PGE_2 promotes Ca^{2+}-mediated epithelial barrier disruption through EP_1 and EP_4 receptors in Caco-2 cell monolayers

M. José Rodríguez-Lagunas, Raquel Martín-Venegas, Juan José Moreno, and Ruth Ferrer *Departament de Fisiologia, Facultat de Farmàcia, Universitat de Barcelona, Barcelona, Spain*

Abstract We recently demonstrated that PGE_2 induces the disruption of the intestinal epithelial barrier function. In the present study, our objectives were to study the role of PGE_2 receptors (EP_1–EP_4) and the signaling pathways involved in this event. Paracellular permeability (PP) was assessed in differenti-ated Caco-2 cell cultures from D-mannitol fluxes and transepithelial electrical resistance (TER) in the presence of different PGE_2 receptor agonists (carbacyclin, sulprostone, butaprost, ONO-AE1-259, ONO-AE-248, GR63799, and ONO-AE1-329) and antago-nists (ONO-8711, SC-19220, AH-6809, ONO-AE3-240, ONO-AE3-208, and AH-23848). The results indicate that EP_1 and EP_4 but not EP_2 and EP_3 might be involved in PP regulation. These effects were mediated through PLC-inositol trisphosphate (IP_3)-Ca^{2+} and cAMP-PKA signaling pathways, respectively. We also observed an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) strengthened by cAMP formation indicating a cross talk interaction of these two pathways. Moreover, the participation of a conventional PKC isoform was shown. The results also indicate that the increase in PP may be correlated with the redistribution of occludin, zona occludens 1 (ZO-1), and the perijunctional actin ring together with an increase in myosin light chain kinase activity. Although the disruption of epithelial barrier function observed in inflammatory bowel disease (IBD) patients has been traditionally attributed to cytokines, the present study focused on the role of PGE_2 in PP regulation, as mucosal levels of this eicosanoid are also increased in these inflammatory processes.

Key words: paracellular permeability; intestine; tight junctions; eicosanoids; ara-chidonic acid cascade

THE STRUCTURAL INTEGRITY OF the epithelium is maintained by three distinct adhesion systems: tight junctions (TJ), adherent junctions, and desmosomes. Of these, TJ are the most apical component and are the rate-limiting step for paracellular per-meability (PP). In addition, TJ constitute the interface (fence) between apical and basolateral membrane domains (32). TJ are multiprotein complexes composed of transmembrane proteins associated with the cytoskeletal perijunctional ring of actin and myosin and with cytosolic proteins involved in cell signaling and vesicle trafficking. Five transmembrane proteins of the junctional complex have been identified in recent years: occlu-din, the claudin family, tricellulin, crumbs, and junctional adhesion molecules. These proteins are associated with a wide spectrum of cytosolic proteins, of which zona occludens (ZO) 1, ZO-2, ZO-3, AF6, and cingulin are described as forming the nexus with cytoskeletal proteins (42). PGE_2 is an inflammatory mediator that has pleiotropic ef-fects on signal transduction and exerts its biological action through binding to four specific membrane receptor subtypes, EP_1, EP_2, EP_3, and EP_4, which are widely distributed and have different tissue expression. PGE_2 stimulation leads to activa-tion of different G proteins, depending on the type of EP subtype engaged, inducing changes in second messengers such as cAMP, Ca^{2+}, and inositol phosphates (44). EP expression has been reported in the intestinal epithelium of various species (16). In rat, EP_1 is expressed in goblet cells of the small intestine, and it is also expressed in other epithelial cells of the large intestine. In rabbit, it is highly expressed in the brush border membrane of differentiated villous cells. EP_2 is expressed in different regions of the intestine, depending on the species, and EP_3, in most rodents, is expressed in goblet cells of the small intestine. EP_4 expression has been detected in mouse mature enterocytes of ileal villi. In humans, EP_2 and EP_3 are expressed at the apex of colonic crypts; and EP_4, on their lateral side.

Recently, we observed that cell differentiation in intestinal Caco-2 cells induces a decrease in PLA_2 activity and cycloox-ygenase-2 expression and, consequently, a decrease in arachi-donic acid release and PGE_2 synthesis in parallel with a reduction in PP. We (30) also demonstrated that the addition of PGE_2 to differentiated Caco-2 cells induces an increase in PP. Several intestinal diseases are associated with the disruption of the epithelial barrier function, particularly inflammatory bowel disease (IBD) (49), characterized by increased mucosal PGE_2 levels (7, 22). Yu and Chadee (54) demonstrated in T-84 colonic epithelial cells that PGE_2 upregulates IL-8 production via EP_4 interaction, thus confirming the proinflammatory role of PGE_2. In contrast, in EP_4 receptor knockout mice, this receptor was described as playing a critical role in keeping mucosal integrity in an experimental model of colitis induced by dextran sodium sulfate, although the role of EP_4 in either epithelial or submucosal cells has not been identified (23). Sim-ilarly, studies in rats using an EP_4 receptor agonist reported the suppression of colitis caused by dextran sodium sulfate treatment through upregulation of an anti-inflammatory cytokine, IL-10 (34). Therefore, the function of EP receptors is still undefined. Dey et al. (16) concluded that EP_1, EP_2, and EP_4 receptors seem to be major determinants in the early stages of intestinal inflam-mation and also tumorigenesis, whereas EP_4 receptors in immune cells may promote the restitution of colitis/inflammation. The main objectives of this study were to study in Caco-2 cells the role of EP receptors in the regulation of epithelial barrier function by PGE_2 and their signaling pathways and the contribution of TJ proteins and the perijunctional actin ring to increased PP.

MATERIALS AND METHODS

Materials. DMEM, trypsin, penicillin, and streptomycin were sup-plied by GIBCO (Paisley, Scotland). Nonessential amino acids, FBS,

BSA, PBS, D-glucose, HEPES, fura 2-AM, PGE_2, butaprost, carba-cyclin, inositol trisphosphate (IP_3), forskolin, U73122, dantrolene, Gö6983, SQ22,536, KT5720, ML-7, and phalloidin-tetramethylrho-damine B isothiocyanate (TRITC-phalloidin), along with other chem-icals, were supplied by Sigma (St. Louis, MO). Dioctanoylglycerol (diC8) was from Molecular Probes (Leiden, The Netherlands). Tissue culture supplies, including Transwells, were obtained from Costar (Cambridge, MA). D-[2-^{3}H]mannitol (specific activity 30 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Biogreen 3 was supplied by Scharlau Chemie (Barcelona, Spain). ONO-8711, ONO-AE3-240, ONO-AE3-208, ONO-AE1-259, ONO-AE1-329, and ONO-AE3-248 were kindly provided by Ono Pharmaceutical (Osaka, Japan), and AH-6809, AH-23848, and GR63799 by Glaxo Wellcome (Stevenage, United Kingdom).

Cell culture. Caco-2 cells were kindly provided by Dr. David Thwaites at the School of Cell and Molecular Biosciences, University of Newcastle-upon-Tyne (United Kingdom), and were cultured as previously described (30). The cells (*passages 107-121*) were rou-tinely grown in plastic flasks at a density of 5×10^4 cells/cm^2 and cultured in DMEM containing 4.5 g/l D-glucose and 2 mM L-glu-tamine, supplemented with 1% (vol/vol) nonessential amino acids, 10% (vol/vol) heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a modified atmosphere of 5% CO_2 in air. Cells grown to ~80% confluence were released by trypsinization and subcultured at a density of 5×10^4 cells/cm^2 in 12-well clusters for intracellular Ca^{2+} determination or at 4×10^5 cells/cm^2 on polycarbonate filters with a pore size of 0.4 μm (Transwells; 12-mm diameter) for PP experiments and confocal immunolocalization. The medium was replaced every 3 days and on the day before the experiment. Experiments were performed in cultures 19–21 days after seeding when cells are differentiated (30).

