Early Alterations in Rat Colonic Mucosal Cyclic Nucleotide Metabolism and Protein Kinase Activity Induced by 1,2-Dimethylhydrazine

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ABSTRACT

The present study examined the acute effects of intrarectal administration of a single dose (25 mg/kg) of the colon carcinogen 1,2-dimethylhydrazine (DMH) on DNA synthesis and cyclic nucleotide metabolism in rat colonic mucosa. DMH initially inhibited DNA synthesis (12 and 24 hr postinstillation) in distal colonic mucosa, at which time there were no detectable alterations in tissue cyclic adenosine 3':5'-monophosphate (cAMP), cyclic guanosine 3':5'-monophosphate (cGMP), or cAMP-dependent protein kinase (PK) activity compared to values in the vehicle control. At three and five days after DMH, [3H]thymidine incorporation into DNA was significantly increased. There was a concurrent reduction in tissue cAMP content and the state of activation (activity ratio) of cytosolic PK. cGMP levels did not change significantly in response to DMH. At three and five days after DMH, there was also an increase in the proportion of the type I isoenzyme of cytosolic PK, a form which has been associated with increased proliferative activity and a less differentiated cellular state in other tissues. DMH-induced increases in DNA synthesis and the PK isozyme pattern were observed in both the superficial and lower crypt (proliferative) epithelial cells isolated from colonic mucosa. DMH-induced changes in DNA, cAMP, and PK were transient, and values had returned to control levels by 10 days. In short-term cultures of rat colonic segments, N6-O2'-dibutyryl cyclic adenosine 3':5'-monophosphate, prostaglandin E2, and vasoactive intestinal peptide, agents which increased colonic mucosal cAMP, all significantly suppressed [3H]thymidine incorporation into DNA in vitro in both tissue with and without exposure to DMH in vivo. By contrast, equimolar N6, O2'-dibutyryl cyclic guanosine 3':5'-monophosphate and 10 μM N-methyl-N'-nitro-N-nitrosoguanidine, a carcinogen which increased endogenous cGMP accumulation in colon mucosa 2-fold, were without acute effects on DNA synthesis in vitro. The present data are consistent with an inhibitory influence of cAMP on the proliferative activity of colonic epithelium and suggest that reductions of cAMP and cAMP-dependent PK activity could play a permissive role in the enhancement of DNA synthesis observed three to five days after DMH.

INTRODUCTION

The cyclic nucleotides cAMP and cGMP may be involved in the regulation of cell growth and the pathogenesis of neoplastic transformation, but their roles in these complex processes remain poorly defined (13, 23, 25, 28, 30, 36, 38, 42). Numerous alterations in the content or metabolism of cAMP and cGMP have been described during normal cell growth and in neoplastic tissue (13, 23, 25, 28, 30, 36, 38, 42). Although considerable attention has been given to the possibility that cAMP serves as a negative signal to cell growth and cGMP serves as a positive signal, no consistent pattern has emerged between changes in cyclic nucleotide economy and cellular proliferative activity. The complexity of the potential relationships is emphasized by observations which indicate that the influence of cAMP on proliferative activity may differ, both as a function of the cell type in question (23, 34, 45) and in the same cell type as a function of the phase of the growth cycle (23). Nevertheless, in colon epithelium, reductions in tissue levels of cAMP and/or in cAMP:cGMP molar ratios have been associated with rapid cell proliferation and neoplastic transformation. Thus, in human colonic carcinomas and polyps (13, 30, 36), and in rodent colonic carcinomas induced by chronic administration of DMH (42), cAMP and/or cAMP:cGMP ratios are reduced, compared to normal colon epithelium. More recent observations have also indicated that the cAMP content of rapidly proliferating normal epithelial cells from the lower colonic crypts is reduced, compared to levels in the nonproliferating superficial epithelium (11). These observations, and the demonstration that exogenous cAMP and agents which increase endogenous cAMP inhibit DNA synthesis in colonic epithelium (1), are consistent with an inhibitory influence of cAMP on the proliferation of this tissue.

In an attempt to assess whether alterations in cyclic nucleotide metabolism might be involved in the initiation of malignant transformation of colonic epithelium, we have previously examined the acute effects of a direct-acting colon carcinogen MNNG on cAMP and cGMP metabolism in this tissue (14, 15, 18, 19). Exposure of colonic epithelium to MNNG in vivo or in vitro produced marked increases in cGMP without detectably altering cAMP (14, 15, 18, 19). However, increases in cGMP induced by MNNG and other agents were not associated with an acute stimulation of cellular proliferative activity, as monitored by the incorporation of [3H]TdR into DNA (18). DMH reliably induces colonic adenocarcinomas in rodents (21) and has been reported to produce early stimulation (within hr) of DNA synthesis in mice (6). Although changes in colonic cyclic nucleotide metabolism have been observed after chronic administration of DMH (42), its acute effects on colonic mucosal cAMP and cGMP metabolism have not been examined. In the

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3 The abbreviations used are: cAMP, cyclic adenosine 3':5'-monophosphate; cGMP, cyclic guanosine 3':5'-monophosphate; DMH, dimethylhydrazine; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; [3H]TdR, [3H]thymidine; i.r., intrarectally; PK, protein kinase; PKI, protein kinase inhibitor; dibutyryl cAMP, N6-O2'-dibutyryl cyclic adenosine 3':5'-monophosphate; dibutyryl cGMP, N6-O2'-dibutyryl cyclic guanosine 3':5'-monophosphate; PGE2, prostaglandin E2; VIP, vasoactive intestinal peptide.
present study, we assessed the early effects of DMH on cAMP and cGMP metabolism in rat colonic epithelium, and we correlated these with changes in DNA synthesis.

