Alterations of the VIP-stimulated cAMP pathway in rat distal colon after abdominal irradiation

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Morel, E., I. Dublineau, F. Lebrun, and N. M. Griffiths. Alterations of the VIP-stimulated cAMP pathway in rat distal colon after abdominal irradiation. Am J Physiol Gastrointest Liver Physiol 282: G835–G843, 2002. First published January 9, 2002; 10.1152/ajpgi.00457.2001.—Ionizing radiation induces hyporesponsiveness of rat colonic mucosa to vasoactive intestinal peptide (VIP). Possible mechanisms responsible for this hyporesponsiveness of the cAMP communication pathway in rat colon were investigated. VIP- and forskolin-stimulated short-circuit current (Isc) responses were studied after a 10-Gy abdominal irradiation in Ussing chambers as well as in single, isolated crypts. Adenylyl cyclase (AC) activity and VIP receptor characteristics were studied after a 10-Gy abdominal irradiation in Ussing chambers as well as in single, isolated crypts. Adenylyl cyclase (AC) activity and VIP receptor characteristics were decreased in mucosal membrane preparations. In addition, alterations in crypt morphology were studied. Impaired secretory responses to VIP and forskolin were observed 4 days after irradiation (decrease of 80%). cAMP analog-stimulated Isc responses were unchanged. In isolated crypts, VIP- and forskolin-stimulated cAMP accumulation was markedly reduced by 80 and 50%, respectively. VIP-stimulated AC activity and VIP receptor number were decreased in membrane preparations. No major change of cellularity was associated with these functional alterations. In conclusion, the decreased secretory responses to VIP of rat colon are associated with reduced cAMP accumulation, decreased AC activity, and diminution of VIP receptor numbers without a marked decrease of crypt cell number.

colonic crypts; radiation; hyporesponsiveness; adenylyl cyclase; short-circuit current

FLUID AND ELECTROLYTE ABSORPTION is a major function of the mammalian colon. It has been proposed that absorptive processes are the constitutive transport processes in the basal state and that secretory processes are stimulated by neurohumoral mediators (48). However, net Na+-dependent fluid absorption represents both a larger Na+ dependent absorptive process and a smaller secretory process (19). Mediators that affect these processes may be locally released agents such as inflammatory mediators (prostaglandins), neurotransmitters (acetylcholine), or neuropeptides [vasoactive intestinal peptide (VIP)]. VIP is a potent secretagogue and is widely distributed in enteric neurons and nerve fibers in all layers along the gut (50). Among other effects on colonic physiology, VIP takes part in the regulation of intestinal motility by its potent relaxing effects on smooth muscle. In addition, VIP is a potent vasodilator and thus may indirectly affect epithelial and smooth muscle functions (20). In epithelial cells, VIP stimulates chloride secretion through a cascade of events initiated by binding to specific G protein coupled receptors associated to the adenylyl cyclase complex located on the basolateral plasma membrane. The result is an increase of intracellular second messenger cAMP levels and the opening of apical chloride channels in colonic epithelial cells leading to chloride secretion (38).

Exposure to ionizing radiation causes severe fluid and electrolyte loss leading to diarrhea. This effect of radiation injury observed for high doses has been attributed to the denudation of the epithelium lining the small intestine, provoked by the disruption of mitosis of the stem cells (23, 43). However, recent studies (34) have reported alterations of fluid and electrolyte transport induced by doses <10 Gy appearing without diarrhea and before significant denudation of the epithelium. An active stimulation of basal fluid secretion and modulation of ion transport (Na+ and Cl−) have been suggested in the small intestine after exposure to ionizing radiation (22). In addition, irradiation seems to modify the tissue response to different stimulatory agents. Indeed, previous studies (24, 36) focused on the small intestine have shown that radiation exposure decreases the responsiveness to theophylline, PGE2, and electrical field stimulation in rat and rabbit ileum. Such modifications of responsiveness could be due to changes in local mediator concentrations. For example, exposure to ionizing radiation results in altered intestinal VIP tissue levels (28).

Secretory capacity of the colon has also been shown to be modified by ionizing radiation. An hyporesponsiveness of the rat or mouse colon to VIP and forskolin has been characterized (16–18). Radiation-induced hyporesponsiveness to secretagogues demonstrated an

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impairment of chloride secretion normally implicated in the intestinal response to luminal aggressions (54) that highlight a suppression of local host defense reactions by radiation. The VIP and forskolin hyporesponsiveness suggests that the cAMP communication pathway is altered by irradiation. Different mechanisms could be responsible for the colonic hyporesponsiveness observed after irradiation to VIP, such as alteration of the binding of VIP to its receptor, impairment of the coupling with regulatory G protein, and transduction from protein G to the catalytic unit of adenylyl cyclase. The intracellular second messenger cAMP level could be affected either by a decreased production or an increased degradation by the phosphodiesterases (PDEs). A dysfunction of protein kinase A (PKA) activation could be also an explanation. The opening of apical Cl– channels, such as cystic fibrosis transmembrane conductance regulator, could be altered.

