Protein Kinase C Regulates the Phosphorylation and Cellular Localization of Occludin*

Received for publication, May 30, 2001, and in revised form, August 6, 2001
Published, JBC Papers in Press, August 13, 2001, DOI 10.1074/jbc.M104923200

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Occludin is an integral membrane phosphoprotein specifically associated with tight junctions, contributing to the structure and function of this intercellular seal. Occludin function is thought to be regulated by phosphorylation, but no information is available on the molecular pathways involved. In the present study, the involvement of the protein kinase C pathway in the regulation of the phosphorylation and cellular distribution of occludin has been investigated. Phorbol 12-myristate 13-acetate and 1,2-diocatanylglycerol induced the rapid phosphorylation of occludin in Madin-Darby canine kidney cells cultured in low extracellular calcium medium with a concomitant translocation of occludin to the regions of cell-cell contact. The extent of occludin phosphorylation as well as its incorporation into tight junctions induced by protein kinase C activators or calcium switch were markedly decreased by the protein kinase C inhibitor GF-10926X. In addition, in vitro experiments showed that the recombinant COOH-terminal domain of murine occludin could be phosphorylated by purified protein kinase C. Ser338 of occludin was identified as an in vitro protein kinase C phosphorylation site using peptide mass fingerprint analysis and electrospray ionization tandem mass spectrometry. These findings indicate that protein kinase C is involved in the regulation of occludin function at tight junctions.

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Occludin comprises four transmembrane domains, two extracellular loops, and three cytoplasmic domains (one intracellular, a short NH2-terminal domain, and a long COOH-terminal domain). Accumulating evidence suggests that occludin plays an important role in tight junctions, although embryonic stem cells lacking occludin are able to form well developed TJs (9). The overexpression of mutant forms of occludin in cultured epithelial cells leads to changes in the barrier and fence function (10, 11). The addition of synthetic peptides corresponding to the extracellular loops of occludin to epithelial cells results in the disappearance of TJ and inhibition of cell adhesion (12–14). Finally, occludin knock-out mice show a complex phenotype, including retarded growth and various histological abnormalities, suggesting that the functions of both TJ and occludin are more complex than was supposed previously (15).

Occludin has been shown to be highly phosphorylated (16, 17). Tight junction assembly induced by calcium switch is paralleled by occludin phosphorylation and incorporation into the TJ of MDCK cells (16, 17). It has also been shown that highly phosphorylated occludin molecules are selectively concentrated at TJs, whereas non- or less phosphorylated occludin is localized in the cytoplasm (16). Occludin becomes phosphorylated and associates with ZO-1 during certain stages of mouse embryonic development, and these processes are proposed to regulate TJ biogenesis and the timing of blastocyst formation (18). In addition, recent results suggest that the phosphorylation of occludin may regulate tight junction permeability in response to histamine and lysophosphatidic acid (19). The findings above led to the conclusion that occludin function at TJ is regulated by phosphorylation. The molecular pathways regulating occludin phosphorylation remain unclear.

In the present study, we analyzed the effect of phorbol 12-myristate 13-acetate (PMA) and 1,2-diocatanylglycerol (diC8), activators of protein kinase C, on the phosphorylation and cellular localization of occludin in monolayers of MDCK cells incubated in low extracellular calcium 2+ medium. PMA and diC8 induced a rapid phosphorylation of occludin and its redistribution to the regions of cell-cell contact. The phosphorylation and incorporation of occludin into tight junctions induced by PMA,
diC8, or calcium switch were inhibited by a PKC inhibitor. Furthermore, Ser336 of the recombinant COOH-terminal domain of murine occludin was found to be phosphorylated in vitro by purified protein kinase C. These findings suggest that the regulation of phosphorylation and cellular distribution of occludin are mediated by protein kinase C.