MATERIALS AND METHODS

Methods

Adult male Sprague Dawley rats (Zivic Miller, Pittsburgh, Pa) were fasted overnight but allowed free access to water. Rats were divided into 2 groups of 3 to 18 each, as indicated in the footnotes to the tables and charts. Each group received 0.5 ml of either DMH (25 mg/kg) or vehicle alone i.r., as described by Chan et al. (6). A 7.5-cm gastric feeding tube with a bulbous protective tip was inserted 5 cm into the colon in order to expose the entire distal colon to the drug, from the colonic flexure to the anus. DMH was dissolved in 50 mm phosphate: 0.85% NaCl solution, and the pH was adjusted to 6.5 with NaOH. The solution was used immediately. Rats were then allowed free access to standard laboratory chow and water. Where indicated, at the times shown after DMH or vehicle treatment, rats received [3H]dThd (50 μCi/rat) i.p. and were sacrificed 2 hr later. Animals were anesthetized with pentobarbital (50 mg/kg i.p.). In some experiments, a portion of the distal colon (1 cm from the anus) was exposed in situ by a distal crosscut and a longitudinal incision along the contramesenteric border. The contents were removed in situ by gentle lavage with 0.85% NaCl solution (37°), and a segment of colon was rapidly resected and frozen within 10 sec between aluminum blocks cooled in liquid N2. Frozen samples were stored at −70°, extracted, and assayed for cAMP and cGMP metabolism. The rate of [3H]dThd incorporation into DNA was monitored in each experiment.

Determinant of cAMP and cGMP. Where indicated, quick-frozen colonic segments were placed on a block of dry ice, and the mucosal surface was scraped with a spatula and extracted immediately in 1.5% HClO4. In other experiments, the scraped mucosa or aliquots of cell suspensions were homogenized in cold 1.5% HClO4, without prior freezing. The extracts were centrifuged at 2000 x g, and cAMP and cGMP content within 2 weeks. The remainder of the distal colon from the colonic flexure to 1 cm above the anal orifice was then removed and placed in chilled 0.85% NaCl solution containing 30 mg penicillin and 5 mg gentamicin per 100 ml. The colons were further processed as described below. In some experiments, specimens of proximal colon were excised adjacent to the cecum for determination of the effects of i.r. DMH on DNA synthesis and cyclic nucleotide metabolism. The rate of [3H]dThd incorporation into DNA was monitored in each experiment.

Determination of cAMP and cGMP. Where indicated, quick-frozen colonic segments were placed on a block of dry ice, and the mucosal surface was scraped with a spatula and extracted immediately in 1.5% HClO4. In other experiments, the scraped mucosa or aliquots of cell suspensions were homogenized in cold 1.5% HClO4, without prior freezing. The extracts were centrifuged at 2000 x g, and cAMP and cGMP content of the supernatant was determined following neutralization with 2 n KOH. The acid-insoluble portion of the extract was extracted and assayed for DNA and protein, as described below. cAMP and cGMP were determined by the radioimmunoassay procedure of Steiner et al. (41), following acetylation of the samples (27), as previously described (13–15, 19). Cross-reactivity of cAMP in the cGMP assay was 0.012%, and cross-reactivity of cGMP in the cAMP assay was 0.008%. The 125I derivatives of the succinylated tyrosyl methyl esters of cAMP and cGMP were prepared as previously described (29). In the absence of added cold cAMP and cGMP, approximately 50% of the labeled nucleotides were bound to the antiserum. Standard curves were plotted as logit-log functions and were linear between 0.0025 and 1 pmol. Validity of the cAMP and cGMP determinations was verified by linearity with sample dilutions, along with destruction of measurable cyclic nucleotides added to the sample extracts. In some experiments, cAMP and cGMP were separated by chromatography on columns of Dowex AG1-X8 formate, as previously described (37). Values for the nucleotides obtained by this procedure did not differ from those obtained from assay of the crude extracts.

PK Activity. PK activity was determined in the 100,000 x g soluble and particulate fractions of incubated epithelial cells, fresh mucosal scrapings, or DEAE-cellulose column fractions, as previously described in detail (9, 16, 17). Briefly, the enzyme assay mixture contained, at final concentration, 12 mm KH2PO4 (pH 6.8), 0.01 m NaF, 4 mm MgCl2, 0.23 mm [γ-32P]ATP (2 x 106 cpm/assay), 6.7 mg of calf thymus histone (type 2A) per ml, and, when present, 2 μM cAMP. Some assay mixtures contained 100 μg of PKI, as indicated. Reaction rates were linear with time for 2 to 7 min and with protein for the 100,000 x g soluble (50 to 100 μg) and particulate (75 to 150 μg) fractions of colonic mucosa. PK activity ratios were calculated from the rates of histone phosphorylation observed in the absence to those obtained in the presence of 2 μM cAMP. The distribution of PK activity between type I and II isoenzymes was determined by DEAE-cellulose chromatography with a linear 0 to 0.4 m NaCl gradient by the procedure of Corbin et al. (8), as previously described (17). PK activity (in the presence of cAMP) in both Peaks I and II was inhibited 80 to 90% by rabbit muscle PKI.