The aim of the present work was to investigate some of these hypotheses by studying the mechanisms leading to chloride secretion stimulated by VIP in rat distal colon. Secretagogues responses were investigated in vitro in isolated rat distal colon in Ussing chambers. In addition, we measured, for the first time, the in situ cAMP accumulation in colonic single crypts isolated by microdissection. Adenylyl cyclase activity and VIP-binding site characteristics were determined in mucosal membrane preparations. Furthermore, an analysis of crypt cellularity was associated with functional studies.

**MATERIALS AND METHODS**

**Treatment of Animals**

Experiments were performed in male Wistar rats weighing ~300 g (CERJ, Le Genest-St-Ie, France). Animals were housed in groups (4/cage) for at least 7 days before the start of the experiments in a ventilated and temperature-controlled room. The day-night cycle was constant at a 12:12-h light-dark cycle. All animals received a standard diet (model 105; UAR, Villemoisson sur Orge, France) and were given tap water ad libitum. These experiments were conducted according to the French regulations for animal experimentation (Ministry of Agriculture, Décret, publication no. 87–848, October 19, 1987).

**Irradiation**

Rats anesthetized with pentobarbital sodium (60 mg/kg ip) were exposed to a single abdominal dose of gamma radiation (60Co; 10 Gy, 0.9 Gy/min). Collimation of the radiation source resulted in a field of 5 × 20 cm. Anesthetized rats were placed on a Plexiglas plate. Only the abdomen (zone betweenxyphoid cartilage and iliac crests) was in the field of irradiation; the rest of the animal was shielded by lead. The control animals were anesthetized and treated as irradiated animals but were not exposed to the source.

**Ussing Chamber Studies**

The distal colon of control or irradiated rats was removed under anesthesia and rinsed with physiological salt solution. Animals were then killed 1, 4, or 7 days after irradiation with an overdose of anesthetic. Samples were used for the following experiments.

For the assessment of transepithelial electrical parameters, the distal colonic tissues were stripped by blunt dissection, mounted in Ussing chambers with a 0.64-cm² aperture (Corning Costar, Cambridge, MA), and bathed on mucosal and serosal surfaces with Ringer solution containing (in mM): 115 NaCl, 25 NaHCO₃, 0.4 KH₂PO₄, 2.5 K₂HPO₄, 1.2 MgCl₂, 1.2 CaCl₂ and 10 glucose. The solution was gassed with 95% O₂-5% CO₂ and maintained at 37°C, pH = 7.4. Basal parameters were determined after an initial steady-state period of 15 min: potential difference (PD), short-circuit current (Iₛₛ), tissue conductance (G,total). Iₛₛ was measured by clamping the voltage at zero using a DVC-100 voltage-current clamp (World Precision Instruments, Hertfordshire, UK). Agonist-stimulated Iₛₛ responses were studied using VIP dose-response curves (5–500 nM) with a washout period of 30 min between agonist additions. In additional experiments, ΔIₛₛ was measured in response to forskolin (10 μM) and cAMP analogs (1 mM 8-Br cAMP and 1 mM dibutyryl-cAMP). The change in ΔIₛₛ was determined as the difference between basal and stimulated conditions.

**Measurement of Adenylyl Cyclase Activity and VIP Receptors**

All of these experimental steps were carried out on ice. The adenylyl cyclase activity was measured on crude homogenate of distal colonic mucosa as described previously by Griffiths et al. (21). In brief, the mucosa was removed by scraping, weighed, and homogenized (Ultra-Turrax, 20,000 rpm, 2 × 30 s) in sucrose buffer (250 mM sucrose, 2 mM Tris·HCl, pH 7.4) containing protease inhibitor 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 2,500 g for 15 min at 4°C, and the pellet was discarded. Supernatant was frozen in liquid nitrogen until adenylyl cyclase activity measurement. Membrane preparations were obtained with an additional centrifugation at 20,000 g for 20 min. Pellets were resuspended in sucrose buffer. The partially purified membrane preparations were frozen in liquid nitrogen and stored at −80°C until determination of VIP receptor characteristics.