PP. PP was estimated from unidirectional apical-to-basal D-man-nitol fluxes and transepithelial electrical resistance (TER) in cells maintained on filters, as described elsewhere (30). After 3-h incuba-tion with PGE_2 or EP_1–EP_4 agonists and antagonists in the apical and basolateral compartments, monolayers were washed with modified Krebs buffer (room temperature), which had 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 10 mM D-glucose, and 10 mM HEPES/Tris (pH 7.4). The filters were then placed in culture wells containing 1.5 and 0.7 ml of modified Krebs buffer in the basolateral and apical compartments, respectively, and TER was determined by a Millicell-ERS Voltohmmeter (Millipore, Bedford, MA). Results are expressed as ohms per centimeter squared monolayer surface area. The resistance of the supporting membrane in filters was subtracted from all readings before calculations. After TER determination, apical medium was replaced by the same volume of modified Krebs buffer containing 0.2 mCi/ml D-[2-^{3}H]mannitol, and the cells were incubated for 5 min at 37°C. At the end of the incubation, basolateral medium was withdrawn, and radioactivity was counted in a scintillation counter (1500 Tri-Carb; Packard, Downers Grove, IL). The concentrations of the PGE_2 antagonists tested were chosen while taking into account their reported inhibitor constant (K_i) values for PGE_2 receptor interaction.

Confocal immunolocalization. Immunolocalization was performed as described elsewhere (36). Caco-2 control or treated monolayers grown on filters were gently washed with PBS and fixed in 3%paraformaldehyde and 2% sucrose in 0.1 M PBS (pH 7.4) for 15 min at room temperature. Cells were washed twice for 5 min in 10 mM PBS containing 20 mM glycine (PBS-glycine) and permeabilized with 0.2% (vol/vol) Triton X-100 for 10 min at room temperature. Cells were washed twice in PBS-glycine and blocked for 20 min in PBS-glycine containing 1% BSA (incubation solution). As primary antibodies, mouse monoclonal anti-occludin (1:500 dilution; Zymed, South San Francisco, CA), rabbit polyclonal anti-ZO-1 (1:250 dilu-tion; Zymed), and rabbit polyclonal anti-EP_1, -EP_2, -EP_3, and -EP_4 (1:500 dilution; Cayman, Ann Arbor, MI) were used. Cells were incubated with the primary antibodies for 1 h at 37°C and washed twice in PBS-glycine for 5 min at room temperature.

Monolayers were then incubated for 1 h at 37°C with Alexa dye-conjugated secondary antibodies (Molecular Probes) and Hoechst 33258 (1:2,000; Sigma) to visualize the nuclei. Finally, the cells were washed for 10 min at room temperature in PBS, mounted in Mowiol (Calbiochem, San Diego, CA), and examined with a confocal laser scanning microscope (TCS 4D; Leica Lasertechnik). Images were taken using a ×63 (numerical aperture 1.3, phase 3, oil) Leitz Plan-Apochromatic objective. Back-ground absorbance (measured by secondary antibody labeling only) was subtracted from all samples. To view the actin subapical ring, cell monolayers were fixed and permeabilized as described above and incubated with TRITC-phalloidin for 1 h at 37°C (1:1,000 dilution).

Intracellular calcium concentration. Intracellular calcium concentration ($[Ca^{2+}]_i$) was monitored using the selective fluorescent Ca^{2+} indicator, fura 2-AM. Caco-2 cells grown on clusters were loaded with 25 μM fura 2-AM in DMEM for 1 h at 37°C. The preloaded monolayers were then washed in modified Krebs buffer (pH 7.4) at 37°C and incubated for 1 h at 37°C to allow fura 2-AM deesterification. The monolayers were washed again to ensure removal of all unloaded indicator and antagonists, and inhibitors were added to the respective wells. Continuous fluorescent signal was monitored with excitation wavelengths of 340 and 380 nm and an emission at 510 nm using a fluorescent microplate reader (FLUOstar OPTIMA; BMG Labtech) before and after the injection of PGE_2, PGE_2 agonists, IP_3, or forskolin. Cells were maintained throughout the experiment at a temperature of 37°C. At the end of the incubation period, the maximal and minimal intracellular probe fluorescent signals were determined by the addition of cell lysis buffer and 20 mM EDTA in Krebs, respectively. $[Ca^{2+}]_i$ was calculated following Grynkiewicz et al. (20) from 340:380 ratio by using a dissociation constant of 224 nM.

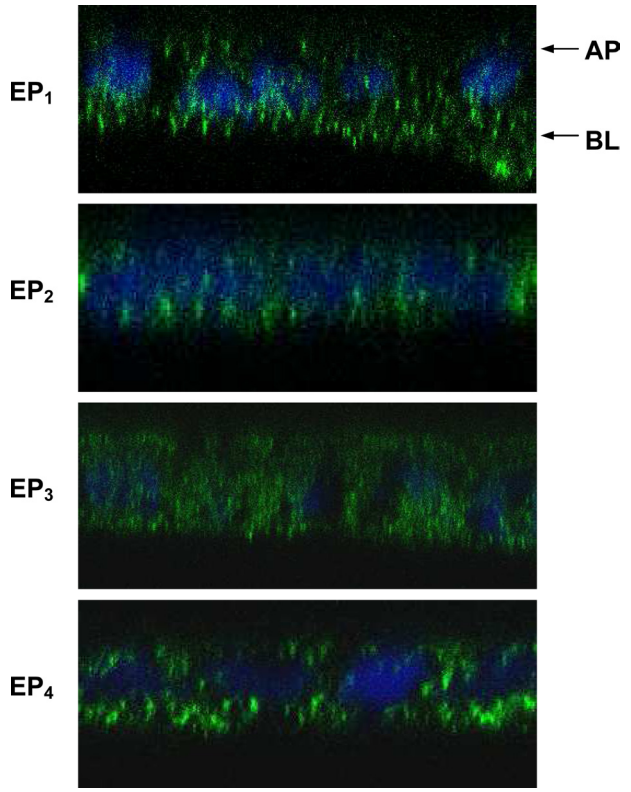

Fig. 1. Immunolocalization of PGE_2 receptors EP_1–EP_4 by confocal analysis. Specific anti-EP_1–EP_4 antibodies were used, as described under MATERIALS AND METHODS, in differentiated Caco-2 cells maintained in filters. Hoechst staining (blue) was used to visualize nuclei. In each case, a representative *x-z* image of sections is shown. Apical (AP) and Basolateral (BL) membrane borders are indicated with arrows.