Adenylate Cyclase Activity. Adenylate cyclase activity was determined by the procedure of Salomon et al. (40). Reaction mixtures contained 2.0 mM ATP, 2 x 106 cpm [α-32P]ATP, 1.0 mM cAMP, 5.0 mM MgSO4, 50 mM Tris-HCl (pH 7.6), 20 mM caffeine, 25 mm creatine phosphate, 0.5 mg creatine kinase per ml, and 1.3 mg bovine serum albumin. The concentration of ATP used gave optimal reaction rates with the colonic mucosal 2000 x g particulate fraction. Incubations were initiated by the addition of the particulate fraction and were conducted for 5 min at 30°. The cAMP formed was isolated by sequential chromatography on Dowex AG50 and neutral alumina (40). Reaction rates were linear from 2 to 10 min with 25 to 100 μg protein per assay.

cAMP Phosphodiesterase Activity. This was determined in the 100,000 x g soluble fraction of fresh colonic mucosal homogenates, using a modification of the procedure of Thompson and Appleman (2, 43). Reaction mixtures contained 5 mM MgCl2, 40 mM Tris (pH 7.4), 4 mM mercaptoethanol, and 10⁻⁴ or 10⁻⁷ M [3H]cAMP (200,000 cpm/assay). Reactions were started by the addition of the 100,000 x g soluble fraction and were conducted for 5 min at 30°. Samples were treated with Ophiopogonus hanna venom, and the [3H]adenosine formed was separated from [3H]cAMP by Dowex AG1-X2 absorption, as previously described (2, 43).

Superficial and Proliferating Epithelial Cell Pools. These were isolated by a modification of the procedure of Weiser (44). Rat distal colons were everted, filled with 0.85% NaCl solution containing 30 mg penicillin and 5 mg gentamicin per 100 ml, and tied at both ends with surgical suture. Each colonic loop was incubated in a 50-ml flask in 10 ml of Medium 199: bicarbonate buffer containing 2 mm ethylene glycyl bia(l-aminoethyl ether) N,N,N',N'-tetraacetic acid, 30 mg penicillin per 100 ml, 5 mg gentamicin per 100 ml, and 10% heat-treated (56° for 60 min) fetal bovine serum which had been equilibrated
with 95% O₂:5% CO₂. After a preliminary incubation for 15 min at 37° that resulted in release of a number of cells which did not exclude trypan blue, the colonic epithelial cells were isolated by timed sequential incubation of the colonic loops in the same buffer with additions and for the times indicated as follows: Incubation 1, 2 mM dithiothreitol, 40 min; Incubation 2, 27 mM citrate, 15 min; Incubation 3, 2 mM dithiothreitol, 15 min; Incubation 4, 2 mM dithiothreitol, 30 min; and Incubation 5, 2 mM dithiothreitol, 30 min. Cells from replicate flasks were pooled and washed 3 times in Krebs-Ringer bicarbonate/glucose buffer containing 1.5 mM Ca²⁺, which had previously been equilibrated with 95% O₂:5% CO₂. The DNA content of a portion of the washed cell suspension was extracted, assayed, and counted for determination of the rate of incorporation of [³H]dThd into DNA. Aliquots (0.4 ml) of the washed cell suspensions were incubated in 5-ml microflasks in Krebs-Ringer bicarbonate/glucose buffer containing 1.5 mM Ca²⁺:95% O₂:5% CO₂ for determination of cAMP content and PK activity, as described above. Approximately 90 to 95% of the isolated cells were epithelial cells, as judged by light microscopy of preparations stained with hematoxylin and eosin or the Papanicolaou stain. Greater than 95% of the cells in each preparation were viable, as determined by trypan blue exclusion. In addition, viability was assessed by the capacity of the cells to oxidize [¹⁴C]glucose, as previously described (20). ¹⁴CO₂ accumulation was progressive when monitored at 30-min intervals for 120 min.

DNA was determined by the method of Burton (3). Protein was determined by the method of Lowry et al. (33). Statistical significance of differences between mean values was determined by Student’s t test for unpaired data.

Materials

DMH, penicillin, gentamicin, dibutyryl cAMP, and dibutyryl cGMP were obtained from Sigma Chemical Co., St. Louis, Mo. [³H]dThd (20 Ci/mmol) was purchased from New England Nuclear, Boston, Mass. Medium 199 and fetal bovine serum were obtained from Grand Island Biological, Co. Grand Island, N.Y. PGE₂ and VIP were gifts of the Upjohn Co., Kalamazoo, Mich., and Dr. George Kitzer, NIH, Bethesda, Md., respectively. The sources of all other chemicals have been described previously (13–15, 19).

RESULTS

As shown in Chart 1, i.r. DMH had biphasic effects on [³H]dThd incorporation into DNA in distal colonic mucosa. After an initial period of suppression observed at 0.5 and 1 day, [³H]dThd incorporation rose approximately 3-fold over control values by 3 days after DMH administration. At 3 days, cAMP content and PK activity ratios of mucosal scrapings from DMH-treated rats were significantly lower than those of the vehicle controls, whereas cAMP values at 0.5 and 1 day after DMH did not differ from control. cGMP content of mucosa from DMH-treated rats did not differ from control values at any of the time points studied (not shown). The type I isoenzyme form of soluble PK, which constituted 20% or less of total activity in mucosa from control rats, increased approximately 2-fold 3 days after DMH administration and represented 40% of soluble PK activity.
PK activity. As is shown in Chart 1, [3H]dThd incorporation into DNA, tissue cAMP content, and PK activity 2 days after DMH administration were not significantly different from values observed in vehicle-treated control rats. Whereas, increases in DNA synthesis and type I PK, and decreases in cAMP and PK activity ratios relative to values at 0.5 and 1 day after DMH were evident. The statistically significant alterations in all of these parameters that were observed by 3 days after DMH administration were still present at Day 5. However, by 10 days after DMH, each of the parameters monitored had returned to control levels. Values in the vehicle control group examined at 0.5 to 10 days did not differ from each other as a function of time or from values in the no-treatment group examined on Day 0. In contrast to the changes found in distal mucosa after i.r. DMH, rates of [3H]dThd incorporation and tissue cAMP content of proximal colonic mucosa from rats treated with DMH in this fashion did not differ from corresponding values in vehicle controls at 1 and 3 days after i.r. administration of test agent.

