PKC-dependent regulation of transepithelial resistance: roles of MLC and MLC kinase

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Departments of Pathology, [1]Wayne State University School of Medicine, Detroit, Michigan 48201; [2]Brigham and Women's Hospital, Boston, Massachusetts 02115; and [3]Emory University, Atlanta, Georgia 30322

Turner, J. R., J. M. Angle, E. D. Black, J. L. Joyal, D. B. Sacks, and J. L. Madara. PKC-dependent regulation of transepithelial resistance: roles of MLC and MLC kinase. *Am. J. Physiol.* 277 (*Cell Physiol.* 46): C554–C562, 1999.—The mechanisms by which protein kinase C (PKC) activation results in increased transepithelial resistance (TER) are unknown [G. Hecht, B. Robinson, and A. Koutsouris. *Am. J. Physiol.* 266 (*Gastrointest. Liver Physiol.* 29): G214–G221, 1994]. We have previously shown that phosphorylation of the regulatory light chain of myosin II (MLC) is associated with decreases in TER and have suggested that contraction of the perijunctional actomyosin ring (PAMR) increases tight junction (TJ) permeability [J. R. Turner, B. K. Rill, S. L. Carlson, D. Carnes, R. Kerner, R. J. Mrsny, and J. L. Madara. *Am. J. Physiol.* 273 (*Cell Physiol.* 42): C1378–C1385, 1997]. We therefore hypothesized that PKC activation alters TER via relaxation of the PAMR. Activation of PKC by the phorbol ester phorbol 12-myristate 13-acetate (PMA) resulted in a progressive dose-dependent increase in TER that was apparent within 15 min (111% of controls) and maximal within 2 h (142% of controls). Similar increases were induced by a diacylglycerol analog, and the effects of both PMA and the diacylglycerol analog were prevented by the PKC inhibitor bisindolylmaleimide I. PMA treatment caused progressive decreases in MLC phosphorylation, by 12% at 15 min and 41% at 2 h. Phosphorylation of MLC kinase (MLCK) increased by 64% within 15 min of PMA treatment and was stable over 2 h (51% greater than that of controls). Thus increases in MLCK phosphorylation preceded decreases in MLC phosphorylation. These data suggest that PKC regulates TER via decreased phosphorylation of MLC, possibly due to inhibitory phosphorylation of MLCK. The decreased phosphorylation of MLC likely reduces PAMR tension, leading to decreased TJ permeability.

tight junction; myosin light chain; perijunctional actomyosin ring; protein kinase C

A MAJOR FUNCTION OF intestinal epithelial cells is to serve as barriers to passive transepithelial solute movement. As the rate-limiting step in the paracellular pathway, the epithelial tight junction (TJ) is the primary determinant of passive transepithelial solute movement. By altering TJ permeability, a variety of physiological stimuli as well as nonphysiological perturbations may influence the permeability of epithelial monolayers.

We have recently used Caco-2 cell monolayers to recapitulate the physiological TJ regulation induced by activation of the Na^+-glucose cotransporter SGLT1 (45). In this model, activation of Na^+-glucose cotransport induces a reversible, size-selective increase in TJ permeability similar to that previously described in isolated mammalian small intestinal mucosa (1, 24, 45). Increased phosphorylation of the regulatory light chain of myosin II (MLC) occurs in conjunction with activation of Na^+-glucose cotransport (45). This MLC phosphorylation is recognized as a biochemical marker of cytoskeletal contraction and correlates with the morphological perijunctional actomyosin ring (PAMR) condensation previously described in small intestinal mucosa after addition of luminal glucose (1, 45). Pharmacological inhibition of MLC kinase (MLCK) prevents MLC phosphorylation and also prevents increased TJ permeability after activation of Na^+-glucose cotransport (45). Thus it appears that Na^+-glucose cotransport-dependent regulation of TJ permeability is mediated by MLC phosphorylation.

Other physiological and pharmacological stimuli also appear to alter TJ permeability via the PAMR. These include disruption of the actin cytoskeleton with cytochalasin (3, 23) and ADP-ribosylation of rho (31), both of which result in nearly complete loss of TJ barrier properties. Additionally, overexpression of a constitutively active MLCK in Madin-Darby canine kidney (MDCK) cells results in monolayers with markedly increased TJ permeability relative to control monolayers (11). Thus a close association between PAMR function and TJ permeability exists.

Protein kinase C (PKC)-activating phorbol esters induce a rapid decrease in TJ permeability (12, 39). Although it has been suggested that the effects of phorbol esters on TJ permeability may be related to alterations of the PAMR (12), the mechanism of action of PKC in mediating these effects is unknown. We undertook this study to test the hypothesis that PKC alters TJ permeability through modification of PAMR contraction. PKC might affect actomyosin contraction by two distinct mechanisms: PKC could potentially phosphorylate MLC at sites that inhibit actomyosin contraction (29, 43) or PKC-mediated phosphorylation of MLCK could inhibit MLC phosphorylation (30). In this study we have investigated the effects of PKC activation on transepithelial resistance (TER) and the phosphorylation of MLC and MLCK in Caco-2 monolayers. Activation of PKC was associated with rapid increases in MLCK phosphorylation and progressive increases in TER and decreases in MLC phosphoryla-

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tion. These data suggest that PKC-mediated phosphorylation may lead to decreased MLCK activity, reduced tension on the PAMR, and decreased TJ permeability.

