PROTEIN KINASE C (PKC) plays an important role in many signaling pathways, including gene expression, cell growth and differentiation, secretion of hormones and neurotransmitters, and membrane functions (24). The signal transduction of these functions is achieved through a variety of isoforms of PKC (23–25). In addition to its many physiological roles, PKC has also been associated with inflammatory disease states.

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with intraluminal instillation of PMA (1.5–3.0 mg/kg in 20% ethanol), according to a method previously described by Freltland 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 I (GR-103920X; Biomol, Plymouth Meeting, PA; 25 ng/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 naive 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-ether 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 a percentage of the total activity in the lane profile as estimated 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 considered significant for all groups.

RESULTS

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

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

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).

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 bioactive 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).
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 PKCe 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 PKCa, PKCb, PKCd, or PKCe 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 PKCe. 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 PKCe are all elevated in response to TNBS treatment, although only PKCe 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 PKCe) 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.

This work was supported by Medical Research Council of Canada Grant MT 6426. J. F. Brown was supported by a Canadian Association of Gastroenterology/Astra Research Initiative Award.

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Received 16 June 1998; accepted in final form 4 November 1998.

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