In separate experiments, adenylyl cyclase activities (2,000 x g particulate fraction) and high-K$_m$ (10$^{-4}$ M cAMP) and low-K$_m$ (10$^{-7}$ M cAMP) phosphodiesterase activities (2,000 x g particulate, 100,000 x g particulate, and 100,000 x g soluble fraction) were examined in distal colonic mucosal scrapings obtained 3 days after administration of DMH or vehicle. Adenylyl cyclase activity of DMH-treated rats [15 ± 3 (S.E.) pmol cAMP per min per mg protein] did not differ significantly from that of the vehicle control (19 ± 4; n = 6). High- or low-K$_m$ cAMP phosphodiesterase activities of soluble and particulate fractions of colonic mucosa from rats examined 3 days after DMH also did not differ from corresponding values in the vehicle controls. Thus, acute reductions in cAMP content of colonic mucosa induced by DMH were not associated with detectable changes in the activities of enzymes of either cAMP synthesis or degradation.

In view of the known lability of tissue cyclic nucleotide content after interruption of the vascular supply, cAMP and cGMP levels were also examined in mucosal scrapings from colonic segments which had been quick frozen in situ (Table 1). Consistent with results shown in Chart 1, cAMP content of quick-frozen specimens obtained 3 or 5 days after DMH administration were significantly suppressed compared to either Time 0 or vehicle control values. This difference was evident when values were calculated on the basis of either tissue protein or DNA content. cAMP content of quick-frozen mucosa obtained 2 days after DMH was variably reduced, and significantly less than vehicle control when expressed on the basis of tissue protein but not DNA content. Both cGMP levels and cAMP:cGMP molar ratios were modestly reduced 3 days after DMH, but these differences were not statistically significant at any of the times examined.

As shown in Table 2, 81 to 87% of total PK activity (in the presence of 2 μM exogenous cAMP in the assay mixture) of homogenates of colonic mucosal scrapings was found in the 100,000 x g soluble fraction under all conditions studied. Total soluble PK activity (in the presence of 2 μM cAMP) was also similar in DMH-treated and control rats at the time points studied. Thus, the decrease in PK activity ratios in the 100,000 x g soluble fraction of mucosal scrapings prepared from rats 3 days after DMH was attributable to the reduction in activity measured in the absence of exogenous cAMP in the assay (without cAMP activity). PKI inhibited soluble PK activity 80 to 85% in each experimental group shown in Table 2. Qualitatively similar reductions in PK activity ratios were found 3 days after DMH when these ratios were corrected for the contribution due to non-cAMP-dependent PK activity, i.e., activity not inhibited by PKI. As is also shown in Table 2, PK and activity ratios in the 100,000 x g particulate fraction were similar in control and DMH-treated rats at each time point examined. Moreover, greater than 80% of the measurable PK activity in the 100,000 x g particulate fraction of DMH-treated and control rats was not cAMP dependent, as judged by the failure of PKI to inhibit enzyme activity in this fraction.

Chart 2 shows the chromatographic distribution of PK activity in control rats (Time 0 and vehicle controls) and as a function of time after DMH administration. Whereas approximately 20% of the activity eluted in the first peak (type I) and 80% in the second peak (type II) in control rats 3 days after DMH, 35 to 40% of the activity eluted in the first peak. Characteristic of the

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**Table 1**

Effects of i.r. administration of DMH on cAMP and cGMP content of rat colonic mucosa quick-frozen in situ

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>cAMP pmol/mg protein</th>
<th>cGMP pmol/mg DNA</th>
<th>cAMP:cGMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.58 ± 1.07$^a$</td>
<td>299 ± 46</td>
<td>14.9 ± 2.4</td>
</tr>
<tr>
<td>DMH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>7.02 ± 0.98$^a$</td>
<td>358 ± 52</td>
<td>13.2 ± 1.5</td>
</tr>
<tr>
<td>2 days</td>
<td>4.32 ± 0.51$^a$</td>
<td>229 ± 38</td>
<td>13.1 ± 1.8</td>
</tr>
<tr>
<td>3 days</td>
<td>3.58 ± 0.58$^a$</td>
<td>153 ± 18$^a$</td>
<td>9.4 ± 1.2</td>
</tr>
<tr>
<td>5 days</td>
<td>3.94 ± 0.48$^a$</td>
<td>168 ± 22$^a$</td>
<td>10.1 ± 2.4</td>
</tr>
<tr>
<td>10 days</td>
<td>6.76 ± 0.92$^a$</td>
<td>324 ± 36</td>
<td>13.8 ± 1.9</td>
</tr>
</tbody>
</table>

$^a$ Means ± S.E. of determinations on 9 (DMH) or 60 (vehicle plus Time 0 controls) rat colons.