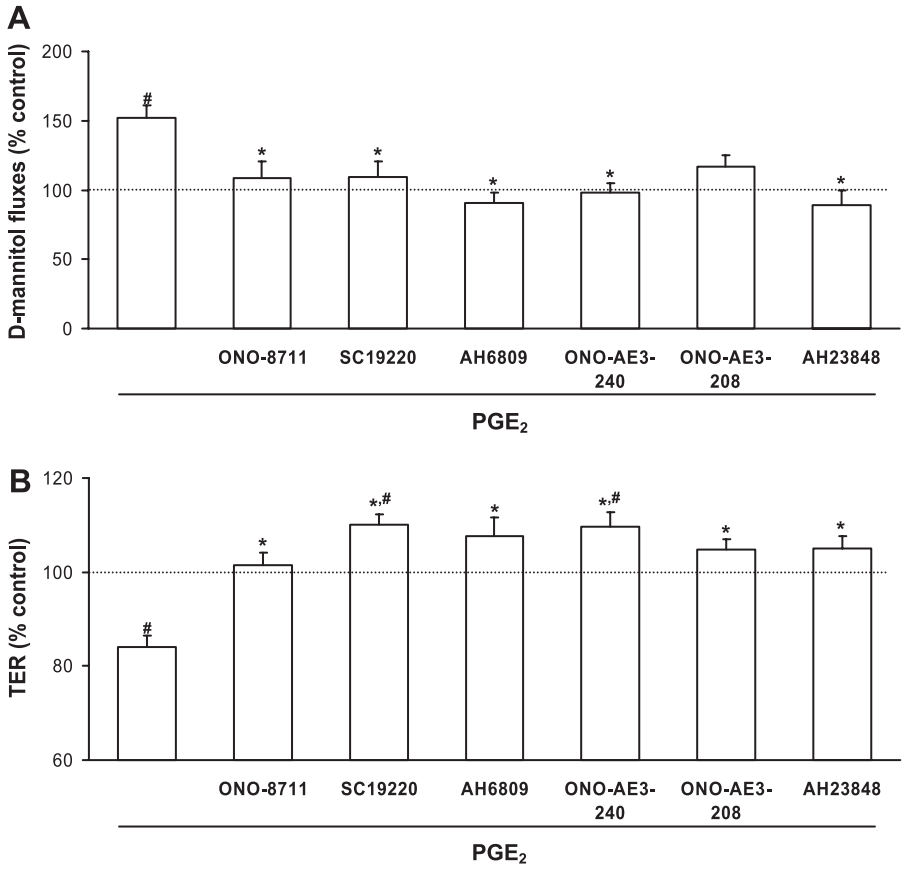

Fig. 2. Effect of EP_1–EP_4 antagonists on epithelial barrier disruption induced by PGE_2. D-mannitol fluxes (*A*) and transepithelial electrical resistance (TER; *B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with PGE_2 (3 nM) and PGE_2 plus ONO-8711 (250 nM), plus SC-19220 (300 nM), plus AH-6809 (3×10^3 nM), plus ONO-AE3-240 (2 nM), plus ONO-AE3-208 (2 nM), or plus AH-23848 (200 nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (4.8 ± 0.48 fmol/cm^2 and 350 ± 32.7 $\Omega \cdot$cm^2). The data were means ± SE of $n = 6-8$. *$P < 0.05$ vs. PGE_2; #$P < 0.05$ vs. control.

Data analysis. Results were expressed as means ± SE. Data were analyzed by one-way analysis of variance followed by Dunnett post hoc test using SPSS software (SPSS, Chicago, IL). $P < 0.05$ was considered to denote significance.

RESULTS

First, the immunolocalization of EP receptors was per-formed, and the results show the presence of the four receptors in differentiated Caco-2 cells (Fig. 1). Then, to investigate the EP receptors involved in the regulation of epithelial barrier function, the ability of different receptor antagonists to prevent the effects of PGE_2 and PGE_2 analogs on D-mannitol fluxes and TER was tested. The results shown in Fig. 2, *A* and *B*, confirm previous data (30), indicating that PGE_2 induces an increase in D-mannitol fluxes and a reduction in TER values. The results also show that all the antagonists tested prevented the effects of PGE_2 on both variables, leading to values no different from those detected under control conditions, except in the case of SC-19220 and ONO-AE3-240, which reverted TER to signif-icantly higher values. Since ONO-8711 and SC-19220 are specific for EP_1 and AH-23848 for EP_4 (Table 1), these results suggest the participation of these two receptors in these events. Nevertheless, since the other antagonists tested (AH-6809, ONO-AE3-240, and ONO-AE3-208) show cross-reaction in-teractions (Table 1), the results do not completely clarify the contribution of EP_2 and EP_3. Therefore, the effect of all these antagonists on specific EP_1–EP_4 agonists was further investi-gated.

First, carbacyclin and sulprostone were tested, and the re-sults show that these PGE_2 analogs significantly increased PP, but to a lesser extent than PGE_2 (Fig. 3, *A* and *B*). The data also show that the effects of carbacyclin, an EP_1 agonist but with some EP_3 activity (Table 1), were prevented by EP_1 antagonists (ONO-8711 and SC-19220), whereas an EP_3 antagonist (ONO-AE3-240) was unable to modify the variables studied. The effects of sulprostone, an EP_3 agonist but with some EP_1 activity, were not significantly affected by ONO-AE3-240, whereas a significant recovery was observed for ONO-8711 and SC-19220. Therefore, all these results obtained for PGE_2 and PGE_2 analogs suggest the participation of EP_1 but not of EP_3 in PP regulation by PGE_2.

As for EP_2 and EP_4 participation, butaprost was tested as an EP_2 agonist but with some EP_4 activity. In this case again, the effect of this analog on PP was lower than that observed for

Table 1. *PGE_2 receptor (EP_1–EP_4) agonists and antagonists tested*

	Agonist	Antagonist
EP_1	Carbacyclin ($EP_1 > EP_3$)[25]	ONO-8711[51] SC-19220[18]
EP_2	Butaprost ($EP_2 > EP_4$)[5,44] ONO-AE1-259[44]	AH-6809 ($EP_1 \approx EP_2$)[52]
EP_3	Sulprostone ($EP_3 > EP_1$)[1,44] ONO-AE-248[44] GR63799[1]	ONO-AE3-240 ($EP_3 > EP_4$)[3,44]
EP_4	ONO-AE1-329[44]	ONO-AE3-208 ($EP_4 > EP_3$)[14,44] AH-23848[1,14]

>, Higher affinity than; ≈, similar affinity to. Superscripted numbers correspond to references.

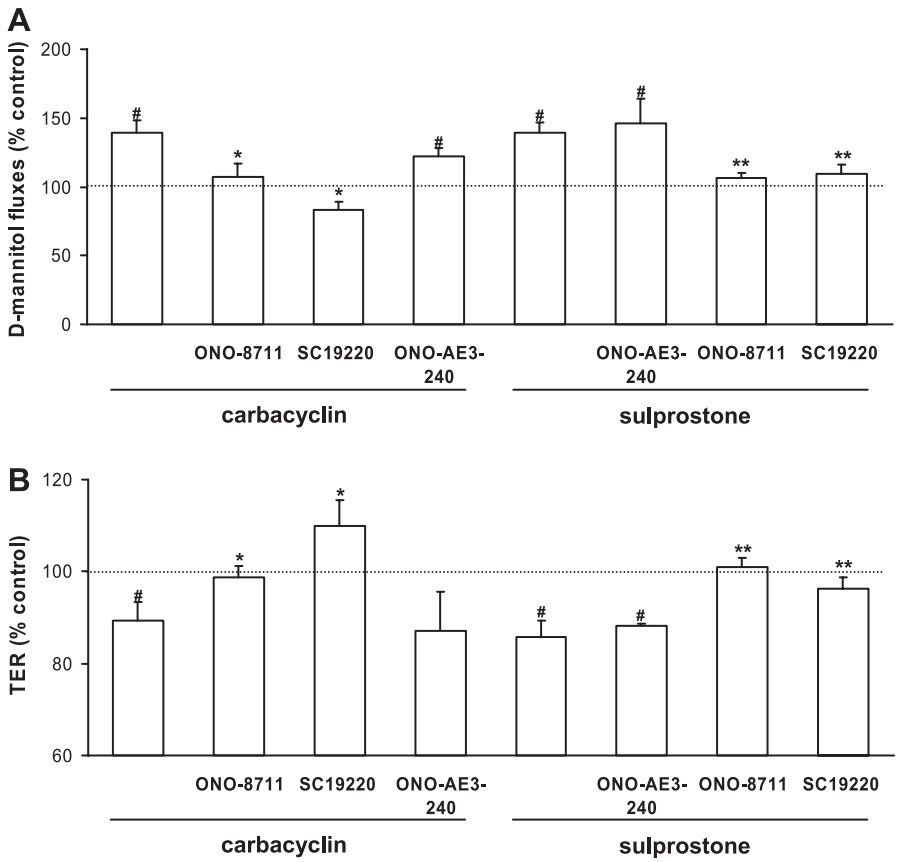

Fig. 3. Effect of EP_1 and EP_3 antagonists on epithelial barrier disruption induced by carbacyclin and sulprostone. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with carbacyclin (300 nM) or sulprostone (2 nM) plus ONO-8711 (250 nM), plus SC-19220 (300 nM), or plus ONO-AE3-240 (2 nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.8 ± 0.38 fmol/cm^2 and 314 ± 20.3 $\Omega \cdot$cm^2). The data were means $\pm$ SE of $n = 6-8$. *$P < 0.05$ vs. carbacyclin; **$P < 0.05$ vs. sulprostone; #$P < 0.05$ vs. control.

