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Dietary modulation of rat colonic cAMP-dependent protein kinase activity

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Abstract

Malignant transformation of cells is associated with enhanced proliferation and alterations in cAMP-dependent protein kinase (PKA) activity. To investigate the role of PKA in normal colonic cell proliferation, PKA was characterized in rat colonic mucosa. In addition, rats were fed diets containing different fats (corn oil, fish oil) and fibers (pectin, cellulose, fiber free) to elicit varying levels of colonic cell proliferation in order to study this signaling system under normal physiologic conditions. Overall, PKA activities were higher in cytosolic compared to membrane fractions. PKA type II (PKA II) isozyme contributed $89 \pm 1\%$ and $96 \pm 1\%$ of total PKA activity in cytosolic and membrane fractions, respectively. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed the presence of mRNA for both the α and β isoforms of the regulatory subunits of PKA II. PKA activities were 21-33% higher in distal membrane and total distal fractions in rats fed a cellulose/corn oil diet compared to animals consuming the other fiber/fat diets. These effects were seen only in the distal colon, where the number of cells per crypt column was elevated only in animals fed the cellulose/corn oil diet relative to other diets. Diet-induced mitogenic responses did not involve significant changes in the relative activity of PKA I and II isozymes. These data demonstrate that dietary effects on PKA activity in the distal colon may be related to changes in cell differentiation as indicated by the number of cells per crypt column.

Keywords: cyclic AMP-dependent protein kinase; Protein kinase; Dietary fat; Dietary fiber; Cell proliferation; (Rat colon)

1. Introduction

Cyclic AMP is an important regulator of a number of cellular processes, including cell growth and differentiation, metabolism, and gene expression [1–6]. Other than the direct regulation of some ion channels, the only known function of cAMP in eukaryotes is the activation of cAMP-dependent protein kinase (PKA; ATP: protein phosphotransferase, EC 2.7.1.37). Binding of two cAMP molecules to each regulatory (R) subunit induces conformational changes and results in the activation of PKA by dissociation of the asymmetric tetrameric holoenyzme into two R subunits and two active catalytic (C) subunits (for reviews see Refs. [7,8]).

Two classes of PKA, designated as type I and type II, containing unique R subunits, RI and RII, respectively, were originally distinguished by their elution patterns from DEAE-cellulose columns [9,10]. To date, four isozymes of the R subunits (RI α , RI β , RII α , RII β), three isozymes of the C subunits (C α , C β , C γ) (for references see Ref. [8]), and splice variants of the C subunit modulates cellular events both at the post-translational level by phosphorylation of cellular sub-strates and at the transcriptional level by phosphorylation of transcription factors which interact with regulatory elements of cAMP-responsive genes. Alterations

Abbreviations: PKA, cAMP-dependent protein kinase; PKA I, type I PKA; PKA II, type II PKA; R, regulatory subunit of PKA; C, catalytic subunit of PKA; PKI, protein kinase inhibitor peptide; RT-PCR, reverse transcriptase-polymerase chain reaction.

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in cAMP levels have been implicated in both the regulation of normal and transformed cell proliferation and normal cell differentiation [2,3,5,13–15]. Cell proliferation can be inhibited or stimulated by PKA, depending on the cell type, state of activation or transformation, the intracellular concentration of cAMP, and the cell environment [5].

The cAMP signaling pathway may be involved in the pathogenesis of colon cancer. Altered levels of cAMP have been associated with human colon carcinomas, as well as chemically induced colon cancer in experimental animal models [16-18]. Cellular growth in colonic cells and organ cultures appears to involve regulation by cAMP [18,19]. The PKA signaling pathway also interacts with other signaling molecules involved in the regulation of colonic cell growth, such as protein kinase C, mitogen activated protein kinase and the ras protooncogene [20-23]. For example, in HT-29 colon cancer cells, protein kinase C activation has been shown to selectively increase mRNA levels for the RI α subunit of PKA [24]. There is also evidence of cross-talk between PKA and protein kinase C at the transcriptional level [25]. We and others have demonstrated that alterations in protein kinase C activity are associated with colonic cell growth [26-31].

Increased colonic cell proliferation is considered to be a risk factor for the development of colon cancer [32]. Evidence implicating PKA involvement in the regulation of colonic mucosal cell proliferation is also provided by the involvement of prostaglandins in tumor growth and formation [33,34]. We have found a positive correlation between the number of cells per crypt and prostaglandin production in distal colons of rats fed diets known to alter colonic cell proliferation [35]. Since prostaglandins both mediate their effects via the PKA cascade and are modulated by PKA, this signaling pathway may also mediate colorectal tumorigenesis.