METHODS

Materials. Tissue culture media and serum were from Life Technologies (Gaithersburg, MD). Phorbol 12-myristate 13-acetate (PMA) was from Sigma (St. Louis, MO) and was prepared as a 1 mg/ml (1.6 mM) stock in anhydrous DMSO and stored in frozen aliquots until use. Bisindolylmaleimide I (GF 109203X) was from Calbiochem (La Jolla, CA) and was prepared as a 10 mM stock in anhydrous DMSO and stored in frozen aliquots until use. 1-Oleoyl-2-acetyl-*sn*-glycerol (OAG) was purchased as 500-µg lyophilized aliquots (Calbiochem) and prepared immediately before use. Monoclonal antibodies to MLC (clone MY-21) and MLCK (clone K36) were from Sigma. Peroxidase-conjugated secondary antibodies for immunoblotting were from ICN (Costa Mesa, CA).

Cell culture. Caco-2 cells with active physiological Na^+-glucose cotransport were generated by transfection, as previously described (44). These were grown as monolayers on Transwell supports (Corning-Costar, Cambridge, MA) and used 20–30 days postconfluence (45). Transwell supports with 0.33- and 5-cm^2 surface areas were used for electrophysiological and biochemical studies, respectively.

Electrophysiology. Electrophysiological measurements of TER and short-circuit current were made with agar bridges, Ag-AgCl and calomel electrodes, and a 50-µA current, as previously described (45).

Quantitative analysis of MLC and MLCK phosphorylation. Monolayers grown on 5-cm^2 Transwell supports were loaded with [^{32}P]orthophosphate (ICN) at 250–300 µCi/ml, as described previously (45). Incubations were terminated by washing the monolayers three times in ice-cold PBS and scraping the cells into 200 µl of lysis buffer [25 mM Tris, pH 8.0, 100 mM sodium pyrophosphate, 100 mM NaF, 250 mM NaCl, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 1% Triton X-100, 10 mM EGTA, 5 mM EDTA, 500 µM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 µM E-64, 1 µM leupeptin, 1 µg/ml aprotinin]. Aliquots were then separated on SDS-PAGE gels and transferred to polyvinylidene difluoride (PVDF). ^{32}P incorporation into MLC or MLCK was assessed by autoradiography, as described previously (45).

Monolayers grown on 5-cm^2 Transwell supports were also used for determination of MLC phosphorylation by two-dimensional urea glycerol-PAGE-SDS-PAGE. For these analyses, monolayers were harvested by being scraped into ice-cold 10% TCA and 10 mM dithiothreitol. The pellets were washed three times with diethyl ether, dried, and solubilized in urea glycerol gel sample buffer (6.7 M urea, 10 mM dithiothreitol, 18 mM Tris, pH 8.6, 20 mM glycine, 5% saturated sucrose, 0.004% bromphenol blue). Urea glycerol gels were performed as described by Persechini et al. (32) using the Mini-Protean II vertical electrophoresis system (Bio-Rad, Hercules, CA) with 0.75-mm gel spacers. After preelectrophoresis at 300 V for 120 min at 25°C, samples were electrophoresed for 150 min at 300 V. After electrophoresis, lanes were excised, equilibrated in 10% SDS and 125 mM Tris, pH 6.8, for 15 min at 25°C, and loaded horizontally across a 1.0 mm thick SDS-15% PAGE gel. After electrophoresis, the SDS-PAGE gels were transferred to PVDF and blotted for MLC as described previously (45).

Determination of PKC activity. PKC activity was measured with a PKC assay kit (Life Technologies), which measures incorporation of ^{32}P from [γ-^{32}P]ATP (ICN) into an acetylated synthetic peptide derived from residues 4–14 of myelin basic protein (Ac-MBP). A synthetic peptide composed of residues 19–36 of PKC was used as a specific PKC inhibitor. Monolayers of Caco-2 cells were harvested by scraping the cells off the Transwell membrane into a solution containing (in mM) 20 Tris, pH 7.5, 0.5 EDTA, and 0.5 EGTA. After homogenization the samples were centrifuged at 20,000 *g* for 30 min at 4°C. The supernatant was considered the cytosolic fraction. The pellet was solubilized in a solution containing 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 0.5% Triton X-100 and recentrifuged, and the supernatant was used as the membrane-associated fraction. PKC was then partially purified from each sample by chromatography over DEAE-cellulose columns, with 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM 2-mercaptoethanol, and 0.2 M NaCl for elution. This partially purified PKC was used in reaction mixtures with 20 mM Tris, pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$, 20 µM [γ-^{32}P]ATP, and 50 µM Ac-MBP, which were incubated at 30°C for 5 min. Reactions were terminated by spotting the mixtures onto phosphocellulose discs. These were washed twice with 1% H_3PO_4 and once with water and dried, and the retained ^{32}P was counted. Preliminary experiments showed that this procedure measured PKC activity within the linear range of the assay, both in terms of amount of PKC added and duration of the reaction.

