Bryostatin-1 enhances barrier function in T84 epithelia through PKC-dependent regulation of tight junction proteins

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Yoo, James, Anthony Nichols, Joshua Mammen, Isabel Calvo, Jaekyung C. Song, Roger T. Worrell, Karl Matlin, and Jeffrey B. Matthews. Bryostatin-1 enhances barrier function in T84 epithelia through PKC-dependent regulation of tight junction proteins. Am J Physiol Cell Physiol 285: C300–C309, 2003. First published March 26, 2003; 10.1152/ajpcell.00267.2002.—Protein kinase C (PKC) is known to regulate epithelial barrier function. However, the effect of specific PKC isozymes, and their mechanism of action, are largely unknown. We determined that the nonphorbol ester PKC agonist bryostatin-1 increased transepithelial electrical resistance (TER), a marker of barrier function, in confluent T84 epithelia. Bryostatin-1, which has been shown to selectively activate PKC-α, -ɛ, and -δ (34), was associated with a shift in the subcellular distribution of the tight junction proteins claudin-1 and ZO-2 from a detergent-soluble fraction into a detergent-insoluble fraction. Bryostatin-1 also led to the appearance of a higher-molecular-weight form of occludin previously shown to correspond to protein phosphorylation. These changes were attenuated by the conventional and novel PKC inhibitor Gö-6850 but not the conventional PKC inhibitor Gö-6976 or the PKC-δ inhibitor röttlerin, implicating a novel isozyme, likely PKC-ɛ. The results suggest that enhanced epithelial barrier function induced by bryostatin-1 involves a PKC-ɛ-dependent signaling pathway leading to recruitment of claudin-1 and ZO-2, and phosphorylation of occludin, into the tight junctional complex.

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bly depending on the cell type and conditions of activation. It is now recognized that specific PKC isoforms can affect the same biological function in either a similar or opposite (counterregulatory) fashion. The pattern of selectivity for target proteins may reflect association of the particular isoform with specific anchoring proteins or other protein-protein interactions.

In the present study we used the human T84 intestinal cell line to study the role of PKC in the regulation of barrier function and subcellular distribution of tight junction proteins. The T84 cell line has been used extensively to study the regulation of epithelial barrier function (3, 21, 23, 28, 30, 31, 39, 44). T84 cells are polarized intestinal epithelial crypt cells that display high TER and have well-developed tight junction structures (31). It was shown previously that prolonged stimulation of PKC by the phorbol ester PMA reduces TER and induces junction disassembly, an effect that appears to be due to sustained activation of PKC-α (5, 34). In the present study, we found that bryostatin-1, a novel nonphorbol PKC agonist derived from a marine sponge, instead increases TER. We found that bryostatin-1, likely through PKC-δ, induces occludin phosphorylation and biochemically defined redistribution of several protein components of the tight junction.

**MATERIALS AND METHODS**

**Cell culture.** T84 cells obtained from American Type Culture Collection (Rockville, MD) were grown in a 5% CO₂ humidified incubator at 37°C on 162-cm² flasks (Corning Costar, Acton, MA) with medium containing a 1:1 mixture of F-12 nutrient mixture (Ham) and Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 6% heat-inactivated fetal bovine serum, 15 mM HEPES, 14.3 mM NaHCO₃, and antibiotics and antimycotics (100 U/ml aqueous penicillin G, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B) at a pH of 7.4. Cells were passaged weekly on reaching confluence. For experiments, cells were plated onto collagen-coated permeable supports, where they were fed every 3 days, maintained until steady-state TER was achieved, and used from days 7–14.

**TER measurements.** Dual voltage-current clamp and apical and basolateral Ag-AgCl and calomel electrodes interfaced with “chopstick” KCl-agar bridges were used to assess TER in confluent monolayers grown on collagen-coated 0.33-cm² Transwell inserts. TER measurements have been used as a measure of paracellular permeability and barrier function in confluent T84 monolayers (17, 34). Baseline levels of TER in confluent T84 monolayers generally exceed 1,000 Ω·cm².