A mixture of PKA type I and II isozymes is present in most tissues [10,36]. It has been hypothesized that the selective expression of these isozymes is a critical component in the control of cell growth and differentiation. Cho-Chung and colleagues [37] have shown that in cultured cells the RI/RII ratio increases with cell growth and decreases with differentiation. Site-selective analogs and antisense oligodeoxynucleotides, which deplete the RI subunit, have been used to inhibit the growth of human cancer cells [37-39]. In vivo, it has been shown that the cytosolic RI/RII ratio was higher in Wilms' tumors relative to normal kidneys [40], suggesting that the isozyme distribution may be a key event in the control of cancer growth. A similar trend has been observed in urethane-induced mouse lung tumors [41]. Recently, it has been demonstrated that colorectal cancers relatively overexpress RI when compared with uninvolved colonic mucosa [42].

We and others have demonstrated that rates of

normal colonic epithelial cell proliferation can be modulated by altering the fat and fiber content of the diet [43-45]. In addition, these manipulations have been shown to modulate protein kinase C activity/levels and prostaglandin synthesis [26,44]. However, the effects of dietary fat and fiber on colonic mucosal PKA activity and RI/RII expression have not been determined. In this study we have characterized PKA activities and isozyme expression in normal rat colonic mucosa fractions. We show that although the relative isozyme expression is not different, PKA activity is altered in rat colonic mucosa following diet induced alterations in normal cell differentiation.

2. Materials and methods

2.1. Materials

PKA assay system, including specific PKA peptide substrate (kemptide) and protein kinase inhibitor $(PKI_{(6-22)})$, was purchased from Gibco (Gaithersburg, MD). $[\gamma^{-32}P]ATP$ (111 TBq/mmol) was purchased from Du Pont New England Nuclear (Boston, MA), and $8-N_3-[\gamma-^{32}P]cAMP$ (1.85 TBq/mmol) was purchased from ICN (Irvine, CA). ATP was from Boehringer Mannheim (Indianapolis, IN), and cAMP was from P-L Biochemicals (Milwaukee, WI). Aprotinin, leupeptin, pepstatin, soybean trypsin inhibitor, 4-(2-aminoethyl) benzenesulfonyl fluoride, 3-isobutyl-1-methylxanthine, sodium fluoride, EDTA, EGTA and Triton X-100 were from Sigma (St. Louis, MO). Cellulose (Avicel Microcrystalline cellulose, type PH 101) was purchased from FMC (Philadelphia, PA). Pectin (citrus pectin, polygalacturonic acid methyl ester) was purchased from US Biochemical (Cleveland, OH). Fish oil (Menhaden oil) was provided by the National Institutes of Health Fish Oil Test Material Program, Southeast Fisheries Center (Charleston, SC). Corn oil was kindly donated by Mr. Sid Tracy of Traco Labs (Seymour, IL). Other reagents were of analytical grade from commercial sources.

2.2. Animals and diets

The animal use protocol was approved by the University Laboratory Animal Care Committee of Texas A&M University and conformed to NIH guidelines. Sprague-Dawley rats were fed standard rat chow for the PKA analysis of normal rat colonic mucosa. In the dietary study, 68 male Sprague-Dawley rats (Harlan Sprague-Dawley, Houston, TX), weighing approx. 250 g, were randomly divided into four dietary groups (17 rats per diet): fiber free/corn oil, pectin/corn oil, cellulose/corn oil, or fiber free/fish oil. These diets have been described in detail [44] and were chosen

based on their ability to elicit proliferation responses in rat colonic mucosa [44,45].

2.3. Measurement of cell proliferation

In vivo cell proliferation was measured by bromodeoxyuridine incorporation into DNA as previously described [45]. Briefly, after the 3 week feeding period, each animal was given an intraperitoneal injection of bromodeoxyuridine in PBS, at a level of 5 mg/kg body weight, exactly 1 h prior to killing by CO₂ overexposure. The first 1 cm from the cecal-proximal colon junction was taken for proximal cell proliferation determination and the first 1 cm proximal to the rectum was taken to determine distal colon proliferation. The incorporation of bromodeoxyuridine into DNA was localized using monoclonal bromodeoxyuridine antibody (Boehringer Mannheim, Indianapolis, IN). The bound antibody was detected using peroxidase-conjugated antibody to mouse IgG (Signet, Dedham, MA). Slides were read for crypt height in number of cells and for the number and position of labeled cells. The mean of 20 crypt heights was calculated from 10 animals in each dietary group for both proximal and distal colon. The labeling index (%) was determined by dividing the number of labeled cells by the total cells in the crypt column, multiplied by 100. Proliferative zone (%), defined as the position of the highest labeled cell divided by the total number of cells in the crypt column, multiplied by 100, was also determined.