$^b$ p < 0.010 compared to control.
DMH (25 mg/kg) or vehicle (50 mM phosphate, pH 8.5, in 0.85% NaCl solution) was administered i.r. to groups of 3 rats each. At the times shown after receiving DMH, rats were anesthetized, and the distal colons were removed and placed in 0.85% NaCl solution. The mucosa derived from each group of 3 rats was scraped with a spatula and pooled. DNA content of a portion (25 mg) of the pooled mucosal surface was extracted, assayed, and counted to determine the rate of \[^{3}H\]dThd incorporation into DNA, as described in Materials and Methods. Values obtained were as follows (dpm/min/\mu g DNA): control, 22 ± 3.6 (S.E.); DMH, 24 hr, 13 ± 2.1; 3 days, 72 ± 12; 10 days, 28 ± 5.4. The remainder of the pooled mucosal surface from each group of rats was homogenized in 5 mM KH2PO4: 2 mM EDTA: 0.5 mM isobutylmethylxanthine, pH 7.0 (500 mg/ml). PK activity was assayed in the 100,000 x g soluble and particulate fractions of the mucosal homogenates, as described in Materials and Methods. Where shown, enzyme reaction mixtures contained 2 uM cAMP and 100 uM PKI. A group of 3 vehicle control rats was studied at each time point indicated. Values for \[^{3}H\]dThd incorporation into DNA and PK were not different in rats which were studied 0, 1, 3, or 10 days after receiving the vehicle alone and were combined. PK activity ratios were calculated from the ratio of enzyme activity obtained in the absence to that observed in the presence of 2 uM cAMP in the assay mixture.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>pmol/min/mg protein</th>
<th>% of total PK activity</th>
<th>cAMP</th>
<th>+cAMP</th>
<th>+cAMP + PKI</th>
<th>-cAMP + cAMP</th>
</tr>
</thead>
<tbody>
<tr>
<td>100,000 x g soluble</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>233 ± 26(^{a})</td>
<td>556 ± 71</td>
<td>103 ± 14</td>
<td>0.42 ± 0.06 (0.29)</td>
<td>82 ± 12</td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>259 ± 31</td>
<td>574 ± 68</td>
<td>92.4 ± 13.1</td>
<td>0.45 ± 0.07 (0.35)</td>
<td>84 ± 16</td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>162 ± 26(^{a})</td>
<td>579 ± 74</td>
<td>114 ± 15</td>
<td>0.26 ± 0.05 (0.10)</td>
<td>81 ± 14</td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>214 ± 32</td>
<td>541 ± 69</td>
<td>91.9 ± 12.4</td>
<td>0.39 ± 0.08 (0.27)</td>
<td>87 ± 11</td>
<td></td>
</tr>
<tr>
<td>10 days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100,000 x g particulate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>97.2 ± 12.3</td>
<td>118 ± 13</td>
<td>101 ± 11</td>
<td>0.82 ± 0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMH</td>
<td>82.3 ± 11</td>
<td>92.1 ± 9.6</td>
<td>76.4 ± 8.3</td>
<td>0.89 ± 0.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 day</td>
<td>88.4 ± 13.2</td>
<td>113 ± 15</td>
<td>94.9 ± 12.3</td>
<td>0.78 ± 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>76.2 ± 8.4</td>
<td>92.8 ± 10.4</td>
<td>67.8 ± 9.4</td>
<td>0.92 ± 0.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 days</td>
<td></td>
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</tbody>
</table>

\(^{a}\) Mean ± S.E. of duplicate determinations on mucosa pooled from 2 (DMH) or 6 (control) groups of 3 rat colons each.

\(^{b}\) Numbers in parentheses, activity ratios corrected for non-cAMP-dependent activity, defined as that not inhibitable by PKI.

\(^{c}\) P < 0.010 compared to control.

Chart 2. Time course of the action of DMH on the distribution of type I and II PK activities in rat colonic mucosa. DMH (25 mg/kg) or vehicle (50 mM phosphate, pH 8.5, in 0.85% NaCl solution) was administered i.r. to groups of 3 rats each. At the times shown after receiving DMH, rats were anesthetized, distal colons were excised, and the mucosal surface was scraped. The 100,000 x g soluble fraction of mucosal homogenates derived from the studies conducted in Table 2 were used. Results shown are means of duplicate determinations on a single column fraction verified in 2 separate studies.

Type I and II enzymes from other tissues (8), activity in the first peak was markedly increased by exposure to 0.5 M NaCl or 0.7 mg histone per ml (80 to 90% increase in the absence of cAMP), whereas activity in Peak II was resistant to both salt and histone activation. Activity eluted in both peaks was inhibited greater than 90% by PKI. Moreover, since total soluble PK activity (in the presence of 2 uM cAMP in enzyme assay mixture) did not change with DMH administration (Table 2) and chromatographic recovery in control and DMH-treated groups was similar (80 to 85%), the altered chromatographic distri-
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...ution of cAMP-dependent PK probably reflects an absolute increase in type I and decrease in type II activity 3 days after DMH.

In order to assess whether the effects of DMH on cAMP and cAMP-dependent PK activity might differ as a function of the proliferative activity of colonic epithelium, these parameters were compared in populations of superficial and proliferating epithelial cells separated by a modification of the sequential washing technique of Weiser (44), described above. To minimize admixture of cell types, only the cells from the first 2 incubations (superficial) and the fifth and final incubation were selected. Histological evaluation indicated that the cells from the fifth incubation represented largely those derived from the lower third of the crypt. As shown in Table 3, the rate of [3H]dThd incorporation into DNA in proliferating lower crypt cells from control rats was approximately 9-fold higher than that of superficial cells from the same rats. Consistent with earlier observations (11), the rate of basal cAMP accumulation in vitro and the activity ratios of soluble PK were significantly reduced in the proliferating compared to the superficial cells, even though total soluble PK activity (in the presence of cAMP in the enzyme assay) was higher in the proliferating cells. The differences in PK activity ratios persisted when corrected for the contribution of non-cAMP-dependent soluble PK activity, as defined as that fraction of total activity not inhibited by PKI (Table 3). In both the superficial and proliferating cells from colonic epithelium, type I represented approximately 20% and type II represented 80% of soluble cAMP-dependent PK, as assessed by DEAE-cellulose chromatography (Table 3; Chart 3). As shown in Table 3, the rate of [3H]dThd incorporation into DNA was significantly increased in both the superficial and proliferating colonic epithelium 3 days after DMH. Absolute rates of [3H]dThd incorporation remained much higher in the proliferating cells. Moreover, compared to corresponding values in cells from control rats, type I PK activity was significantly increased and type II activity was decreased in both the superficial and proliferating cell population 3 days after DMH (Table 3; Chart 3). However, in contrast to the findings in mucosal scrapings (Chart 1; Tables 1 and 2), cAMP accumulation and PK activity ratios of superficial and proliferating cells from DMH-treated rats were not detectably different from corresponding values from control rats, when assessed after cell isolation and in vitro incubation (Table 3).