**MATERIALS AND METHODS**

**Reagents, Antibodies, and Cells**—Protease inhibitors and protein A-Sepharose were from Sigma (Taufkirchen, Germany). Protein kinase C, PMA, t-32P-ATP, and a PKC inhibitor were purchased from Alexis Biochemicals (Gräfenhain, Germany). Mouse anti-occludin monoclonal antibodies 3D9 and 5D1 were purchased from Life Technologies GmbH (Technologiepark Karlsruhe, Germany). Cy3- and horseradish peroxidase-conjugated goat anti-rabbit monoclonal antibodies and rabbit polyclonal anti-occludin antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA). MDCR cells were obtained from Dr. Swaroop (MDCR, Berlin, Germany). The pMAL-c2x plasmid (Clontech, Palo Alto, CA) was provided by Dr. G. Herling (Institute of Biochemistry, University of Karlsruhe, Germany). Occludin cDNA was purchased from Promega GmbH (Mannheim, Germany). 5'-32P-t-32P-ATP and the blotting detection system, Bio-Rad (Hercules, CA) were purchased from Amersham Pharmacia Biotech Europe GmbH (Freiburg, Germany).

**Occludin Expression Construct and in Vitro Phosphorylation of Recombinant Occludin**—Total RNA was isolated from 50 mg of murine kidney using TRIzol reagent and reverse transcription was performed using Moloney murine leukemia virus reverse transcriptase and random hexamer primers. A 777-base pair fragment encoding amino acids 264–521 of murine occludin was amplified from the primers 5'-AGATCGAACAACCGAAGAAGATGACG-3' and 5'-TTCTGACGTAAGTTTCCGTCGTACATGC-3' (BamHI and SalI sites are underlined). The amplified product was cloned using the TOPO-TA cloning kit (Invitrogen, Carlsbad, CA). Several clones were sequenced (Taq DyeDeoxy-Terminator cycle sequencing kit, Applied Biosystems, Weiterstadt, Germany); a clone containing no mutations was selected, and its BamHI-SalI fragment was subcloned into the pMAL-c2x plasmid to produce a plasmid coding for the COOH-terminal domain of occludin fused with maltose-binding protein. The fusion protein was overexpressed at Escherichia coli and purified over an anion exchange column according to the manufacturer's instructions.

The in vitro phosphorylation of occludin was performed as follows. Purified recombinant occludin fragment (5 μg) was mixed with 16 ng of protein kinase C in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM MgCl2, 2 mM CaCl2, 1 mM dithiothreitol, 0.2 mM ATP, 5 nM PMA, and 5 μCi of 5'-32P-t-32P-ATP in a reaction volume of 40 μl. In other experiments GF-109203X at a concentration of 5 μM was added to inhibit PKC. Phosphorylation of MBP-β-galactosidase (the expression product of the pMal-c2x plasmid) was performed as for occludin, but double the amount of PKC was used.

**Cell Culture, Low Calcium Medium Culture, and Calcium Switch**—MDCR cells were cultured in MEM with 10% FCS. Low Ca2+-medium (LC) was prepared from S-MEM (calcium-free MEM) and 5% FCS (the resulting extract is referred to as the Triton X-100-insoluble fraction). One-dimensional SDS-PAGE (8% gel) was performed as for occludin, but double the amount of protein was loaded. For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose sheets, which were then incubated with the antibodies. The antibodies were detected with a blotting detection kit. To detect protein phosphorylation, samples were blotted as above and the 32P signal was detected by autoradiography. Proteins transferred on the nitrocellulose membrane were stained by silver staining.

**Occludin Immunoprecipitation and Alkaline Phosphatase Treatment**—MDCR cells cultured on 8.8-cm² Petri dishes were washed twice with ice-cold PBS and extracted with 1 ml of ice-cold immunoprecipitation buffer (25 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% SDS), boiled for 5 min, and centrifuged for 10 min at 14,000 × g (the resulting extract is referred to as the Triton X-100-insoluble fraction). One-dimensional SDS-PAGE (5% gel) was performed according to Laemmli (20). For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose sheets, which were then incubated with the antibodies. The antibodies were detected with a blotting detection kit. To detect protein phosphorylation, samples were blotted as above and the 32P signal was detected by autoradiography. Proteins transferred on the nitrocellulose membrane were stained by silver staining.