2.4. Tissue preparation

For normal rat colon, the entire colon was resected and divided into proximal and distal segments which were approximately equal in length. After the proximal and distal segments were removed for histology (see above), the remaining colon segments were flushed clean with ice-cold PBS. The colonic mucosa was separated from the muscle and serosa by scraping the mucosal surface with a microscope slide as previously described [45]. The mucosa from two to three animals was combined, weighed, and homogenized in 6 vol. of buffer containing 5 mmol/l Tris-HCl (pH 7.2), 250 mmol/l sucrose, 50 µmol/l NaF, 2 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l 2-mercaptoethanol, 1 μ g/ml soybean trypsin inhibitor, 35 μ g/ml 4-(2aminoethyl) benzenesulfonyl fluoride, and 25 mg/l of aprotinin, pepstatin, and leupeptin, using six strokes in a Potter-Elvehjem type homogenizer. All extractions were carried out on ice. The homogenates were centrifuged at $100\,000 \times g$ for 30 min at 4°C using a Beckman L8-M ultracentrifuge (Palo Alto, CA). The supernatant, which represented the cytosol fraction, was collected. The pellet was resuspended in 4 vol. of homogenization buffer containing 1% Triton X-100.

After incubation on ice for 10 min, the solubilized pellet was centrifuged at $100\,000 \times g$ for 30 min at 4°C. The resulting supernatant was designated as the membrane fraction. Whole brain extract was prepared by homogenizing rat brain tissue in homogenization buffer containing 1% Triton X-100 and centrifuging as described above. Rat liver cytosol was prepared in the same manner as rat colonic mucosa cytosolic extracts.

Human colon tissue was obtained from the Cooperative Human Tissue Network, University of Alabama at Birmingham. Sections of normal colon obtained during surgery were removed, flushed with cold sterile saline, slit open and the mucosa scraped, weighed and placed in vials containing homogenization buffer. Samples were frozen, shipped on dry ice and stored at -80° C upon arrival. Samples were processed as described above for rat tissue to obtain cytosolic and membrane extracts.

2.5. Determination of PKA activity

The PKA activity in cytosol and membrane fractions was determined by measuring the phosphorylation of a specific peptide substrate (kemptide, Leu-Arg-Arg-Ala-Ser-Leu-Gly) using a commercial PKA assay system. Phosphorylation of kemptide by PKA and its specific inhibition by a protein kinase inhibitor peptide $(PKI_{(6-22)})$ form the basis of this method. The reaction mixture (40 μ l per microfuge tube) contained 50 mmol/l Tris-HCl at pH 7.5, 10 mmol/l MgCl₂, 100 μ mol/l [γ -³²P]ATP (4 nmol per assay tube), 50 μ mol/l kemptide substrate, and an aliquot of the PKA extracts. For specific activation of PKA, assays contained 10 μ mol/l cAMP; for specific inhibition assays, 1 μ mol/l PKI was added. The reaction mixture was incubated at 30°C for 5 min and stopped by spotting a 20 μ l aliquot onto phosphocellulose paper. The paper was gently washed twice with 1% phosphoric acid (4 min, 10 ml/disc paper) and then washed with water twice (4 min, 20 ml/disc paper). Phosphorylated PKA substrate, bound to the washed discs, was measured by liquid scintillation counting (Beckman Model LS-3801, Palo Alto, CA). Phosphotransferase activity was determined in the presence and absence of cAMP and/or inhibitory peptide (PKI) specific for PKA. Specific PKA activity was calculated as the difference between the amount of ³²P incorporated in the presence and absence of PKI. PKA activity derived from unactivated holoenzyme was determined by calculating the difference in ³²P incorporation in the presence and absence of cAMP. Less than 10% of total activity was derived from endogenous activated PKA (data not shown). All assays were performed in duplicate. Variation in duplicate incubations and between experiments was less than 10%. Protein concentration was determined by BCA protein assay as per manufacturer's instructions (Pierce, Rockford, IL). Bovine serum albumin was used as the standard.

2.6. Separation of type I and type II isoenzymes

PKA I and II were separated using either gradient or stepwise elution from DEAE-cellulose columns (DE-52, Whatman, Hillsboro, OR). Cytosol or membrane fractions (1.5 mg) were loaded onto the cellulose column (bed volume approx. 1 ml, $0.9 \text{ cm} \times 2 \text{ cm}$) previously equilibrated with column buffer (10 mmol/l K_2 HPO₄, pH 7.2). For gradient chromatography, the column was rinsed with 10 ml of column buffer and eluted with a linear gradient of 0-0.3 mol/l NaCl in column buffer, at a flow rate of 0.75 ml/min. 1 ml fractions were collected and assayed for PKA activity as described above. The elution profile obtained was used to determine NaCl concentrations required for the stepwise elution of the isozymes. PKA I was eluted in four 3.5 ml washes of 0.09 mol/l NaCl in column buffer and PKA II with four washes of 0.4 mol/l NaCl in column buffer. Fractions were concentrated using Centricon-30 concentrators (Amicon, Beverly, MA) and assayed as described above. The known elution profiles of rat liver and brain [10] were used as standards for both linear gradient and stepwise elution profiles of rat colonic mucosa protein extracts. Elution patterns were confirmed by photoaffinity labeling (Fig. 1) and immunoblot analysis (Fig. 2).

