Strain induces Caco-2 intestinal epithelial proliferation and differentiation via PKC and tyrosine kinase signals

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Han, Okhee, Guang Di Li, Bauer E. Sumpio, and Marc D. Basson. Strain induces Caco-2 intestinal epithelial proliferation and differentiation via PKC and tyrosine kinase signals. *Am. J. Physiol.* 275 (*Gastrointest. Liver Physiol.* 38): G534–G541, 1998.—Although the intestinal epithelium undergoes complex deformations during normal function, nutrient absorption, fasting, lactation, and disease, the effects of deformation on intestinal mucosal biology are poorly understood. We previously demonstrated that 24 h of cyclic deformation at an average 10% deformation every 6 s stimulates proliferation and modulates brush-border enzyme activity in human intestinal Caco-2 cell monolayers. In the present study we sought potential mechanisms for these effects. Protein kinase C (PKC) activity increased within 1 min after initiation of cyclic deformation, and the PKC-α and -ζ isoforms translocated from the soluble to the particulate fraction. Cyclic deformation also rapidly increased tyrosine kinase activity. Tyrosine phosphorylation of several proteins was increased in the soluble fraction but decreased in the particulate fraction by cyclic deformation for 30 min. Inhibition of PKC and tyrosine kinase signals by calphostin C, G-06967, and erbstatin attenuated or blocked cyclic deformation-mediated modulation of Caco-2 DNA synthesis and differentiation. These results suggest that cyclic deformation may modulate intestinal epithelial proliferation and brush-border enzyme activity by regulating PKC and tyrosine kinase signals.

alkaline phosphatase; dipeptidyl dipeptidase; protein kinase C-α isoform; protein kinase C-ζ isoform; tyrosine phosphorylation

ALTHOUGH THE INTESTINAL mucosa is exposed to complex patterns of physical deformation during peristalsis, villous motility, and diverse disease states (8, 11, 21, 23, 25–28, 33, 35–37), the effects of deformation on intestinal epithelial biology are poorly understood. Because it is difficult to regulate physical deformation in the intestinal mucosa in vivo without altering the entire regulatory milieu, we used a subclone of the human intestinal Caco-2 cell line (29) as a model to study the effects of deformation on the intestinal epithelial cell. These cells spontaneously differentiate at confluence into cells exhibiting many of the morphological and functional characteristics of mature enterocytes and represent a useful in vitro model to study normal human intestinal epithelium (18). To simulate physical deformation, we utilized a computer-regulated deformation apparatus that modulates the physical deformation and relaxation of cultured intestinal epithelial cell monolayers.

We have previously reported that 24-h exposure of human intestinal Caco-2 cells to an average 10% deformation at 10 cycles/min stimulates cell proliferation and modulates brush-border enzyme activity, dipeptidyl dipeptidase (DPDD), and alkaline phosphatase (AKP) (4). The frequency and amplitude of deformation are within the range of deformation on mucosa during peristalsis (28) or villous motility (36). Protein kinase C (PKC) and tyrosine kinase signals are known to regulate important cellular events, including gene expression, proliferation, and differentiation of enterocytes in vivo (10, 19) and in vitro (2, 3, 7). Furthermore, rhythmic deformation of endothelial and smooth muscle cell monolayers has been observed to modulate PKC and tyrosine kinase activity (30, 38).

In this study we therefore sought to determine whether rhythmic deformation modulates PKC and tyrosine kinase activity in Caco-2 cells and whether cyclic deformation-mediated alterations of proliferation and brush-border enzyme activity of Caco-2 cells are regulated by modulating PKC and tyrosine kinase-mediated signals.

MATERIALS AND METHODS

Materials. Tissue culture medium, FCS, phenylmethylsulfonyl fluoride (PMSF), leupeptin, 1-chloro-3-tosylamido-7-amino-L-2-hepatone (TLCK), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), and sodium orthovanadate were obtained from Sigma Chemical (St. Louis, MO). Transferrin, anti-phosphotyrosine peroxidase, biotin-labeled synthetic tyrosine kinase substrates, phosphopeptide standard, piceatannol, 2,2′-azino-bis-(3-ethylbenz-thiazolin-6-sulfonic acid) diammonium salt (ABTS), and streptavidin-coated microtiter plates were purchased from Boehringer Mannheim (Indianapolis, IN). PepTag C1 peptide, phosphatidyl serine, and gel solubilization solution were purchased from Promega (Madison, WI). Polyclonal antibody to PKC-ζ, G-06967, genistein, and erbstatin were obtained from GIBCO (Gaithersburg, MD). Monoclonal antibody to PKC-α was obtained from Upstate Biotechnology (Lake Placid, NY). Calphostin C was purchased from Calbiochem (La Jolla, CA). Nitrocellulose membranes, enhanced chemiluminescence (ECL) kits for Western blotting protein detection, and the peroxidase-coupled sheep anti-mouse and donkey anti-rabbit antibodies were purchased from Amersham (Arlington Heights, IL). [^{3}H]thymidine was obtained from Dupont-NEN (Boston, MA). Unless otherwise noted all other reagents were purchased from Sigma Chemical or Fisher Scientific (Springfield, NJ).

Cells. The Caco-2 cells used for these studies were a clonal subpopulation selected for enterocytic differentiation (29). Stock cultures were maintained in DMEM containing 10% FCS, 10 μg/ml transferrin, 25 mmol/l glucose, 2 mmol/l glutamine, 1 mmol/l pyruvate, 15 mmol/l HEPES, 100 U/ml penicillin G, and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air-5% CO_2.

