# Assembly of the Tight Junction: The Role of Diacylglycerol

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***Abstract.*** Extracellular $Ca^{2+}$ triggers assembly and sealing of tight junctions (TJs) in MDCK cells. These events are modulated by G-proteins, phospholipase C, protein kinase C (PKC), and calmodulin. In the present work we observed that 1,2-dioctanoylglycerol (diC8) promotes the assembly of TJ in low extracellular $Ca^{2+}$, as evidenced by translocation of the TJ-associated protein ZO-1 to the plasma membrane, formation of junctional fibrils observed in freeze-fracture replicas, decreased permeability of the intercellular space to [$^3$H]mannitol, and reorganization of actin filaments to the cell periphery, visualized by fluorescence microscopy using rhodamine-phalloidin. In contrast, diC8 in low $Ca^{2+}$ did not induce redistribution of the Ca-dependent adhesion protein E-cadherin (uvomorulin). Extracellular antibodies to E-cadherin block junction formation normally induced by adding $Ca^{2+}$. diC8 counteracted this inhibition, suggesting that PKC may be in the signaling pathway activated by E-cadherin-mediated cell-cell adhesion.

In addition, we found a novel phosphoprotein of 130 kD which coimmunoprecipitated with the ZO-1/ZO-2 complex. Although the assembly and sealing of TJs may involve the activation of PKC, the level of phosphorylation of ZO-1, ZO-2, and the 130-kD protein did not change after adding $Ca^{2+}$ or a PKC agonist. The complex of these three proteins was present even in low extracellular $Ca^{2+}$, suggesting that the addition of $Ca^{2+}$ or diC8 triggers the translocation and assembly of preformed TJ subcomplexes.

THE intercellular space between epithelial cells is bridged by a set of specialized structures: tight junctions (TJs)[1] or *zonula occludens*, the *zonula adherens*, desmosomes and gap junctions. TJs, the most apical structure of the junctional complex, act as a diffusion barrier regulating the passage of ions and molecules through the paracellular pathway and, in addition, prevent the intermixing of lipids and proteins between the outer leaflets of the apical and basolateral plasma membrane (reviewed by Cereijido et al., 1989; Stevenson et al., 1988; Schneeberger and Lynch, 1992).

Two proteins in association with the cytoplasmic side of TJs have been identified: zonula occludens-one (ZO-1), a 210-220-kD protein (Stevenson et al., 1986; Anderson et al., 1988) whose two isoforms (Willott et al., 1992) exhibit a differential cellular distribution (Kurihara et al., 1992; Balda and Anderson, 1993), and cingulin, a 140-kD protein located farther from the plasma membrane than ZO-1 (Citi et al., 1988; Stevenson et al., 1989*a*). In addition, three other proteins have been identified but remain less well characterized: BG9.1 antigen, a 192-kD protein (Chapman and Eddy, 1989), ZO-2, a 160-kD protein which coimmunoprecipitates with ZO-1 (Gumbiner et al., 1991), and 7H6 antigen, a 175-kD protein (Zhong et al., 1993).

The morphology and tightness of TJs vary among epithelia, and are modified in response to physiological conditions and pharmacological challenge (reviewed by Cereijido et al., 1988; Bentzel et al., 1991; Scheneeberger and Lynch, 1992). Although the cause of these variations are far from being elucidated, several lines of evidence indicate they involve complex cellular signaling mechanisms.

Extracellular $Ca^{2+}$ triggers the assembly and sealing of TJs in monolayers of MDCK cells that have been preincubated in the absence of this ion ("Ca-switch") (Gonzalez-Mariscal et al., 1985, 1990; Contreras et al., 1992). This suggests the existence of a mechanism for transducing the $Ca^{2+}$ signal from the extracellular to the cytoplasmic side where the changes associated with the assembly of TJs are known to occur; e.g., reorganization of actin filaments (Meza et al., 1980) and translocation of ZO-1 to the plasma membrane (Siliciano et al., 1988). It has been suggested that the initial requirement for $Ca^{2+}$ in junction assembly is in permitting homotypic intercellular binding of the Ca-dependent adhesion molecule E-cadherin (Gumbiner, 1991). The Ca-induced assembly of TJs can be inhibited by blocking the formation of cell-cell adhesion with antibodies to the extracellular domain of E-cadherin (Vestweber and Kemler, 1985; Gumbiner et al., 1988) suggesting a role for E-cadherin in the transduction of the Ca-signal across the plasma

