell types^{7,22)}. However, neither immunohistochemical nor biochemical studies seem to have been done in the small intestine.

Noda and Mitsui have examined the localizations of cytoskeletal proteins such as actin and vinculin during the development of chick duodenum, and

suggested a relation between these cytoskeletal proteins and the morphogenesis of the duodenum. However, the detailed mechanism is not clear²³⁾. Recent studies^{8,10,11,27,28)} have indicated that vinculin can be a substrate of protein kinase or tyrosine kinase that has a major impact on the growth and differentiation of cells. We were interested to investigate whether actin or vinculin is a substrate of PKC in the duodenum to clarify the function of PKC and actin and vinculin.

In this study, as a first step, we examined the immunohistochemical localization of PKC in chick duodenal villi contained in cultured duodena using monoclonal antibodies that recognize types 1, 2 and 3 at the light and electron microscopic levels. We discuss the function of PKC enzyme in the duodenal villi.

Materials and Methods

Materials and organ culture: The duodena of 19 and 20-day-old embryo and chick duodena just after hatching and later, were used. The duodena of 8-day-old hatched chicks were used for the organ

culture study. The excised duodena were quickly placed in warm Eagle's minimal essential medium (MEM) and cut into fragments of 3–5 mm in length. Half of these fragments retained their ring-like shape, and the others were slit open lengthwise (sheet-like shape) with a sharp stainless-steel pincette. Tissues were cultured in MEM for 20 min in 51 mm culture dishes in an atmosphere of 5% CO₂ in air.

Antibodies and other chemicals: Mouse monoclonal antibodies to types 1, 2 and 3 PKC enzymes from rabbit brain (PKC 1, 2, 3 staining kit) were obtained from Seikagaku Kogyo Industries, Japan. The 15 nm of gold labelled goat anti-mouse IgG (Fc) (Auroprobe EM) was obtained from Amersham International plc.

Western immunoblotting: Duodenal epithelium from 10-day-old chicks was isolated over ice and homogenized in a Teflon-glass homogenizer with 10 volumes of the homogenized buffer, then the crude soluble extract was obtained according to a modification of the method of Kosaka et al.¹³⁾ Isolated crude extracts were incubated in the sample buffer and subjected to 5–20% sodium dodecyl sulfate polyacrylamide gel electrophoresis by the method of Laemmli¹⁵⁾ in the presence of 2% β -mercaptoethanol. The protein bands separated by electrophoresis were transferred to a Clearblot-P membrane (ATTO), which was then processed for a mixture of type 2 and 3 PKC antibodies staining by the ABC method²³⁾ (Fig. 1).

Light microscopic immunohistochemistry: All preparations were fixed in 4% paraformaldehyde solution, pH 7.2, for 1–2 hr at 4°C, dehydrated in a graded series of ethanols, cleared with xylene and embedded in paraffin. Sections (4 μ m) on glass slide were deparaffinized in xylene, and rehydrated by passage through a graded series of ethanols. During this process, the sections were transferred to absolute methanol containing 2% hydrogen peroxide, to remove traces of paraffin. Sections were indirectly immunostained by the PKC 1, 2, 3 staining kit method and then incubated in the medium for peroxidase, which contained 0.05% 3,3-diaminobenzidine tetrachloride and 0.01% H₂O₂ in Tris-HCl, pH 7.6. Counterstaining with methyl green to study the reactivity of the nucleus was not done.

Control sections treated with normal serum instead of the primary antibody in the kit and having no reactivity to type 1 PKC antibody, showed that the staining was specific for the appropriate antigen (Fig. 2).

Electron microscopic immunocytochemistry: Pre-embedding [1] and post-embedding [2] were performed in this study.