Application of rhythmic deformation. Caco-2 cells were seeded at a density of 30,000 cells/cm^2 on collagen I-coated flexible-bottomed six-well plates (Flexercell, McKeesport, PA). Either at confluence (for enzyme activity and detection of PKC isoforms and phosphorylated tyrosine) or 30–40% con-

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fluence (for cell number and [^{3}H]thymidine uptake), plates were placed in a deformation unit (Flexercell) consisting of a vacuum manifold in a tissue culture incubator (5% CO_2, 37°C) which was regulated by a solenoid valve controlled by a computer with a timer program. The membrane bottoms were deformed by a known percent elongation by application of a precise vacuum. On release of the vacuum, the membrane bottoms returned to their original conformation. Because cells adhere to the flexible surface of the culture well, the cell experiences the same deformation that is applied to the culture well (4). The deformation on the flexible well during stretch at various vacuum levels (i.e., increasing levels of deformation) has been calculated mathematically by finite element analysis and empirically by measuring with a micrometer the distance between concentric circles (radial deformation) or diametric axes (axial deformation) marked on the membrane. Very little change is observed in the latter; hence, the force on the attached cells is uniaxial. Indeed, we have previously demonstrated that Caco-2 cells subjected to such deformation regimens on these membranes remain adherent and experience uniaxial deformation consistent with deformation of the membrane (4).

In the present study Caco-2 cells were subjected to an average deformation of 10% at 10 cycles/min (3 s of deformation alternating with 3 s in neutral conformation) for the indicated times. Unstretched Caco-2 cell monolayers served as controls. In some experiments, blockers of PKC and tyrosine kinase or the appropriate vehicle (DMSO) were added to the Caco-2 cells before the initiation of cyclic deformation. When calphostin C was added to cells, it was preactivated by exposure to light for 2 h.

Cell number. Subconfluent (30–40%) Caco-2 cells grown on a collagen-coated flexible membrane were subjected to an average 10% deformation with 10 cycles/min for 24 h in the presence and absence of either staurosporine (20 ng/ml) or genistein (200 μmol/l). Caco-2 cell monolayers were washed with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution and trypsinized. Cell number was then determined by Coulter counter analysis (Coulter Instruments, Hialeah, FL). The cell viability was higher than 98%.

[^{3}H]thymidine incorporation. After cells were attached to the membrane, cells were exposed to cyclic deformation for 24 h with 1 μCi [^{3}H]thymidine/well, and [^{3}H]thymidine incorporation was assessed by scintillation counting of the TCA-soluble, sodium hydroxide-precipitable fraction of the cell lysate as previously described (4). Calphostin C (10 nmol/l), G-06967 (100 nmol/l), and erbstatin (3 μmol/l) were added simultaneously with [^{3}H]thymidine.

Digestive enzyme activity. AKP and DPDD were assayed as previously described by measuring hydrolysis of the synthetic substrates *p*-nitrophenyl phosphate and L-alanine-*p*-nitroaniline, respectively (4).

PKC assay. Caco-2 cell monolayers were washed twice with ice-cold 10 mmol/l phosphate buffer and homogenized by 10 strokes with a type B pestle in 20 mmol/l Tris·HCl (pH 7.4), 1 mmol/l EGTA, 1 mmol/l dithiothreitol (DTT), 1 mmol/l PMSF, 0.2 mmol/l leupeptin, and 5 U/ml aprotinin. The homogenates were centrifuged for 5 min at 600 *g*, and supernatants were used for PKC assay. PKC activity was assessed by measuring phosphorylation of a synthetic colored substrate, PepTag C1 peptide (P-L-S-R-T-L-S-V-A-A-K; Promega), using highly purified PKC enzyme from rat brain (Promega) as a standard.

Tyrosine kinase assay. Caco-2 cells were washed twice with ice-cold phosphate buffer and harvested in lysis buffer containing 50 mmol/l Tris·HCl (pH 8.0), 150 mmol/l NaCl, 1 mmol/l DTT, 0.5 mmol/l EDTA, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mmol/l sodium orthovanadate, 100 μg/ml PMSF, 1 μg/ml aprotinin, and 2 μg/ml leupeptin and homogenized with 10 strokes using a type B pestle. The homogenates were centrifuged at 10,000 *g* for 10 min at 4°C, and the supernatants were used for tyrosine kinase assay. Tyrosine kinase activity was determined by measuring the transfer of the γ-phosphate group from ATP to the biotin-labeled tyrosine kinase substrate (K-V-E-K-I-G-E-G-T-Y-V-V-Y-K-amide), which corresponds to a partial amino acid sequence of cell division kinase p34cdc2, by ELISA as recommended by the manufacturer's instructions (Boehringer Mannheim).

Detection of PKC-α and -ζ isoforms and phosphorylated tyrosine by Western blotting. All samples for Western blotting were preliminarily assayed for protein (bicinchoninic acid; Pierce, Rockford, IL) and diluted with buffer to equal protein concentrations before loading. Equivalency of loading across the lanes of the gel was also routinely verified by Coomassie blue staining.