Confluent T84 monolayers were equilibrated in HEPES-phosphate-buffered Ringer solution [HPBR; containing (in mM) 135 NaCl, 5 KCl, 3.33 NaHPO₄, 1 CaCl₂, 1 MgCl₂, 10 glucose, and 5 HEPES at pH 7.4] for 30 min before further treatment. Bryostatin-1 (100 nM) was applied to the basolateral compartment of confluent T84 monolayers, and TER was measured over a 4-h time course. Selective PKC inhibitors (Gö-6855, 5 μM; Gö-6976, 5 μM; röttlinerin, 10 μM) were added 30 min before treatment with bryostatin-1.

**Triton X-100-soluble and -insoluble fractions.** Triton X-100-soluble and -insoluble fractions are operational definitions that have been used to biochemically define the localization of tight junction proteins, and this method has been used in a number of studies (1, 3, 30, 31, 44). Proteins found in the Triton X-100-insoluble fraction have been associated with the tight junction complex.

T84 monolayers grown on 4.7-cm² Transwell inserts were washed twice in ice-cold PBS and then lysed with a 1% Triton X-100-based lysis buffer (1% Triton X-100, 50 mM Tris·HCl, pH 7.5, 140 mM EGTA, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, and complete protease inhibitor cocktail tablets). The supernatant was completely removed and was designated the Triton X-100-soluble fraction. The remaining filter-associated cellular residue was solubilized with heated (95°C) 1% SDS-based lysis buffer (1% SDS, 50 mM Tris·HCl, pH 7.5, 140 mM EGTA, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, and complete protease inhibitor cocktail tablets). The cells were scraped from the filter with a rubber policeman, collected, and heated at 95°C for 5 min followed by brief sonication. This was designated the Triton X-100-insoluble fraction. Protein concentrations were measured by the Lowry method, and protein concentrations in Triton X-100-soluble and -insoluble fractions were equalized separately.

**Ca²⁺ switch.** Confluent T84 monolayers grown on collagen-coated 0.33-cm² Transwell inserts were equilibrated in HPBR. After a 30-min equilibration period, apical and basolateral buffer was switched to HPBR containing no Ca²⁺ with EGTA (2 mM). Buffer was later switched back to Ca²⁺-containing HPBR in the absence of EGTA. TER was measured over time. T84 monolayers grown on collagen-coated 4.7-cm² Transwell inserts were used for parallel Western blotting experiments to assess occludin in both Triton X-100-soluble and -insoluble fractions.

**Immunoprecipitation.** Confluent T84 monolayers on 4.7-cm² Transwell inserts were treated with bryostatin-1 (100 nM) for up to 4 h followed by two washes in ice-cold PBS. Extraction of Triton X-100-soluble proteins occurred by a 30-min incubation with lysis buffer containing 1% Triton X-100, 50 mM Tris·HCl, pH 7.5, 140 mM EGTA, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, and complete protease inhibitor cocktail tablets. The resulting extract was completely aspirated and constitutized the Triton X-100-soluble fraction. The remaining filter-associated cellular residue was next used for the extraction of Triton X-100-insoluble proteins, which were added by 0°C lysis buffer containing 1% SDS, 50 mM Tris·HCl, pH 7.5, 140 mM EGTA, 30 mM sodium pyrophosphate, 50 mM NaF, 100 μM Na₃VO₄, and complete protease inhibitor cocktail tablets. The filters were scraped with a rubber policeman, heated at 95°C for 5 min, and sonicated. Samples were normalized to a concentration of 1.25 mg/ml and were incubated overnight at 4°C in the presence of monoclonal antibody to occludin. The Triton X-100-insoluble proteins were diluted 1:5 with 1% Triton X-100 lysis buffer to avoid degradation of antibody. Immune complexes were precipitated with protein A agarose beads (2-h incubation) and washed three times, Laemmli sample buffer with 0% β-mercaptoethanol was added, and samples were boiled for 5 min. Supernatants were subjected to one-dimensional SDS-PAGE, and gels (8%) were blotted with phosphospecific antibodies.

**Gel electrophoresis and Western blotting.** Samples were loaded at equal concentrations as determined by the Bradford assay after addition of Laemmli sample buffer containing 10% β-mercaptoethanol and boiling for 5 min. Proteins were separated by electrophoresis on 8–12% gels and transferred on nitrocellulose membranes, followed by a 1-h incubation at room temperature in blocking buffer containing 20 mM Tris (pH 7.5), 500 mM NaCl, 5% nonfat dry milk, 0.2% Tween 20, a 1-h incubation with blocking buffer containing primary antibody, a 30-min rinse in wash buffer (20 mM Tris,
pH 7.5, 500 mM NaCl, 0.2% Tween 20), a 1-h incubation with blocking buffer containing secondary antibody, and another 30-min rinse in wash buffer. Bands were detected with enhanced chemiluminescence (ECL) detection reagents.