Phosphoamino acid analysis. MLC was excised from SDS-PAGE gels on the basis of the alignment of the gel with an autoradiograph. Only gels in which the MLC band was well separated from other low-molecular weight radiolabeled phosphoproteins were used for MLC purification. After excision of the MLC band, gels were reexposed to confirm that only the MLC band had been excised. The excised gel fragments were washed three times in 25% isopropanol and then in 10% methanol. The slices were then minced and dried under a heat lamp. The dried gel pieces were rehydrated in 100 mM NH_4CO_3, pH 8.0. After 24 h, the supernatant was removed and replaced with fresh NH_4CO_3 buffer. The supernatants were pooled and lyophilized. A portion was analyzed by SDS-PAGE to verify the purity of the preparation. The remainder was hydrolyzed in 6 M HCl at 110°C for 2 h. Phosphoamino acids were resolved by thin-layer electrophoresis in 7% (vol/vol) formic acid as described previously (19). Phosphoamino acid standards were detected by ninhydrin staining. ^{32}P-labeled phosphoamino acids were imaged with a model 425E PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Statistical analysis. Differences between conditions were compared by Student's *t*-test. All experiments shown were performed multiple times. When duplicate or triplicate samples were present in the same experiment, results are shown as means ± SE.

RESULTS

Phorbol esters induce dose-dependent increases in TER. It has been previously shown that increases in TER occur shortly after activation of PKC by phorbol esters (12, 39). We evaluated the effects of increasing doses of the PKC-activating phorbol ester PMA, ranging from 60 nM to 16 µM, on TER in Caco-2 monolayers. There was a dose-dependent increase in TER, relative to control monolayers, which was insignificant with 60 nM PMA. Small increases in TER were apparent after 2 h of treatment with 170 nM PMA (17 ± 10%; $P < 0.05$). Increases in TER were maximal at 42 ± 7% after 2 h of treatment with 1.6 µM PMA (Fig. 1; $P < 0.01$) and were

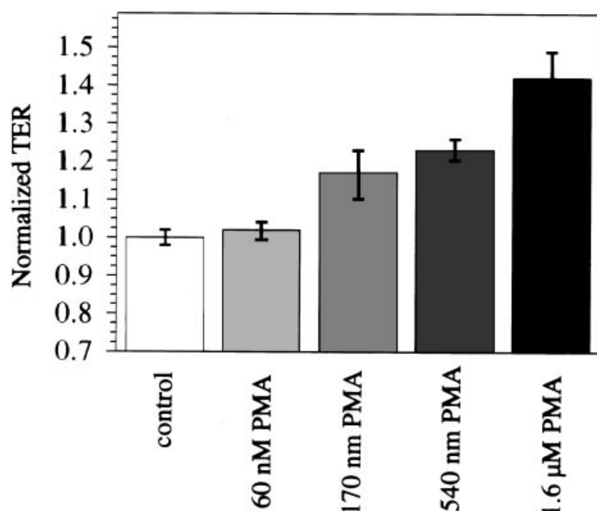

Fig. 1. Maximal increases in transepithelial resistance (TER) after phorbol 12-myristate 13-acetate (PMA) treatment. TER of Caco-2 cell monolayers was measured after exposure to PMA for 2 h at doses indicated. Results are shown as TER ± SE normalized to control monolayers (without PMA) at 2 h. Baseline TER was typically between 175 and 250 Ω·cm².

similar to the changes induced by up to 16 μM PMA (data not shown). While small increases in TER were evident within 15 min after PKC activation with PMA at 1.6 μM or greater (e.g., 11 ± 3% increase after 15 min with 1.6 μM PMA), these changes were not maximal until 2 h of PMA treatment. At lower PMA doses (<540 nM), no increases in TER were apparent until at least 60 to 120 min. Thus both the magnitudes and the intervals over which the PMA-induced increases in TER occurred were dose dependent.

Phorbol esters induce translocation of PKC to the membrane. To determine if PKC activation and increases in TER occurred simultaneously, we directly measured PKC activity in membrane and cytosol fractions of PMA-treated Caco-2 monolayers. The addition of 1.6 μM PMA for 15 min increased the membrane-associated fraction of PKC activity from 42 ± 9 to 69 ± 1% of total cellular PKC activity ($P < 0.05$). This increase was apparent for at least 3 h after PMA addition. As an additional marker of PKC activity, an endogenous 80-kDa phosphoprotein with an isoelectric point of ~4.5, consistent with identification as myristoylated alanine-rich C kinase substrate (MARCKS), was noted to be intensely phosphorylated in lysates of ^{32}P-labeled, PMA-stimulated monolayers analyzed by two-dimensional isoelectric focusing-SDS-PAGE. MARCKS is widely used as an endogenous marker of PKC activation, and the phosphorylation of MARCKS in Caco-2 cells after PKC activation has been previously described (38). Thus, together with the effect of PMA on TER, these data show that increases in membrane-associated PKC activity occur concurrently with the initiation of increases in TER.

Activation of PKC by diacylglycerol analogs increases TER. Given that maximal effects of PMA were seen at relatively high doses (1.6 μM), we considered the possibility that the effects of PMA were due to supraphysiological activation of PKC. To determine whether more physiological activators of PKC could regulate TER, monolayers were treated with the diacylglycerol analog OAG. OAG caused a transient increase in TER of 31 ± 2% ($P < 0.01$) relative to that of control monolayers (Fig. 2). However, in contrast to the progressive increases in TER seen after PMA addition, the effect of OAG is short-lived, with TER returning to near baseline 90 min after OAG addition. This is consistent with the instability and rapid metabolism of OAG. Thus the effects of PMA on TER can be reproduced by the diacylglycerol analog OAG.