**Occludin Phosphorylation by PKC**—In vitro phosphorylation of occludin was performed as follows. Purified recombinant occludin fragment (5 μg) was mixed with 16 ng of protein kinase C in 50 mM Tris-HCl buffer, pH 7.5, containing 10 mM MgCl2, 2 mM CaCl2, 1 mM dithiothreitol, 0.2 mM ATP, 5 μM PMA, and 5 μCi of 5'-32P-t-32P-ATP in a reaction volume of 40 μl. In other experiments GF-109203X at a concentration of 5 μM was added to inhibit PKC. Phosphorylation of MBP-β-galactosidase (the expression product of the pMal-c2x plasmid) was performed as for occludin, but double the amount of PKC was used.

**Identification of Phosphorylation Sites by Mass Spectrometry**—For MALDI analysis, 15 μl of occludin solution taken from the phosphorylation reaction were resolved by SDS-PAGE. The proteins were detected by staining with a Colloidal Blue staining kit (Novex, San Diego, CA). Phosphorylated and non-phosphorylated occludin bands were excised from the stained gels, washed with 50% (v/v) acetonitrile in water, then dried under vacuum. The samples were re-dissolved in 30 μl of 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 45 min at 55 °C. Alkylation was performed by replacing the dithiothreitol solution with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. After a 20-min incubation at 25 °C in the dark, the gel pieces were washed with 50–100 μl of 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, 10 mM ammonium bicarbonate, dehydrated in acetonitrile, and dried in a vacuum centrifuge. Diisulfide bonds were reduced by incubation in 30 μl of 10 mM dithiothreitol in 100 mM ammonium bicarbonate for 45 min at 55 °C. Alkylation was performed by replacing the dithiothreitol solution with 55 mM iodoacetamide in 100 mM ammonium bicarbonate. After a 20-min incubation at 25 °C in the dark, the gel pieces were washed with 50–100 μl of 50% (v/v) acetonitrile in 25 mM ammonium bicarbonate, 10 mM ammonium bicarbonate, dehydrated in acetonitrile, and dried in a vacuum centrifuge. Diisulfide bonds were reduced by incubation in 30 μl of 10 mM dithiothreitol in 100 mM ammonium bicarbonate, containing 300 ng of endoproteinase Lys-C (sequencing grade, Roche Diagnostics, Mannheim, Germany). After 15 min, 5 μl of 50 mM ammonium bicarbonate was added to keep the gel pieces moist during Lys-C cleavage (37 °C, overnight). To extract the peptides, 15 μl of 0.5% (v/v) trifluoroacetic acid acetonitrile in acetonitrile was added, and the samples were sonicated for 5 min. The extraction liquid was dried under vacuum and redissolved in 10 μl of 0.1% (v/v) trifluoroacetic acid in water. The peptides were purified over a C18 reversed-phase minicolumn filled in a microipette tip (ZipTip C18, Millipore, Bedford, MA) for mass spectrometry analysis. Purification was performed according to the manufacturer's manual, except that peptides were eluted with 3 μl of 60% (v/v) acetonitrile, 0.2% (v/v) trifluoroacetic acid or with 5 μl of 60% (v/v) acetonitrile, 0.2% (v/v) formic acid for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and nanoelectrospray tandem mass spectrometry (nanoESI-MS/MS), respectively.

**MALDI-MS measurements** were performed on a Voyager-DE STR BioSpectrometry work station MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA). One μl of the analyte solution was mixed with 1 μl of α-cyano-4-hydroxy-cinnamic acid matrix solution consisting of 10 mg of matrix dissolved in 1 ml of 0.3% trifluoroacetic acid in acetonitrile-water (1:1, v/v). One μl of the resulting mixture was applied to the sample plate. Samples were air-dried at ambient temperature (24 °C). Measurements were performed in the reflection mode at an acceleration voltage of 20 kV, 70% grid voltage, and a delay of 200 ns. Each spectrum obtained was the mean of 256 laser shots.