2.7. Immunoblotting of colonic extracts

Human colonic homogenates were treated with $3 \times$ SDS sample buffer (62.5 mmol/l Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol (pH 6.8)) and subjected to SDS-PAGE in 8% pre-cast minigels (Novel Experimental Technologies, San Diego, CA) as per the method of Laemmli [46]. Following electrophoresis, gels were soaked for 15 min in transfer buffer and electroblotted onto PVDF membranes (Millipore, Bedford, MA) using a Hoefer Mighty Small Transphor unit (San Francisco, CA) at 200 mA for 3.5 h. Following transfer, the membrane was processed by the method of Sheng and Schuster [47]. Primary antibody to human RI α and antisera to RII α (generously provided by Dr. Jahnsen, Institute of Medical Biochemistry, University of Oslo) were diluted 1/500-1/1000 and the membrane incubated for 2 h at room temperature with rocking. Alkaline phosphatase-conjugated secondary antibody (Jackson, West Grove, PA) was subsequently incubated for 2 h at room temperature, followed by development to visualize PKA bands.

2.8. Photoaffinity labeling

Incorporation of $8-N_3-[\gamma-^{32}P]cAMP$ into PKA was performed as previously described [36,48]. The reaction

mix (final volume of 100 μ l) contained 20 mmol/l Tris-HCl (pH 7.2), 1 mmol/l ATP, 1 mmol/l 3-isobutyl-1-methylxanthine, 10 mmol/l MgCl₂, 1 mmol/l 2-mercaptoethanol, $25 \,\mu g/ml$ aprotinin, $25 \,\mu g/ml$ leupeptin, 1 μ mol/l (185 kBq) 8-N₃-[γ -³²P]cAMP, and up to 200 μ g of protein. After preincubation for 60 min with or without 100 μ mol/l cAMP in the dark at 4°C, the samples were irradiated with a wavelength of 256 nm for 10 min at a distance of 8 cm to covalently bind 8- N_3 -[γ -³²P]cAMP to the R subunit of PKA. The reaction was stopped with 50 μ l of a solution containing 62.5 mmol/l Tris-HCl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol (pH 6.8) and heated at 100°C for 3 min. The entire sample was subjected to SDS-PAGE, using a 4% stacking gel and 10% separation gel [48]. After soaking for 1 h in 10% glycerol, gels were dried overnight and exposed to Kodak X-Omat film (Kodak, Rochester, NY) for 2–18 h.

2.9. RNA extraction and RT-PCR

Colon, brain and testis RNA was isolated using RNAzolTM as per manufacturer's instructions (Biotecx, Houston, TX). RNA was reverse transcribed into cDNA with Stratagene's First Strand Synthesis kit (La Jolla, CA) using oligo-dT primers. cDNA generated from brain and testes RNA was used as a positive control for the PCR reactions, and incubations containing no reverse transcriptase were used as negative controls. Forward and reverse PCR primers used for RII α were 5'-GGAGAGCGAATAATCACTCAGGG-3' and 5'-TCTGGGTGTGTGTTCTTGTGGCTG-3' and for RII β were 5'-AGTGGGCTCAACAATATC-CATGTT-3' and 5'-CAAGAAGCCTGCAAAGACA-TCC-3'. For RII α , 35 cycles of denaturation (93°C, 30 s), annealing (55°C, 45 s), and extension (74°C, 45 s) were conducted in a DNA thermal cycler (Perkin-Elmer 9600, Norwalk, CT) in a volume of 50 μ l. For RII β , 35 cycles of denaturation (93°C, 30 s), annealing (54°C, 45 s), and extension (74°C, 45 s) were conducted followed by a second round of 35 cycles with an annealing temperature of 55°C using 5–10 μ l of the reaction from the first round of amplification. PCR products were visualized on a 1.2% agarose gel to confirm their correct size. PCR products were then subcloned into either BluescriptTM (Stratagene, La Jolla, CA) or pCRTMII using a commercial kit (InVitrogen, San Diego, CA), and sequenced.