Inhibition of PKC prevents the effects of PMA and OAG on TER. To determine if the effects of PMA and OAG were mediated by PKC activation-dependent events, Caco-2 monolayers were preincubated for 30 min with the PKC inhibitor bisindolylmaleimide I (GF 109203X; 5 μM). This treatment had no effect on baseline TER. These monolayers were then stimulated with PMA or OAG. In the absence of GF 109203X, TER increased by 22 ± 5 and 25 ± 0% after the addition of PMA or OAG, respectively (Fig. 3). In contrast, GF 109203X prevented TER increases in monolayers treated with PMA or OAG ($P < 0.01$ for PMA or OAG). These data suggest that the increased TER is dependent on PKC activation.

Activation of PKC results in decreased phosphorylation of MLC. We have previously shown that physiological increases in TJ permeability associated with Na$^+$-glucose cotransport require the phosphorylation of MLC (45). This phosphorylation is likely mediated by MLCK, which phosphorylates MLC at Ser-19, resulting in increased actomyosin contraction (9, 20, 22). PKC can phosphorylate MLC at Ser-1, Ser-2, and Thr-9 (2, 15, 27, 28, 43). The phosphorylation at Ser-1 and Ser-2 appears to be involved in cytokinesis (42, 51), whereas the phosphorylation at Thr-9 decreases actomyosin contraction and decreases the rate of MLC phosphorylation at Ser-19 by MLCK (27, 43). Thus we considered the hypothesis that PKC might effect relaxation of the

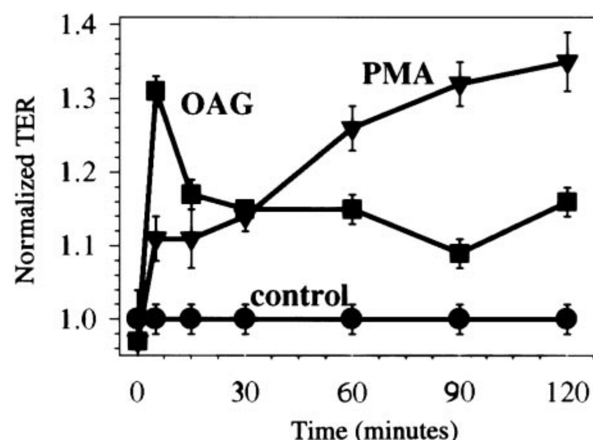

Fig. 2. Protein kinase C (PKC)-activating diacylglycerol analogs induce transient increases in TER. Monolayers of Caco-2 cells were exposed to 1.6 μM PMA or 40 μM 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). Results are mean TER ± SE normalized to control monolayers at each time point. Data were compiled from 3 separate experiments.

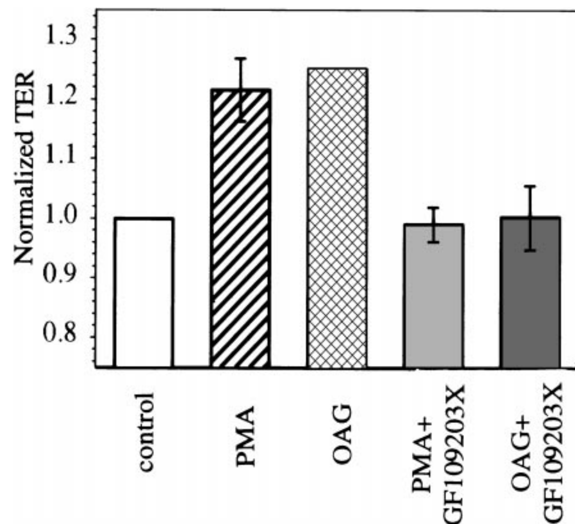

Fig. 3. PKC inhibition prevents PMA- and OAG-induced increases in TER. Monolayers of Caco-2 cells were exposed to 540 nM PMA or 40 μM OAG, with or without a 30-min preincubation with 5 μM GF 109203X. In monolayers preincubated with GF 109203X, drug was also present during incubation with PMA or OAG. GF 109203X by itself had no effect on TER. Results are mean TER ± SE normalized to control monolayers.

PAMR and increased TER via phosphorylation of MLC at Thr-9.

To determine if PKC activation increased the phosphorylation of MLC at Thr-9, we began by measuring ^{32}P incorporation into MLC. Small decreases in MLC phosphorylation (12%) were evident within 15 min of treatment with 1.6 μM PMA. As was true of TER increases, decreases in MLC phosphorylation continued over time until ^{32}P incorporation into MLC was decreased by 41% after 2 h of PMA treatment ($P < 0.05$; Fig. 4).

To evaluate the stoichiometry of MLC phosphorylation, we also separated MLC by phosphorylation state (charge) by urea glycerol-PAGE (32) in the first dimension, followed by SDS-PAGE in the second dimension. MLC was then detected in the two-dimensional gels by immunoblotting. Only monophosphorylated MLC was detectable in lysates of control monolayers (Fig. 5). Thus 100% of MLC was monophosphorylated in control monolayers. Neither diphosphorylated nor nonphosphorylated isoforms were detected (Fig. 5). In contrast, only 74% of MLC was monophosphorylated after treatment with PMA ($P < 0.05$). No diphosphorylated MLC was detected in PMA-treated monolayers. Thus PKC activation results in decreased phosphorylation of MLC, with a shift from the monophosphorylated to the nonphosphorylated isoform.