**Measurement of Adenylyl Cyclase Activity**

Adenylyl cyclase activity was measured as described previously for pig jejunal plasma membranes (21). Colonic mucosa from two rats was pooled and used for each sample. Homogenate protein (40 μg) was incubated for 15 min at 30°C in 80 mM Tris-maleate buffer containing 1 mM IBMX, 100 μM guanosine 5’-O-(3-thiotriphosphate), 100 mM creatine phosphate, 100 mM creatine kinase, and 1 mM ATP. Incubation was terminated by the addition of 1 M HCl. Samples were neutralized with 1 M NaOH and stored at −20°C before cAMP measurement by a competitive binding protein assay (RIA; Amersham Pharmacia Biotech). Adenylyl cyclase activity was measured in the absence (basal activity) and presence of VIP 0.5 μM or forskolin 10 μM.

**Determination of VIP Receptor Characteristics**

VIP specific binding sites were characterized on partially purified colonic membrane preparations (100 μg protein/40 μl) using the displacement of 125I-labeled VIP by increasing concentrations of unlabeled VIP. Samples were incubated in a 25 mM Tris·HCl buffer, pH = 7.5, containing 5 mM MgCl₂, 0.5% bovine serum albumin, 0.1% bacitracin, 500 KIU/ml aprotinin, and 55–80 nM 125I-labeled VIP and 0.05–10 nM unlabeled VIP to a final volume of 225 μl. Specific binding was determined as the difference between 125I-labeled VIP bound in the absence and presence of 1 μM unlabeled VIP. Free and bound 125I-labeled VIP was separated by rapid
filtration over GF/F filters presoaked in 0.3% polyethylenimine followed by washing (5 x 4 ml) with 50 mM Tris-HCl buffer, pH = 7.5. The apparent dissociation constant (Kd) and maximum number of VIP-binding sites (Bmax) was calculated with Scatchard analysis.

In situ cAMP Accumulation Measurement in Single Colonic Crypts

To isolate crypts and to keep them alive, we have modified the method used to measure cAMP in renal tubules previously described by Chabardès et al. (9). The distal colonic mucosa was removed by scraping and kept on ice in a dissection buffer containing (in mM): 120 NaCl, 5 KCl, 0.33 Na2HPO4, 0.44 KH2PO4, 10 CH3COONa, 4 NaHCO3, 1 MgCl2, 0.8 MgSO4, and 0.5 CaCl2 with 5 mM glucose, 10 mM HEPES, 5 mM glutamine, 0.1% BSA, 1 μM indomethacin, 70 units of adenosine deaminase, and 0.1% bacitracin. pH = 7.2. Incubation buffer has the same composition as the dissection buffer except for the presence of 1 mM IBMX. The osmotic pressure (determination based on measurement of the freezing point depression, microosmometer, Roelbling) was around 310 mosmol/l for the dissection buffer and 320 mosmol/l for the incubation buffer containing (in mM): 120 NaCl, 5 KCl, 0.33 Na2HPO4, 0.44 KH2PO4, 10 CH3COONa, 4 NaHCO3, 1 MgCl2, 0.8 MgSO4, and 0.5 CaCl2 with 5 mM glucose, 10 mM HEPES, 5 mM glutamine, 0.1% BSA, 1 μM indomethacin, 70 units of adenosine deaminase, and 0.1% bacitracin. pH = 7.2. Incubation buffer has the same composition as the dissection buffer except for the presence of 1 mM IBMX. The osmotic pressure (determination based on measurement of the freezing point depression, microosmometer, Roelbling) was around 310 mosmol/l for the dissection buffer and 320 mosmol/l for the incubation one. Crypts were individualized by hand microdissection with needles under stereomicroscopic observation and transferred with a volume of 2 μl with a Hamilton syringe between two concave glass slides. After tight covering, each crypt was kept at ice-cold temperature and length was measured under inverted microscope (×10 magnification). After a 5-min preincubation period at 30°C, crypts were incubated in a 2-μl solution containing either incubation buffer alone or incubation buffer with forskolin (10 μM) or increasing amount of VIP (from 0.1–100 nM) and were incubated 4 min at 35°C. Incubation was stopped by rapidly transferring crypts together with 1 μl of incubation solution into a glass tube containing 20 μl of formic acid in absolute ethanol (5% vol/vol). After an overnight evaporation to dryness at 40°C, samples were stored at −20°C until cAMP accumulation measurements.

cAMP contained in a single crypt was quantified by a radioimmunoassay (125I-labeled RIA kit, NEN) slightly modified by Chabardès et al. (9). Potassium phosphate buffer (25 μl of 50 mM), pH 6.2, was added to the dry extracts before the acetylation procedure. Under these conditions, the standard curves range from 1 to 156 fmol/tube for cAMP and the method allows measurement with accuracy 2–80 fmol of cAMP.