2.10. Statistical analysis

Data were analyzed by analysis of variance (ANOVA). When the P values were less than 0.05 for the individual effects of diet or fraction but not for interactions, total means were separated using Duncan's Multiple Range test [49]. Data are presented as mean \pm S.E.

3. Results

In normal chow-fed rats, PKA phosphotransferase activity in colonic mucosa proximal cytosol was significantly higher than in either of the membrane fractions (proximal or distal), with the distal cytosol level being intermediate. Total PKA activity for proximal cytosol, distal cytosol, proximal membrane, and distal membrane was 5.8 ± 1.1 , 3.8 ± 0.4 , 2.1 ± 0.6 , and 2.3 ± 0.4 pmol phosphate transferred/min per μ g protein, respectively (n = 4). Overall, the cytosolic fractions had significantly higher levels of PKA activity relative to the membrane fractions (P = 0.0017). These findings were confirmed in the diet study (results below).

Using rat liver and brain, which are known to contain predominantly type I and type II PKA, respectively [10], the identity of the fractions eluted from DEAE-cellulose columns was determined. PKA type I and II eluted at approx. 0.08 mol/l and 0.18 mol/l NaCl, respectively, as evidenced by the elution profiles using gradient anion-exchange chromatography (Fig. 1a). Stepwise anion-exchange chromatography (Fig. 1b) was performed based on the NaCl concentrations that eluted PKA types I and II from the gradient columns. The identities of the eluted fractions (with 0.09 mol/I and 0.4 mol/l NaCl) was confirmed by photoaffinity labeling with $8-N_3-[\gamma-^{32}P]cAMP$ followed by SDS-PAGE. As shown in the autoradiogram in Fig. 1c, bands incorporating the $[\gamma^{-32}P]cAMP$ were observed in lanes 1 and 2, corresponding to PKA type I eluted with 0.09 mol/l NaCl, and in lanes 5 and 6, corresponding to PKA type II, eluted with 0.4 mol/l NaCl.



Fig. 1. Separation of PKA I and II isozymes. (a) Gradient elution of protein extracts from rat liver and brain using a DEAE-cellulose column. 1 ml fractions were collected and assayed for phosphotransferase activity as described in Materials and methods. Briefly, phosphotransferase activity was determined in the presence and absence of cAMP and/or inhibitory peptide (PKI) specific for PKA. PKA activity was defined as the difference in the PKI inhibited phosphotransferase activity in the presence and absence of cAMP. ———, pmol of phosphate transferred/min/ml; — ——, concentration of NaCl gradient in mol/l. (b) Stepwise elution of the rat liver and brain extracts used in (a) with a DEAE-cellulose column. 3.5 ml fractions were collected, concentrated, and assayed for PKA activity. Fractions 1–4 were eluted with 0.09 mol/l NaCl, fractions 5–8 were subsequently eluted with 0.4 mol/l NaCl. (c) Fractions obtained in (b) were photoaffinity labeled with 8- N_3 -[γ - 32 P]cAMP, and subjected to 10% SDS-PAGE and autoradiography. (d) Autoradiogram of fractions 1 and 5 from (c) run side-by-side indicating the differences in molecular masses of PKA I and PKA II. The size of a known marker at 46 kDa is indicated. Specificity of PKA labeling was demonstrated in samples that were incubated in the presence of 100 μ mol/l unlabeled cAMP. No incorporation of 8- N_3 -[γ - 32 P]cAMP was observed in the presence of excess cAMP, as shown in the lanes labeled + cAMP.

The difference in size was readily apparent when fractions 1 (PKA I, approx. 48 kDa) and 5 (PKA II, approx. 52-54 kDa) were run side-by-side (Fig. 1d). Specificity of PKA labeling was shown by incubating samples with an excess (100 μ mol/l) of unlabeled cAMP, which prevented the labeling of PKA, as shown in lanes designated + cAMP (Fig. 1d). Further confirmation of the elution patterns was obtained by immunoblot analysis of human colonic tissue that had been subjected to anion-exchange chromatography (Fig. 2). As shown in the immunoblot in Fig. 2a, bands reacting with human RI α antibody were present in the pre-column tissue extract (lane 3) and in pooled fractions 1-3 which were eluted with 0.09 mol/l NaCl (lane 1), but absent in pooled fractions 5-7 which were eluted with 0.4 mol/l NaCl (lane 2). In contrast, with human RII α antiserum (Fig. 2b), immunoreactive bands were visible in the pre-column tissue extract (lane 3) and in pooled fractions 5-7 (lane 2), but not in pooled fractions 1-3 (lane 1), confirming that the 0.09 mol/l NaCl column eluate contained only PKA I, and the subsequent 0.4 mol/l NaCl eluate contained only PKA II.