[1] Duodena fixed in 4% paraformaldehyde solution were immersed in graded sucrose solutions and

embedded in O.C.T. compound (Lab-Tek Products), then frozen in a mixture of dry ice and absolute ethanol. 8- μ m sections were cut and collected on gelatin-coated glass slides, immunostained for light microscopy, then postfixed with osmium tetroxide buffered with 0.1 M sodium phosphate pH 7.4 (PBS) and dehydrated with graded ethanols. After embedding in Quetol 812, the glass slides were detached from the tissue sections. Ultra-thin sections were cut with an ultramicrotome (Porter Blum MT2B) and observed without staining with uranyl acetate or lead citrate.

[2] The duodena were dissected quickly, cut into small blocks and fixed in 4% paraformaldehyde solution. After washing in PBS, tissues were dehydrated in a graded series of dimethylformamide (DMF), infiltrated with a mixture of DMF and Lowicryl K4M (K4M), then embedded in fresh 100% K4M in BEEM capsules followed by exposure to UV irradiation for about 4–6 hr. All steps were performed at 4°C. It took about 8 to 10 hr to finish the whole procedure by the method of rapid embedding¹⁾. Ultra-thin sections were cut and placed on nickel grids mounted with carbon-coated formvar films and immunostained according to the modified method of Lorenzsonn et al.¹⁷⁾. Gold labelled goat-anti-mouse IgG conjugate (Auroprobe EM) was used as an appropriate secondary antibody-colloidal gold probe. After immunostaining the sections were stained with uranyl acetate and lead citrate.

Results

Western immunoblotting. Figure 1 shows a western immunoblot analysis of type 2 and 3 PKC enzymes from an extract of duodenal epithelial cells of 10-day-old chicks. Two major immunoreactive bands migrated about 80-KD. And the appearance of these two bands is presumably the result of reaction with a mixture containing type 3 PKC antibody, together with study on the enzymological characteristics of type 3 PKC by Kosaka et al.¹³⁾. Some faint bands visible in this figure seemed to be proteolytic products during the extraction procedures²⁴⁾.

Light microscopic immunohistochemistry: Type 2 and 3 PKC enzymes in the absorptive epithelial cells of the villi approximated the time course after hatching (Figs. 4–8), but were weak and partial just before hatching (Fig. 3). The epithelial cells of crypts seemed to lose their stain before and after the time of hatching (Fig. 6). In this study reactivity to type 2 PKC antibody was about three times more intense than that to type 3 PKC antibody, and reactivity to type 1 PKC antibody was not found (Fig. 2). After the time of hatching, the positive portions of type 2

and 3 PKC were similar to each other, and were observed clearly in the cytoplasm and nucleus of the absorptive epithelial cells in the upper region of the villi (Figs. 4–8). The apical region or basal side of the absorptive epithelial cells sometimes stained as a line.

In the cultured duodenal fragments, changes in the distribution of types 2 and 3 enzymes were evident on the sheet-like duodenal fragments, and immunoreaction was mainly observed at the basal side of the absorptive epithelial cells (Fig. 10). In contrast, this change in staining was not revealed on the ring-like duodenal fragments (Fig. 9).

The lamina propria mucosae in Figs. 3 and 10 shows a weak reaction, and some blood cells in it were also stained. However, these details were unclear in this study.

Electron microscopic immunocytochemistry: Positive immunoreaction seemed to be found in the endoplasmic reticulum, nuclear membrane and condensed chromatin (Figs. 11–16), and sometimes in the terminal web near or adjacent to cell junctions. Unidentified parts in the cytoplasm of cells seemed to react. PKC near the basal side of cells seemed to come from the absorptive epithelial cells, as shown in Fig. 13, but this was not clear. It is necessary to study the relation between the PKC of the basal side and the lamina propria mucosae. Further detailed electron immunocytochemical studies, are being carried out.

Discussion

In this work we found that type 2 and 3 PKC enzymes existed in the chick duodenum. These two proteins seemed to be functionally related not with the proliferation but with the differentiation of absorptive cells, because they appeared after the time of hatching and were not localized in the cells of the crypt but in the absorptive epithelial cells of the villi. However, the functional difference between the two PKC enzymes was unclear except for the extent of immunoreactivity.