**Materials.** Transwell inserts were purchased from Corning Costar. Bryostatin-1 was obtained from Biomol (Plymouth Meeting, PA). Tissue culture reagents and agarose beads were purchased from Life Technologies (Gaithersburg, MD). Gel electrophoresis and gel blotting reagents were purchased from Bio-Rad (Hercules, CA). Antibodies to ZO-2, occludin, phosphoserine, phosphotyrosine, and claudin-1, -2, -3, and -5 were purchased from Zymed (San Francisco, CA). Anti-phosphotyrosine antibody and antibody to ZO-1 were purchased from Transduction Laboratories (Lexington, KY).

**Statistical analysis.** Data are expressed as means ± SE. Statistical analysis was performed by Student’s t-test, one-way ANOVA, and Tukey pairwise multiple-comparison test, with P < 0.05 considered statistically significant. All n ≥ 3.

**RESULTS**

Bryostatin-1 increases TER through nPKC activation. Confluent T84 monolayers were treated with bryostatin-1 (100 nM) over a 4-h time period, and TER was measured (Fig. 1A). Bryostatin-1 led to an increase in TER that was statistically significant by 2 h (120 ± 3% control; P < 0.05) and which increased in a time-dependent fashion (at 4 h, 131 ± 4% control; P < 0.05). We showed previously (34) by both subcellular fractionation and Western blot as well as by in vitro kinase assay that bryostatin-1 induces rapid and sustained activation of the δ and ε nPKC isozymes in T84 cells after 30 min. In contrast, activation of the cPKC-α isozyme occurs only after 3–4 h, followed by downregulation (34). This implicates a nPKC isozyme—either ε or δ—in the initial increase in TER evoked by bryostatin-1. Indeed, the effect of bryostatin-1 on TER was blocked by a 30-min pretreatment with the cPKC and nPKC inhibitor Gö-6976 (5 μM; at 4 h, bryostatin-1 + Gö-6976 = 107 ± 3% control, P < 0.05 compared with bryostatin-1 alone) but not by the cPKC inhibitor Gö-6976 (5 μM; at 4 h, bryostatin-1 + Gö-6976 = 142 ± 6% control) (Fig. 1B). Thus, by both time course and selective inhibitor criteria, the effect of bryostatin-1 on TER appears to be due to activation of a nPKC isozyme. Neither Gö-6850 nor Gö-6976 had any effect on baseline resistance. However, the PKC-δ inhibitor rottlerin induced a substantial fall in TER at a concentration we have shown to inhibit PKC-δ but not PKC-ε or PKC-α. Because of this effect, we could not determine whether rottlerin specifically blocked the bryostatin-1 effect and could not, by the inhibitor strategy alone, distinguish between PKC-ε and PKC-δ.

Occludin exists in Triton X-100-soluble and -insoluble fractions. The assembly of structural proteins into the tight junctional complex is a dynamic process that involves changes in their association with components of the cytoskeleton. Biochemically, this association or assembly event can be operationally defined by changes in detergent (Triton X-100) solubility (1, 3, 30, 31, 44).

Occludin has been shown to partition into both Triton X-100-soluble and -insoluble fractions (Refs. 1, 7, 30, 31; Fig. 2A). The Triton X-100-soluble fraction appears to represent cytoplasmic and basolaterally associated forms of occludin, whereas the Triton X-100-insoluble fraction, characterized by higher-molecular-weight (HMW) forms reflecting enhanced occludin phosphorylation, is associated with the tight junction complex (1, 4, 9, 32). Because of these phosphorylated forms, occludin exhibits a molecular weight in the range of ~65–85 on Western blot. Occludin was immunoprecipitated from Triton X-100-soluble and -insoluble fractions followed by Western blot analysis with

