Protein kinase C mediates experimental colitis in the rat

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Protein kinase C (PKC) plays an important role in the cell signal transduction of many physiological processes. In contrast to these physiological responses, increases in PKC activity have also been associated with inflammatory disease states, including ulcerative colitis. The objective of this study was to examine the role of PKC as a causative mediator in initiation of experimentally induced colitis in the rat. Colitis was induced in rats by intrarectal instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS; 75 mg/kg in 50% ethanol) or the PKC activator phorbol 12-myristate 13-acetate (PMA; 1.5–3.0 mg/kg in 20% ethanol). Gross and histological mucosal damage, mucosal neutrophil infiltration, mucosal PKC activity, and PKC protein content for PKC isoforms α, β, δ, and ε were assessed 2 h to 14 days after an inflammatory challenge. Both PKC activity and mucosal injury increased significantly within 4 h of TNBS treatment. PKC activity was maximal at 7 days and declined at 14 days, whereas mucosal damage became maximal at 1 day and declined after 7 days. In contrast, neutrophil infiltration as assessed by myeloperoxidase activity only increased 12 h after TNBS treatment, became maximal 1 day after TNBS administration, and declined thereafter. PKCβδ, -δ, and -ε were increased in response to TNBS, whereas PKCα protein content was decreased. The PKC antagonists staurosporine and GF-109203X (25 ng/kg iv) reduced TNBS-induced changes in mucosal PKC activity and the degree of mucosal damage. In contrast, neutropenia induced by antineutrophil serum treatment did not significantly affect the degree of injury or mucosal PKC activity. Furthermore, activation of mucosal PKC activity with PMA also induced mucosal damage, which was also inhibited by pretreatment with a PKC antagonist. In conclusion, these results suggest that increases in PKC activity play a causative role in TNBS-induced colitis. The PKC-mediated response to TNBS does not appear to involve neutrophil infiltration.

2,4,6-trinitrobenzenesulfonic acid; inflammation; phorbol 12-myristate 13-acetate; myeloperoxidase protein kinase C antagonist

Activation of PKC activity is associated with inflammation in a number of tissues, including skin, cartilage, and epithelial cells of the airway and glomerulus (10, 31, 35, 36). PKC has also been shown to play a role in the sensitization of endothelial cells to bacterial endotoxin challenge (20). PKC activation has also been implicated in bile salt-mediated injury to hepatocytes (16) and radiation-induced apoptosis of thymocytes (44). Similarly, PKC activity has been shown to be elevated in a variety of cell types in response to a number of inflammatory challenges, including treatment with platelet-activating factor, cytokines, and exposure to oxidant stress (4, 15, 17, 30, 41). PKC also regulates a variety of signal transduction events implicated in the pathogenesis of inflammation, including the biosynthesis of nitric oxide, inflammatory cytokines, superoxide production, and the activation of phospholipase A2 (14, 34).

In the intestine, PKC activity was found to be elevated in biopsy samples taken from patients with ulcerative colitis (33). PKC activation has also been shown to increase intestinal endothelial and epithelial permeability in vivo and in vitro (3, 27, 29). Furthermore, intraluminal instillation of PKC activators such as phorbol 12-myristate 13-acetate (PMA) has been shown to result in ileal and colonic injury in experimental animals (9, 28). However, the primary nature of PKC activation in experimentally induced intestinal mucosal injury is unknown.

In the present study, we examine the role of PKC as a causative mediator in the initiation of colonic mucosal damage in response to intraluminal instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS). In addition, we examine whether PKC plays a critical role in PMA-induced injury in the rat colon, and we also examine the role of neutrophils in the inflammation produced by activation of PKC activity.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 180–200 g were purchased from Canada Breeding Labs (St. Constant, PQ) and were maintained in a temperature-controlled environment (22°C) on a 12:12-h light-dark cycle with standard laboratory chow and tap water available ad libitum. All studies were approved by the University of Western Ontario Animal Care Committee, and all animals were treated according to the guidelines set out by the Canadian Council on Animal Care.

Treatments

Induction of colonic inflammation. The rats were randomized into treatment groups, and colitis was induced using two methods. First, colitis was induced with TNBS (75 mg/kg in 50% ethanol), according to a modified version of a method described by Morris et al. (22). Second, colitis was induced...
with intraluminal instillation of PMA (1.5–3.0 mg/kg in 20% ethanol), according to a method previously described by Fretland et al. (9). In some studies, the negative control compound for PMA, 4α-phorbol 12-myristate 13-acetate (4α-PMA) (1), was used in the concentration of 3.0 mg/kg. Briefly, in these studies, a rubber catheter was inserted rectally into the colon, such that the tip was ~8 cm proximal to the anus. A volume of 0.6 ml of either TNBS, PMA, or 4α-PMA was instilled into the lumen of the colon through the catheter. Control animals received either 0.6 ml of 20% ethanol, 50% ethanol, or 0.9% saline, administered as described above. Vehicle- and TNBS- or PMA-treated animals were always housed in separate cages.