For nanoESI-MS/MS, 5 μl of the peptide mixture were lyophilized and dissolved in 5 μl of methanol, 1% formic acid (1:1, v/v). The MS/MS measurements were performed with a nanoelectrospray hybrid quadrupole mass spectrometer (Q-ToF, Micromass, Manchester, United Kingdom). The collision gas was argon at a pressure of 6.0 × 10−7 mbar in the collision cell.
RESULTS

Effect of PKC Activators on the Localization of Occludin in MDCK Cells in Low Calcium Medium—In view of the fact that diacylglycerols are known to induce the formation of tight junction strands in MDCK cells cultivated in low calcium medium (4), we assumed that occludin phosphorylation and recruitment into tight junctions are mediated by PKC. To verify this hypothesis, we investigated the effect of PKC activators on the phosphorylation and cellular distribution of occludin in MDCK cells cultured in low calcium medium, which disrupts tight junctions. In accordance with literature data (7, 10), occludin was intensively stained at cell-cell contacts in confluent MDCK cells in normal Ca^{2+} medium (4), indicating that continuous TJs were formed (Fig. 1A). After incubation of the cells in LC, occludin became localized almost exclusively in the cytoplasm, indicating a down-regulation of TJs (Fig. 1B). When PMA at 2 nM was added for 2 h to the cells in LC, a portion of the occludin was translocated to the plasma membrane at regions of cell-cell contact (Fig. 1C, arrows). Staining of occludin at cell-cell contacts was not as continuous or intensive as in the cells grown in NC (Fig. 1A). Prolongation of the incubation period up to 4 h further increased the amount of occludin translocated to the regions of cell-cell contact (Fig. 1D). When 25 nM PMA was used to stimulate cells, occludin translocated to the periphery showed a more discontinuous and intensive staining as compared with samples treated with 2 nM PMA (Fig. 1E). Most of the occludin was still localized in the cytoplasm, a significant portion in large granular structures (Fig. 1E, arrowheads). Prolongation of the incubation time up to 4 h did not change the distribution pattern significantly (Fig. 1F), although the decrease in the amount of occludin localized at cell-cell contacts was notable. The treatment of MDCK cells in LC with the another protein kinase C activator diC8 resulted in the redistribution of occludin, which was similar to the effect of 2 nM PMA. Fragmentary staining of occludin at cell-cell contacts was evident 2 h after the addition of diC8 (Fig. 1G). The amount of occludin translocated to cell-cell contacts further increased when the incubation time was prolonged up to 4 h (Fig. 1H).

Effect of PKC Activators on the Triton X-100 Solubility and Phosphorylation of Occludin in MDCK Cells in LC—Previous studies have shown that occludin localized in TJs cannot be extracted with non-ionic detergents from confluent epithelial cells and that it shows a decreased electrophoretic mobility due to extensive phosphorylation (16, 17, 21). In accordance with literature data (17), occludin extracted with Triton X-100 from MDCK cells grown in NC migrated as a single band with an apparent molecular mass of ±60 kDa, whereas that from the Triton X-100-insoluble fraction migrated as multiple unresolved bands with apparent molecular masses ranging between 60 and 82 kDa (Fig. 2A, lane 1). When MDCK cells were incubated in LC in order to down-regulate TJs, the high molecular mass bands disappeared from the Triton X-100-insoluble fraction and the amount of Triton X-100-insoluble occludin decreased significantly (Fig. 2A, lane 2). When TJs re-assembly was induced by calcium switch, the banding pattern of Triton X-100-insoluble occludin was restored (Fig. 2A, lane 3). Four hours after the addition of 2 nM PMA to the cells in LC, the amount of Triton X-100-insoluble occludin increased significantly and a portion of occludin underwent an upward band shift (Fig. 2A, lane 4). Treatment of cells in LC with 25 nM PMA

FIG. 1. PMA and diC8 induce the translocation of occludin from the cytoplasm to the lateral cell membrane in MDCK cells incubated in low Ca^{2+} medium. MDCK cells were incubated in low calcium medium for 20 h after which PMA, diC8, or Ca^{2+} was added. Cells cultivated in normal calcium medium show continuous staining of occludin at cell-cell contacts (A). After cultivation of cells in low calcium medium, the occludin signal was detected mainly in small granular structures in the cytoplasm (B). Two hours after the addition of 2 nM PMA, staining of occludin at cell borders (arrows) was evident (C). Prolongation of the incubation period to 4 h further increased the amount of occludin migrating to the cell borders (D). When 25 nM PMA was used to stimulate the cells for 2 h, large granular structures were found in the cytoplasm (arrowheads) and occludin staining at the cell borders became discontinuous (E). Prolongation of the incubation period (25 nM PMA) up to 4 h led to some decrease in the amount of occludin found at cell-cell contacts (F). Two hours after the addition of 0.5 mM diC8, staining of occludin at cell borders was evident (G). Prolongation of the incubation period to 4 h further increased the amount of occludin at cell borders (H). Bar, 10 μm.