1. *Abbreviations used in this paper*: diC8, 1,2-dioctanoylglycerol; LC, low calcium medium; NC, normal calcium medium; PKC, protein kinase C; TER, transepithelial electrical resistance; TJ, tight junction; ZO-1, zonula occludens-one.

membrane. How the signal propagates inside the cell is not clear but based on pharmacological studies seems to involve G-proteins, phospholipase C, protein kinase C (PKC), and calmodulin (Balda et al., 1991). The finding that protein kinase inhibitors prevent disassembly of TJs induced by low extracellular calcium (Citi, 1992) further supports the importance of protein kinases in the regulation of TJs.

In the present work, we analyzed the effect of the PKC agonist 1,2-dioctanoylglycerol (diC8) on the assembly of TJs in monolayers of MDCK cells in low extracellular $Ca^{2+}$. We present evidence that diC8 induces translocation of ZO-1 from the cytoplasm to the plasm membrane, the reorganization of the actin cytoskeleton, the appearance of TJ fibrils, and that it reduces the permeability of the paracellular pathway to mannitol. Despite these effects, diC8 did not induce E-cadherin redistribution in low $Ca^{2+}$. The inhibition by anti-E-cadherin antibodies of the translocation of ZO-1 and of the formation of a tight monolayer could be counteracted by diC8, indicating that E-cadherin regulates the assembly of TJs via a pathway activated by a diacylglycerol analogue, possibly through PKC. We also found a new protein of 130 kD which interacts with the ZO-1/ZO-2 complex. The stoichiometry within this complex is the same in low and normal extracellular $Ca^{2+}$, suggesting that TJs are assembled from preformed subcomplexes. Furthermore, our results indicate that during both $Ca^{2+}$ and diC8-induced TJ assembly, the level of phosphorylation of these three proteins does not change.

## Materials and Methods

### Cell Culture

Strain II MDCK cells were used. The culture conditions, Ca-switch protocol and treatments with drugs were as described previously (Gonzalez-Mariscal et al., 1985; Gumbiner et al., 1988; Balda et al., 1991).

### Immunofluorescence

Cells were grown on round glass coverslips in multi-well plates. They were fixed with 3% paraformaldehyde (10 min) and permeabilized with 0.5% Triton X-100 in PBS (5 min). Monolayers were washed with PBS, blocked with 0.2% BSA and 20 mM glycine in PBS for 30 min, and incubated for 1 h with the primary antibody (1:100 of anti-ZO-1 rabbit polyclonal, 7445 [Willott et al., 1992]; or 1:800 anti-E-cadherin rat monoclonal [DECMA-1; Sigma Chemical Co., St. Louis, MO] [Vestweber and Kemler, 1985]). After three washes with PBS, cells were incubated for 1 h with the secondary antibody (1:250, FITC anti-rabbit, or TRITC anti-rat; Sigma Chemical Co.) and, in some experiments with rhodamine-phalloidin (1:500, Molecular Probes, Inc., Eugene, OR). After washing three times, coverslips were mounted with Mowiol (Calbiochem Biochemical, San Diego, CA).

### Transepithelial Electrical Resistance and Paracellular Permeability for Mannitol

Cells were plated at confluent density on filter culture inserts (0.6 $cm^2$, Falcon; Becton Dickinson, Lincoln Park, NJ) coated with rat tail collagen. The degree of sealing of the TJ was assessed by measuring transepithelial electrical resistance (TER) using an EVOM (World Precision Instruments, New Haven, CT). TER values were obtained by subtracting the contribution of the filter and bathing solution.

Paracellular permeability for mannitol was determined as described elsewhere (Rochat et al., 1988) by adding [$^3$H]mannitol (1 $\mu$Ci/ml, 30 Ci/mmol; NEN-Dupont, Wilmington, DE) to the apical side of the monolayer. The unidirectional flux of tracer was determined by serially sampling the basal compartment. To normalize the results of different experiments, the paracellular permeability for mannitol of monolayers in low $Ca^{2+}$ was taken as 100%.

### Freeze-Fracture EM

Freeze-fracture replicas were obtained and analyzed as described elsewhere (Gonzalez-Mariscal et al., 1985).