PGE_2 (Fig. 4, *A* and *B*). The results revealed no effect of AH-6809, here used as an EP_2 antagonist, but did show the capacity of two EP_4 antagonists (ONO-AE3-208 and AH-23848) to completely prevent these effects. To confirm EP_4 participation, increasing concentrations of a specific agonist for this receptor (ONO-AE1-329: 10, 100, 10^3, and 10^4 nM) were tested (Fig. 5, *A* and *B*), and the results showed an increase in D-mannitol fluxes for the highest concentration, although no effects on TER values were detected. Finally, to confirm the lack of EP_2 and EP_3 involvement, the effect of increasing concentrations of an EP_2 (ONO-AE1-259: 10, 100, 10^3, and 10^4 nM) and two EP_3 agonists (ONO-AE-248: 10, 100, 10^3, and 10^4 nM and GR63799: 2, 20, 200, and 2×10^3 nM) were determined. The results obtained revealed no effect on D-mannitol fluxes or on TER values for either of the concentra-tions tested. Figure 5, *A* and *B*, shows the values obtained for the highest concentration tested. Therefore, all these results together suggest EP_1 and EP_4, but not EP_2 and EP_3, involve-ment in PP regulation by PGE_2.

The participation of EP_1 and EP_4 was further investigated by analyzing the signaling pathways involved, especially the changes in $[Ca^{2+}]_i$. EP activates a G_q protein that induces PLC activation. PLC cleaves phosphatidylinositol 4,5-bisphos-phate into diacylglycerol (DAG) and inositol trisphosphate (IP_3) and the interaction of IP_3 with its receptors at the endoplasmic reticulum results in an increase in $[Ca^{2+}]_i$ (44). Therefore, coupling of PGE_2 to EP_1 results in PKC activation. In contrast, EP_4 activates a G_s protein, which stimulates cAMP formation by adenylate cyclase (44). The results shown in Fig. 6*A* confirm the capacity of PGE_2 to increase $[Ca^{2+}]_i$ in Caco-2 cell cultures, an effect that was prevented by ONO-8711, U73122 (PLC inhibitor), and dantrolene (an inhibitor of intracellular Ca2□ release from the endoplasmic reticulum). The changes in [Ca2□]i induced by carbacyclin were not so pronounced, and the maximum obtained for this variable was delayed (Fig. 6B). This effect was also prevented by ONO-8711, U73122, and dantrolene. IP3 was tested, too, and the results also revealed a delayed peak but a similar increase in [Ca2□]i as in PGE2-treated cells.

Phosphorylation of IP_3 receptors by PKA is considered an important locus for cross talk between PLC-IP_3-Ca^{2+} and cAMP-PKA pathways and has been put forward as having major functions in diverse Ca^{2+}-regulated events, such as neural activity, epithelial cell fluid secretion, and modulation of insulin secretion (11, 13, 21, 37, 46). For this reason, the effect of butaprost on $[Ca^{2+}]_i$ was also tested. Interestingly, the results revealed a significant increase in this variable (Fig. 6*C*), an effect that was mimicked by forskolin (adenylate cyclase activator) and prevented, in both cases, by dantrolene. The addition of KT5720 (PKA inhibitor) to butaprost and to PGE_2 was also tested and, after 5-min treatment, $[Ca^{2+}]_i$ was reduced by 84 and 64%, respectively (data not shown).

The participation of both transduction pathways in PGE_2-induced epithelial barrier disruption was also tested. The re-sults revealed that the increase in D-mannitol fluxes and the reduction in TER induced by PGE_2 were prevented by U73122, dantrolene, and Gö6983 (PKC inhibitor; Fig. 7, *A* and *B*). Moreover, IP_3 and diC8 (a DAG analog) significantly increased D-mannitol fluxes and reduced TER values.

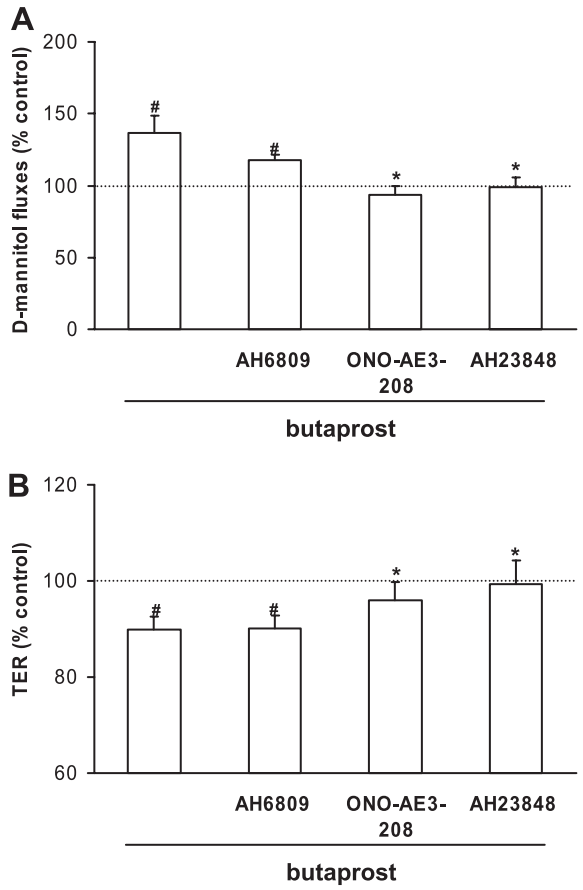

Fig. 4. Effect of EP_2 and EP_4 antagonists on epithelial barrier disruption induced by butaprost. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with butaprost (250 nM) and butaprost plus AH-6809 (3×10^3 nM), plus ONO-AE3-208 (2 nM), or plus AH-23848 (200 nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.7 ± 0.3 fmol/cm^2 and 295 ± 28.4 $\Omega \cdot$cm^2). The data were means $\pm$ SE of $n = 6$–12. $^*P < 0.05$ vs. butaprost; $\#P < 0.05$ vs. control

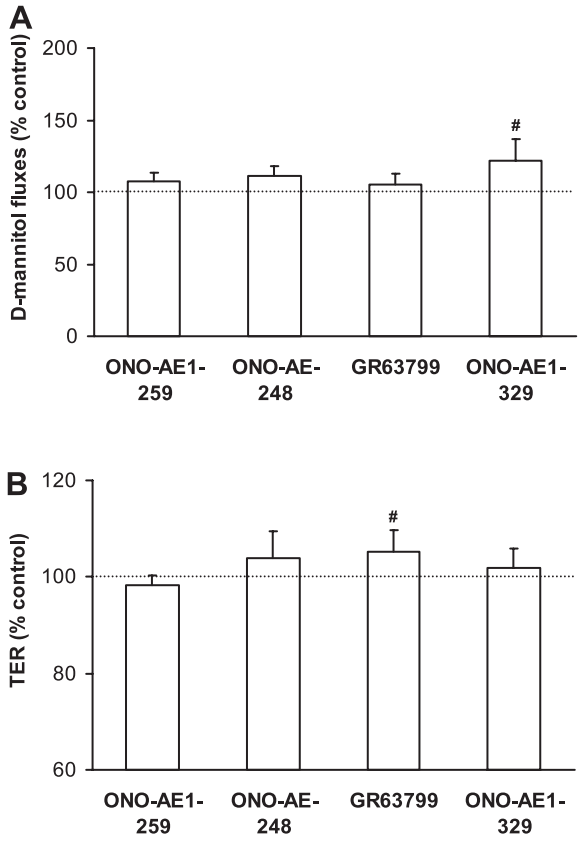

Fig. 5. Effect of EP_2, EP_3, and EP_4 agonists on paracellular permeability (PP). D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with ONO-AE1-259 (10^4 nM), ONO-AE-248 (10^4 nM), GR63799 (2×10^3 nM), or ONO-AE1-329 (10^4 nM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.2 ± 0.2 fmol/cm^2 and 254 ± 11 $\Omega \cdot$cm^2). The data were means $\pm$ SE of $n = 5$–11. $\#P < 0.05$ vs. control.