Drugs and agents. Before induction of colitis, animals were subdivided to receive the following treatments: 1) antineutrophil serum (ANS; Accurate Chemical and Scientific; 100 µl ip; 2 h before induction of colitis) at a dose that has previously been demonstrated to reduce circulating neutrophil concentrations to <5% of control concentrations within 2 h of injection (38, 2) the PKC antagonists staurosporine or bisindolylmaleimide (m-109203X; Biomol, Plymouth Meeting, PA; 25 µg/kg iv; 30 min before induction of colitis). The doses of these antagonists have previously been used to inhibit PKC activity in vivo (21, 26).

Assessment of Colonic Injury and PKC Activity

Tissue damage. The colon was removed under sodium pentobarbital anesthesia, opened by longitudinal incision, rinsed under tap water, and pinned to an “ice-cold” wax block. Slide photographs were taken, and damage to the colon was assessed macroscopically by three naïve observers using criteria described by Bell et al. (2). The mean of the three individual assessments was used for statistical comparisons. Immediately after the samples were photographed, the mucosa was removed by scraping with a blunt spatula. Samples were snap frozen in liquid nitrogen for later determination of myeloperoxidase and PKC activities.

In some studies, tissues from TNBS-treated rats were fixed in formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and were examined by light microscopy. The thickness of colonic wall was determined using a micrometer by measuring the distance from the serosal surface to the luminal surface of the mucosa. Measurements were done in triplicate.

Measurement of myeloperoxidase activity. Mucosal myeloperoxidase (MPO) levels were measured to provide an index of polymorphonuclear leukocyte infiltration. MPO activity was determined as described by Wallace (40). Briefly, frozen samples from mucosal scrapings were suspended in 50 mM phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide (pH 6.0; Sigma) at a tissue concentration of 50 mg/ml. Samples were homogenized three times for 30 s each, frozen and thawed three times in an aceton-dry ice bath, and centrifuged at 40,000 g for 15 min at 4°C. MPO activity in the supernatant was determined by adding 100 µl of the supernatant to 2.9 ml of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine hydrochloride (Sigma) and 0.0005% wt/vol hydrogen peroxide. The change in absorbance at 460 nm over a 3-min period was measured. MPO activity is presented as moles of hydrogen peroxide converted to water in 1 min at 22°C.

Measurement of PKC activity. Frozen mucosal scrapes were resuspended in 50 mM Tris-Cl buffer (pH 7.4) containing 5 mM EDTA, 10 mM EGTA, 50 µg/ml phenylmethylsulfonyl fluoride (PMSF), 10 mM benzamidine, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.3% wt/vol β-mercaptoethanol, and 10 mM okadaic acid. Tissue was homogenized at full speed on ice in an Ultra-Turrax tissue homogenizer for 15 s. A 25-µl aliquot was removed for determination of PKC activity using a commercially available kit (RPN 77, Amersham International) that measures the transfer of [γ-32P]ATP to a peptide specific for PKC.

Measurement of PKC Protein Content

Materials. Affinity-purified rabbit polyclonal anti-PKCα, anti-PKCβ1, anti-PKCδ, and anti-PKCε were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibody was a goat anti-rabbit antibody conjugated to horse-radish peroxidase (HRP) from Amersham. Rainbow electrophoresis molecular weight marker, the enhanced chemiluminescence (ECL) kit, Hybrid ECL nitrocellulose membrane, and Hyperfilm ECL were purchased from Amersham (Oakville, Canada).