FIG. 2. PMA and diC8 induce the phosphorylation of occludin in MDCK cells incubated in low Ca^{2+} medium. Panel A, immunoblot analysis of occludin in the Triton X-100-soluble (S) and -insoluble (I) fractions of MDCK cells incubated in normal Ca^{2+} medium (lane 1) or in low Ca^{2+} medium (lane 2). MDCK cells cultivated in low calcium medium were subjected to a calcium switch (lane 3) or treated with 2 nM (lane 4), 25 nM PMA (lane 5), or 0.5 mM diC8 for 4 h (lane 6). Treatment of MDCK cells cultivated in low calcium medium with PMA or diC8 induced an upward band shift. B, immunoblot analysis of the Triton X-100-insoluble fraction of immunoprecipitated occludin from MDCK cells cultivated in low calcium medium and stimulated with 10 nM PMA for 20 min. Samples were either left untreated (lane 1) or subjected to treatment with alkaline phosphatase (lane 2), resolved by electrophoresis, and then analyzed by immunoblotting. Alkaline phosphatase treatment completely abolished the upward band shift caused by PMA treatment. The arrow shows the position of non-phosphorylated occludin, the dash shows the position of antibody heavy chain, and a bracket denotes the position of multiple phosphorylated forms of occludin.
for 4 h resulted in an additional increase in the amounts of Triton X-100-insoluble occludin and in the degree of upward band shift, with a concomitant decrease in the amount of Triton X-100-soluble occludin (Fig. 2A, lane 5). The decrease is probably due to increased proteolysis, since high concentrations of PMA are known to cause degradation of occludin (21, 22). An increase in Triton X-100-insoluble occludin and an upward band shift were also evident in cells treated with 0.5 mM diC8 for 4 h (Fig. 2A, lane 6).

The multiple bands revealed by immunoblotting analysis probably represent differently phosphorylated occludin molecules. In order to show that the upward band shift induced by PMA was due to phosphorylation, MDCK cells were treated with PMA and occludin immunoprecipitated from the Triton X-100-insoluble fraction was subjected to in vitro phosphatase treatment. Fig. 2B shows that the additional high molecular weight occludin bands disappeared on alkaline phosphatase treatment. Therefore, the upward band shift of occludin induced by PMA is due to phosphorylation of occludin, and changes in the cellular distribution of occludin induced by the treatment of MDCK cells in LC by PMA or diC8 are paralleled by its phosphorylation.

Fig. 3 shows the time and concentration dependence of PMA-induced phosphorylation of occludin. As high molecular weight bands corresponding to phosphorylated occludin were invariably found to be Triton X-100-insoluble, only Triton X-100-insoluble fractions were analyzed. Addition of PMA to MDCK cells incubated in LC medium resulted in a dose-dependent phosphorylation of occludin (Fig. 3A). When PMA was added for 1 h, the phosphorylation was evident at 2.5 mM PMA and reached a maximum at 40 mM PMA. The time dependence of occludin phosphorylation in MDCK cells in LC induced by 25 mM PMA is shown on Fig. 3B. The upward band shift became evident after 5 min and reached a maximum 40 min after PMA addition.