Type II PKA was the predominant isozyme in rat, as well as human (data not shown), colonic mucosa. Results shown in Fig. 3 of the distal cytosolic fraction of rat mucosa are representative of all fractions examined (proximal cytosol, distal cytosol, proximal membrane, and distal membrane). The level of PKA II in rat colonic mucosa ranged from 90 to 98% of total PKA, as determined by PKA activity of fractions eluted using DEAE-cellulose (Fig. 3a). Photoaffinity labeling of the anion-exchange eluates confirmed the predominance of PKA II in rat colonic mucosa (Fig. 3b). RT-PCR was performed to determine the subtype of the RII isozymes present in normal rat colonic tissue. As shown in Fig. 4, PCR products derived from mRNA for both RII α and RII β were detected in rat proximal and distal colonic mucosa. It should be noted that RT-PCR analysis is not quantitative, and therefore these results are qualitative only.



Fig. 3. Separation of PKA type I and type II isozymes in rat colonic mucosa distal cytosol by (a) stepwise elution anion-exchange chromatography and (b) SDS-PAGE of PKA photoaffinity labeled with $8-N_3$ - $[\gamma-^{32}P]$ cAMP, as described in Fig. 1.

In the diet study, the pattern of PKA activities in different fractions of the colonic mucosa was similar to that found in chow-fed rats. PKA activity for proximal cytosol, distal cytosol, proximal membrane, and distal membrane was 5.5 ± 0.3 , 5.3 ± 0.4 , 2.7 ± 0.2 , and 4.2 ± 0.3 pmol phosphate transferred/min/µg protein, respectively (n = 20-24 per fraction). Both proximal and distal cytosol fractions had higher PKA levels than the membrane fractions (P < 0.001), with 67% and 55% of the total PKA activity in proximal and distal colonic



Fig. 2. Immunoblot analysis of human colonic mucosa using (a) RI α antibody or (b) RII α antiserum. Protein extracts were applied to DEAE-cellulose columns and PKA type I was eluted using 0.09 mol/l NaCl and PKA type II was collected in the 0.4 mol/l NaCl eluate in a stepwise manner. Fractions were concentrated and immunoblot analysis was performed as described in Materials and methods.



Fig. 4. Characterization of RT-PCR products from rat brain, proximal coion and distal colon. mRNA was incubated with (+) and without (-) reverse transcriptase (RT), and the cDNA synthesized was used for PCR analysis as described in Materials and methods. PCR products were subjected to agarose gel (1%) electrophoresis. Size of known markers in base pairs is indicated. Expected sizes of the PCR products using specific primers for (a) PKA RII α and (b) PKA RII β were 434 and 815 base pairs, respectively.

mucosa being associated with the proximal and distal cytosol, respectively. In addition, differences between the distal membrane fraction and the other fractions were significant in the diet study (P < 0.01), presumably because of the larger n value in this data set compared to the data obtained from chow-fed animals (above).

Although the relative pattern of PKA activities in colonic mucosa fractions was not altered by diet, PKA activities in rats fed the cellulose/corn oil diet com-

Table 1 Effect of diet on PKA levels in rat colonic mucosa extracts

pared to the other diets were higher in the distal membrane and total distal fractions of the colonic mucosa (Table 1). Total distal activity was calculated by dividing the total phosphotransferase activity of the distal cytosol and membrane fractions by the total mg of protein in the distal fractions. In total distal colonic mucosa (cytosol plus membrane fraction), PKA activity was 21-33% higher in rats on the cellulose/corn oil diet compared to the animals on the other diets. A similar trend was seen in distal cytosol, with a P value

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	Cellulose/corn oil	Fiber free/corn oil	Fiber free/fish oil	Pectin/corn oil			
Proximal colon		······································		······································			
Cytosol	5.26 ± 0.99	5.38 ± 0.26	5.12 ± 0.94	6.16 ± 0.39			
Membrane	2.65 ± 0.27	2.87 ± 0.62	2.66 ± 0.29	2.54 ± 0.50			
Total	4.15 ± 0.76	4.30 ± 0.40	3.93 ± 0.75	4.62 ± 0.33			
Distal colon							
Cytosol	6.13 ± 0.83	5.12 ± 0.52	5.67 ± 1.02	4.09 ± 0.16			
Membrane	5.06 ± 0.48 ^a	3.24 ± 0.19 °	3.64 ± 0.45 ^{b,c}	$4.88 \pm 0.54^{a,b}$			
Total	5.67 ± 0.45 ^a	4.25 ± 0.33 b	4.67 ± 0.57 ^b	4.42 ± 0.28 ^b			

Cytosol and membrane extracts were prepared as described in Materials and methods. Activity in the total cytosol and membrane fractions was calculated by dividing the total phosphotransferase activity of the cytosol or membrane fractions by the total μg of protein in the cytosol or membrane fractions, respectively. Phosphotransferase activity is expressed as pmol of phosphate transferred/min per μg protein (n = 4-6). Means within fractions having different letters are significantly different from each other (P < 0.05). See Fig. 1 for determination of PKA activity.