PKC activation does not cause phosphorylation of MLC Thr-9. Although the total phosphorylation of MLC was decreased after PKC activation, we considered the possibility that this was due to a PKC-mediated increase in the phosphorylation of MLC at Thr-9 and a greater decrease in phosphorylation at Ser-19. This might result in decreased total phosphorylation of MLC as well as a greater-than-anticipated decrease in actomyosin ATPase activity, because PKC-mediated phosphorylation of MLC at Thr-9 inhibits actomyosin ATPase activity, while MLCK-mediated phosphorylation of MLC at Ser-19 activates actomyosin ATPase activity. To determine if any phosphorylation of MLC at Thr-9 occurred after PKC activation, we analyzed the phosphoamino acid composition of ^{32}P-labeled MLC in control and PMA-stimulated monolayers. This analysis detected phosphoserine, but not phosphothreonine, in MLC from either control or PMA-stimulated monolayers (Fig. 6). Tryptic peptide analysis of MLC from PMA-stimulated monolayers also failed to identify phosphorylation at Thr-9; only Ser-19 phosphoryla-

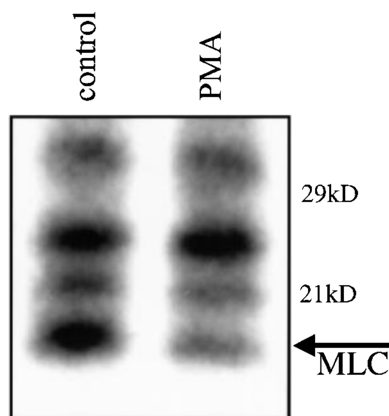

Fig. 4. Myosin light chain (MLC) phosphorylation is decreased after PMA-induced activation of PKC. MLC phosphorylation was assessed in lysates of ^{32}P-labeled control or PMA-treated monolayers harvested 2 h after addition of 1.6 μM PMA. Lysates were separated by SDS-15% PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After autoradiography, quantitative MLC immunoblots were used for localization and assessment of MLC for each sample. This corresponded to a 41% decrease in MLC phosphorylation after PMA addition, relative to control monolayers.

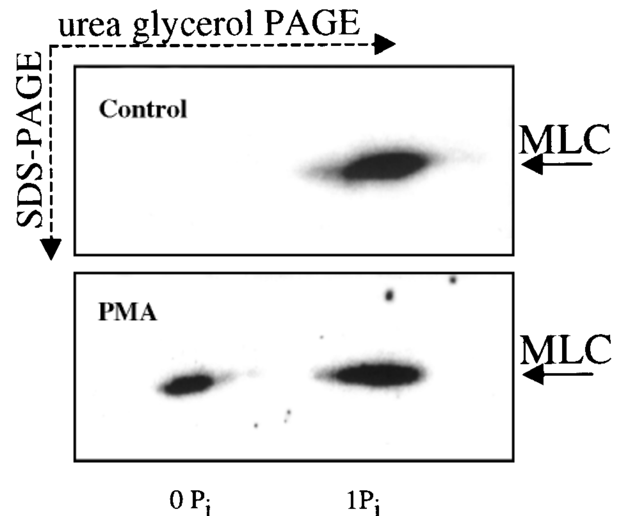

Fig. 5. MLC shifts from monophosphorylated ($1P_i$) to nonphosphorylated ($0P_i$) state after PMA-induced activation of PKC. MLC phosphorylation in control or PMA-treated monolayers harvested 2 h after addition of 1.6 μM PMA was assessed. TCA precipitates were separated by 2-dimensional urea glycerol-PAGE-SDS-PAGE, transferred, and immunoblotted with anti-MLC. Spots corresponding to monophosphorylated and nonphosphorylated MLC were detected in lysates from PMA-treated monolayers, but only monophosphorylated MLC was detected in lysates from control monolayers.

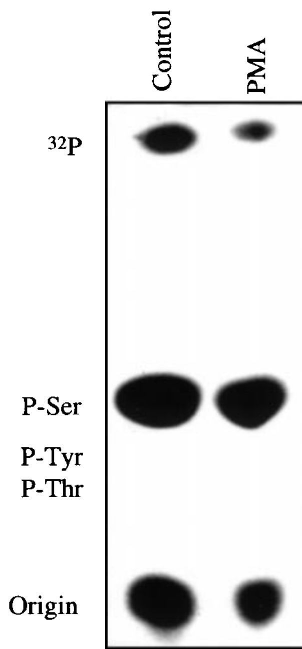

Fig. 6. MLC is phosphorylated on serine, but not threonine, residues in Caco-2 monolayers, regardless of PKC activity. ^{32}P-labeled MLC was purified by SDS-15% PAGE from control monolayers or monolayers incubated with 1.6 μM PMA for 2 h. After acid hydrolysis, phosphoamino acids were separated by thin-layer electrophoresis. Positions of migration of phosphoamino acid standards, determined by ninhydrin staining, are indicated. In MLC purified from control or PMA-treated monolayers, only phosphoserine (P-Ser) was detected.

tion was detected (data not shown). Thus phosphorylation of MLC in monolayers of Caco-2 intestinal epithelial cells occurred primarily at Ser-19, and increased phosphorylation of MLC at Thr-9 did not occur after PKC activation. These data show that the mechanism of PKC-dependent increases in TJ permeability does not include phosphorylation of MLC on Thr-9.