Phosphorylation and Redistribution of Ocludin Induced by Ca\(^{2+}\) Switch PMA and diC8 Is PKC-dependent—The striking effects of the PKC activators PMA and diC8 on the phosphorylation and distribution of occludin suggest that PKC may be a physiological modulator of occludin function. To investigate the involvement of PKC in the regulation of occludin incorporation into TJs, the assembly of tight junctions was induced by the calcium switch procedure in the presence or absence of the PKC inhibitor GF-109203X and occludin localization was assessed by immunofluorescence microscopy. Four hours after switching to NC, continuous occludin staining was evident at intercellular contacts, indicating the recruitment of occludin into tight junctions (Fig. 4A). Inhibition of PKC with 5 mM GF-109203X resulted in very weak staining of occludin at the intercellular contacts but a prominent staining of occludin in cytoplasm (Fig. 4B). It can be concluded from these data that PKC participates in the targeting of occludin into TJs. Furthermore, GF-109203X inhibited the redistribution of occludin to the regions of cell-cell contacts induced by PMA (Fig. 4, C and D) and diC8 (Fig. 4, E and F).

In order to study the involvement of PKC in the phosphorylation of occludin, the effect of GF-109203X on calcium switch-induced occludin phosphorylation was investigated. The characteristic multiple-band pattern of occludin is seen on the immunoblot in Triton X-100-insoluble fractions of MDCK cells incubated in NC medium (Fig. 5, NC). High molecular weight bands disappeared when cells were incubated in LC medium, indicating the dephosphorylation of occludin (Fig. 5, LC). Four hours after switching to NC, a profound upward band shift of occludin was visible on the immunoblot, reflecting the increased phosphorylation of occludin (Fig. 5, +Ca\(^{2+}\)). When the calcium switch was performed in the presence of 5 mM GF-109203X, the extent of occludin phosphorylation was markedly decreased, indicating the involvement of PKC. Similarly, the increase in phosphorylation of occludin induced by PMA and diC8 was significantly decreased by GF-109203X, again indicating the involvement of PKC (Fig. 5, +PMA and +diC8).

Ocludin as a Substrate of PKC—To investigate whether occludin may serve as a substrate for protein kinase C, recombinant occludin (MBP fusion protein of the soluble cytoplasmic COOH-terminal domain of murine occludin or MBP-occludin) was incubated in vitro with purified PKC (a mixture of \(\alpha, \beta, \beta\), and \(\gamma\) isoforms of PKC) in the presence of [\(\gamma\)-32P]ATP. As shown on Fig. 6 (lane 1), an autoradiography signal was detected corresponding to a protein of ~74 kDa (the predicted molecular mass of MBP-occludin is 73.6 kDa), implying that MBP-occludin was phosphorylated by protein kinase C. No
Occludin phosphorylation has been implicated in the regulation of tight junction function (16, 19, 21), but the molecular pathways involved remain unclear. The present study establishes the importance of the protein kinase C pathway in the phosphorylation and cellular localization of occludin.

The redistribution of occludin from the cytoplasm to the lateral plasma membrane has been observed after the treatment of MDCK cells in LC with 2 nM PMA or 0.5 mM of more specific PKC activator diC8. This probably reflects the insertion of occludin into TJs, since the formation of tight junction fibrils was reported under similar conditions (4). The amount of occludin translocated to the lateral plasma membrane increases with PMA concentration, but the staining of occludin becomes discontinuous. This observation is in agreement with studies reporting the disruption of TJs and increase in occludin proteolysis in response to the treatment of MDCK cells with very high (0.1–1 μM) concentrations of PMA (21, 22).

The results presented here clearly indicate that a Triton X-100-insoluble pool of occludin is phosphorylated upon the addition of the protein kinase C activators PMA or diC8 to MDCK cells incubated in low calcium medium. Previously, the time course of occludin phosphorylation induced by a calcium switch was shown to be paralleled by TJ formation (16). In this study, a low but easily distinguishable level of upward shift of occludin caused by the 4-h incubation of MDCK cells with 2 nM PMA correlates well with the levels of occludin translocated to the lateral cell membrane. Correspondingly, the increased phosphorylation caused by 25 nM PMA is paralleled by the increased amounts of occludin translocated to TJs. These observations support the assumption that the phosphorylation of occludin in response to PMA or diC8 is involved in the regulation of TJ assembly. The degree and rate of occludin phosphorylation induced by PMA are the same or even higher compared with those induced by the calcium switch; phosphorylation is induced by rather low concentrations of PKC, indicating that diacylglycerols, along with calcium ions, are physiological regulators of occludin phosphorylation. High molecular weight forms of occludin that appeared after the treatment of the cells in LC by PMA or diC8 were invariably not extractable with Triton X-100. It can be concluded that the phosphorylation regulates the association of occludin with the cytoskeleton and/or detergent-resistant membrane microdomains (24). Future investigations should clarify which detergent-insoluble structures interact with occludin and how the phosphorylation of occludin regulates these interactions.