### Metabolic Labeling, Immunoprecipitation, and Protein Analysis

Cells were plated at confluent density on filter culture inserts (4.9 $cm^2$, Falcon) coated with rat tail collagen. After 1 h the cells were washed three times with PBS without calcium (450-1,300, GIBCO-BRL, Gaithersburg, MD) and transferred to S-MEM without calcium (430-2,300, GIBCO-BRL), referred to as low-calcium medium (LC) (<5 $\mu$M), or DMEM (450-1,600, GIBCO-BRL), referred to as normal calcium medium (NC) (1.8 mM). 20 h later the experimental protocol was started by washing twice with 150 mM NaCl/10 mM Tris, pH 7.4. Thereafter, the cells were incubated for 60 min in MEM without phosphate (300-9,050, MEM select amine kit, GIBCO-BRL). Labeling was done in the same medium supplemented with 100 $\mu$Ci/ml of [$^{32}$P]orthophosphate (acid free, 0.8 mCi/ml; Amersham Corp., Arlington Heights, IL) for 3 h at 37°C in a $CO_2$ incubator. $Ca^{2+}$ or drugs were added to the phosphate-free labeling medium, as indicated.

In some experiments, cells were metabolically labeled overnight with [$^{35}$S]methionine and [$^{35}$S]cysteine (tran$^{35}$S-label; ICN Radiochemicals, Irvine, CA) using 0.2 mCi/ml in methionine-cysteine free growth medium (300-9,050; GIBCO BRL).

After labeling, the cells were washed with PBS, scraped, and collected by centrifugation at 4°C. Cells from a filter insert (4.9 $cm^2$) were extracted with 1 ml of 1% Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 150 mM NaCl in 10 mM Hepes, pH 7.4 (Gumbiner et al., 1991). All buffers contained the following protease and phosphatase inhibitors: 1 mM PMSF, 0.5 mM iodoacetamide, 10 $\mu$g/ml antipain, 1 $\mu$g/ml pepstatin A, 0.5 mM benzamidine, 25 $\mu$g/ml leupeptin, 10 $\mu$g/ml aprotinin, 1 mM diisopropylfluorophosphate, 10 $\mu$g/ml trypsin-chymotrypsin inhibitor, 2 mM EDTA, 0.4 mM $Na_3VO_4$, 10 mM NaF, and 5 mM of $Na_4P_2O_7$. The cell extracts were passed 10 times through a 25-gauge needle. In some experiments the extraction of ZO-1 was done under more denaturing conditions by boiling for 15 min in 1% SDS as described elsewhere (Matter and Hauri, 1991). The insoluble material was removed by centrifugation at 15,000 rpm for 20 min at 4°C and the supernatant was transferred to tubes containing immunobeads and incubated on an end-over-end shaker at 4°C. The immunoaffinity matrix was prepared as previously described (Matter and Hauri, 1991) using a rabbit polyclonal antibody anti-ZO-1 (7,445, Willott et al., 1992) bound to protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ). After washing four times with extraction buffer, the immunoprecipitates were analyzed on a 7% polyacrylamide-SDS gel by the method of Laemmli (1970) and transferred to nitrocellulose (Towbin et al., 1979).

[$^{32}$P]phosphate labeling of ZO-1 was detected by autoradiography and total ZO-1 protein was determined in the same gel lane by immunoblotting using a polyclonal anti-ZO-1 antibody (7,445, Willott et al., 1992) and a chemiluminescence detection system (ECL, Amersham). Autoradiographs were quantified by computer-aided digitalization with a Kodak Visage 2000 image processor (Eastman Kodak Co., Rochester, NY) supported by a Sun Microsystem Bioimage workstation (MilliGen/Biosearch, Ann Arbor, MI). The ratio of $^{32}$P-ZO-1/total ZO-1 protein represents the relative specific activity of ZO-1.

## Results

### diC8 Induces Translocation of ZO-1 to the Plasma Membrane

Induction of TJ formation by the addition of $Ca^{2+}$ seems to occur via signal transduction across the plasma membrane (Gonzalez-Mariscal et al., 1990) and can be potentiated by the diacylglycerol analogue diC8 (Balda et al., 1991). Therefore, we tested whether stimulating MDCK cells in low $Ca^{2+}$ with diC8 is able to mimic the $Ca^{2+}$ effect on translocation of ZO-1 from the cytoplasm to the plasma membrane.