Morphological Study of Rat Colonic Distal Crypts

Single crypts were isolated by hand microdissection with needles as described above. Crypts were immediately transferred in a paraformaldehyde 4% solution for 30 min at room temperature. Crypts were rinsed by transfer into two successive PBS baths and permeabilized by immersion for 10 min in a Triton X-100 solution (0.1%). Crypts were rinsed twice in PBS before being transferred for 30 min at room temperature in a nuclear staining solution containing PBS, an excess of propidium iodide (12.5 μM) and RNaseA. Each sample was transferred onto a microscope coverslip in mounting solution (fluoromount G) and analyzed by confocal microscopy (Bio-Rad MRC 1024, objective ×40). After excitation at 568 nm, pictures of the fluorescence intensity emitted by the crypt cell nucleus were recorded from crypt surface every 5 μm parallel to the crypt axis. Pictures of the crypt surface were used to estimate the cell number per 100 μm2 at the base and at the upper part of the crypt (see Fig. 5, B and E). Pictures in the median plan of the crypt were used to determine the length, the width, and the cell number on the perimeter of the crypt (see Fig. 5, C and F). The whole cell number per crypt was estimated considering the crypt as a cylinder (upper part of the crypt) with one extremity closed by a half-sphere (base of the crypt). Analyses were made on 10 to 15 crypts per animal for control and irradiated rats.

Materials

Forskolin was obtained from Calbiochem-Novabiochem, guanosine 5’ triphosphate and adenosine deaminase were from Boehringer, and fluoromount-G was from Southern Bio-technology Associates. All other chemicals, enzyme substrates, and salts were from Sigma.

Statistics

For each condition, the results are given as the mean ± SE of number of animals, except for Fig. 3A, which shows a representative experiment and where n is the number of crypts from one animal (n = 8–10). Comparison between control and irradiated animals was performed by unpaired t-test except for Ussing chamber data which were analyzed by one-way ANOVA followed by a Tukey-Kramer test. A P level of 0.05 or less was considered statistically significant.

RESULTS

Ussing Chamber Studies

Basal values of electrical parameters. Determination of the PD, Isc, and Gt in basal conditions provides information about the integrity of the epithelial tissue. Table 1 indicates values obtained in control and irradiated animals. The control values measured on days 1 (D1), 4 (D4), and 7 (D7) were not significantly different and were pooled [for example, PD in (mV): D1, −6.6 ± 1; D4, −7.1 ± 0.8; D7, −8.9 ± 1.4]. Irradiation did not induce modification of basal values one day after exposure. Four days after exposure, PD and Isc were markedly reduced. A strong, significant 2.3-fold increase of conductance was observed. Seven days after exposure, PD and Isc values returned toward the control values; however, the conductance remained significantly elevated.

Effect of VIP stimulation on Isc. To investigate the capacity of colonic tissue to respond to a secretagogue, VIP dose-response curves were performed in control and irradiated rats at 1, 4, and 7 days after exposure (Fig. 1). In all cases, the maximal ΔIsc was obtained with a VIP concentration around 100 nM. No change of dose-response curves was observed at D1 after irradiation.

| Table 1. Basal electrical parameters of stripped distal colonic tissues |
|-----------|--------|--------|--------|
|           | Control| Day 1  | Day 4  | Day 7  |
| n         | 38     | 14     | 20     | 10     |
| PD, mV    | −7.3 ± 0.6 | −6.8 ± 0.8 | −1.6 ± 0.2† | −4.4 ± 0.5* |
| Isc, μA/cm² | 91 ± 5 | 95 ± 9 | 58 ± 8* | 113 ± 9 |
| Gt, mS/cm² | 17.9 ± 1.6 | 13.5 ± 1.5 | 42.2 ± 3.6† | 26.9 ± 3.6† |

Values are means ± SE for the number (n) of animals. Gt, tissue conductance; PD, potential difference; Isc, short-circuit current. Data were analyzed by ANOVA followed by a Tukey-Kramer test. *P < 0.05; †P < 0.01.
Hyporesponsiveness observed at 4 days is thus characterized by a decrease of the maximal response capacity without modification of sensitivity \((EC_{50})\). The decreased maximal response may result from a reduced number of different units per unit of membrane area implicated in the cAMP pathway (VIP receptors, protein G and/or of adenylyl cyclase). The lack of \(EC_{50}\) modification suggests that the coupling of these different elements was functional. A reduced maximal response without modification of \(EC_{50}\) has been already observed by Griffiths et al. (21) after irradiation.