Regarding EP_4 participation, the effect of butaprost on D-mannitol fluxes was also prevented by dantrolene, although no effects on TER values were detected (Fig. 8, *A* and *B*). Moreover, PGE_2 effects on both D-mannitol fluxes and TER were prevented by the addition of SQ22,536 (adenylate cyclase inhibitor) and KT5720, thus suggesting cAMP involvement associated with Ca^{2+} mobilization. Forskolin also induced a significant increase in PP, an effect again prevented by the addition of dantrolene.

The contribution of TJ proteins and cytoskeletal actin to PP regulation by PGE_2 was also investigated. The results of TJ protein immunofluorescent staining show in control conditions occludin and ZO-1 mainly located at the cell border (Fig. 9). The treatment of Caco-2 cell monolayers with PGE_2, carbacy-clin, or butaprost resulted in a redistribution of occludin with adjacent diffuse intracellular staining and granular appearance, mainly in carbacyclin-treated cells. The effect on ZO-1 loca-tion was not so pronounced; only the presence of cytosolic diffuse fluorescence was detected in carbacyclin- and butap-rost-treated cells. Morphological assessment of subapical actin showed characteristic perijunctional rings in control monolayers.

Treatment with PGE_2 induced a complete disorganization of the F-actin belt. In the case of carbacyclin and butaprost, the images revealed a general reduction in fluorescent signal, more pronounced in the case of carbacyclin, showing brighter foci alternating with areas of reduced labeling, accompanied by the presence of adjacent diffuse intracellular fluorescent material. Myosin light chain (MLC) kinase (MLCK) activation in-duces the phosphorylation of the regulatory MLC and thus the contraction of the subapical actomyosin ring leading to an increase in PP (29). The results shown in Table 2 revealed that ML-7, an MLCK inhibitor, completely prevented the effects of PGE_2 and butaprost on D-mannitol fluxes and TER, thus suggesting MLCK involvement.

DISCUSSION

Cultures of differentiated Caco-2 cells form a highly polar-ized epithelium with many of the properties of the intestinal villous absorptive cells and constitute an in vitro experimental model, currently used to evaluate intestinal epithelial PP. Human colon cancer cells express EP receptors. In this sense, Shoji et al. (43) analyzed EP_1–EP_4 expression in different cell lines including Caco-2 cells and observed the presence of EP_1, EP_2, and EP_4 in most of them. Accordingly, Löffler et al. (27)

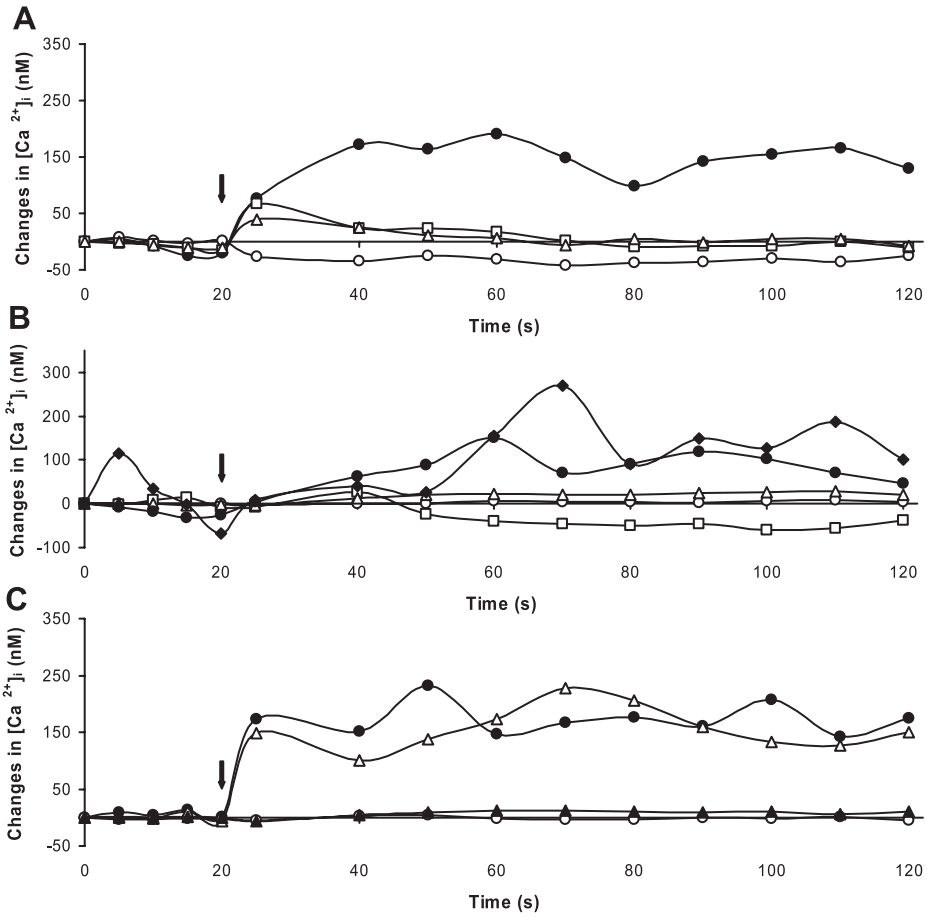

Fig. 6. Changes in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) induced by PGE_2 and PGE_2 agonists. Changes in $[Ca^{2+}]_i$ were determined in differentiated Caco-2 cell monolayers using fura-2 AM, as described under MATERIALS AND METHODS. Cells were incubated for 120 s in the presence of *A*: PGE_2 (●; 13 μM) and PGE_2 plus ONO-8711 (□; 250 nM), plus U73122 (△; 0.1 μM), or plus dantrolene (○; 50 μM); *B*, inositol trisphosphate (IP_3; ◆; 45 μM), carbacyclin (●; 70 μM), carbacyclin plus ONO-8711 (□; 250 nM), plus U73122 (△; 0.1 μM), or plus dantrolene (○; 50 μM); and *C*: butaprost (●; 250 μM), butaprost plus dantrolene (○; 50 μM), forskolin (△; 450 μM), or forskolin plus dantrolene (▲; 50 μM). Each plot corresponds to a representative profile obtained for $n = 3$. The arrow indicates the injection of PGE_2 or PGE_2 agonist, IP_3, or forskolin. Antagonists and inhibitors were preincubated for 30 min.

detected the expression of these EP receptors in intestinal HT-29 cells. Here, we demonstrate the presence of EP_1–EP_4 receptors in differentiated Caco-2 cells, suggesting a higher basolateral localization. Moreover, EP receptors were described to be involved in the regulation of various cellular processes in Caco-2 cells (cell proliferation and adhesion, interleukin synthesis, and PP) (15, 26, 27, 31, 43, 45).

The first step to investigate the involvement of PGE_2 recep-tors in the regulation of epithelial barrier function was to test the capacity of different receptor antagonists to prevent PGE_2 effects. ONO-8711 and SC-19220 are specific antagonists for EP_1, and AH-23848 is for EP_4; therefore, their capacity to prevent PGE_2 epithelial barrier disruption was considered the first proof of EP_1 and EP_4 participation in these events. The results obtained with AH-6809 also suggest the participation of EP_1, although this antagonist has greater affinity for EP_2. Moreover, the capacity of ONO-AE3-240 and ONO-AE3-208 to prevent PGE_2 effects may indicate the participation of EP_3 and/or EP_4. Thus our first hypothesis was to consider the participation of at least EP_1 and EP_4. Nevertheless, the cross-reactivity of some of the antagonists used led us to test the effect of these antagonists on changes in PP induced by other, more specific agonists than PGE_2. Carbacyclin and sulprostone were used to test the contribution of EP_1 and EP_3, and butap-rost of EP_2 and EP_4. The results obtained show the capacity of ONO-8711 and SC-19220 to prevent both carbacyclin and sulprostone effects, and no changes were detected for ONO-AE3-240, which in this case only interacts with EP_3. These results may allow both the rejection of EP3 involvement and confirmation of EP1 contribution.