Immunoblotting of colonic homogenate. A suspension of colonic tissue was obtained as described above. Samples were taken from animals treated 4 h to 14 days previously with TNBS. Samples were resuspended in ice-cold homogenization buffer that consisted of 50 mM Tris·HCl (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 1 mM EGTA, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 1 µg/ml soybean trypsin inhibitor, 50 µg/ml PMSF, and 10 mM β-mercaptoethanol with 10% Triton. Homogenized samples were centrifuged at 25,000 g for 30 min. The supernatant was mixed with an equal volume of 2% SDS sample buffer (125 mM Tris, pH 6.8, 20% glycerol, and 10% mercaptoethanol) and heated at 95°C for 5 min. The protein concentration of each extract was subsequently determined. Each sample of 12–15 µg homogenized protein was subjected to 10% SDS-PAGE. After electrophoresis, the gels were soaked for 30 min in transfer buffer and electrotblotted onto nitrocellulose membranes using Mini Trans-Blot (Bio-Rad) for 75 min at 100 V. After transfer, the membrane was incubated for 1 h with 10% nonfat dry milk in PBS and blots were incubated with either specific PKCα antibody (1:1,500) for 2 h or PKCβ1, PKCδ, or PKCe antibodies (1:1,000) for 3 h at room temperature. HRP-linked secondary antibody (1:6,000) incubation was for 1 h at room temperature. The blots were washed three times (10 min each time) between each antibody step with 0.1% Tween-20 in PBS. The ECL kit was used to visualize the immunoreactive bands according to the manufacturer’s protocols. The density of the immunoreactive bands on the autoradiogram was measured by Image Master VDS (Pharmacia Biotech). Band intensity was quantified by measurement of the absolute integrated optical density, which estimates the volume of the band in the lane profile as calculated by Image Master VDS software.

Statistical Analysis

Results are presented as means ± SE for n animals. Data were analyzed by ANOVA and Dunnett’s or Newman-Keuls tests for multiple comparisons or by Student’s t-tests for unpaired data; P < 0.05 was the minimum accepted level of significance for all groups.

RESULTS

Effect of TNBS or Ethanol on Mucosal Damage, PKC Activity, and MPO Activity

Intracolonic administration of TNBS (75 mg/kg) caused a time-related and significant (P < 0.05) increase in PKC activity between 4 h and 1 wk after administration (Fig. 1A). Elevated PKC activity sub-
sided between 7 and 14 days after administration of TNBS. Administration of TNBS also caused a time-related increase in MPO activity commencing 12 h after instillation. Activity, which was maximal at 1 day after TNBS administration, declined over the next 14 days (Fig. 1B). Activity, which was maximal at 1 day after TNBS administration, declined over the next 14 days (Fig. 1B). Activity, which was maximal at 1 day after TNBS administration, declined over the next 14 days (Fig. 1B).

Similarly, macroscopic mucosal damage was evident as early as 4 h after TNBS instillation, with the degree of injury increasing to its maximum 1 day after administration of the hapten TNBS (Fig. 1C). Mucosal damage declined from that time so that by day 14 the extent of damage was not significantly different from that observed in response to vehicle treatment alone.

In contrast, intracolonic treatment with the ethanol vehicle did not affect the activity of PKC in colonic mucosal homogenates (Fig. 2A). Both MPO activity and macroscopic mucosal damage were significantly elevated at 1 day (Fig. 2B and C). Neither parameter was significantly affected at any other time point.

Effect of Staurosporine, GF-109203X, and Neutropenia on TNBS-Induced PKC Activity and Mucosal Damage

Administration of either staurosporine or GF-109203X (25 ng/kg iv; 30 min before induction of colitis by TNBS) each inhibited the increase in PKC activity observed 1 day after TNBS treatment (Fig. 3A). Furthermore, administration of the nonselective PKC antagonist staurosporine and the selective antagonist GF-109203X 30 min before induction of colitis with TNBS significantly reduced the macroscopic damage score assessed 24 h later (Fig. 3B). The extent of damage after staurosporine or GF-109203X treatment was significantly greater than that observed in the vehicle control group. Treatment of rats with ANS did not significantly affect either PKC activity or the extent of mucosal injury as determined 1 day after TNBS treatment.

Effect of PKC Inhibitor on Mucosal MPO Levels

Intravenous administration of either staurosporine or GF-109203X 30 min before TNBS treatment both significantly inhibited the increase in MPO levels observed in response to TNBS (1 day) alone, although these levels were still significantly greater than the MPO activity detected in vehicle control samples (Fig. 4).

Effect of PMA on Mucosal Damage

Intracolonic instillation of the PKC activator PMA in the concentrations of 1.5 and 3.0 mg/kg resulted in a
dose-dependent increase in mucosal injury. Preadministration of the PKC antagonist staurosporine significantly reduced PMA-induced colonic damage (Fig. 5). The bioinactive PMA analog 4α-PMA was without effect in these studies. As detailed in Fig. 6, colonic wall thickness was increased in rats examined 1 day after TNBS or PMA treatment. This was observed to be due primarily to edema, although in some animals muscularis thickness was enhanced as well. Staurosporine treatment significantly reduced wall thickness in TNBS-treated rats, although this parameter was still greater than in control animals. Staurosporine treatment significantly reduced wall thickness of PMA-treated rats to control levels (Fig. 6).