Effect of forskolin- and cAMP analogs on \(\Delta I_{sc}\). To localize at day four the origins of eventual alterations of the cAMP pathway, effects of agonists that act at different steps of this communication pathway, were investigated in Ussing chambers (Fig. 2). Addition of forskolin, which activates directly the catalytic unit of the colonic epithelium. Dose-response curves for cAMP accumulation in isolated crypts were performed using increasing amounts of VIP (Fig. 3). Without VIP stimulation, the basal production of cAMP was close to the detection threshold for the two groups. Under VIP stimulation, a plateau was obtained with a dose of 10 nM for control group and the value of maximal amount of cAMP per crypt for 4 min incubation was 12.8 ± 2.9 fmol/crypt. In the irradiated group, maximal response was obtained for a VIP concentration of 100 nM and cAMP production in isolated crypts from irradiated rats was markedly decreased by 80%. Diminution of the response was associated with decrease in sensitivity, because \(EC_{50}\) for the irradiated animals was greater than that of the controls (2.8 ± 1.9 nM for the irradiated vs. 0.3 ± 0.07 nM for the control). Figure 4 shows the effect of irradiation on VIP and forskolin-stimulated cAMP accumulation in crypts. The maximal response observed after a 10⁻⁸ M VIP stimulation (see Fig. 3) is indicated for comparison. Addition of forskolin increased cAMP accumulation in induced at D4, a marked decrease of 80% of maximal \(\Delta I_{sc}\) response to both VIP and forskolin. In contrast no significant differences of \(\Delta I_{sc}\) between control and irradiated groups were obtained when cAMP analogs, dibutyryl-cAMP or 8-Br-cAMP, were used. These results suggest that the cAMP communication pathway is altered by radiation upstream of the PKA activation, and may be at the step of cAMP production by adenylyl cyclase.

In situ cAMP accumulation measurement in single colonic crypt. The above data suggest that there is an impaired cAMP production after exposure to ionizing radiation. cAMP accumulation has been measured in single isolated crypts, the morphological and functional unit of the colonic epithelium. Dose-response curves for cAMP accumulation in isolated crypts were performed using increasing amounts of VIP (Fig. 3). Without VIP stimulation, the basal production of cAMP was close to the detection threshold for the two groups. Under VIP stimulation, a plateau was obtained with a dose of 10 nM for control group and the value of maximal amount of cAMP per crypt for 4 min incubation was 12.8 ± 2.9 fmol/crypt. In the irradiated group, maximal response was obtained for a VIP concentration of 100 nM and cAMP production in isolated crypts from irradiated rats was markedly decreased by 80%. Diminution of the response was associated with decrease in sensitivity, because \(EC_{50}\) for the irradiated animals was greater than that of the controls (2.8 ± 1.9 nM for the irradiated vs. 0.3 ± 0.07 nM for the control). Figure 4 shows the effect of irradiation on VIP and forskolin-stimulated cAMP accumulation in crypts. The maximal response observed after a 10⁻⁸ M VIP stimulation (see Fig. 3) is indicated for comparison. Addition of forskolin increased cAMP accumulation in

\[ \Delta I_{sc} = \text{dose response curves of short-circuit current} \]

\[ \text{of short-circuit current} \Delta I_{sc} \text{ after} \]

\[ \text{vasoactive intestinal peptide (VIP) stimulation in rat distal colon.} \]

\[ \Delta I_{sc} \text{ was determined in the presence of increasing concentrations of VIP (5.10^{-5} \text{ to } 5.10^{-7} \text{ M}) in stripped distal colonic tissue of control (} \bullet; \text{ } n = 34 \text{ and in irradiated rats, 1 day (} \odot; \text{ } n = 13 \text{, 4 days (} \odot; \text{ } n = 14 \text{, and 7 days (} \odot; \text{ } n = 12 \text{) after irradiation. Data are expressed as the means ± SE for number of animals and analyzed by ANOVA followed by a Tukey-Kramer test (}^{*}P < 0.05; \text{ }^{* * }P < 0.01).} \]

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isolated crypts of control animals (12-fold). In irradiated animals, forskolin-stimulated cAMP increase was only 50% of that observed in control group. cAMP production in response to VIP stimulation was thus more altered by irradiation than the response to forskolin.