To probe the effect of diC8 on the intracellular location of ZO-1, we incubated MDCK monolayers in LC with the drug and studied the distribution of ZO-1 by immunofluorescence.

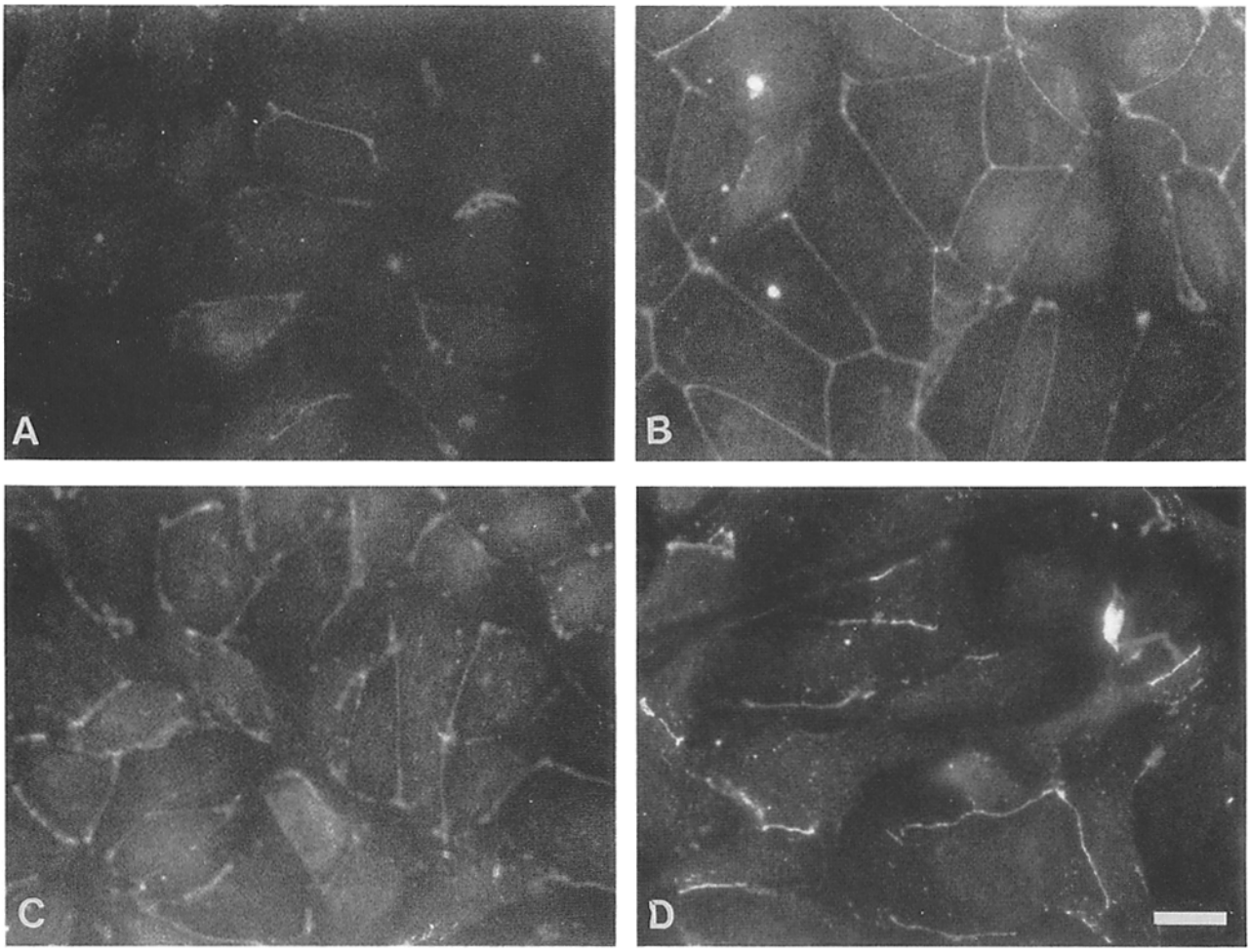



*Figure 1.* diC8 induces translocation of ZO-1 to the plasma membrane. MDCK cells were incubated in LC for 20 h, and then transferred to LC (*A*), NC (*B*), LC + 0.5 mM diC8 (*C*), or LC + 50 nM staurosporine + diC8 (*D*) for 2 h. Indirect immunofluorescent of ZO-1 was performed as described in Materials and Methods. Bar, 6 $\mu$m.