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**Fig. 1.** Bryostatin-1 increases transepithelial electrical resistance (TER) in T84 epithelia through novel (n) protein kinase C (PKC) activation. A: confluent T84 monolayers on Transwell supports were treated with bryostatin-1 (Bryo; 100 nM) over 4 h, and TER was measured. Bryostatin-1 treatment led to a time-dependent increase in TER: at 1 h, 106 ± 3% control (P = 0.0945); at 2 h, 120 ± 3% control (P < 0.0001); at 3 h, 125 ± 3% control (P < 0.0001); at 4 h, 131 ± 4% control (P < 0.0001). Data (analyzed by unpaired t-test, 1-way ANOVA, and Tukey pairwise multiple-comparison test) are expressed as means ± SE % control; n = 7 in triplicate. B: effect of bryostatin-1 on TER could be blocked with a 30-min pretreatment with the conventional (c) PKC and nPKC inhibitor Gö-6850 (5 μM) but not by the cPKC inhibitor Gö-6976 (5 μM). Data are expressed as means ± SE % control; n = 3 in triplicate. *P < 0.05.
forms of occludin are primarily phosphorylated on threonine residues and less so on tyrosine residues. By Western blot with antibody to phosphotyrosine, phosphoserine, and phosphothreonine residues. The HMW claudin appears to be phosphorylated predominantly on phosphospecific antibodies. By this method, HMW occludin appears to be phosphorylated predominantly on threonine and tyrosine residues (Fig. 2B).

**Bryostatin-1 treatment is associated with an increase in HMW forms of occludin.** Confluent T84 monolayers were treated with bryostatin-1 (100 nM) over 4 h, and occludin was assessed in both Triton X-100-soluble and -insoluble fractions. Bryostatin-1 treatment of confluent T84 monolayers was associated with an increase in HMW forms of occludin in both the Triton X-100-soluble and -insoluble fractions (Fig. 3A). This effect was evident by 2 h, which correlated with the time course of increase in TER, and was sustained over a 4-h time period. Bryostatin-1 did not affect total protein levels of occludin as assessed by Western blot (data not shown), suggesting that the new HMW forms seen after bryostatin-1 treatment occurred through post-translational modification of occludin. The bryostatin-1-associated increase in HMW occludin was selectively sensitive to PKC inhibitors. These changes were attenuated by the cPKC and nPKC inhibitor Gö-6850 (5 μM) but not by the cPKC inhibitor Gö-6976 (5 μM) or by the PKC-δ-specific inhibitor röttlerin (10 μM) (Fig. 3B). This pattern of inhibitor sensitivity suggests that PKC-ε may be the key PKC isozyme responsible for occludin phosphorylation.

**Bryostatin-1 recruits claudin-1 to Triton X-100-insoluble fraction.** Confluent T84 monolayers were treated with bryostatin-1 (100 nM) for 4 h, and claudin-1, -2, -3, and -5 were assessed in both Triton X-100-soluble and -insoluble fractions. Within the claudin family of proteins we examined, bryostatin-1 selectively affected the biochemical localization of claudin-1 (Fig. 4A). Bryostatin-1 treatment led to a roughly 50% reduction in the amount of claudin-1 found in the Triton X-100-soluble fraction (bryostatin-1 treated = 54 ± 5% control; P = 0.0014; Fig. 5A), with a parallel increase of claudin-1 in the Triton X-100-insoluble fraction (174 ± 13% control; P = 0.0024; Fig. 5B). The distribution of claudin-2, -3, and -5 between Triton X-100-soluble and -insoluble fractions was unaffected by bryostatin-1 treatment.

To establish a time course for the shift in claudin-1, T84 cells were treated with bryostatin-1 (100 nM) and claudin-1 was assessed in Triton X-100-soluble and -insoluble fractions at 1, 2, and 4 h. The shift in claudin-1 occurs somewhere between 2 and 4 h (Fig. 4B), which, like occludin, was consistent in time with the bryostatin-1-induced increase in TER. Bryostatin-1 did not affect total protein levels of claudin-1 as assessed by Western blot (data not shown). The bryostatin-1-induced decrease in Triton X-100-soluble claudin-1 displayed the same pattern of sensitivity to PKC inhibitors (Fig. 5A), being attenuated by the cPKC and nPKC inhibitor Gö-6850 (5 μM; bryostatin-1 + Gö-6850 = 87 ± 4% control, bryostatin-1 alone = 54 ± 5% control; P = 0.0015) but not by the cPKC inhibitor Gö-6976 (5 μM; bryostatin-1 + Gö-6976 = 41 ± 6% control) or the PKC-δ-specific inhibitor röttlerin (10 μM; bryostatin-1 + röttlerin = 37 ± 7% control). Similarly, the parallel increase of claudin-1 in the Triton X-100-insoluble fraction (Fig. 5B) was also blocked by Gö-6850 (bryostatin-1 + Gö-6850 = 98 ± 13% control, bryostatin-1 alone = 174 ± 13% control; P = 0.0042) but not by Gö-6976 (bryostatin-1 + Gö-6976 = 193 ± 22% control) or röttlerin (bryostatin-1 + röttlerin = 214 ± 41% control).
Bryostatin-1 treatment increases ZO-2 in Triton X-100-insoluble fraction.