Estimation of the length and crypt cell number of rat colonic distal crypts. This study was undertaken to ascertain whether the diminution of cAMP production was related to a decrease in the crypt cell number. Pictures of individualized crypts from control and irradiated rats are shown on Fig. 5. Parameters of the morphological study are reported on Table 2. Irradiated crypts were significantly longer than the control crypts with a 30% increase. There were no significant differences of the crypt width (49 ± 1 μm for control vs. 46 ± 2 μm for irradiated group) and of the cell number on the perimeter of the median plan (84 ± 2 cells for control vs. 78 ± 5 cells for irradiated group). The cell number per 100 μm² at the bottom and at the upper part of the crypt were significantly decreased for the irradiated animal by 47 and 33%, respectively. Moreover, in crypts of control animals, the cell number per 100 μm² was significantly higher at the bottom than in the upper part of the crypt. This difference was not evident in crypts from irradiated animals 4 days after exposure. This study shows a number of crypt cells for the control animals comparable with values reported from the literature for the rat descending colon from 620 to 1,300 cells per crypt (44, 53). Finally, the estimated whole cell number per crypt was only slightly decreased in crypts from irradiated animals (~25%, not significant).

Adenylyl cyclase activity measurements. Further biochemical measurements were performed to investigate the adenylyl cyclase activity 4 days after irradiation (Fig. 6). In the control group, adenylyl cyclase activity was increased by 5.2-fold under VIP or forskolin stimulation compared with basal activity. After irradiation,
VIP-stimulated adenylyl cyclase activity was decreased by 50%, whereas the activity stimulated by forskolin was only slightly decreased (~20%). Basal activity was increased by 4.3- and 5.9-fold under VIP and forskolin stimulation, respectively. The decreased adenylyl cyclase activity, more important with VIP than with forskolin stimulation, suggests modification of VIP receptors by irradiation.

**VIP-receptor characteristics.** VIP specific binding sites were characterized in membrane preparations of control and irradiated rats 4 days after exposure (Table 3). In both groups, Scatchard analysis of the saturation-binding curve fits with high and low affinity binding sites model. These data are in agreement with previous studies showing two sites of VIP receptors in the rat and human colon (12, 32). Irradiation did not alter affinity values of either the high or low affinity binding sites. In contrast, receptor numbers were significantly reduced in irradiated animals by a factor of 2 for both binding sites. This reduction of VIP receptor number without affinity modification is in agreement with the decreased secretory response without altered EC50 values observed in Ussing chambers.

**DISCUSSION**

In the present study, we have shown that 4 days after total abdominal irradiation (10 Gy) Ia responses to VIP and forskolin are markedly attenuated in rat distal colon. Moreover responses to cAMP analogs were similar in tissues from irradiated and sham-irradiated animals (Fig. 2), which suggests an alteration proximal to activation of PKA and chloride channels. Thus the decreased responses observed in Ussing chamber experiments may stem from among other causes: 1) altered VIP receptor binding and/or coupling of receptor-G protein; 2) modified adenylyl cyclase activity; 3) modified cAMP production and/or degradation; 4) altered crypt cell number. Several approaches were thus employed to determine which factors may contribute to the observed hyporesponsiveness.

In membrane preparations, VIP-stimulated adenylyl cyclase activity was significantly decreased (similar to observations in Ussing chambers), whereas forskolin-stimulated activity was only slightly decreased. Effect of irradiation on adenylyl cyclase activity has already been observed in a different model. A reduction of both VIP and forskolin-stimulated adenylyl cyclase activity has been reported (21) (47 and 27%, respectively) in membranes isolated from pig jejunum seven days after an irradiation of 6 Gy. The adenylyl cyclase dysfunction induced by irradiation may have several origins such as direct effect of irradiation, oxidative stress, hypoxia, or inflammatory mediators.

Chatterjee et al. (11) have shown that a high dose of irradiation could have a direct effect at the protein level: a gamma irradiation dose-dependent diminution of the activity of the enzyme (300 Gy) has been observed in Escherichia coli. However, in our model, a direct effect of irradiation on the adenylyl cyclase seems unlikely at an irradiation dose of 10 Gy.

Irradiation generates reactive oxygen species that provoke peroxidation of membrane lipids leading to a modification in the lipid membrane composition associated with a change in membrane microviscosity. These microenvironmental modifications may modify enzyme functionality of the adenylyl cyclase and may affect the receptor-coupled regulatory G protein (31, 49).