Both the phosphorylation of occludin and its incorporation into tight junctions induced by calcium switch were markedly inhibited by the PKC inhibitor GF-109203X. These observations indicate that PKC participates in the regulation of occludin function during tight junction assembly induced by calcium switch. Previously, the application of the PKC inhibitor has been shown to inhibit the development of transepithelial electrical resistance, a functional measure of tight junction biogenesis (25). Occludin, the only known transmembrane protein of TJ whose localization in tight junctions is dependent on its phosphorylation state, may be a key target in the PKC-mediated regulation of tight junctions. Other authors have noted similarities between occludin and connexins in terms of phosphorylation and structure (16). The data presented here extend the similarities further; connexin43 is phosphorylated by PKC in response to PMA, and the phosphorylation dramatically changes gap junction channel conductivity (26). Possibly, the assembly of the junctional complex is regulated by PKC via the phosphorylation of connexin43 and occludin to coordinate the formation of a functionally active barrier and the function
of intercellular channels. The phosphorylation and redistribution of occludin induced by PKC activators were strongly inhibited by the application of GF-109203X. These observations further substantiate the specificity of the regulation of occludin functions by PKC.

The simplest explanation for the observed occludin phosphorylation induced by PMA or diC8 is that PKC directly phosphorylates occludin when stimulated by PMA, although the possibility that PKC activates an intermediate kinase(s) cannot be ruled out. Therefore, the in vitro phosphorylation of the recombinant occludin COOH-terminal domain by PKC was investigated. The carboxyl-terminal domain of occludin has been recently shown to be responsible for the targeting of occludin to the TJs (27) and for its interaction with other proteins (28, 29). The results suggest that the COOH-terminal domain of occludin may be phosphorylated by classical isoforms of PKC, which are known to be expressed in MDCK cells (30). To date, only casein kinase 2 has been reported to phosphorylate the COOH-terminal domain of occludin in vitro, whereas a number of tested kinases did not phosphorylate occludin (31). However, there are no indications of the involvement of casein kinase 2 in the regulation of TJ functions.

Despite the use of four different proteases to produce various peptides of occludin, Ser338 of occludin was the only phosphorylation site for classical PKCs that was positively identified by MS. On the other hand, the presence of multiple phosphorylated forms of occludin in vivo was demonstrated by two-dimensional gel electrophoresis (19). The appearance of multiple phosphatase-sensitive occludin bands resulting from treatment of MDCK cells with PMA indicates the multiple phosphorylation of occludin. Whereas it is possible that some phosphorylation sites might not have been identified by MS, the absence of additional phosphorylation sites suggests that other kinase(s) must phosphorylate occludin in vivo. These may include non-classical PKC isoforms, some of which are known to be associated with TJs (32, 33) and which have different substrate specificities.
sequence motifs from those of classical PKCs (34), or an intermediate kinase(s) which is activated by PKC-dependent phosphorylation and subsequently phosphorylates occludin at different positions. Ser338 is located within a ~100-amino acid stretch of occludin, which was shown to be dispensable for the binding to ZO-1 (29) and for the targeting of occludin to TJs (27). Therefore, the significance of the phosphorylation on Ser338 is not clear.

Taken together, our data outline the importance of the protein kinase C pathway in the regulation of occludin function at tight junctions. Further analysis of the PKC pathway will lead to a better understanding of the functional biochemistry of occludin and tight junctions.

Acknowledgments—We thank Gislinde Hartmann and Heidemarine Lerch for technical assistance. We appreciate the assistance of John Dickson in preparation of this manuscript. We are grateful to Dr. Reiner Haseloff for help in the radioactivity experiments.

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