To examine the regulation of subcellular distribution of PKC-α and -ζ isoforms by rhythmic deformation, Caco-2 cells were fractionated into the soluble and particulate fractions in extraction buffer containing 25 mmol/l Tris·HCl, pH 7.6, 5 mmol/l EGTA, 0.7 mmol/l $CaCl_2$, 1 mmol/l PMSF, 10 μmol/l leupeptin, 0.1 mmol/l TLCK and 0.1 mmol/l TPCK as described by Bissonnette and colleagues (7). Aliquots of soluble and particulate fractions were heated to 100°C for 5 min in Laemmli SDS buffer. SDS-treated samples were then separated by SDS-PAGE using a 7.5% resolving and 3.5% stacking gel and electroblotted to a nitrocellulose membrane. To block nonspecific antibody binding, the blots were incubated in TBST (50 mmol/l Tris·HCl, 150 mmol/l NaCl, and 0.05% Tween 20, pH 7.4) containing 5% nonfat dry milk at room temperature. After a 1-h incubation, the blots were washed three times for 10 min with fresh TBST. The blots were then incubated with either PKC-α or -ζ antibody in TBST for 1 h at room temperature. The concentrations of antibodies to PKC-α and -ζ were 0.1 and 0.15 μg/ml, respectively. After five washes in TBST, the blots were incubated with a 1:3,000 final dilution of appropriate peroxidase-coupled secondary antibodies (sheep anti-mouse IgG for PKC-α and donkey anti-rabbit IgG for PKC-ζ) at room temperature. After a 1-h incubation, the blots were washed five times and the PKC-α and -ζ were detected by ECL assay (Amersham) and quantitated by densitometric analysis (SigmaScan/Image, Jandel Scientific, Anaheim, CA).

Phosphotyrosine was also detected by Western blot. Briefly, Caco-2 cells were harvested in a hypotonic lysis buffer containing 10 mmol/l Tris·HCl, pH 8.0, 5 mmol/l KCl, 1 mmol/l DTT, 1.5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 100 μmol/l sodium orthovanadate, 100 μg/ml PMSF, 1 μg/ml aprotinin, and 2 μg/ml leupeptin and homogenized by ten strokes with a type B pestle. Soluble and particulate fractions were prepared by centrifugation, and sample proteins were separated by SDS-PAGE as previously described. The tyrosine phosphorylation of proteins was detected by incubating with a 0.2 μg/ml anti-mouse phosphotyrosine monoclonal antibody (Transduction, Lexington, KY) and a 1:3,000 final dilution of a peroxidase-coupled sheep anti-mouse secondary antibody (Amersham).

All densitometry was performed within the linear range, demonstrated by densitometry of multiple exposures of the same gel. However, we have chosen to present darker gel images here for purposes of illustration. PKC-α translocation was also demonstrated in phorbol 12-myristate 13-acetate (PMA)-treated Caco-2 cells as a positive control for the PKC

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studies and changes in Caco-2 tyrosine phosphorylation in response to epidermal growth factor as a positive control for the tyrosine phosphorylation Western blots (data not shown).

Analysis of data. Data are presented as means ± SE. Data were analyzed using a paired *t*-test with a Bonferroni correction. $P < 0.05$ was considered a significant difference.

RESULTS

Cyclic deformation rapidly induced PKC activity. We initially examined whether cyclic deformation regulates PKC activity in Caco-2 cells. Confluent Caco-2 cells grown on collagen-coated flexible membranes were exposed to an average 10% deformation at 10 cycles/min for 0–24 h. Basal PKC activity in Caco-2 cells at confluence was 2.6 ± 0.1 U/mg protein. Cyclic deformation rapidly increased cellular PKC activity to a maximum of 80 ± 31% (Fig. 1, $P < 0.05$, $n = 12$) by 1 min after initiation of deformation. After 24 h of exposure to cyclic deformation, PKC activity was still slightly but statistically significantly elevated (17 ± 13%, $P < 0.05$, $n = 12$).

Cyclic deformation rapidly induced and redistributed PKC-α and -ζ isoforms in subcellular fractions. Because activated PKC translocates from the cytosol to the membrane fraction, we next examined the effects of cyclic deformation on the subcellular distribution of PKC-α and -ζ, the predominant PKC isoforms in Caco-2 cells (7) by Western blotting. Our preliminary studies also confirmed the lack of detection of PKC-β and -ϵ isoforms (not shown). Because PKC activity was rapidly increased by deformation with the peak at 1 min, we examined the distribution of PKC isoforms from 6 s to 5 min after initiation of deformation. Cyclic deformation rapidly translocated PKC-α from the soluble to the particulate fraction. The peak of translocation of PKC-α occurred at 30 s (Fig. 2*A*). Densitometric analysis demonstrated that 28 ± 9% of PKC-α was associated with the particulate fraction and 72 ± 9% was within the soluble fraction in static cells ($n = 4$). However, cyclic deformation for 30 s increased the level of the PKC-α isoform in the particulate fraction up to 47 ± 9% ($P < 0.05$, $n = 4$) with a corresponding decrease in the soluble fraction to 53 ± 9% ($P < 0.05$, $n = 4$). Similarly, cyclic deformation also rapidly translocated the PKC-ζ isoform from the soluble to the particulate fraction (Fig. 2*B*). In static cells 56 ± 7% of the PKC-ζ isoform was located in the particulate fraction and 44 ± 7% was in the soluble fraction ($n = 4$). Cyclic deformation for 30 s rapidly increased the PKC-ζ isoform from 56 ± 7 to 76 ± 7% ($P < 0.05$, $n = 4$) in the particulate fraction with a decrease from 44 ± 7 to 24 ± 7% ($P < 0.05$, $n = 4$) in the soluble fraction. As a positive control for these studies, we also replicated the finding of Bissonnette and colleagues (7) that the exposure of Caco-2 cells to 1 μmol/l of PMA for 20 min completely ($P < 0.05$, $n = 4$) translocated PKC-α but not PKC-ζ from the soluble to the particulate fraction (data not shown).