In order to examine directly the influence of cAMP and cGMP on the proliferative activity of colonic epithelium of control rats and DMH-exposed rats, the effects of several agents on [3H]dThd incorporation into DNA was tested in vitro in incubated colonic segments. As shown in Chart 4, the rate of incorporation of label into DNA in vitro was linear for 6 hr in incubated segments from both control and rats given DMH 3 days earlier. [3H]dThd incorporation in vitro was higher in tissue from DMH-treated rats. Addition of 10−5 M PGE2 significantly suppressed [3H]dThd incorporation into DNA in both groups. However, absolute incorporation rate remained higher during the 6-hr period of study in the DMH group (Chart 4).

Table 3 shows the effects of several test agents on colonic mucosal DNA synthesis and cyclic nucleotide content in vitro. PGE2 and VIP significantly increased tissue cAMP content and suppressed [3H]dThd incorporation in both control and DMH-exposed mucosa. Neither PGE2 nor VIP altered cGMP. Dibutyryl cAMP also inhibited [3H]dThd incorporation, whereas equimolar concentrations of dibutyryl cGMP were without ef-

Table 3

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>[3H]dThd (dpm/μg DNA)</th>
<th>cAMP (pmol/mg protein)</th>
<th>cAMP activity (pmol/min/mg protein)</th>
<th>% of type I enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial</td>
<td>3.42 ± 0.48α</td>
<td>3.72 ± 0.51α</td>
<td>245 ± 29α</td>
<td>15 ± 6c</td>
</tr>
<tr>
<td>Proliferating</td>
<td>29.7 ± 3.8d</td>
<td>2.13 ± 0.59d</td>
<td>228 ± 36d</td>
<td>19 ± 6d</td>
</tr>
<tr>
<td>DMH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Superficial</td>
<td>18.4 ± 2.3α</td>
<td>3.84 ± 0.69</td>
<td>241 ± 38</td>
<td>39 ± 10d</td>
</tr>
<tr>
<td>Proliferating</td>
<td>83.7 ± 9.4d α-1</td>
<td>2.25 ± 0.48d</td>
<td>209 ± 32</td>
<td>44 ± 9d</td>
</tr>
</tbody>
</table>

Mean ± S.E. of determinations on 9 incubations pooled from 3 separate experiments (cAMP) or duplicate determinations on 3 soluble fractions from 3 separate experiments (PK activity). Numbers in parentheses, PK activity ratios corrected for the contribution due to non-cAMP-dependent PK activity. i.e., that portion of enzymatic activity not inhibited by PKI. The percentage of type I enzyme is the mean ± S.E. of 3 separate chromatographic separations. p < 0.01 compared to corresponding value in superficial cells from the same treatment group.
Chart 3. Effect of DMH on the distribution of type I and II PK activities in superficial and proliferating epithelial cells from rat colon. Rats received DMH (25 mg/kg) or vehicle i.r. and were studied 3 days later. The 100,000 x g soluble fractions of incubated superficial and proliferating epithelial cell pools obtained from the studies described in the footnote to Table 3 were used. Type I and II PK activities were separated on columns of DEAE-cellulose with a linear (0 to 0.4 M) NaCl gradient. PK activity was measured in the presence of 2 μM cAMP. Results shown are means of duplicate determinations on a single column fraction verified in 3 separate studies.

DISCUSSION

Consistent with earlier observations (6), administration of DMH i.r. results in rapid inhibition, followed by stimulation of DNA synthesis in distal colonic mucosa. This biphasic response pattern has been described in target organs following administration of single doses of other carcinogens (12, 39). It may represent an initial cytotoxic effect of the carcinogen that is followed by repair or compensatory DNA synthesis (12, 39). Initiation of this sequence in colonic mucosa appears to reflect a direct or local action of DMH, since changes were evident in the distal but not in the proximal mucosa after i.r. administration of the drug. The capacity of DMH to suppress DNA synthesis in vitro in colonic mucosa, observed in the present (Table 4) and other studies (35), is also consistent with a direct action of DMH on colonic mucosal DNA synthesis. In contrast to the findings of Chan et al. (6) after i.r. DMH in mice, but similar to the findings of Löhrs et al. (32) after a single s.c. injection of DMH, enhancement of DNA synthesis was observed in both surface and lower crypt cells harvested from rats 3 to 5 days after DMH administration (Table 3). The explanation for these differences is not clear, but alterations in the isoenzyme pattern of soluble cAMP-dependent PK after DMH also support a biological action of the drug on the surface epithelium (Table 3).