**Fig. 6.** Effect of irradiation on adenylyl cyclase activity of rat distal colon. Basal, VIP (5.10−7 M), and forskolin (10−5 M) stimulated adenylyl cyclase activities were determined in control (●) and irradiated (■) animals 4 days after exposure as described in MATERIALS AND METHODS. Results are expressed as pmol cAMP produced per milligram protein per 15-min incubation time and are given as the means ± SE for 11 animals. Analysis of statistical differences was performed by unpaired Student’s t-test. (**P < 0.01 and ***P < 0.001, differences between control and irradiated groups; ###P < 0.001, differences between basal and stimulated control groups).
A previous study has shown that hypoxia may also inhibit adenyl cyclase activity: a significant reduction in forskolin- and VIP-stimulated cAMP production and stimulated Cl\(^{-}\) secretion was observed in T84 cells after an hypoxic period of 72 h (52). Thus hypoxia seems to have a pronounced effect on cAMP-dependent responses. In our model, we cannot exclude that dysfunction of the cAMP pathway may be due, in part, to hypoxia induced by abdominal irradiation.

Hyporesponsiveness to secretagogues has been observed in irradiated rat colon and ileum (17, 36), as well as in cases of experimental colitis (2, 5), or of human inflammatory bowel disease (26, 45). Inflammatory reactions after ionizing radiation exposure are characterized by neutrophil infiltration, increased adhesion molecule expression, and increased levels of nitric oxide (NO) (7, 18, 35, 37, 40). With regard to the involvement of NO, it has been shown in mouse colon that after irradiation, epithelial hyporesponsiveness to electrical field stimulation and forskolin was reversed by inhibition of inducible NO synthase (iNOS) in vitro (18). NO has been reported to have a direct inhibitory effect on adenyl cyclase activity (39). Preliminary experiments performed in rat colon using an iNOS inhibitor (L-NIL) have shown partial recovery (~40%, not significant) of VIP- and forskolin-stimulated Δc across responses in tissue from irradiated animals (E. Morel unpublished observations). However, the responses remained much lower than those obtained from tissue of control animals that suggests a weak involvement of NO in our conditions. The ability of NO to inhibit adenyl cyclase stimulation seems to be dependent on the nature of the isoform present (27). The types of isoform present in rat distal colon may be different from those found in mouse colon and may respond differently in function of irradiation.

Impaired VIP-stimulated adenyl cyclase activity and reduced VIP-stimulated cAMP production induced by irradiation is associated with a 50% decrease of the VIP receptor number without modification of their affinity. Modifications of VIP receptor characteristics have already been observed after irradiation (21). Increase of VIP concentration in colon may be responsible for modification of VIP receptor characteristics. Höckerfelt et al. (29) described an increase in VIP concentration and a number of VIP immunoreactive nerve fibers in human colonic mucosa after irradiation. VIP increased levels could lead to desensitization and internalization of the VIP receptors (6). In the present study, the decrease of VIP receptor number observed after irradiation is unlikely to be due to an internalization, because results were obtained on whole membranes of the colonic mucosa. However, a degradation of the internalized VIP receptor by lysozymes could be postulated as a maximal increased lyosomal activity has been reported from 40–72 h after irradiation in the rat intestine (4).

Impaired adenyl cyclase activity and VIP receptor functionality are in agreement with our results of a marked reduction of cAMP quantity measured in crypts. In the present work, measurement of very low amounts of cAMP has been achieved for the first time in single, isolated and individualized colonic crypts. Measurements were made on intact and complete crypts composed of epithelial cells at different stages of differentiation. In isolated crypts, VIP dose-response curves of cAMP accumulation are obtained for a 10% lower VIP concentration than VIP concentration necessary to obtain maximal secretory response in a Ussing chamber (compare Fig. 1 with Fig. 3). This discrepancy could be explained by an easier access of VIP to receptors, a less important non-specific binding, and a lower degradation of the agonist on isolated crypt than in the stripped tissue mounted in Ussing chambers. This difference could be also due to the intervention of the steps downstream of the cAMP production (such as PKA activation or opening of the chloride channels) that are not considered in isolated crypts. Experiments with an epithelial monolayer in Ussing chambers have shown similar threshold stimulation for VIP (0.1 nM) and maximal effect (10 nM) (15). Thus data obtained in single crypts are in agreement with these values.