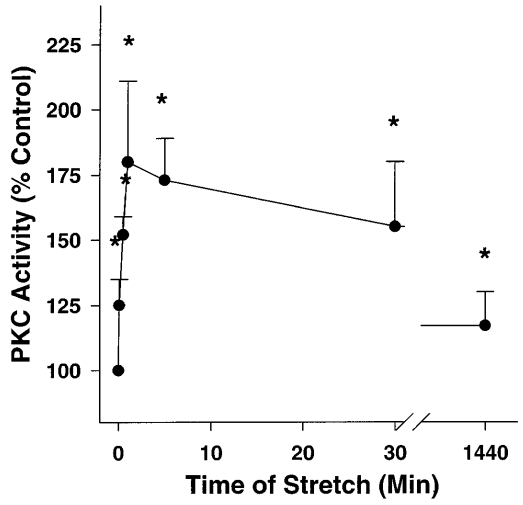


Fig. 1. Cyclic deformation rapidly increases protein kinase C (PKC) activity in Caco-2 intestinal cells. An average 10% deformation was applied for 6 s to 24 h at 10 cycles/min to confluent Caco-2 cells grown on collagen-coated flexible membranes at 37°C. At the indicated times cells were lysed, and PKC activity was assessed by measuring phosphorylation of a synthetic colored substrate. Data shown represent means ± SE as percent of static control for 4 separate experiments with 3 wells per experiment. *Values significantly different ($P < 0.05$) from static controls.

Rhythmic deformation of Caco-2 monolayer also rapidly stimulated tyrosine kinase activity. We next examined the effects of repetitive deformation on tyrosine kinase activity. As in the PKC study, cyclic deformation was applied to confluent Caco-2 cells for 0–24 h before tyrosine kinase activity assay. At 100% confluence, cellular tyrosine kinase activity in Caco-2 cells was 2.1 ± 0.3 mU/mg protein. Cyclic deformation rapidly increased cellular tyrosine kinase activity. The peak occurred at 5 min after initiation of deformation with a 41.0 ± 13.5% ($P < 0.05$, $n = 15$) increase compared with that in control (Fig. 3). Tyrosine kinase activity then rapidly decreased toward control levels after 10–30 min of deformation. However, chronic exposure of Caco-2 cells to cyclic deformation for 24 h demonstrated a sustained increase (49.2 ± 18.0%, $P < 0.05$, $n = 15$) in tyrosine kinase activity compared with that in static control cells.

Repetitive deformation rapidly increased phosphorylation of tyrosine residues in several proteins in soluble fraction but decreased phosphotyrosine in particulate fraction. We next directly examined the effect of deformation on the tyrosine phosphorylation of cellular proteins. Western blotting revealed that exposure to cyclic deformation rapidly increased tyrosine phosphorylation in several cellular proteins in the soluble fraction with peaks at 30 min (Fig. 4). Densitometric analysis demonstrated that cyclic deformation for 30 min increased tyrosine phosphorylation by 1,397 ± 290, 389 ± 98, 176 ± 37, and 92 ± 18% in 50-, 60-, 70-, and 125-kDa molecular mass proteins, respectively ($P < 0.05$ for each, $n = 4$), above that in control cells. In contrast, tyrosine phosphorylation of cellular proteins in the particulate fraction was decreased by 73 ± 21, 92 ± 8, 78 ± 17, and 89 ± 10% in 50-, 60-, 70-, and 125-kDa proteins, respectively, by cyclic deformation for 30 min (Fig. 4; $P < 0.05$ for each, $n = 4$). In two additional

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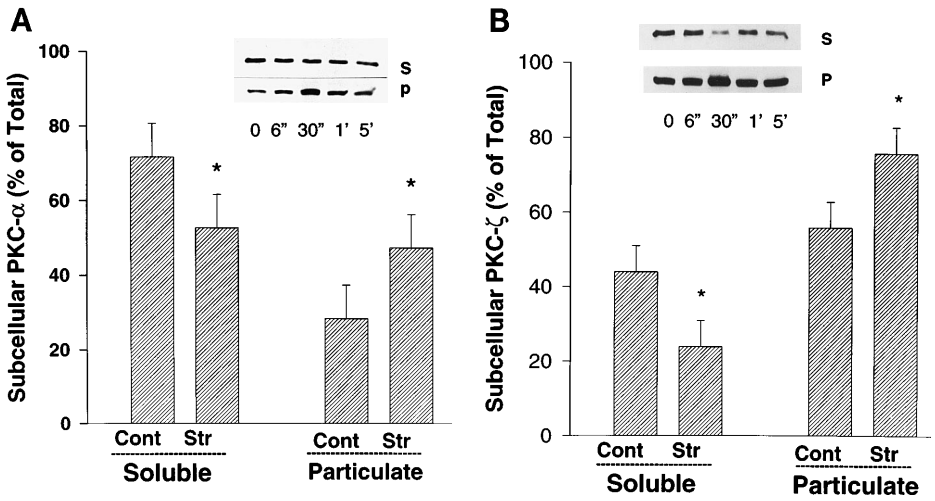


Fig. 2. Cyclic deformation alters the subcellular distribution of PKC-α (*A*) and -ζ (*B*) isoforms in Caco-2 cells. An average 10% deformation was applied for 6 s to 5 min at 10 cycles/min to confluent Caco-2 cells grown on collagen-coated flexible membranes at 37°C. At indicated times, cells were lysed and fractionated into soluble and particulate fractions by differential centrifugation. Samples were solubilized in Laemmli SDS buffer, resolved by 7.5% SDS-PAGE, and electroblotted to a nitrocellulose membrane. PKC-α and -ζ isoforms were detected by Western blotting using specific antibodies for these isoforms. Data are expressed as the particulate percentage of total isoform-specific PKC immunoreactivity at 30 s after initiation of cyclic deformation. Values are means ± SE for 4 independent experiments. *Values significantly different ($P < 0.05$) from static control cells. Cont, control; Str, stretched. *A, inset*: PKC-α in particulate (P) and soluble (S) fractions for indicated times from representative experiment. *Lane 1*, static control; *lane 2*, 6 s; *lane 3*, 30 s; *lane 4*, 1 min; *lane 5*, 5 min. *B, inset*: PKC-ζ in particulate (P) and soluble (S) fractions for indicated times from representative experiment. *Lane 1*, static control; *lane 2*, 6 s; *lane 3*, 30 s; *lane 4*, 1 min; *lane 5*, 5 min.

independent experiments, we compared phosphorylation of these tyrosine phosphoproteins at the 0- and 30-min time points in multiple lanes on the same gels and observed similar effects, demonstrating that the observed differences in band intensity were not artifacts of lane position on the gels (data not shown).