Effect of TNBS on PKC Isoforms

Immunoblot analysis of PKC revealed that TNBS treatment increased PKCβ, PKCδ, and PKCe protein. In contrast, PKCa protein was not augmented by TNBS treatment (Fig. 7). Densitometric analysis indicated that, although protein for the PKCβ, PKCδ, and PKCe isoforms increased 1 day after TNBS treatment, administration of the ethanol vehicle had no significant effect in this regard (Fig. 8). PKCa protein did not increase significantly over the 14 days of observation. Densitometric analysis of PKCa revealed that this isoform was significantly decreased from 4 to 7 days after TNBS treatment and returned to control levels on day 14 (Fig. 9A). PKCβ, PKCδ, and PKCe protein content all increased within 1 day after TNBS treatment, whereas PKCe was increased as early as 4 h after TNBS treatment (Fig. 9D). Protein levels for PKCβ, PKCδ, and PKCe remained elevated even at 14 days after TNBS treatment.

![Fig. 3. Effect of TNBS treatment (75 mg/kg intracolonically) alone or with pretreatment with antineutrophil serum (ANS; 100 µl/kg ip), staurosporine (St; 25 ng/kg iv), or GF-109203X (GFX; 25 ng/kg iv) on means ± SE of mucosal PKC activity (A) and macroscopic damage score (B). Tissues were examined 1 day after TNBS treatment. **Significant difference vs. vehicle (50% ethanol) control, ††significant difference vs. TNBS treatment alone by ANOVA and Newman-Keuls test for multiple comparisons (n = 6–8 animals/group; P < 0.05).](http://ajpgi.physiology.org)

![Fig. 4. Effect of TNBS treatment alone or with pretreatment with either staurosporine or GF-109203X (each at 25 ng/kg iv; 30 min before TNBS) on MPO activity (means ± SE). Tissues were examined 1 day after TNBS treatment. *Significant difference vs. vehicle control, †significant difference vs. TNBS alone by ANOVA and Newman-Keuls test for multiple comparisons (n = 6–7 animals/group; P < 0.05).](http://ajpgi.physiology.org)

![Fig. 5. Effect of intracolonic instillation (0.6 ml) of PKC activator phorbol 12-myristate 13-acetate (PMA; 1.5–3.0 mg/kg in 20% ethanol) on macroscopic mucosal damage score (means ± SE). In some experiments, animals were pretreated with PKC inhibitor staurosporine (25 ng/kg iv). In other studies, rats were treated with bioinactive PMA analog 4α-phorbol 12-myristate 13-acetate (4α-PMA; 3.0 mg/kg). Tissues were examined 1 day after PMA treatment. *Significant increase over respective controls, †significant reduction from PMA treatment alone as determined by ANOVA and Dunnett’s test (n = 6–8 animals/group; P < 0.05).](http://ajpgi.physiology.org)
DISCUSSION

In this study of experimentally induced colitis, we have attempted to determine whether there is a direct association between the severity of inflammation as estimated by myeloperoxidase activity, gross mucosal injury, and activation of PKC in the colonic mucosa. The study revealed that intrarectal instillation of TNBS resulted in a progressive increase in the severity of colitis commencing 2–4 h after instillation of TNBS, becoming maximal 1 day after induction, and then declining over the next 14 days. This pattern of colonic injury in response to TNBS has been described previously (2, 22).

This study also demonstrated that, in response to TNBS, mucosal PKC activity was elevated between 2 and 4 h and increased over the next 7 days. Furthermore, protein content for PKCε was also increased as early as 4 h after TNBS treatment. Treatment of rats with the ethanol vehicle alone did not affect PKC activity or the protein levels of PKCα, PKCβ, PKCδ, or PKCε at any time point. Furthermore, significant mucosal damage was only evident on day 1, and this level was significantly less than that observed on day 1 after TNBS treatment. Therefore, ethanol-mediated mucosal injury was relatively short-lived and not dependent on PKC activation.