Table 2

Modulation of colonic cell proliferation by diet in rat proximal and distal colon (for further details, see Table 1)

	Cellulose/corn oil	Fiber free/corn oil	Fiber free/fish oil	Pectin/corn oil
Proximal colon				
No. of cells per crypt column	28.2 ± 0.4	27.4 ± 0.3	27.0 ± 0.4	28.4 + 0.6
Labeling index (%)	11.1 ± 0.57 ^ь	14.4 ± 0.76 ^a	$8.9 \pm 0.5^{\circ}$	15.7 ± 0.81^{a}
Proliferative zone (%)	41.4 ± 0.96 ^{b,c}	$43.1 \pm 1.13^{a,b}$	39.0 ± 0.77 °	44.8 ± 0.90^{a}
Distal colon				
No. of cells per crypt column	37.6 ± 0.6^{a}	35.4 ± 0.7 ^b	34.2 ± 0.6^{b}	34.8 ± 0.8 ^b
Labeling index (%)	9.8 ± 0.99 ^ь	$11.1 \pm 0.98^{a,b}$	8.4 ± 0.83 b	12.8 ± 0.91^{a}
Proliferative zone (%)	32.2 ± 1.60	33.0 ± 0.75	31.2 ± 1.76	35.0 ± 1.31

for a diet effect of 0.07. PKA activities either in the proximal cytosol, proximal membrane, or total proximal fractions were not significantly affected by diet.

Consistent with our previous reports, colonic cell proliferation was altered in rats fed different dietary fats and fibers [45]. Table 2 describes the effect of diet on selected indices of cell proliferation. In the proximal colon, rats receiving pectin/corn oil and fiber free/corn oil diets had a higher labeling index than rats fed cellulose/corn oil or fiber free/fish oil diets. The proliferative zone followed the same pattern (pectin/corn oil > fiber free/corn oil > cellulose/corn oil > fiber free / fish oil). The values for pectin / corn oil were significantly greater than those for either cellulose/corn oil of fiber free/fish oil. There was no effect of diet on the number of cells per crypt column in the proximal colon. In contrast, in the distal colon, cellulose/corn oil feeding resulted in a greater number of cells per crypt column than any other diet (Table 2). The labeling index in the distal colon followed a similar pattern to that found in the proximal colon, with pectin/corn oil resulting in higher labeling index than either cellulose/corn oil or fiber free/fish oil. Fiber free/corn oil produced an intermediate result. There was no statistically significant effect of diet on the percentage of the mucosa occupied by the proliferative zone in the distal colon.

Interestingly, the number of cells per crypt column in rats fed the different diets followed a pattern similar to PKA activity in colonic mucosa, i.e., in the distal colon there was an effect of diet with the cellulose/corn oil fed animals having both a higher number of cells per crypt and a higher PKA activity relative to the other diets. In the proximal colon, diet had no effect on either the number of cells per crypt column or PKA activity.

There was no effect of diet on the relative activity of PKA I and II in rat colonic mucosa. The mean contribution of type II isozyme activity in proximal and distal cytosol and membrane fractions was 91.6 ± 1.1 , 91.8 ± 2.0 , 91.2 ± 1.5 , and $91.4 \pm 2.0\%$, for animals on the cellulose/corn oil, pectin/corn oil, fiber free/corn oil, and fiber free/fish oil diets, respectively. Across all diets, the activity level of the type II isozyme in proximal cytosol, distal cytosol, proximal membrane, and distal membrane was 88.2 ± 1.4 , 89.2 ± 1.2 , 96.0 ± 0.8 , $95.2 \pm 1.1\%$, respectively (n = 8-12 per fraction). Cytosolic fractions were significantly different from membrane fractions (P < 0.001).

4. Discussion

Normal rat colonic mucosa possesses substantial PKA activity, with levels comparable to that found in rat liver (data not shown). PKA activity was detected in

both the cytosolic and membrane fractions, with slightly more activity (55–67% of total) located in the cytosolic fraction. Since phosphotransferase activity was measured in the presence of excess cAMP, activity levels likely reflect the steady-state levels of PKA. As demonstrated by anion-exchange chromatography and photoaffinity labeling, the majority of the PKA activity was type II PKA in both the cytosol (89% of total) and membrane (96% of total) fractions. RT-PCR analysis revealed the presence of mRNA for both the RII α and RII β isozymes of PKA in rat colonic mucosa. Although we have previously shown that our colonic mucosal isolation technique selectively removes the mucosa, leaving the muscle and serosal layers intact [35], we cannot rule out the possibility that a small amount of contamination by other cells could have influenced RT-PCR data, due to the high sensitivity of this assav.