The deformation-induced increase in cell number was blocked by PKC and tyrosine kinase inhibitors. We next directly examined the effects of inhibitors of PKC and tyrosine kinase activity on the mitogenic effect of deformation. Subconfluent Caco-2 cells were exposed to cyclic deformation (average 10% deformation at 10 cycles/min) for 24 h, and cell numbers were counted by a Coulter counter after trypsinization. Cyclic deformation increased cell number by 44.5 ± 7.8 to 54 ± 7% ($P < 0.05$, $n = 6$) above that (890,000 ± 73,800 cells) in static controls (Fig. 5). This deformation-mediated stimulation of cell number was completely blocked by the PKC inhibitor staurosporine (Fig. 5*A*) and by the tyrosine kinase inhibitor genistein (Fig. 5*B*). Because of the less than optimal specificity of staurosporine and genistein, we used more specific inhibitors of PKC and tyrosine kinase activity for the following studies.

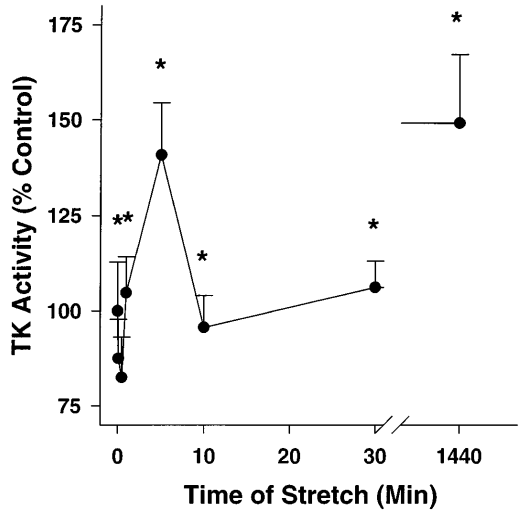


Fig. 3. Cyclic deformation modulates tyrosine kinase (TK) activity in Caco-2 cells. Confluent Caco-2 cells were exposed to the cyclic deformation for 6 s to 24 h and homogenized in Tris·HCl buffer (pH 8.0) containing orthovanadate. Tyrosine kinase activity was determined by measuring phosphorylation of a synthetic tyrosine kinase substrate by ELISA. Data are means ± SE as percent of static controls from 5 independent experiments with 3 wells per experiment. *Values significantly different ($P < 0.05$) from static control cells.

Blockade of PKC and tyrosine kinase inhibited deformation-mediated stimulation of [^{3}H]thymidine incorporation in Caco-2 cells. Caco-2 cells at 30–40% confluence were used for [^{3}H]thymidine incorporation studies. In static control monolayers Caco-2 cells accumulated 168,400 ± 7,238 counts/min [^{3}H]thymidine/well during 24-h incubation at 37°C (Table 1). Exposure of Caco-2 cells to an average 10% deformation at 10 cycles/min for 24 h increased [^{3}H]thymidine incorporation by 26.4 ± 5.3% ($P < 0.05$, $n = 12$). This change is similar to what we have previously observed (4). Deformation-mediated stimulation of Caco-2 [^{3}H]thymidine incorporation was completely inhibited by the PKC inhibitor calphostin C (10 nmol/l) and by the tyrosine kinase inhibitor erbstatin (3 μmol/l) but was potentiated by the PKC-α inhibitor G-06967 (100 nmol/l). Although neither calphostin C nor erbstatin affected [^{3}H]thymidine uptake in static control cells, G-06967 slightly but significantly ($P < 0.05$) increased [^{3}H]thymidine uptake in static control cells.

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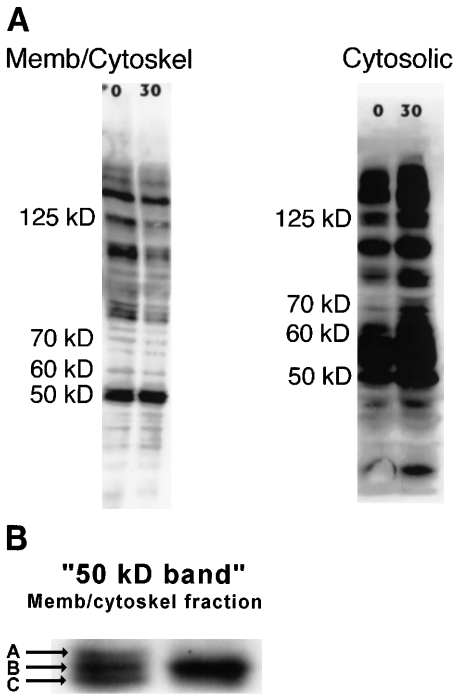