As for butaprost results, ONO-AE3-240 and AH-23848 were found to prevent the changes induced by PGE_2 in PP, which remained unchanged with AH-6809 (used in this case as an EP_2 antagonist), thus suggesting the lack of EP_2 participation while confirming EP_4 involvement. The participation of EP_4 and the lack of EP_2 and EP_3 involvement were further confirmed with specific receptor agonists such as ONO-AE1-259 for EP_2, ONO-AE-248 and GR63799 for EP_3, and ONO-AE1-239 for EP_4.

Recently, Tanaka et al. (45) investigated the mechanisms underlying the effect of PGE_2 on PP, but they did so in nondifferentiated Caco-2 cells, using a high PGE_2 concentra-tion that is hard to reach in physiological/pathological condi-tions. They conclude that EP_1 and EP_2 were involved in PGE_2 effects from the results obtained with a specific EP_1 agonist and butaprost, respectively. However, they did not consider that butaprost is also an EP_4 agonist (44), and they did not test any PGE_2 antagonist. Therefore, these data in addition to our results support EP_1 participation and the lack of EP_3 contribu-tion, but different conclusions can be drawn concerning EP_2 and/or EP_4 involvement.

We must consider that PP change induced by PGE_2 could be consequence of PGE_2 binding to other prostanoid receptors such as DP, FP, IP, and TP. In this sense, PGD_2 is a prostanoid showing higher affinity for DP and FP receptors than PGE_2 (9, 39, 40), very low and similar affinity as PGE_2 for IP and TP receptors (33, 39), and very low affinity for EP receptors (39,44). However, we have observed that PGD2 did not have any effect either on D-mannitol fluxes (control: 100 □ 7.7, PGD2: 116.4 □ 22.9%, n 4–5; P 0.05) or TER (control: 100 □ 1.6, PGD2: 101.4 □ 10.2%, n 4–5; P 0.05).

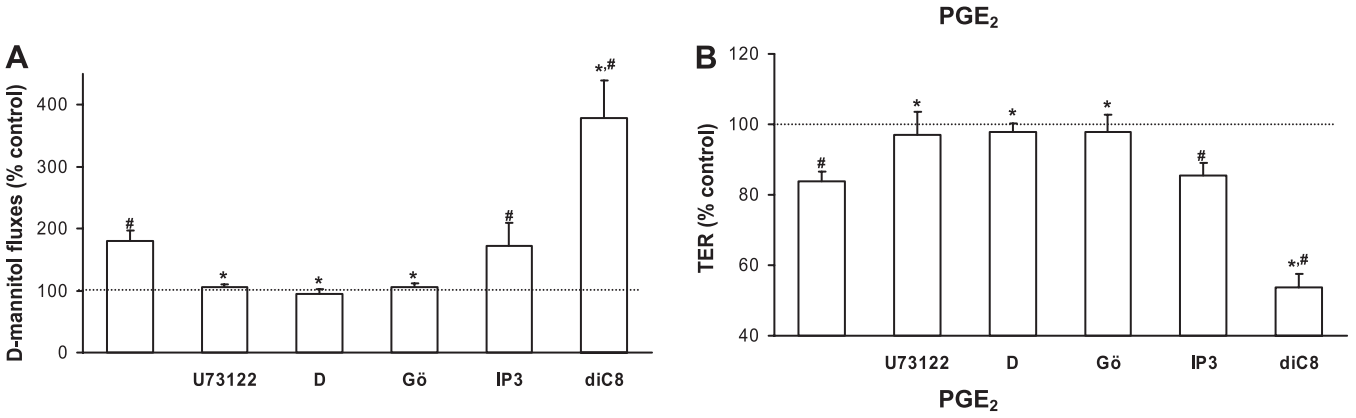

Fig. 7. PLC-IP_3-Ca^{2+}-PKC pathway involvement in epithelial barrier disrup-tion induced by PGE_2. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with PGE_2 (3 nM) or PGE_2 plus U73122 (0.1 μM), plus dantrolene (D; 50 μM), or plus Gö6983 (Gö; 1 μM), IP_3 (18 μM), or dioctanoylglycerol (diC8; 0.5 mM) in the apical and basolat-eral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (4.0 ± 0.24 fmol/cm^2 and 302 ± 47 Ω·cm^2). The data were means ± SE of n = 5–11. *$P < 0.05$ vs. PGE_2; #$P < 0.05$ vs. control.

Consequently, these findings report that, in our experimental condi-tions, a DP/FP/IP/TP agonist such as PGD_2 did not modify PP, suggesting that the effect of PGE_2 on PP is not mediated by its interaction with the above mentioned prostanoid receptors.

The participation of EP_1 and EP_4 in epithelial barrier func-tion regulation was further investigated through the study of intracellular signaling pathways, especially changes in $[Ca^{2+}]_i$. As expected from PGE_2 interaction with EP_1 receptor, an increase in $[Ca^{2+}]_i$ was observed after the treatment of the cells with this prostanoid, an effect that was prevented by an EP_1 antagonist. The results also indicate the participation of the PLC-IP_3-Ca^{2+} signaling pathway, since both $[Ca^{2+}]_i$ and PP were increased by IP_3. Moreover, the increase in $[Ca^{2+}]_i$ and PP induced by PGE_2 and carbacyclin was prevented by an EP_1 antagonist and by the inhibition of PLC or intracellular Ca^{2+} release from the endoplasmic reticulum.

PKC consists of a family of Ser/Thr-specific kinases that includes 12 known isozymes that can be classified into 3 subfamilies:conventional(, 1, 2, and=), novel (δ, ε, θ, η, and μ), and atypical (λ, τ, and ζ). Conventional isoforms are both Ca^{2+}- and DAG-dependent, novel isoforms are Ca^{2+}-independent but DAG-dependent, whereas atypical PKC isoforms are both Ca^{2+}- and DAG-independent (19). Although contradictory results are reported on PKC contribution to the regulation of epithelial barrier function, it is accepted that conventional isoforms participate in TJ disassembly, whereas novel isoforms regulate TJ formation (4). The results reported here indicate that the increase in PP induced by PGE_2 was prevented by Gö6983, which is described as a pan-PKC inhib-itor (19). Given the results obtained with dantrolene, indicating the contribution of Ca^{2+} to PKC activation and the capacity of IP_3 and diC8 to increase PP, the participation of a conventional PKC isoform in the disruption of epithelial barrier function by PGE_2 should also be considered.

As for EP_4 underlying intracellular mechanisms, the protec-tive effect detected by adenylate cyclase and PKA inhibition confirm the expected participation of the cAMP-PKA pathway. Nevertheless, a significant increase in $[Ca^{2+}]_i$ was observed after butaprost incubation, an effect that was mimicked by forskolin and prevented, in both cases, by the addition of dantrolene, thus suggesting the involvement of intracellular Ca^{2+} stores. Interestingly, the profile of $[Ca^{2+}]_i$ changes shows a rapid and sustained increase for PGE_2, butaprost, and fors-kolin, whereas a more delayed peak was observed for carba-cyclin and IP_3. Therefore, the profile of $[Ca^{2+}]_i$ changes is different, whether cAMP is involved or not.