Caco-2 intestinal epithelial cell monolayers express a 215-kDa MLCK. Because direct inhibitory phosphorylation, at Thr-9, of MLC did not occur, we considered the possibility that PKC regulated the activity of MLCK. To test this hypothesis, we first assessed the presence of MLCK in Caco-2 monolayers. Cell lysates were separated by SDS-PAGE, and blots were probed with a monoclonal anti-MLCK antibody specific for the NH_2 region that reacts with residues 29–80 of chicken gizzard MLCK and also detects mammalian smooth muscle MLCK (7). A single band of ~215 kDa was detected (Fig. 7). Although significantly larger than MLCK proteins identified in smooth muscle and platelets, this protein is similar in mass to a 214-kDa MLCK protein recently cloned from human endothelial cells (8). The endothelial MLCK gene has a coding region with >95% sequence homology to the coding region of the rabbit and bovine smooth muscle MLCK genes. The amino terminus of this protein reacts with the same monoclonal antibody we used for detection of MLCK in Caco-2 cells. Although regulation of this MLCK by PKC has not been demonstrated, phosphorylation by protein kinase A (PKA) has been shown to decrease the activity of endothelial MLCK (8). The calmodulin-binding domain, which is conserved between endothelial and smooth muscle MLCK isoforms, contains both PKC and PKA phosphorylation sites, and phosphorylation at these sites appears to inhibit MLCK activity by interfering with calmodulin-dependent enzyme activation (29, 43).

Caco-2 intestinal epithelial MLCK is phosphorylated after PKC activation. To determine whether the Caco-2 MLCK is phosphorylated after activation of PKC, ^{32}P-labeled lysates or MLCK immunoprecipitates from control or PMA-stimulated Caco-2 monolayers were separated by SDS-PAGE (Fig. 7). The immunoblot shows the 215-kDa MLCK expressed in Caco-2 monolayers and also shows that comparable amounts of MLCK are present in the samples from control and PMA-stimulated Caco-2 monolayers. Increased MLCK phosphorylation occurred within 15 min (64% increase over control; $P < 0.05$) in PMA-stimulated monolayers and was stable for at least 2 h (51% increase over control; $P < 0.01$) after PMA addition. Increased MLCK phosphorylation after PMA addition was prevented by the PKC inhibitor GF 109203X ($P < 0.01$; Fig. 7), suggesting that activation of PKC is required for increased phosphorylation of MLCK.

DISCUSSION

We have previously shown that monolayers of SGLT1-transfected Caco-2 cells are capable of physiological Na^+-glucose cotransport (44). In this system, activation of Na^+-glucose cotransport increases both TJ permeability and MLC phosphorylation, suggesting that increased tension on the PAMR is the mechanism by which MLC phosphorylation increases TJ permeability and decreases TER (45). Similar associations between MLC phosphorylation, PAMR contraction, and TJ permeability have been described for models utilizing pathophysiological and nonphysiological stimuli in epithelial and endothelial cell monolayers (9, 11, 25, 50, 52).

One such agonist, phorbol ester-mediated activation of PKC, has been previously shown to acutely decrease

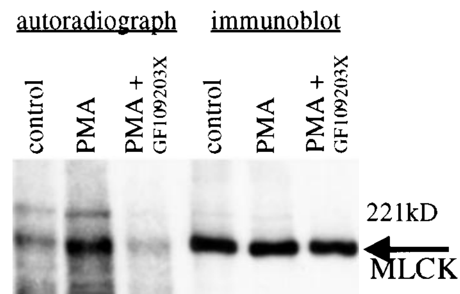

Fig. 7. MLC kinase (MLCK) phosphorylation increases after PMA-induced activation of PKC. MLCK phosphorylation in lysates of ^{32}P-labeled monolayers was assessed. Monolayers were harvested 2 h after addition of 1.6 μM PMA, with or without GF 109203X pretreatment. Lysates were separated by SDS-5% PAGE and transferred to PVDF membranes. After autoradiography (*left*), quantitative MLCK immunoblots (*right*) were used for localization and assessment of MLCK content for each sample. A 51% increase in MLCK phosphorylation, relative to control monolayers, occurred after PMA addition. Results were similar after 15 min of PMA treatment (see text).

TJ permeability and increase TER in intestinal epithelial cells (12, 39). TER then decreases after prolonged phorbol ester treatment, most likely because of PKC downregulation (12, 39). Because phorbol ester-induced changes in TER were associated with ultrastructural perturbations of the actomyosin cytoskeleton (12), we sought to determine if the effects of phorbol esters were related to MLC phosphorylation. We tested the hypothesis that PKC activation results in decreased TJ permeability via relaxation of the PAMR. Two alternative mechanisms were considered. The first, that PKC phosphorylates MLC at Thr-9, was refuted. Thr-9 phosphorylation of MLC has been shown to inhibit actomyosin contraction and decrease the suitability of MLC as a substrate for phosphorylation by MLCK in nonepithelial cells (13, 17, 29, 43). In our study of intestinal epithelial cells, MLC phosphorylation was only detected on serine residues after PKC activation. Thus an alternative mechanism, that phosphorylation of MLCK decreases its activity, was considered (8, 10, 16, 30, 41). Intestinal epithelial cell MLCK was found to be a 215-kDa protein that was rapidly phosphorylated after PKC activation. This was associated with progressive decreases in MLC phosphorylation and concomitant increases in TER. These data suggest that PKC-mediated phosphorylation of MLCK may trigger a sequence that leads to increased TER via PAMR relaxation.