Fig. 4. Effects of cyclic deformation on tyrosine phosphorylation of proteins in the subcellular fractions. Confluent Caco-2 cells were exposed to an average 10% deformation with 10 cycles/min for indicated times. Cells were lysed and fractionated into soluble and particulate fractions by differential centrifugation, and proteins were resolved by 7.5% SDS-PAGE and electroblotted to nitrocellulose membranes before Western blotting for phosphotyrosine. *A*: immunoblot analysis of tyrosine phosphorylation of proteins in the particulate (Memb/Cytoskel) and soluble (Cytosolic) fractions from representative experiment. *Left lane* (0), derived from cells before rhythmic deformation. *Right lane* (30), derived from cells after 30 min of rhythmic deformation. Several bands are reproducibly altered in intensity, including those at 125, 70, 60, and 50 kDa, in general exhibiting decreased band intensity in the membrane/cytoskeletal fraction. *B*: magnified view of the 50-kDa band resolved from the membrane/cytoskeletal fraction in the experiments in *A* demonstrates that this band is actually a triplet which exhibits a differential response in band intensity in response to 30 min of strain (*right lane*) compared with band intensities in cells lysed and fractionated before strain (*left lane*). Although the phosphorylation of *band B* increases in response to strain, the phosphorylation of *band A* and *band C* decreases in response to strain. Whether this represents a shift in differentially phosphorylated forms of the same molecular species or three differentially regulated individual tyrosine phosphoproteins in the particulate fraction awaits further study.

PKC and tyrosine kinase inhibition completely prevented cyclic deformation-mediated modulation of DPDD brush-border enzyme activity and attenuated AKP activity. To examine the effects of cyclic deformation on differentiation, we exposed confluent Caco-2 cells to cyclic deformation for 24 h. Cyclic deformation increased DPDD activity by 38 ± 9% ($P < 0.001$, $n = 12$) compared with that in static cells (Table 2). However, the stretch-associated increase in DPDD activity was completely blocked by PKC inhibition with either calphostin C or G-06967, as well as by the tyrosine kinase inhibitor erbstatin. Neither PKC nor tyrosine kinase inhibition influenced DPDD activity in static control cells. In contrast to DPDD, AKP activity in stretched cells was decreased by 33.5 ± 2.6% ($P < 0.0005$, $n = 12$) compared with their static controls (Table 3). The addition of PKC, PKC-α, or tyrosine kinase inhibitors decreased AKP activity in static controls by 13.0 ± 6.1 to 21.9 ± 1.6% ($n = 12$, $P < 0.05$). However, treatment with each inhibitor prevented any further decrease in AKP activity by cyclic deformation (Table 3).

DISCUSSION

Intestinal epithelial cells are exposed to various conditions, including fasting, intestinal motility, feeding, altered luminal nutrient concentrations, ionic com-

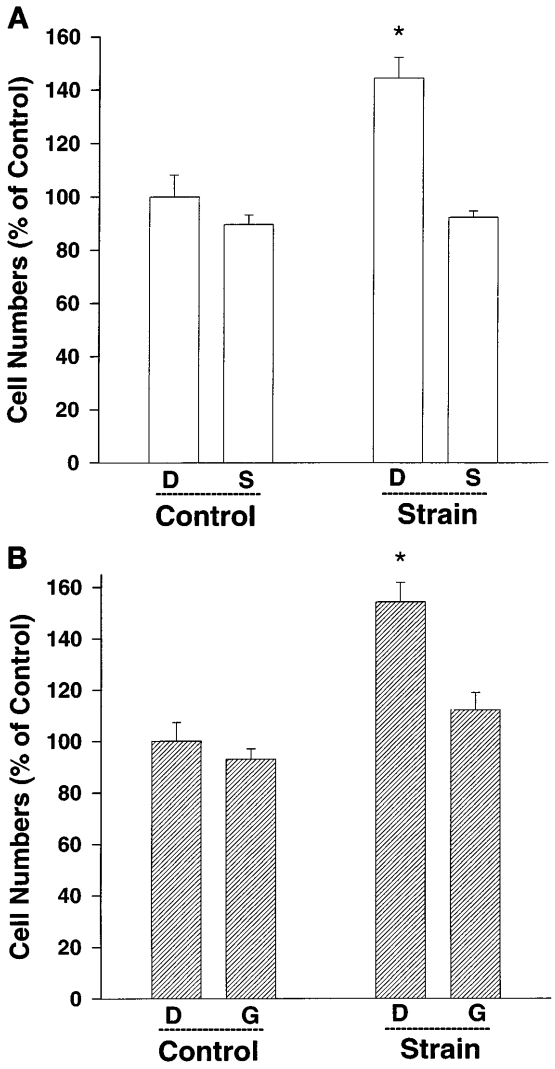


Fig. 5. Effects of inhibitors of PKC and tyrosine kinase on the cyclic deformation-mediated increase in cell numbers in Caco-2 cells. Subconfluent (30–40%) Caco-2 cells grown on collagen-coated flexible membranes were subjected to an average 10% deformation with 10 cycles/min for 24 h in the presence of DMSO alone (D) or with 20 ng/ml staurosporine (S) or 200 μmol/l genistein (G). Monolayers were then washed and trypsinized so that cell numbers could be counted by a Coulter counter. Data shown represent means ± SE as percent of static control from 2 independent experiments with 3 wells per experiment. *Values significantly different ($P < 0.05$) from the static control. *A*: PKC inhibitor staurosporine completely blocked the strain-mediated increase in cell number. *B*: tyrosine kinase inhibitor genistein completely prevented the strain-induced increase in cell number.