The present results demonstrate that the transient enhancement of DNA synthesis which follows carcinogen exposure is associated with a significant reduction in cAMP content and cAMP-dependent PK activity of distal mucosa. These changes were evident 3 to 5 days after administration of DMH, even though the drug is largely metabolized within 24 hr (22). Moreover, exposure of colonic mucosa to DMH in vitro did not alter cAMP or cGMP levels (Table 4). Thus, in contrast to
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contain only type I PK (31). Type II activity increases progressively with testicular maturation and differentiation, reaching a maximal level in 25-day-old rats, when maximal capacity for spermatogenesis and steroidogenesis is also expressed. Similarly, in mouse heart the type I:type II PK activity ratio declines from 3.0 in the 7-day-old neonate to 1 in the adult heart (26). Isoproterenol stimulation of cardiac hypertrophy in the rat is also associated with an increase in type I activity (4). The appearance of type I PK has been observed with viral transformation of 3T3 cells (24). Type I PK is elevated during mitosis and relatively depressed during the G1 and S phase of the growth cycle of Chinese hamster ovary cells (10). Conversely, type II activity is low during mitosis and increases at the G1:S border (10). Stimulation of mitogenesis in human lymphocytes has also been correlated with activation of type I PK (5), while an increase in type II activity has been identified as an early event in cAMP- or ovariotectomy-induced arrest of growth in rat mammary carcinoma (7). Thus, several observations have correlated the emergence or dominance of type I PK with active cell growth, cell transformation, or an early stage of differentiation. In normal colonic epithelium, the type I PK clearly did not vary as a function of proliferative activity or differentiation, since the proportion of type I activity in proliferating cells from the lower crypts was not detectably different from that of the normal surface cells (Table 3). While DHM exposure increased the proportion of type I activity in both superficial and lower crypt cells, the markedly enhanced rates of DNA synthesis found in lower crypt cells after DMH were not associated with type I activity in excess of that observed in the surface cells. Accordingly, a close relationship between proliferative activity and type I PK activity was not apparent in colonic epithelium before or after exposure to DMH.

suppression of DNA synthesis, it seems unlikely that the reduction in tissue cAMP content and cAMP-dependent PK activity represents direct actions of DMH. The reduction in cAMP found in rapidly processed mucosal specimens from rats exposed to DMH were not associated with detectable alterations in either adenylate cyclase or cAMP phosphodiesterase activities examined in subcellular fractions of homogenates of mucosa. In addition, with prolonged tissue processing in vitro, as occurred during preparation and incubation of colonic epithelial cells or incubation of colonic segments, a reduction in cellular cAMP from in vivo exposure to DMH was no longer apparent. These observations are consistent with the possibility that reductions in colonic mucosal cAMP in DMH-treated rats were mediated in vitro by changes in local neurohumoral or other modulators of cAMP, the effects of which were labile and reversible in vitro.

The reduction in the activity ratio of soluble cAMP-dependent PK from distal colonic mucosa of rats exposed to DMH 3 to 5 days previously provides evidence that lower cAMP levels found in this tissue are associated with a detectable decrease in the biological action of cAMP. In addition, a significant alteration in the isoenzyme pattern of soluble cAMP-dependent PK occurred in both superficial and proliferating colonic epithelium 3 to 5 days after DMH administration, at a time when DNA synthesis was also enhanced. Earlier studies in other tissues have also described alterations in soluble PK activity as a function of cell growth rate, differentiation, or the phase of the cell growth cycle. Thus, testes from fetal and 2-day-old rats contain only type I PK (31). Type II activity increases progressively with testicular maturation and differentiation, reaching a maximal level in 25-day-old rats, when maximal capacity for spermatogenesis and steroidogenesis is also expressed. Similarly, in mouse heart the type I:type II PK activity ratio declines from 3.0 in the 7-day-old neonate to 1 in the adult heart (26). Isoproterenol stimulation of cardiac hypertrophy in the rat is also associated with an increase in type I activity (4). The appearance of type I PK has been observed with viral transformation of 3T3 cells (24). Type I PK is elevated during mitosis and relatively depressed during the G1 and S phase of the growth cycle of Chinese hamster ovary cells (10). Conversely, type II activity is low during mitosis and increases at the G1:S border (10). Stimulation of mitogenesis in human lymphocytes has also been correlated with activation of type I PK (5), while an increase in type II activity has been identified as an early event in cAMP- or ovariotectomy-induced arrest of growth in rat mammary carcinoma (7). Thus, several observations have correlated the emergence or dominance of type I PK with active cell growth, cell transformation, or an early stage of differentiation. In normal colonic epithelium, the type I PK clearly did not vary as a function of proliferative activity or differentiation, since the proportion of type I activity in proliferating cells from the lower crypts was not detectably different from that of the normal surface cells (Table 3). While DHM exposure increased the proportion of type I activity in both superficial and lower crypt cells, the markedly enhanced rates of DNA synthesis found in lower crypt cells after DMH were not associated with type I activity in excess of that observed in the surface cells. Accordingly, a close relationship between proliferative activity and type I PK activity was not apparent in colonic epithelium before or after exposure to DMH.

### Table 4

<table>
<thead>
<tr>
<th>Test agents</th>
<th>C(\text{H})dThd incorporation into DNA (dpm/\mu g DNA)</th>
<th>cAMP (pmol/mg protein)</th>
<th>cGMP (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>DMH</td>
<td>Control</td>
</tr>
<tr>
<td>None</td>
<td>117 ± 13a</td>
<td>204 ± 36b</td>
<td>2.94 ± 0.41</td>
</tr>
<tr>
<td>PGE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>65.4 ± 8.7c</td>
<td>97.2 ± 14.6abc</td>
<td>9.41 ± 1.03c</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>79.5 ± 9.3c</td>
<td>131 ± 29abc</td>
<td>8.74 ± 1.22c</td>
</tr>
<tr>
<td>Dibutyryl cAMP</td>
<td>61.2 ± 8.1c</td>
<td>111 ± 16abc</td>
<td>10.2 ± 1.9c</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>124 ± 14</td>
<td>212 ± 28b</td>
<td>2.94 ± 0.41</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>113 ± 16</td>
<td>223 ± 34a</td>
<td>2.76 ± 0.41</td>
</tr>
<tr>
<td>MNNG</td>
<td>53.8 ± 7.3c</td>
<td>61.8 ± 9.2c</td>
<td>2.61 ± 0.39</td>
</tr>
<tr>
<td>10⁻⁴ M</td>
<td>104 ± 12</td>
<td>193 ± 26</td>
<td>2.97 ± 0.36</td>
</tr>
<tr>
<td>10⁻⁵ M</td>
<td>110 ± 14</td>
<td>193 ± 25</td>
<td>2.89 ± 0.36</td>
</tr>
</tbody>
</table>

*Mean ± S.E. of 9 determinations pooled from 3 separate experiments.