Cells in LC showed only very occasional staining in areas of intercellular contact (Fig. 1 *A*). In contrast, 2 h after transferring the cells to media containing 1.8 mM $Ca^{2+}$, staining of ZO-1 revealed a reticular pattern around all cells indicating translocation to the plasma membrane at regions of cell-cell contact (Fig. 1 *B*). After 2 h of incubation with diC8 in LC medium, ZO-1 was found at the plasma membrane in most areas of intercellular contact although the staining was not as continuous as after the Ca-switch (Fig. 1 *C*). This effect was not enhanced by longer periods of stimulation with diC8 and was partially blocked by the protein kinase inhibitors staurosporine (50 nM, Fig. 1 *D*) and H7 (50 $\mu$M, not shown). Similar or higher concentrations of these kinase inhibitors have been shown in vivo to block the effect of PKC agonists (Sheu et al., 1989; Linder et al., 1992). The effect of diC8 on the redistribution of ZO-1 could also be reproduced with low concentration (0.1–1.0 ng/ml) of PMA (not shown). These results suggest diC8 may induce translocation of ZO-1 by activating PKC.

### *diC8 Reorganizes Actin Filaments*

We next addressed the question of whether diC8 induces other structural changes known to occur during the Ca-switch. Cytochalasin B, a drug that affects f-actin organization, lowers the electrical resistance in established MDCK monolayers and completely prevents sealing of tight junctions during the Ca-switch (Meza et al., 1980, 1982). This suggests that microfilament integrity is essential for the maintenance and assembly of TJs.

To test whether the effect of diC8 on ZO-1 redistribution correlates with changes in the actin cytoskeleton we studied the f-actin distribution using rhodamine-phalloidin. In LC, most of the actin appears concentrated around the nuclear area (Fig. 2 *A*). After 2 h in the presence of $Ca^{2+}$, most of the actin redistributed along the periphery of the cells, concentrated at areas of cell-cell contact (Fig. 2 *B*). If diC8 was added to cells kept in LC, cells spread and microfilaments redistributed along the periphery of the cells (Fig. 2 *C*). This effect was also seen with the PKC agonist PMA (0.1–1.0 ng/ml, not shown). Neither drug induced cell spreading to the same degree as did $Ca^{2+}$, perhaps because of the absence of Ca-dependent cell–cell adhesion. Thus, diC8 not only induces translocation of ZO-1, but also triggers the reorganization of actin filaments.

### *diC8 Induces Tight Junction Fibril Formation*

A characteristic of TJ structure, observed by freeze-fracture EM, is a distinctive pattern of fibrils surrounding the cell at