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Table 1. *Effect of PKC and tyrosine kinase inhibitors on cyclic deformation-mediated stimulation of [^{3}H]thymidine incorporation in Caco-2 cells*

Inhibitors	[^{3}H]Thymidine Incorporation	
	Static condition	Stretched condition
DMSO	100.0 ± 9.1^{d}	126.4 ± 5.3^{b}
Calphostin (10 nM)	90.2 ± 6.2^{d}	90.2 ± 9.5^{d}
G-06967 (100 nM)	112.0 ± 4.1^{c}	146.8 ± 6.7^{a}
Erbstatin (3 μM)	103.3 ± 5.0^{d}	94.5 ± 5.1^{d}

Values are means ± SE, expressed as percent of controls from 4 experiments with 3 wells per treatment. To examine impact of modulation of protein kinase C (PKC) and tyrosine kinase activity on cyclic deformation-mediated stimulation of [^{3}H]thymidine incorporation, subconfluent (30–40%) Caco-2 cells were exposed to average 10% deformation at 10 cycles/min in medium containing 1 μCi of [^{3}H]thymidine/well in presence of inhibitor of PKC or tyrosine kinase activity. DMSO was used as vehicle control. After 24 h [^{3}H]thymidine incorporation by Caco-2 cells was quantitated. Superscripts indicate significant differences ($P < 0.05$) within treatments.

Table 3. *Effect of inhibition of PKC and tyrosine kinase on deformation-mediated modulation of AKP activity in Caco-2 intestinal cells*

Inhibitors	AKP Activity	
	Static condition	Stretched condition
DMSO	100.0 ± 3.5^{a}	66.5 ± 2.6^{c}
Calphostin (10 nM)	78.1 ± 1.6^{bc}	80.9 ± 3.9^{bc}
G-06967 (100 nM)	86.8 ± 3.2^{b}	77.3 ± 4.7^{bc}
Erbstatin (3 μM)	87.0 ± 6.1^{b}	71.5 ± 4.2^{bc}

Values are means ± SE as percent of static control for 4 experiments with 3 wells per treatment for each experiment. To study the mechanism of cyclic deformation effects on alkaline phosphatase (AKP) activity, confluent Caco-2 cells cultured on collagen-coated flexible membrane were subjected to average 10% deformation with 10 cycles/min for 24 h with indicated inhibitors. DMSO was used as vehicle control. Superscripts indicate significant differences ($P < 0.05$) within groups.

position, pressure, and villous contraction or motility, which cause diverse deformation patterns of the intestinal mucosa (11, 21, 23, 28, 33, 35, 36). Modulation of intestinal epithelial proliferation and differentiation in vivo under such conditions are well described (12, 13, 15, 24), but it remains unknown whether the modulation of enterocyte biology by these factors is deformation mediated. Because it is difficult to regulate deformation frequency and amplitude in the intestinal mucosa in vivo and to isolate the effects of deformation from other physiological parameters, we have previously used a computer-regulated deformation apparatus to repetitively deform cultured human intestinal epithelial Caco-2 cell monolayers (4). Caco-2 cells are derived from a colon cancer but spontaneously differentiate into polarized cells with many morphological and functional properties of mature enterocytes and are a common in vitro model for intestinal epithelial biology (9, 18, 29).

Intestinal epithelial deformation is likely to represent a complex summation of peristaltic contraction, physical interaction of the mucosa with luminal contents, alterations in villus shape, and alterations in cell shape. Intestinal circular smooth muscle contraction transmits contractile forces across the mucosa to luminal contents. Because luminal contents are largely noncompressible, this would increase pressure on the mucosa and change intestinal epithelial shape (6, 26). Longitudinal smooth muscle contraction may also induce mucosal deformation as small bowel segments themselves move and bend within the abdomen.

Table 2. *Cyclic deformation-mediated stimulation of DPDD activity is blocked by inhibitors of PKC and tyrosine kinase*

Inhibitors	DPDD Activity	
	Static condition	Stretched condition
DMSO	100.0 ± 4.2^{b}	138.0 ± 7.0^{a}
Calphostin C (10 nM)	88.5 ± 4.1^{b}	92.2 ± 10.6^{b}
G-06967 (100 nM)	92.0 ± 4.2^{b}	105.4 ± 1.8^{b}
Erbstatin (3 μM)	95.0 ± 5.6^{b}	106.0 ± 11.9^{b}

Values are means ± SE and are pooled data from 4 experiments from 12 wells expressed as percent of control values. To study the mechanism of cyclic deformation effects or depeptidyl peptidase (DPDD) activity, confluent Caco-2 cells were exposed to average 10% deformation at 10 cycles/min for 24 h in presence of various inhibitors. DMSO was used as vehicle control. Superscripts indicate significant differences ($P < 0.05$) within treatments.

Villus contraction may also induce intestinal epithelial deformation. Spontaneous repetitive intestinal villus contraction is mediated by muscularis mucosa smooth muscle fibers oriented along the villus. Individual villi contract 0–15 times/min (36). Videomicroscopy (37) suggests a dramatic decrease in villus length. Water absorption, vagal stimulation, amino acids, and fatty acids increase contractile frequency; vagotomy and sympathetic stimulation inhibit it (8, 36, 37). Villus and epithelial deformations also occur during lactation (22) and ingestion of fiber (33) and after small bowel resection (35).

Enterocytes also change shape during normal gut function and during contact with luminal contents or opposing mucosal surfaces in vitro (25), presumably because of compression from within the lumen by the passage of a bolus of noncompressible luminal contents as well as friction or drag forces from luminal contents which move individual villi and further induce strain on the intestinal epithelial cells (23). Finally, intestinal epithelial cells actively deform during lipid absorption (11) or during restitution (5).