Recently, several authors described the cross-talk relation-ship between IP_3 receptors/Ca^{2+} release and the cAMP-PKA signaling pathway. Phosphorylation of IP_3 receptors by PKA is the locus where these two signal transduction pathways converge and is involved in diverse Ca^{2+}-regulated physiological processes, such as neuronal activity, epithelial fluid secretion, and modulation of insulin secretion (11, 13, 21, 37, 46). These authors have demonstrated that PKA phosphorylation results in a significant enhancement of IP_3-induced $[Ca^{2+}]_i$ from either extracellular or intracellular origins. Chaloux et al. (13) showed that PKA enhances IP_3-induced Ca^{2+} release in endo-crine cells by increasing IP_3 binding affinity. More recently, Wagner et al. (50) demonstrated that PKA phosphorylation increases the sensitivity of the IP_3 receptor to IP_3. They explained this effect as the ability of IP_3 to gate the channel by increasing Ca^{2+} sensitivity of the receptor. Thus our results show the capacity of butaprost and forskolin to increase $[Ca^{2+}]_i$ in the absence of any IP_3-stimulating Ca^{2+} release stimulus. In line with this, Chaloux et al. (13) found that forskolin also increased $[Ca^{2+}]_i$ in the absence of any Ca^{2+}-mobilizing agonist. They ascribed this phenomenon to the ability of basal intracellular levels of IP_3 to simulate IP_3 receptors. Therefore, this effect was exerted by low IP_3 doses and, accordingly, they found that a maximum IP_3 dose was unable to modify Ca^{2+} release. Thus our hypothesis to explain the capacity of butaprost and forskolin to enhance $[Ca^{2+}]_i$ is that basal IP_3 levels in Caco-2 cell monolayers induce Ca^{2+} release under conditions in which cAMP levels were increased. This proposed mechanism may explain the ability of dan-trolene to prevent the increase in PP induced by PGE_2, butap-rost, and forskolin. These results corroborate those of Gu et al.(21), who also described such an effect for PGE_2 in cultured rat vagal sensory neurons. Therefore, the effect of PGE_2 on PP mediated through EP_4 interaction may result, in Caco-2 cells, in increased $[Ca^{2+}]_i$ strengthened by cAMP formation, thus

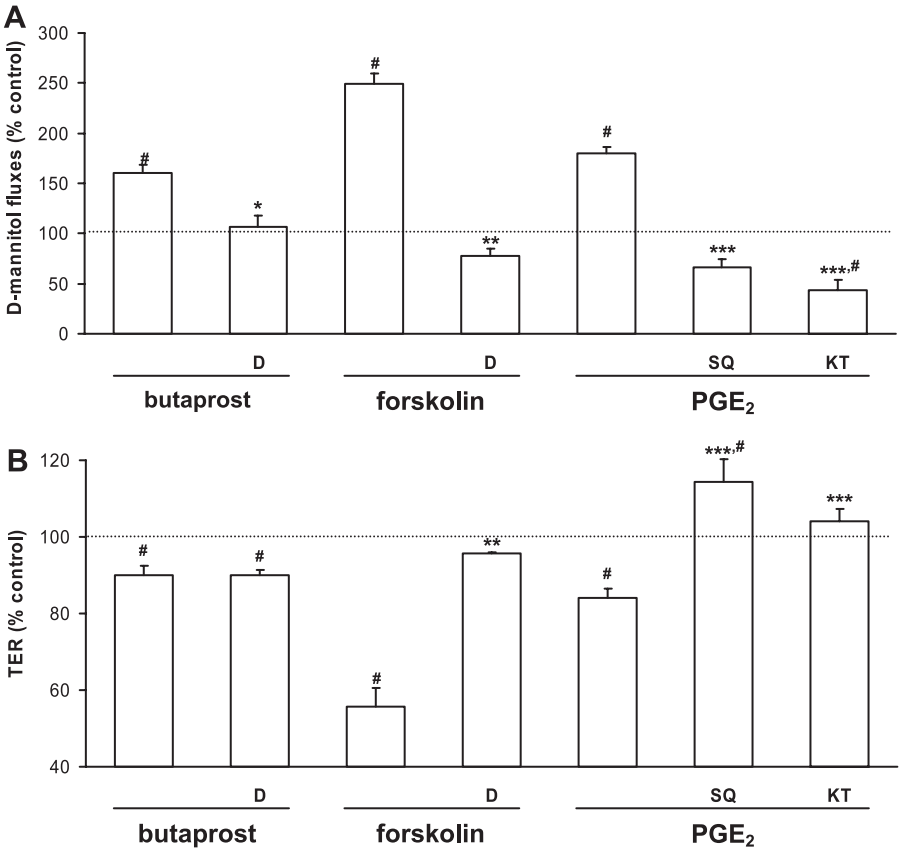

Fig. 8. cAMP-PKA pathway involvement in epithelial barrier disruption induced by PGE_2. D-mannitol fluxes (*A*) and TER (*B*) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with butaprost (250 nM), butaprost plus dantrolene (D; 50 μM), forskolin (10 μM), forskolin plus dantrolene (D; 50 μM), PGE_2, or PGE_2 plus SQ22,536 (SQ; 10 μM) or plus KT5720 (KT; 10 μM) in the apical and basolateral compartments. Results were expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.0 ± 0.28 fmol/cm^2 and 456 ± 24.3 Ω·cm^2). The data were means ± SE of $n = 4$–7. *$P < 0.05$ vs. butaprost; **$P < 0.05$ vs. forskolin; ***$P < 0.05$ vs. PGE_2; #$P < 0.05$ vs. control.

confirming the cross talk interaction of these two signaling pathways in the intestinal epithelium.

Previous studies indicated that MLCK plays an important role in the regulation of intestinal TJ permeability. The increase in PP induced by TNF-, IFN-=, IL-1, bile acids, cytochalasins, ethanol, Na^+-glucose transport, and extracellu-lar Ca^{2+} has been described as mediated by increased MLCK activity (2, 6, 28, 41, 48, 53). Moreover, the overexpression of MLCK in Caco-2 cells induces the reorganization of perijunc-tional actin and increases PP (41). In fact, MLCK expression is significantly enhanced in the mucosa of IBD patients (8). In addition, the protective action of glucocorticoids on epithelial barrier function has been ascribed to the suppression of TNF--induced increase in MLCK activity (10). Our results show-ing the ability of an MLCK inhibitor to prevent the effects induced on both D-mannitol fluxes and TER by PGE_2 and butaprost confirm these data. Since MLCK is activated by the Ca^{2+}-calmodulin complex (19), these results also confirm the above data by suggesting that the butaprost effect may be mediated by an increase in $[Ca^{2+}]_i$. In this respect, the reduc-tion in TER induced by bile acids in Caco-2 cells through increased MLCK activity was described as mediated at least in part by an increase in cyclooxygenase and PKC activities (6). It is not the first time that a relationship between PKC and MLCK has been posited in TER regulation, but different conclusions were drawn. Turner et al. (47) found in Caco-2 cells that MLCK can be inhibited via PKC phosphorylation, thus reducing MLC phosphorylation and increasing TER. In contrast, the increase in PP induced in T-84 cells by infection with enterohemorrhagic *Escherichia coli* and in brain endothelial cells with HIV-1 envelope glycoprotein gp120 was descirbed by a process that includes the activation of both PKC and MLCK (24, 35), as appears to be the case of PGE_2 in our experimental conditions.