An increase in cAMP catabolism by PDE is another hypothesis that could explain the decreased cAMP accumulation. However, the same 80% decreased response to VIP observed both in Ussing chambers and in isolated crypt under the PDE inhibitor IBMX and the discrepancy of VIP and forskolin responses observed in isolated crypt may suggest that PDE is not involved in our conditions and reinforces the implication of the receptor-regulatory G protein-adenyl cyclase coupling. Nevertheless, measurements of cAMP accumulation performed in single crypts did not allow the investigation of mechanisms leading to an increased cAMP degradation, because IBMX, a large-spectrum PDE inhibitor, was used in these experiments. Theoretically, the threshold of the PKA activation can be reached either by activation of adenyl cyclase or by inhibition of cAMP dependent-PDE. According to Houslay and Milligan (30), two modes of regulation have been defined depending on both the type of isoform of adenyl cyclase isoforms and PDE present: 1) one with high activity of the adenyl cyclase and PDE in which a response is triggered by PDE inhibition alone and 2) one with low activity of the adenyl cyclase and PDE in which a stimulation of the adenyl cyclase is necessary. In our model, the lack of increase of cAMP in basal conditions under PDE inhibition may be due to the second mode. However, further experiments are needed to understand whether the regulation of cAMP pathway is localized on adenyl cyclase and/or on PDE.

Hyporesponsiveness to VIP of rat distal colon in vitro observed in the present study could be related, in part, to a loss of crypt cell numbers and a modification of the characteristics of the crypt cells.

A slight decrease of crypt cell number was associated with an increased crypt length without modification of the width that suggests an increase of cell size. Similarly unchanged crypt cellularity and increased length have been described in the rabbit ileum after irradiation (24, 25). These observations are associated with a change in cell shape from columnar to cuboidal. An alteration of cell shape has...
also been observed after exposure of rabbit colon in vitro to acidic medium that led rapidly to increased cell size and to recovery of epithelial integrity (33). Thus the marked reduction of cAMP production in isolated crypts after irradiation is not associated with a significant decrease of crypt cell number. Nevertheless, this small 25% reduction of the cell number may concern an important number of secretory cells, leading to a significant loss of secretory response. Indeed, the proportion of cryptal cells of different types has been shown to vary differently after an irradiation (8). The hyporesponsiveness to VIP is transitory, because I_max responses returned to normal values at 7 days after exposure. Temporal evolution of radiation effects may be explained by the crypt cell turnover time (4). The cell population originating from stem cells located at the base of the crypt produces by division new proliferative and differentiating cells. Sunter et al. (51) have calculated a total crypt cell production rate of 7.3 new cells per hour in rat descending colon and a total number of crypt cells ~735, which may lead to a crypt renewal in about 4 days. These data suggest that most of the crypt cell replacement has been achieved 4 days after irradiation. Thus alteration in VIP response observed after irradiation should be due to modifications of cells that appeared after exposure and suggests that impaired secretory capacity to VIP stimulation of crypt cells at 4 days may be the result of changes in the differentiation pathway or to a less mature state as proposed by Gunter-Smith (24). Irradiation is known to have an effect on proliferation and on differentiation (42, 47). Colonic cells covering the whole crypt epithelium from irradiated animals may be at stages of differentiation and maturation different from that of nonirradiated animals and thus could be poor in VIP-receptor number or equipped with nonfunctional receptors. Indeed, cell surface VIP receptor expression and activity has been hypothesized as an indicator of intestinal cell differentiation (10). A gradient of cAMP production has been reported along the villus-to-crypt axis; i.e., cAMP production is greater in duodenal crypts than in villus enterocytes in basal and VIP- and forskolin-stimulated conditions suggesting a relationship between cAMP communication pathway and differentiation (1). Craven and DeRubertis (13) have shown that 1) the basal cAMP content and the cAMP protein kinase activity was lower in cell populations harvested from colonic crypt zones compared with superficial cells and 2) the cyclic nucleotide PDE activity was markedly higher in cells from crypt base with respect to lumen cell preparations (46). Furthermore, Defer et al. (14) have shown that the expression of adenylyl cyclase isoforms with their different regulation mode may vary with the degree of cell differentiation. These observations reinforce the interest to undertake further work to investigate the localization of adenylyl cyclase isoforms along the crypt to understand effect of irradiation on the cAMP communication pathway.

In conclusion, the present work clearly shows that an abdominal gamma irradiation markedly attenuates VIP-stimulated ΔLac responses of the rat colonic mucosa 4 days after exposure. A part of the mechanisms leading to this impaired secretion has been elucidated. Functional and morphological aspects of this study show that the hyporesponsiveness to VIP of the rat colon is associated with a reduced cAMP accumulation, a decreased adenylyl cyclase activity, and a diminution of VIP binding site number without change of cellularity. Moreover, for the first time, measurement of cAMP accumulation has been achieved in single isolated crypts.

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