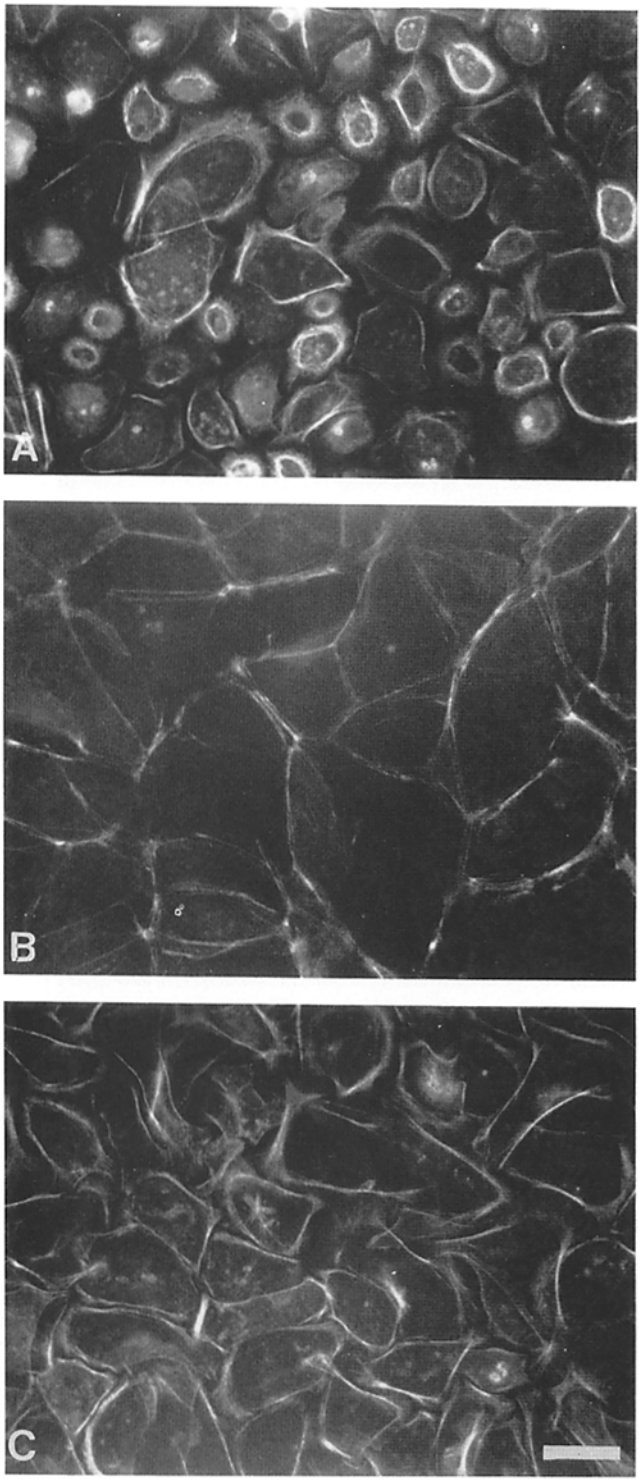



*Figure 2.* diC8 stimulates reorganization of actin filaments. MDCK cells were incubated in LC for 20 h, and then transferred to LC (*A*), NC (*B*), or LC + 0.5 mM diC8 (*C*) for 2 h. Rhodamine-phalloidin was used to stain actin filaments as described in Materials and Methods. Bar, 6 μm.

the zone of cell-cell contact. If cells in LC were treated for 15 min with diC8 they acquired the typical pattern of continuous fibrils (Fig. 3 *A*) similar to that observed 15 min after a Ca-switch (Gonzalez-Mariscal et al., 1985). In control cells in LC without diC8 continuous fibrils were not observed (Fig. 3 *B*), although an occasional knot of filaments was observed, as previously documented (Gonzalez-Mariscal et al., 1985). The observation that diC8 also triggers the assembly of typical TJ fibrils suggests that PKC stimulation in low extracellular $Ca^{2+}$ not only stimulates cytoplasmic rearrangements but also intramembrane events.

### *diC8 Decreases Paracellular Permeability*

In a previous study, we observed that diC8 potentiates the increase in TER normally observed following a Ca-switch (Balda et al., 1991). Therefore, we tested whether diC8 is able to stimulate the development of TER in MDCK cells remaining in LC. There was no significant increase in the TER under these conditions (Fig. 4 *A*). Nevertheless, TER increased faster if $Ca^{2+}$ was added to cells pretreated with diC8 for 2 h (Fig. 4 *A*). This suggests that the pretreatment with diC8 induces a step toward the assembly, e.g., translocation of tight junction components to the plasma membrane. The development of TER, however, seems to require at least one additional step which could not be induced with diC8.

TER is a very sensitive indicator of the paracellular barrier and a small defect in TJ sealing results in negligible values. For example, if only 2% of all TJs of a monolayer are open, the value of TER is reduced by 95% (Gonzalez-Mariscal et al., 1990). In contrast, permeability for the non-transported tracer, mannitol, has been used to measure the integrity of TJs in epithelia of low TER. Therefore, we determined whether diC8 in LC decreases the paracellular flux of [$^3$H]mannitol from the apical to the basolateral side (Fig. 4 *B*). For comparison, the permeability for mannitol of monolayers in LC is referred to as 100%. 26 h (NC) or 6 h calcium switch (CS) of incubation with $Ca^{2+}$ decreased mannitol permeability by 90 and 70%, respectively. After 6 h of incubation with diC8 in LC permeability for mannitol was significantly ($p < 0.05$) reduced by 30%. The same reduction could already be detected after 2 h (not shown). Thus, diC8 in LC induces a partial sealing of the intercellular space.