Confluent T84 monolayers were treated with bryostatin-1 (100 nM) over 4 h, and the tight junction proteins ZO-1 and ZO-2 were assessed in Triton X-100-soluble and -insoluble fractions. Bryostatin-1 did not appear to alter ZO-1 in either Triton X-100-soluble or -insoluble fractions (Fig. 6A). Bryostatin-1 also did not appear to affect the amount of ZO-2 found in the Triton X-100-soluble fraction over the 4-h time course (111 ± 3% control at 4 h; Fig. 6A) but did increase the amount of ZO-2 found in the Triton X-100-insoluble fraction (Fig. 6A) (At 4 h, bryostatin-1-treated = 182 ± 11% control; P = 0.002). Similar to its effects on occludin and claudin-1, this effect was evident after 2 h of bryostatin-1 treatment (Fig. 6B) and was sustained over 4 h, which again correlated in time with the bryostatin-1-induced increase in TER. Similar to occludin and claudin-1, total ZO-2 protein was not...
affected by bryostatin-1 as assessed by Western blot (data not shown). The increase of ZO-2 in the Triton X-100-insoluble fraction (Fig. 7B) was blocked by the cPKC and nPKC inhibitor Gø-6850 (5 μM; bryostatin-1 + Gø-6850 = 111 ± 7% control, bryostatin-1 alone = 182 ± 11% control; P = 0.018) but not by the cPKC inhibitor Gø-6976 (5 μM; bryostatin-1 + Gø-6976 = 190 ± 11% control) or by the PKC-δ-specific inhibitor röttlerin (10 μM; bryostatin-1 + röttlerin = 167 ± 5% control).

**DISCUSSION**

PKC is known to regulate epithelial barrier function (1, 37), although isozyme specificity has not yet been defined. We have demonstrated that activation of

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**Fig. 5.** The shift in claudin-1 is selectively sensitive to PKC inhibitors. Confluent T84 monolayers were treated with bryostatin-1 (100 nM) for 4 h in the presence or absence of various PKC inhibitors. The decrease in Triton X-100-soluble claudin-1 (A) and the parallel increase in Triton X-100-insoluble claudin-1 (B) were both inhibited by Gø-6850 but not by Gø-6976 or röttlerin. Graphs on right depict densitometric analysis. n = 3.

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**Fig. 6.** Bryostatin-1 increases ZO-2 in the Triton-X-100-insoluble fraction. A: confluent T84 monolayers were treated with bryostatin-1 (100 nM) for 4 h, and the tight junction proteins ZO-1 and ZO-2 were assessed in Triton X-100-soluble and -insoluble fractions. Bryostatin-1 did not appear to affect the detergent solubility of ZO-1 but increased the amount of ZO-2 in the Triton-X-100-insoluble fraction. Data shown are representative of multiple experiments (n ≥ 3). B: ZO-2 was assessed in Triton X-100-soluble and -insoluble fractions at various times after treatment with bryostatin-1 (100 nM). The increase in Triton X-100-insoluble ZO-2 was evident after 2 h and was sustained over the 4-h time period. Arrows indicate the increase in ZO-2 seen in the Triton X-100-insoluble fraction after bryostatin-1 treatment. Data shown are representative of multiple experiments (n ≥ 3).
nPKC isoforms by bryostatin-1 increases TER in T84 human intestinal epithelia. This increase in TER was paralleled in time by selective changes in occludin, claudin-1, and ZO-2, whereas the other tight junction proteins studied (ZO-1, claudin-2, -3, and -5) were not affected by bryostatin-1 treatment.