Diet had a significant effect on PKA activity. Specifically, the cellulose/corn oil diet resulted in higher PKA activity in the distal mucosa relative to the other diets. The cellulose/corn oil diet also significantly increased the number of cells per crypt column in the distal colon. The significance of the relationship between an increased number of cells per crypt and increased PKA activity is not known. An increased number of cells per crypt column, in the absence of an increased labeling index or expanded proliferative zone, suggests an increase in differentiated cells. This would suggest that PKA is more active in differentiated relative to proliferative cells. Other investigators have reported higher levels of cAMP in differentiated cells compared to proliferating or transformed cells [17,50]. Confirmation of this hypothesis would require the specific labeling of differentiated cells with markers such as the lectins dolichos biflorus agglutin (DBA) and soybean agglutinin (SBA).

Differences in PKA activity in the colonic mucosa from rats on select diets may, therefore, be associated with alterations in the distribution of proliferating and differentiated cells which are known to be affected by diet. Interestingly, in a previous study [44], we reported an association between diet-induced proliferation and prostaglandin synthetic capacity in the distal, but not proximal, colon. The distal specific differences in PKA activity and proliferation as found herein may also involve prostaglandins, since prostaglandin effects are mediated in part via the PKA signaling system.

In previous studies we have also demonstrated that diet-induced changes in colonic cell proliferation were associated with alterations in protein kinase C activity and subcellular distribution [26,44]. Other studies have also implicated roles for protein kinase C [26–31] and prostaglandins [33,34] in colonic epithelial cell proliferation and colon carcinogenesis. Mutations in the *ras* protooncogene have also been found to be an early

event in the development of colonic tumors [51,52]. Interestingly, protein kinase C, prostaglandins, and *ras* have been shown to interact with the PKA signaling pathway in various cell systems [20–24], suggesting that the cAMP signaling pathway may be involved in the pathogenesis of colon cancer. In addition, cAMP levels appear to be altered in human colon carcinomas, as well as in experimental animal models of colon cancer [16–18]. Modulation of intracellular cAMP levels also appears to regulate colonic cell growth [18,19]. It is possible, therefore, that diet modulates select mediators of the PKA signaling pathway (e.g., protein kinase C, prostaglandins, *ras*), which in turn alter colonic epithelial cell proliferation and differentiation.

While the reason for the apparent differences in PKA activity between proximal and distal colons is not known, we have previously demonstrated that the proximal and distal colons are differentially affected by diet [45]. Recently, we have also shown that the proximal and distal colons exhibit distinctive patterns of protein kinase C isozyme expression. Protein kinase C α was found in higher levels in the proximal colon, whereas the δ and ζ isozymes predominated in the distal colon [53].

With respect to PKA isozymes, dietary intervention did not alter colonic PKA I and II activity ratios. This is in contrast to studies indicating that certain cells with increased proliferative rates, associated with malignant transformation, display increased levels of PKA I relative to PKA II [37-42]. The lack of any change in this ratio in response to the diet-induced alterations in cell proliferation in this study suggests that under conditions of normal cell proliferation, the relative levels of PKA type I and type II remain constant. This is consistent with previous work on rat colon [50], where it was found that cells in the lower end of the crypt column compared to those at the top of the column did not have different relative activities of PKA I and II, even though colonic epithelial cells at the upper end of the crypt column were more differentiated than those found at the lower end of the column.

It has recently been reported that a heterodimer of PKA consisting of RI α and RI β is present at low levels in a neoplastic B cell line (Reh), which contains predominantly type I PKA homodimers [54]. Since this heterodimeric form of PKA has been shown to co-elute with PKA type II on DEAE columns, it is possible that a portion of the second peak eluted from the anion-exchange column in the present study may have contained some of this unique heterodimer. However, unlike Reh cells, rat colonic mucosa contains very little homodimeric type I PKA and it is therefore unlikely that heterodimeric type I is present in large amounts in this tissue.

In conclusion, we have shown that rat colonic mucosa contains PKA activity that is predominantly present as the type II isozyme. The distribution of PKA varies between proximal and distal colon and cytosolic and membrane fractions, with proximal and distal cytosolic fractions containing higher relative levels of PKA phosphotransferase activity. Diet-induced changes in PKA activity may be related to changes in cell proliferation and differentiation patterns. The ability of diet to alter PKA levels and its significance in relation to alterations in signal transduction pathways in colon carcinogenesis remains to be elucidated.

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