The extent of mucosal injury in TNBS-treated animals was inhibited when animals were pretreated with the nonselective PKC antagonist staurosporine. Staurosporine treatment also reduced the increase in mucosal thickness in colonic tissues in which PKC activity had been augmented. Staurosporine has previously been shown to reduce injury induced in response to a number of inflammatory challenges in nongastrointestinal tissues and to inhibit release of certain proinflammatory mediators (12, 13, 21). In contrast to staurosporine, which inhibits not only PKC but also protein kinase A (PKA), protein kinase G, and myosin light chain kinase activity, GF-109203X is a potent and selective inhibitor of PKC activity with no effects on PKA or tyrosine kinase activities (39). In the present study, GF-109203X also reduced the extent of damage and inhibited PKC activity in tissue excised from TNBS-challenged animals. These data suggest that PKC plays a direct role in this type of experimentally induced colitis.

This suggestion is reinforced by our demonstration that intraluminal instillation of the PKC activator PMA could also produce mucosal damage, which was also inhibited by the antagonist staurosporine. PMA is a potent activator of inflammatory cells (36) that has
been used to model various intestinal diseases. Intraluminal PMA instillation has been shown to produce colonic mucosal inflammation (3, 5, 9) and ileal microvascular injury (28), and recently PMA treatment has been associated with gastric ulcer formation (37). In the present study, PMA acted via a PKC-dependent mechanism as assessed using the PKC-inactive phorbol analog 4α-PMA. This confirms and extends findings by Berin and Buell (3) in which PMA but not the bioinactive analog increased colonic epithelial permeability.

Changes in PKC activity have been associated with inflammation of a number of tissues and cells, including skin, cartilage, and epithelial cells of the airway, glomerulus, and liver (10, 31, 35, 36). A number of isoforms of PKC are expressed in the colon (6), and PKC activity has been shown to be increased in tissue from ulcerative colitis patients (33). Furthermore, increased PKC activity is also linked with the increase in mesenteric venular leakage in response to ischemia-reperfusion injury to the intestine (43). PKC activation has also been shown to be associated with an increase in intestinal endothelial and epithelial permeability in vivo and in vitro (3, 27, 29). Those data together with the present results strongly suggest a causative role for PKC in the initiation of colonic mucosal injury.

The mechanisms through which increases in PKC activity mediate TNBS-induced colitis are unknown. PKC has been shown to regulate a variety of signal transduction events involved in inflammation, including induction of NO synthase activity, production of cytokines, oxidants, and activation of phospholipase A2 (14, 34). PKC activation via PMA has also been shown to cause activation of neutrophils (18), resulting in an oxidative burst (8, 18). Although neutrophil infiltration has been associated with TNBS-induced colitis (2), neutrophil depletion has not been shown to effectively reduce acetic acid- or TNBS-induced colonic damage (5, 42). In our study, although TNBS treatment resulted in an increase in MPO activity, induction of neutropenia via antineutrophil serum administration did not ameliorate the TNBS-mediated colonic injury. Furthermore, neutropenia did not significantly reduce mucosal PKC activity. Similarly, in PMA-induced colitis, neutropenia has been shown to be ineffective in reducing the degree of damage (5). In contrast, the PKC antagonists staurosporine and GF-109203X reduced the increase in MPO levels in colonic mucosal samples from TNBS-treated rats. These data suggest that, in TNBS-induced colitis, neutrophil infiltration is secondary to tissue damage and the response is not mediated by PKC. However, the ability of the PKC antagonists to reduce tissue injury may be attributed in part to a reduction in neutrophil infiltration as well as a reduction in tissue PKC activity.

Normal colonic mucosa expresses a number of PKC isoforms with distinct subcellular distributions for each (6). In the present study using tissue from TNBS-challenged animals, we detected protein for PKCα, PKCβ, PKCδ, and PKCε. These PKC isoforms have been associated with cytotoxicity in some cell types (7), and these isoforms can be activated by proinflammatory cytokines (19). Furthermore, the results of work done in tissues such as liver, brain, and peripheral nerves and in cells such as macrophages indicate that an inflammatory challenge can result in changes in the expression of various PKC isoforms (16, 32, 34). The present results indicate that PKCβ, PKCδ, and PKCε are all elevated in response to TNBS treatment, although only PKCε protein is increased before the onset of colonic damage. PKCα content is decreased as early as 4 h after treatment and only returns to control levels at 14 days. It may be suggested that the elevated isoforms (PKCβ, PKCδ, and PKCε) play roles at various
stages of TNBS-induced inflammation. The return of PKCα to control levels at day 14 suggests that this isoform contributes to the reduced PKC activity measured at that time point and may play a role in the restitution process.

In summary, our results provide the first evidence that PKC is directly involved in colonic mucosal inflammation. This study also supports the proposal that neutrophils do not mediate the inflammatory effects of TNBS and are not involved in the colonic response to PKC activation.

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