The involvement of the PAMR in PKC-dependent intestinal epithelial TJ regulation has been documented previously (12). In a study of the effect of prolonged phorbol ester exposure on TJ permeability in monolayers of the intestinal epithelial cell line T84, Hecht et al. (12) showed that downregulation of PKC correlated with decreased TER and disruption of both the PAMR and basal stress fibers. Notably, both that study and a related study by Tai et al. (12, 39) documented increased TER shortly after the addition of phorbol esters to monolayers of intestinal epithelial cells. This effect is similar to the results we have reported and coincides with PKC activation. In the studies by Hecht et al. (12) and Tai et al. (39), TER of phorbol ester-treated monolayers only decreased below that of controls after >8 h of treatment. These data are similar to ours but contrast sharply with those reported by Stenson et al. (38). Stenson et al. noted 50% decreases in TER 2 h after the addition of 50 nM PMA to Caco-2 cell monolayers (38). We did not detect any effect of 50 nM PMA on TER of Caco-2 cell monolayers, and noted increases in TER at doses of PMA >170 nM. The most likely explanations for the disparity in results between our study and that of Stenson et al. have to do with the specific Caco-2 cell lines used and the techniques used for preparation of the monolayers. We used a subclone of Caco-2 cells derived from the Caco-2 BBe line (34), which forms polarized monolayers with well-developed microvilli and expresses microvillus brush-border-associated proteins (34). Our Caco-2 cells also express the intestinal Na^+-glucose cotransporter SGLT1, demonstrate vectorial glucose-dependent Na^+ transport, are capable of physiological TJ regulation, and manifest a high level of MLC phosphorylation when cultured in typical growth media with 25 mM glucose (44, 45). Our Caco-2 monolayers were used at least 20 days postconfluence, an interval associated with homogeneous phenotypic differentiation of Caco-2 cells toward absorptive enterocytes (33, 46, 47). In contrast, Stenson et al. (38) studied a different clone of Caco-2 cells at 7 days postconfluence, at which time cell polarization, differentiation, and brush-border protein expression are heterogeneous (46). Therefore, the functional states of the Caco-2 monolayers used in our study and that of Stenson et al. were likely very different. A discrepancy in the effects of PMA on TJ regulation in various clones of the LLC-PK_1 renal epithelial cell line has also been reported (5). Thus the effects of PMA on TJ permeability may vary, depending on the cell type, tissue of origin, differentiation status, and degree of basal MLC phosphorylation in the monolayers studied.

An additional mechanism by which phorbol esters may have differential effects in various cell types could be heterogeneity in PKC isoform expression and localization. Rabbit ileal enterocytes express PKC-α, PKC-ζ, and PKC-ϵ (14). An atypical PKC isotype-specific interacting protein and PKC-λ have also been colocalized to the TJ in rat intestinal epithelium (18). Similarly, Caco-2 cells contain PKC-α and PKC-ζ (6), with the PKC-ζ isoform localizing to or near the TJ (4). This spatial pattern of expression might lead to the hypothesis that PKC-ζ is responsible for PMA-dependent TJ regulation. However, the observations that chronic phorbol ester treatment downregulates PKC-α but not PKC-ζ in Caco-2 cells (6) and that downregulation of PKC activity is associated with increased TJ permeability (12) suggest that PKC-α may be the critical regulatory isoform. This is supported by the observation that overexpression of PKC-α in LLC-PK_1 cells, which increases TJ permeability in response to phorbol esters, made the monolayers more sensitive to phorbol esters (35). However, overexpression of PKC-δ also increased transepithelial permeability in LLC-PK_1 cells (26). Thus the possibility that several PKC isoforms are capable of regulating TER via the PAMR cannot be excluded.

The conclusion that PKC-induced regulation of TJ permeability is mediated via decreased MLC phosphorylation and PAMR relaxation is consistent with a growing body of data supporting the role of MLC phosphorylation in TJ regulation. For example, we have shown that reversible phosphorylation of MLC occurs during Na^+-glucose cotransport-dependent TJ regulation (45). The dependence of this process on MLCK activation is demonstrated by the fact that inhibitors of MLCK prevent both MLC phosphorylation and TJ regulation (45). Similarly, MLC phosphorylation occurs in association with increased TJ permeability after infection of model intestinal epithelia by enteropathogenic *Escherichia coli* (52). These changes are also preventable by MLCK inhibitors (52). Finally, the most direct evidence supporting the role of MLC phosphorylation in TJ regulation comes from the transfection of MDCK cells with a mutant MLCK. The

calmodulin-binding domain was genetically removed from this MLCK, resulting in a constitutively active truncated MLCK. MDCK cells that express this constitutively active MLCK develop TERs that are <10% of that for control monolayers (11).