In the transmission electron microscopic studies, PKC in the terminal web or near the cell junctions seemed to be related to cytoskeletal proteins such as actin and vinculin^{8,10,11,23,27,28}). The change in the distribution of PKC found in the basal side of cultured duodenal absorptive epithelial cells (Fig. 10) seemed to affect neighboring cellular interactions. We found the same change in the distribution of cytoskeletal proteins in our previous organ culture study (unpublished data). PKC and cytoskeletal proteins seemed to be related with each other, but the details of the relationship were not clear in this study.

The positive immunoreaction of the nucleus in this study was very interesting. PKC had not been found in the nucleus until recently^{19,21}). Nevertheless, recent studies documented its nuclear localization in various cell types, and indicated the functional role of PKC at the site of nuclei^{2–4,7,12,14,16,18,25,26,29}). The nuclear localization of PKC seems to be accepted. Cambier et al.³) proposed that PKC in the nucleus is translocated from the cytosol. On the other hand, Masmoudi et al.¹⁸) proposed the endogenous presence of the enzyme in the nucleus. In this study, we found its nuclear localization in duodenal absorptive epithelial cells, but its characterization and functional role were unclear.

We have described only the immunohistochemical localization of PKC enzymes in the chick duodenal epithelium. Further detailed studies using biochemical analysis are needed to determine the functional mechanism of type 2 and 3 PKC enzymes in the duodenum.

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Explanation of Figures

Plate I

Fig. 1. Western immunoblot analysis of type 2 and 3 PKC (A), and proteins staining with Coomassie Brilliant Blue (B) from a crude extract of 10-day-old chick duodenum.

Figs. 2–6. Light micrographs of immunohistochemical staining with anti-type 1 PKC (2) and anti-type 2 PKC (3–6) enzymes of chick embryonic (3) and chick (2, 4–6) duodenal villi.

Transverse sections of the duodena of 20-day-old embryo (3), just-hatched chick (4), and 1-day- (5, 6), 3-day- (2) old hatched chicks.

C, crypt; La, lamina propria mucosae; Lu, lumen.

Figs. 2, 4, 5 and 6. ×240, Fig. 3. ×480

Plate II

Figs. 7–10. Light micrographs of immunohistochemical staining with anti-type 2 PKC (7, 8) and anti-type 3 PKC (9, 10) enzymes of the normal chick duodenal villi (7, 8), the cultured ring-like duodenal villi (9) and cultured sheet-like duodenal villi (10). Transverse sections of the duodena of 1-day- (7) and 8-day- (8, 9, 10) old hatched chicks. La, lamina propria mucosae; Lu, lumen.
Fig. 7. $\times 480$, Figs. 8, 9 and 10. $\times 240$

Plate III

Figs. 11–14. Transmission electron micrographs of immunocytochemical staining with anti-type 2 PKC enzyme of the duodena of 8-day-old hatched chicks by the pre-embedding method (see “Materials and Methods”).

Fig. 11. The upper region of absorptive epithelial cell. Immunoreaction is observed between mitochondria and endoplasmic reticulum.

Figs. 12–14. Portions surrounding and under the nuclei of absorptive epithelial cells. The nuclear membrane, condensed chromatin and endoplasmic reticulum seem to show positive reactions.

Bs, basal side of cell; Mt, mitochondria; N, nucleus.

Figs. 11, 12 and 13. $\times 20,000$, Fig. 14, $\times 12,000$

Plate IV

Figs. 15 and 16. Transmission electron micrographs of immunocytochemical staining with anti-type 2 PKC (15) and anti-type 3 PKC (16) enzymes of the apical region of absorptive epithelial cells by the post-embedding method (see "Materials and Methods"). Positive gold particles were evident in the terminal web near or adjacent to the cell junctions. $\times 26,000$
Lu, lumen; Mv, microvilli; Tw, terminal web; Aj, adherent junction.