Confocal microscopy findings revealed an alteration in oc-cludin, ZO-1, and actin distribution. ZO-1 constitutes the bridge between the perijunctional actin ring and TJ occludin (17). Therefore, a correlation between the disturbance of peri-junctional actin filaments and occludin and ZO-1 location may be established. Nevertheless, ZO-1 is the TJ protein that shows the fewest alterations in distribution; the main change observed after incubation with carbacyclin and butaprost was the pres-ence of cytosolic fluorescence. Similarly, Bruewer et al. (12) detected that, despite the dramatic redistribution of TJ trans-membrane proteins following IFN-=exposure, ZO-1 was only minimally affected, and most of it remained at the TJ. More-over, Tanaka et al. (45) found that the incubation of nondif-ferentiated Caco-2 cells with PGE_2 significantly affected E-cadherin, -catenin, claudin-1, and occludin distribution but was unable to alter ZO-1 location. Regarding the formation of fluorescent clumps, Ma et al. (28) attributed their formation in Caco-2 cells treated with ethanol to a multifocal aggregation of cytoskeletal elements, including actin. They also suggested a central role for actin-myosin contraction in the formation of these aggregates (29). In this respect, the presence of cytosolic occludin is associated with protein internalization by endocy-tosis. In the case of barrier loss induced by TNF-, occludin internalization was described as a MLCK-dependent process (38). The results obtained here in Caco-2 cells indicate that the increase in PP may correlate with the redistribution of TJ

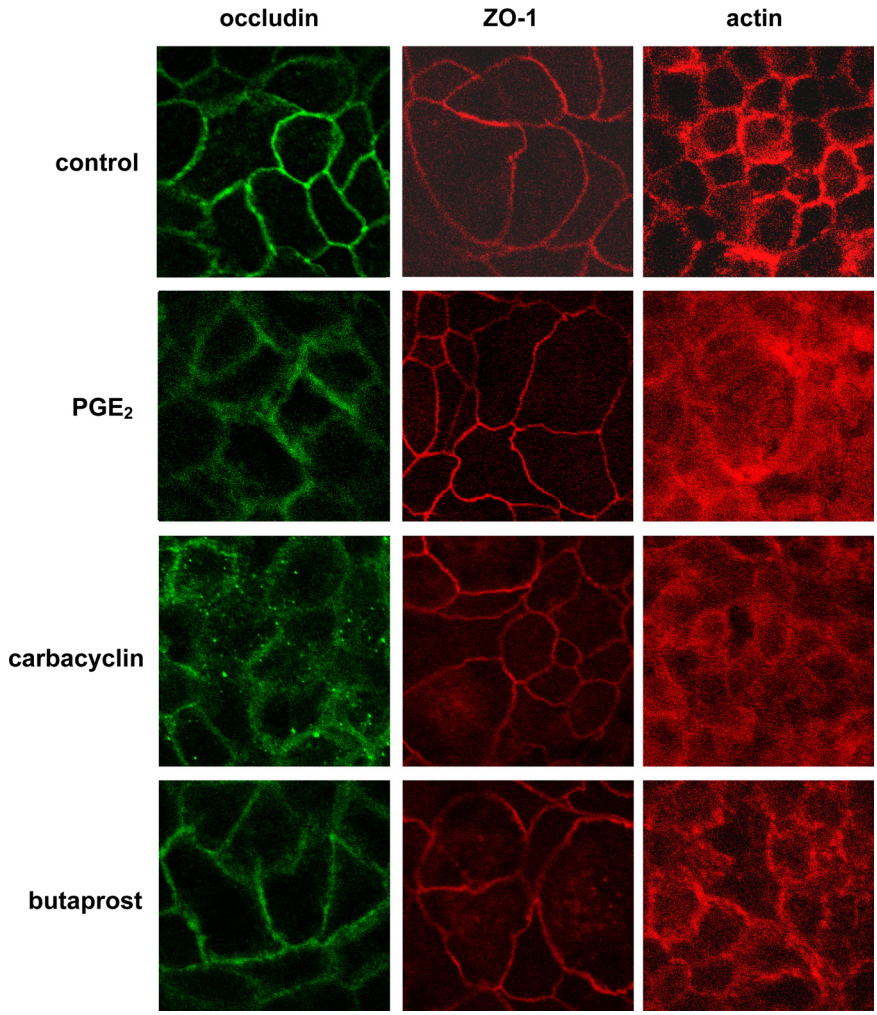

Fig. 9. Changes in occludin, zona occludens 1 (ZO-1), and perijunctional actin distribution induced by PGE_2 agonists. Confocal analysis was performed using specific occludin and ZO-1 antibodies and phalloidin-tetramethylrhodamine B isothiocyanate (TRITC-phalloidin), as described under MATERIALS AND METHODS, in cells incubated for 3 h with PGE_2 (3 nM), carbacyclin (300 nM), or butaprost (250 nM). In each case, a representative *x-y* image of sections close to the apical cell side is shown.

proteins (mainly occludin), the perijunctional actin ring, and MLCK activation.

All the results obtained lead us to conclude that the regulation of the epithelial barrier function by PGE_2 is mediated by interaction with EP_1 and EP_4 receptors, which activate PLC-IP_3-Ca^{2+} and cAMP-PKA pathways, respectively, and lead to a common increase in $[Ca^{2+}]_i$. Moreover, PKC and MLCK may be involved in the regulation of PP by PGE_2. Furthermore, these events are accompanied by the redistribution of TJ

Table 2. *Effect of ML-7 on changes induced by PGE_2 or butaprost on D-mannitol fluxes and TER*

	D-Mannitol Fluxes, % Control	TER, % Control
PGE_2	162.7 ± 24.3‡	84 ± 2.6‡
PGE_2 + ML-7	77.4 ± 18.6*	100.2 ± 4.6*
Butaprost	136.9 ± 11.9‡	89.1 ± 3.57‡
Butaprost + ML-7	89.5 ± 35.9†	111.1 ± 3.4†

Values are means ± SE of n = 3–5. D-Mannitol fluxes and transepithelial electrical resistance (TER) were determined in differentiated Caco-2 cell monolayers, as described under MATERIALS AND METHODS. Cells were incubated for 3 h with PGE_2 (3 nM), PGE_2 plus myosin light chain kinase inhibitor ML-7 (50 μM), butaprost (250 nM), or butaprost plus ML-7 (50 μM). Results are expressed as the percentage of D-mannitol fluxes and TER values obtained in control conditions (3.5 ± 0.4 fmol/cm^2 and 326 ± 66 Ω·cm^2). *$P < 0.05$ vs. PGE_2; †$P < 0.05$ vs. butaprost; ‡$P < 0.05$ vs. control.

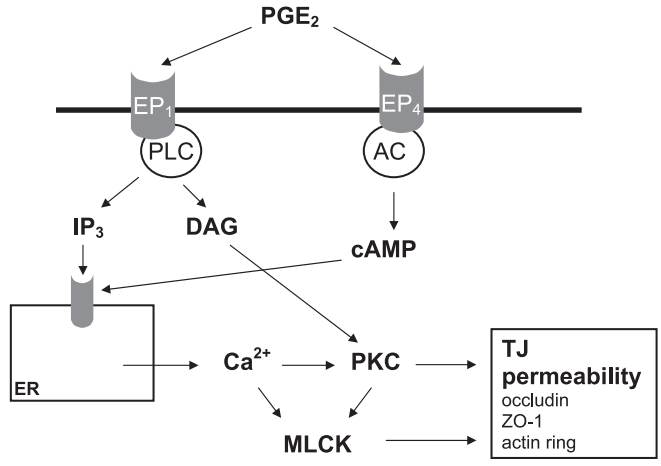

Fig. 10. Mechanisms involved in the regulation of PP by PGE_2. PGE_2 interaction with EP_1 activates PLC, which induces IP_3 and diacylglycerol (DAG) formation. IP_3 induces Ca^{2+} release from endoplasmic reticulum (ER). Moreover, PGE_2 binding to EP_4 induces cAMP synthesis by adenylate cyclase (AC). The enhancement of cAMP levels stimulates IP_3-induced Ca^{2+} release. These events are involved in PP enhancement associated with an increase in myosin light chain kinase (MLCK) activity and changes in the distribution of the tight junction (TJ) proteins, occludin and ZO-1, and the perijunctional actin ring.

proteins and the perijunctional actin ring (Fig. 10). Although the disruption of epithelial barrier function observed in IBD patients has been traditionally attributed to the effect of TNF-α and IFN-γ, the present study goes deeper into the function of PGE_2 in the inflammatory process, thus opening up new therapeutic strategies.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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