Relatively little information on the regulation of MLCK in intestinal epithelia is available. This study represents the first report of a 215-kDa MLCK expressed in intestinal epithelial cells. This MLCK was detected with an NH_2 region-specific anti-MLCK monoclonal antibody that reacts with chicken gizzard and mammalian smooth muscle MLCK (7). Two other high-molecular-mass MLCK molecules have been described. The first, a 208-kDa MLCK that is distinct from both smooth muscle and nonmuscle MLCK, is expressed in embryonic tissues and some cultured cells (7). This MLCK does not contain the NH_2 terminus sequence of smooth muscle MLCK and is not recognized by the monoclonal antibody we used. A distinct 214-kDa MLCK molecule that has sequence homology to mammalian smooth muscle and avian nonmuscle MLCK has been cloned from human endothelial cells (8). This endothelial MLCK can be immunoprecipitated by the same monoclonal antibody used in our study (8). Immunoprecipitates of this endothelial MLCK have displayed kinase activity in in vitro assays (8), and we have shown that the immunoprecipitated 215-kDa intestinal epithelial MLCK possesses MLC kinase activity and is responsible for the majority of MLCK activity in Caco-2 cell extracts (unpublished data). Phosphorylation of human endothelial MLCK is increased by PKA activation, resulting in decreased kinase activity (8). Thus it appears that phosphorylation of endothelial MLCK may be a physiologically relevant mechanism for kinase downregulation. This report provides evidence that a similar phenomenon may explain the regulation of TER by PKC in intestinal epithelium. As shown in Fig. 8, it is clear that MLCK phosphorylation occurs rapidly and that the response is complete within 15 min. This coincides with the maximal activation of PKC. In contrast, the observed decreases in MLC phosphorylation occur progressively, with only small changes apparent at 15 min. Two hours are required for development of the complete response. These data are consistent with inhibition of MLCK by PKC-mediated phosphorylation and a resulting progressive loss of phosphorylated MLC. The progressive TER increases correlate with the progressive decreases in MLC phosphorylation (Fig. 8), lending further support to this hypothesis. Unfortunately, we have been unable to confirm decreased activity of phosphorylated epithelial MLCK by an in vitro assay. This may be due to our inability to reproduce spatial localization within cells in an in vitro assay, to the phosphorylation of a specific subset of MLCK, to differences in ion or calmodulin composition between the in vitro assay and intracellular reaction mixtures, or to the unavailability of intestinal epithelial MLC as an in vitro substrate. Nonetheless, it should be noted that small changes in in vitro MLCK activity have been associated with significant changes in MLC phosphorylation and actomyosin function in intact cells (21). Alternatively, we cannot exclude the possibility that PKC-dependent MLCK phosphorylation does not alter the activity of epithelial MLCK. In that case, the decreased phosphorylation of MLC must be explained by other mechanisms. These might include activation of MLC phosphatase or of other cellular phosphatases. Further analysis of the specific molecular mechanisms by which PKC alters MLC phosphorylation and TER is needed.

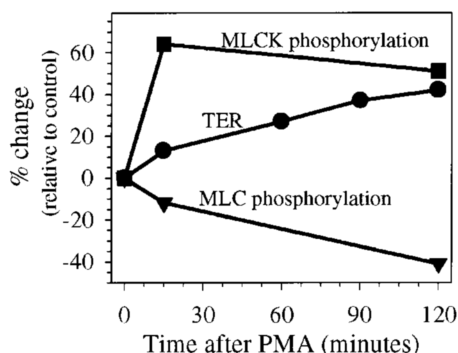

Fig. 8. PKC activation induces rapid changes in MLCK phosphorylation and progressive changes in TER and MLC phosphorylation. Monolayers of Caco-2 cells were exposed to 1.6 μM PMA. TER, MLC phosphorylation, and MLCK phosphorylation were assessed at indicated times after PMA addition. Values are percent changes (positive or negative) relative to control monolayers, which did not have PMA added.

In addition to MLCK, a variety of other TJ-associated proteins are potential targets for PKC and other serine/threonine kinases as well as tyrosine kinases. Threonine phosphorylation of the TJ protein occludin, which is involved in intercellular TJ interactions, correlates with TJ localization of occludin and decreased TJ permeability (36, 49). No occludin kinase has yet been identified. Tyrosine phosphorylation may also be involved in TJ regulation. Increased TJ permeability after epidermal growth factor receptor tyrosine kinase activation, expression of v-Src tyrosine kinase, or treatment of epithelial monolayers with tyrosine phosphatase inhibitors has also been described (37, 40, 48). Potential tyrosine kinase targets include the TJ proteins ZO-1 and ZO-2.

In summary, we have shown that the progressive increases in TER that follow PKC activation are accompanied by progressive decreases in MLC phosphorylation. An acute increase in MLCK phosphorylation that occurs concurrently with PKC activation precedes changes in MLC phosphorylation and TER. These data are consistent with a model in which PKC-mediated phosphorylation decreases the activity of the 215-kDa intestinal epithelial MLCK. MLCK inactivation may then lead to decreased MLC phosphorylation, relaxation of the PAMR, and increased TER. Thus this study represents the initial characterization of a molecular mechanism by which PKC activation may alter PAMR tension and TJ permeability.

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