Although intestinal epithelial deformation in vivo is complex, we chose to study the effects on cultured Caco-2 cells of a regular and rhythmic deformation pattern of physiologically relevant amplitude and frequency (28, 36) to facilitate reproducible analysis of intracellular signaling at acute time points. We found that cyclic deformation rapidly stimulated PKC activity and translocated PKC-α and -ζ from a soluble to a particulate subcellular fraction. Deformation also rapidly stimulated intracellular tyrosine kinase activity. The tyrosine phosphorylation of several cellular proteins rapidly increased in the soluble fraction and tyrosine phosphoprotein content in the particulate

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fraction decreased in parallel. PKC and tyrosine kinase inhibitors blocked the effects of deformation on Caco-2 proliferation and differentiation.

The finding that deformation initiated rapid PKC and tyrosine kinase signals in Caco-2 cells is consistent with observations in vascular endothelial cells (30) and myocytes (31), albeit at very different deformation parameters. Interestingly, colonic mucosal PKC activity is altered by dietary fat and fiber (10). Although these agents could act directly on the colonocyte, alterations in bowel contractility and mucosal deformation could also contribute to such PKC modulation. Because PKC activation is often accompanied by PKC isozyme translocation (20), we next examined the subcellular distribution of the PKC-α and -ζ isoforms. In static Caco-2 cells, one-third and two-thirds of total PKC-α, a Ca^{2+}-dependent PKC isoform, were located in the particulate and soluble fraction, respectively, whereas two-thirds and one-third of total PKC-ζ, a Ca^{2+}-independent PKC isoform, were found in the particulate and soluble fraction, respectively, consistent with previous observations (7). However, deformation rapidly translocated both the PKC-α and -ζ isoforms from the soluble to the particulate fraction. Thus cyclic deformation may stimulate Caco-2 PKC via both Ca^{2+}-dependent and -independent PKC isozymes.

Altered PKC and tyrosine kinase activity is associated with altered intestinal mucosal proliferation and differentiation in vivo (10, 19) and in cultured Caco-2 cells (2, 3). Cyclic deformation for 24 h increased both cell number and thymidine uptake in proliferating Caco-2 cells and modulated Caco-2 brush-border enzyme specific activity in confluent cells. Although intestinal brush-border enzyme activities may increase together in differentiation (18), intestinal marker enzyme activities may also be selectively increased or decreased by hormones and peptides (12, 17), PKC inhibition (3), and refeeding (15). We therefore next examined whether deformation-mediated alterations of Caco-2 proliferation and differentiation were sensitive to modulation of PKC and tyrosine kinase activity.

Indeed, deformation-stimulated increases in cell number were significantly inhibited by staurosporine and genistein, which inhibit PKC and tyrosine kinase activity, respectively. Although thymidine uptake does not necessarily mirror proliferation in all settings and must therefore be interpreted cautiously, we have previously reported that deformation increases cell number and thymidine uptake equivalently (4). The hypothesis that the PKC and tyrosine kinase signals induced by deformation might mediate the mitogenic effects of deformation may thus also be supported by observations that calphostin C (a more specific PKC inhibitor) and erbstatin (a tyrosine kinase inhibitor with different specificity) blocked deformation-mediated increases in thymidine uptake without altering control monolayer thymidine uptake.

Intestinal brush-border enzyme specific activity is a common intestinal epithelial differentiation marker (18). Deformation substantially increased Caco-2 DPDD activity and decreased AKP activity. PKC and tyrosine kinase signaling may also mediate deformation effects on Caco-2 differentiation, because PKC or tyrosine kinase blockade prevented these effects. Although 1 μmol/l calphostin C stimulated DPDD and inhibited AKP in static cells in another study (3), the 10 nmol/l calphostin C used here did not significantly affect DPDD.

The mechanism of the deformation-induced decrease in AKP activity awaits elucidation. Because enterocytes secrete intestinal AKP (1), the apparent downregulation of cellular AKP by deformation could reflect increased secretion of this protein. However, we could not demonstrate such a phenomenon by Western blot (M. D. Basson and D. H. Alpers, unpublished data).

G-06967, which inhibits PKC-α, increased thymidine uptake in static cells and potentiated deformation-induced thymidine uptake. G-06967 augmentation of static Caco-2 proliferation is consistent with suggestions that PKC-α induction inhibits vascular smooth muscle cell proliferation (32). PKC-α, -δ, and -ζ are downregulated in proliferative colonic adenomas and carcinomas compared with colonocytes (34). Although the role of PKC-α in modulating static and deformation-induced Caco-2 proliferation awaits further study, these data suggest that PKC-α is unlikely to be a critical mediator of the mitogenic effects of deformation in Caco-2 cells. G-06967 also decreased AKP in static cells and slightly but not significantly decreased DPDD. However, the effects of deformation on brush-border enzyme activity were substantially attenuated by G-06967 treatment and did not achieve statistical significance. Thus a PKC-α signal may be involved in the effects of deformation on Caco-2 differentiation.

In summary, cyclic deformation rapidly stimulates PKC activity in human intestinal Caco-2 cells, translocating both Ca^{2+}-dependent (PKC-α) and Ca^{2+}-independent (PKC-ζ) isoforms from the soluble to the particulate fraction. Cyclic deformation also rapidly modulates tyrosine kinase activity and tyrosine phosphorylation of proteins in the soluble and particulate fractions. Certainly, chronic cyclic deformation could conceivably modulate the expression of other PKC isoforms in a signal cascade by regulating a specific PKC isoform, stimulate interaction between one or more PKC isozymes and tyrosine kinases, or regulate other intracellular signals. Deformation-associated PKC and tyrosine kinase signals may also modulate other intestinal epithelial characteristics since, for instance, PKC modulates transepithelial chloride and glucose transport (14, 16). However, these results suggest that deformation-mediated PKC and tyrosine kinase signals may influence proliferation and differentiation of human intestinal Caco-2 cells. PKC-α, in particular, may be involved in deformation effects on differentiation but not proliferation.

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