* p < 0.01 compared to corresponding value in the control group.

* p < 0.01 compared to corresponding value in the absence of test agent.

Groups of 9 rats each received either DMH (25 mg/kg) or vehicle i.r. and were studied 3 days later. For the determination of the rate of C(\text{H})dThd incorporation into DNA, segments (1 x 1 cm) were prepared from the distal colon and preincubated for 60 min in Medium 199 in the presence or absence of test agents, as shown. [C(\text{H})dThd (0.5 pCi/ml) was then added, and the incubation was continued for an additional 5 hr. At the end of 5 hr, the mucosa was scraped and placed in 1.5% HCO3, and the DNA content was extracted, assayed, and counted as described in Materials and Methods. (*For determination of cAMP, segments of rat colon (1 x 1 cm) were incubated for 60 min in Medium 199. Where indicated, test agents were present for the final 5 min. At the end of the incubation, the mucosa was quickly scraped (30 sec), placed in 1.5% HCO3, homogenized, and centrifuged at 2000 x g. cAMP content of the acid-insoluble fraction was determined, as described in Materials and Methods.*

The reduction in the type I:cAMP content ratio was not apparent in colonic epithelium from 3.0 in the 7-day-old neonate to 1 in the adult heart (26). Isoproterenol stimulation of cardiac hypertrophy in the rat is also associated with an increase in type I activity (4). The appearance of type I PK has been observed with viral transformation of 3T3 cells (24). Type I PK is elevated during mitosis and relatively depressed during the G1 and S phase of the growth cycle of Chinese hamster ovary cells (10). Conversely, type II activity is low during mitosis and increases at the G1:S border (10). Stimulation of mitogenesis in human lymphocytes has also been correlated with activation of type I PK (5), while an increase in type II activity has been identified as an early event in cAMP- or ovariotectomy-induced arrest of growth in rat mammary carcinoma (7). Thus, several observations have correlated the emergence or dominance of type I PK with active cell growth, cell transformation, or an early stage of differentiation. In normal colonic epithelium, the type I PK clearly did not vary as a function of proliferative activity or differentiation, since the proportion of type I activity in proliferating cells from the lower crypts was not detectably different from that of the normal surface cells (Table 3). While DHM exposure increased the proportion of type I activity in both superficial and lower crypt cells, the markedly enhanced rates of DNA synthesis found in lower crypt cells after DMH were not associated with type I activity in excess of that observed in the surface cells. Accordingly, a close relationship between proliferative activity and type I PK activity was not apparent in colonic epithelium before or after exposure to DMH.
The finding of reduced tissue cAMP content and cAMP-dependent PK activity in vivo during the period of enhanced DNA synthesis in DMH-exposed colonic mucosa obviously does not establish a functional relationship between the change in cAMP metabolism and proliferative activity. Indeed, it is quite possible that the alterations in cAMP are secondary to changes in proliferative activity rather than involved in their pathogenesis. However, in vitro studies in short-term cultures of rat colonic segments are consistent with the proposal that the increase in DNA synthesis in DMH-exposed colonie mucosa 24 hr after in vivo administration of cGMP. Furthermore, it has been shown that DMH-exposed colonie segments are consistent with the proposal that the increase in proliferative activity which followed DMH exposure. Thus, dibutyryl cAMP, PGE₂, or VIP, agents which increase the endogenous cAMP content of colonie mucosa, significantly suppressed [³H]Tdhth incorporation into DNA in the short-term cultures of both control and colon segments exposed to DMH in vivo (Table 4). By contrast, dibutyryl cGMP and 10 μM MNNG, which increased endogenous cGMP approximately 2-fold (Table 4), were without detectable effects on DNA synthesis. A higher concentration of MNNG (0.1 mm), which also increased cGMP, inhibited DNA synthesis. Acute inhibition of colonie mucosal DNA was previously observed following i.r. administration of a large dose (2 mg) of MNNG and was associated with an increase in cGMP in vivo (18). However, as shown in Table 4, inhibition of DNA synthesis by addition of 1 mM DMH to cultured segments was not correlated with acute changes in either cAMP or cGMP. This observation, the absence of associated changes in cAMP and cGMP in colonie mucosa 24 hr after in vivo administration of DMH when DNA synthesis was suppressed (Chart 1, Table 1), and the lack of an inhibitory effect of exogenous dibutyryl cGMP all fail to support a role for these cyclic nucleotides in carcinogen-induced suppression of DNA synthesis.

Accordingly, the present data are consistent with an inhibitory influence of cAMP on the proliferative activity of colonie epithelium and with the possibility that a reduction in mucosal cAMP has at least a permissive effect on the transient acceleration of proliferative activity which followed DMH exposure. The present results provide no new insights into the biological actions of cGMP in control of colonie mucosal proliferation or into the functional significance of the striking increases in cGMP induced by the colonic carcinogen MNNG.

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REFERENCES


Early Alterations in Rat Colonic Mucosal Cyclic Nucleotide Metabolism and Protein Kinase Activity Induced by 1,2-Dimethylhydrazine

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