Bryostatin-1 treatment led to time-dependent changes in the phosphorylation of occludin in both Triton X-100-soluble and -insoluble fractions. The phosphorylation of occludin regulates its association with the cytoskeleton or with detergent-insoluble glycolipid rafts (DIGs) (1, 31) and appears to be a key step in tight junction assembly (1, 4, 9, 32). This has been confirmed in Ca2+ switch experiments (data not shown), in which the presence of HMW forms of occludin correlated with increases in TER. Although we were able to show further increases in HMW occludin forms, we were unable to identify increases in phosphorylation from baseline levels on tyrosine or threonine residues with occludin immunoprecipitation and Western blot with phosphospecific antibodies. A more sensitive technique (e.g., metabolic labeling for phosphorylation analysis) is likely required to demonstrate these changes.

Although a role for PKC in the regulation of occludin phosphorylation has been suggested on the basis of earlier studies (4), there have been contradictory findings regarding the phosphorylation state and its relationship to TER (1, 7, 25, 34). It is likely that these seemingly paradoxical findings are due to the simultaneous activation of multiple PKC isoforms that may have opposing effects on tight junction structure and function. It is increasingly well established that different PKC isoforms can exert opposing effects on the same biological function. For example, we previously demonstrated (34) that PKC-α and PKC-ε have opposing effects on Cl− secretion and cytoskeletal structure in the T84 cell line.

The present study used bryostatin-1 to demonstrate an increase in TER associated with occludin phosphorylation and suggests a role for PKC-ε in this response. In contrast, previous work suggests that activation of PKC-α decreases TER. On the basis of these observations, we propose that PKC-α and PKC-ε exert opposing actions on the macroscopic property of junctional permeability (6, 21, 25, 34, 35). Although bryostatin-1 does activate PKC-α, it does so only transiently and after an extended incubation (~4 h) and is followed by rapid downregulation of this isozyme (34). Bryostatin-1, in contrast to PMA, does not induce the long-term junctional disassembly seen with phorbol ester (17) and in fact antagonizes this action, presumably because of its ability to downregulate PKC-α (5). Although the precise mechanism of this PMA/PKC-α effect remains to be determined, we suspect that it is probably unrelated to acute changes in occludin phosphorylation because PMA induces changes in occludin phosphorylation similar to those found here with bryostatin-1 (unpublished observations) and, as in the case for bryostatin-1, this phosphorylation is not sensitive to Go6976 (although the PMA-induced fall in TER is completely blocked by this agent; Ref. 34).

Bryostatin-1 treatment also shifted claudin-1 from the Triton X-100-soluble to the insoluble fraction in a parallel fashion. This apparent shift in the biochemical defined localization of claudin-1 suggests that a cytoplasmic or membrane-associated pool (Triton X-100-soluble fraction) was being mobilized. Claudin-1 contributes to epithelial barrier function (11, 19, 41).

Fig. 7. The increase in Triton X-100-insoluble ZO-2 is selectively sensitive to PKC inhibitors. A: ZO-2 in the Triton X-100-soluble fraction did not appear to be affected by bryostatin-1 treatment (representative Western blot with corresponding densitometric analysis of 3 separate experiments). B: increase in ZO-2 in the Triton X-100-insoluble fraction after bryostatin-1 treatment was inhibited by Go6850 but not by Go6976 or rottlerin (representative Western blot with corresponding densitometric analysis of 3 separate experiments).
and its subcellular distribution can be regulated by PKC (24). Overexpression of claudin-1 in MDCK cells increased TER and decreased paracellular flux to FITC-dextran of different molecular sizes (19). In this MDCK model, claudin-1 and occludin colocalized in the tight junction and in cytoplasmic vesicles, suggesting that these proteins may be processed and targeted to the tight junction together.

Bryostatin-1 also increased the recruitment of ZO-2 to the Triton X-100-insoluble fraction. ZO-2 has also been demonstrated to be a target of phosphorylation by PKC (2). Unlike occludin, phosphorylated ZO-2 may not be entirely targeted to the tight junction but may also appear in non-junction-associated locations (2). It is not known whether bryostatin-1 treatment additionally affects the phosphorylation state of ZO-2 in our system.

Although PKC-ε activation appears to be associated with changes in the subcellular localization and phosphorylation state of several tight junction proteins, the precise mechanism whereby PKC-ε exerts this effect remains unclear. Although our data suggest that the novel isozyme PKC-ε is the key isozyme involved in these changes, this conclusion must be considered tentative because it is largely based on sensitivity to pharmacological inhibitors. A PKC-ε-specific translocation inhibitor peptide failed to reliably block bryostatin-1-induced PKC-ε translocation in T84 cells; antisense oligonucleotide downregulation of PKC-ε was also unsuccessful in this cell line. Our results suggest, but do not prove, that barrier function of epithelia may be dynamically regulated by PKC-ε.

aPKC isozymes such as PKC-ζ have been identified as potential regulators of barrier function because they colocalize with tight junctions in a number of cell lines (MDCK, LLC-PK1, Caco-2, and T84) (6, 8, 38, 43). However, there is no evidence to suggest that PKC-ζ is involved in bryostatin-1-induced tight junction protein regulation in our model system. Bryostatin-1 activates the α, δ, and ε PKC isozymes but does not activate PKC-ζ in our T84 cell line (34). Similarly, the PKC inhibitors used in this study have no inhibitory effect on the aPKC isozymes. Therefore, the specific effects of bryostatin-1 on TER and tight junction protein regulation are not likely to be due to PKC-ζ.

There is some evidence that PKC-ε may also localize to the tight junction in intestinal epithelia (33). We previously demonstrated (35) that in the T84 cell line bryostatin-1-induced activation of PKC-ε leads to its translocation to the basolateral membrane, but we have not yet established colocalization with the tight junction complex. Preliminary colocalization and communoprecipitation studies for PKC-ε with the tight junction proteins occludin and ZO-2 after bryostatin-1 treatment have failed to provide evidence of a direct interaction (unpublished observations). Thus it is unclear whether PKC-ε interacts indirectly via an intermediate kinase or phosphatase or another target. The intermediate signaling pathways following PKC-ε activation have not yet been identified, although the bryostatin-1-induced increase in TER was inhibited by PD-98059 (50 μM), an inhibitor of the p42/p44 MAPK pathway (data not shown). It is possible that PKC-ε acts on tight junction proteins either directly at the tight junction complex or on cytoplasmic and/or membrane-associated pools, which are then targeted to the tight junction complex.

The observation that bryostatin-1 specifically affects occludin, claudin-1, and ZO-2 (and not other protein components) in concert suggests that a specific protein stoichiometry may be required for junctional assembly and proper function. Imbalances in protein components may affect the integrity of this complex, as has been suggested both in overexpression models (27) and with functional loss of one component of the tight junction complex (30). Conversely, in disease states in which tight junction components are disturbed, accumulation and stabilization of one part of this complex may attenuate the degree of tight junction disruption. This was demonstrated in the MDCK line, where constitutively active Rho, which regulates the actin cytoskeleton as well as the phosphorylation state of occludin (15, 18), partially prevented tight junction disruption during ATP depletion (15). Maintaining the molecular organization of both the tight junction strands and the link via ZO family proteins to the actin cytoskeleton is likely to be critical for the functional properties of the tight junction complex.

Most studies concerning the regulation of tight junction proteins have generally fallen into three main categories. Tight junction assembly and disassembly has been studied most extensively with the Ca²⁺ switch model (1, 22, 32, 37). The relative importance of specific tight junction proteins has been studied through deletion, transfected overexpression, or incorporation of a structurally modified protein into the tight junction complex (11, 27, 29). Finally, the modification of tight junction proteins by known toxins or enteropathogenic bacteria has been used to identify important structural regulators of epithelial permeability (3, 23, 30, 36, 44). For example, the Clostridium perfringens enterotoxin has been shown to remove claudin-4 from tight junction strands, which correlated in time with a decrease in tight junction strand number, a decrease in TER, and an increase in paracellular flux (36). Our approach in the present study is novel in that it involves the augmentation of TER in an already confluent epithelial monolayer. Furthermore, although PKC as a broad family of proteins has been identified as an important regulator of epithelial permeability, this is the first study to suggest that a novel isozyme—probably PKC-ε—may play a role in the steady-state regulation of tight junction integrity. We speculate that PKC-ε activation can augment barrier function by enhancing the recruitment of key proteins to the tight junction complex. Also, similar to their mutually antagonistic effects on other biological functions, PKC-ε and PKC-α may also exert opposing effects on epithelial barrier function.
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