### *Stimulation with diC8 Induces ZO-1 Translocation to the Plasma Membrane in an E-cadherin-independent Manner*

Antibodies against E-cadherin, a Ca-dependent adhesion molecule, inhibit the assembly and sealing of TJs when added to the extracellular medium before the Ca-switch. It has been proposed that interaction of cadherin molecules is a prerequisite for the formation of TJs (Gumbiner et al., 1988), and that Ca-dependent cell-cell contact triggers the activation of PKC which in turn regulates the assembly and sealing of TJs (Balda et al., 1991).

To test this hypothesis, we first asked whether diC8 treatment in LC induces E-cadherin to concentrate along the lateral plasma membrane. In LC, only diffuse staining for E-cadherin over the entire cell was observed as reported before (data not shown). After 2 h in NC, E-cadherin was detected in areas of cell-cell contact (Fig. 5 *A*) colocalizing with ZO-1 (Fig. 5 *C*). No lateral plasma membrane staining by anti-E-cadherin antibodies was detected in cells treated with diC8 in LC (Fig. 5 *B*), in contrast to the redistribution of ZO-1 (Fig. 5 *D*). Thus, diC8 induces redistribution of

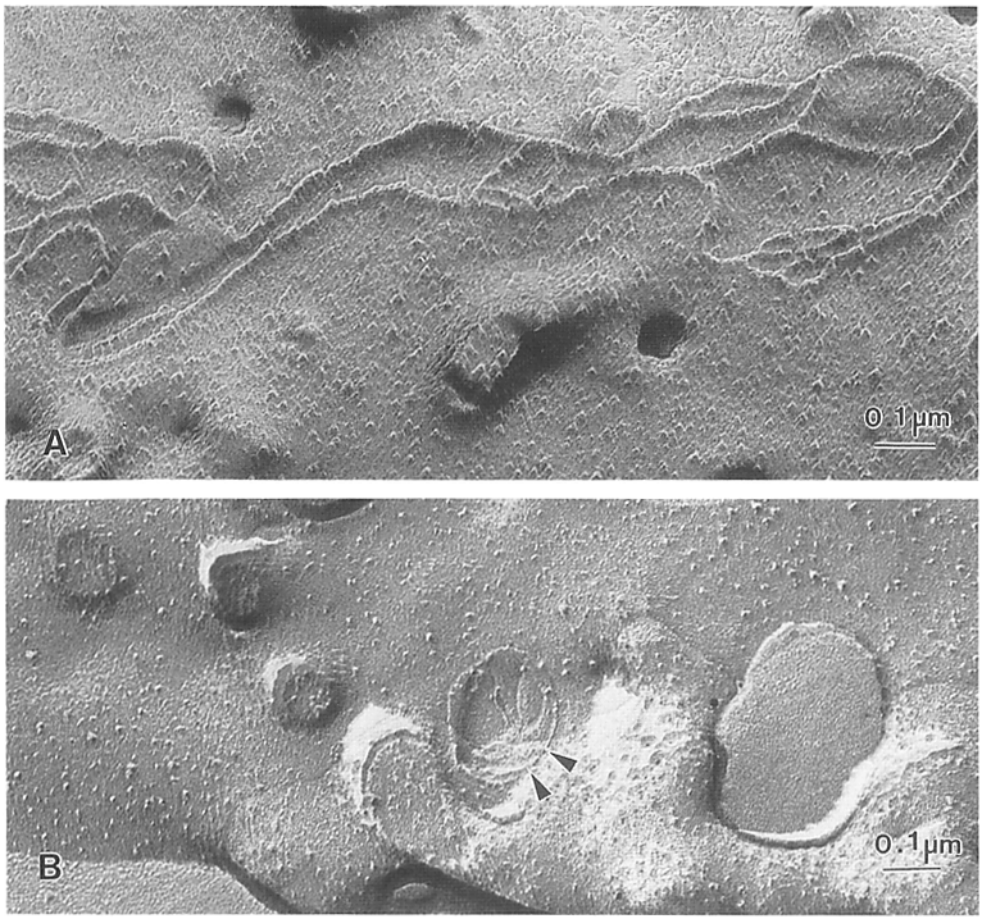
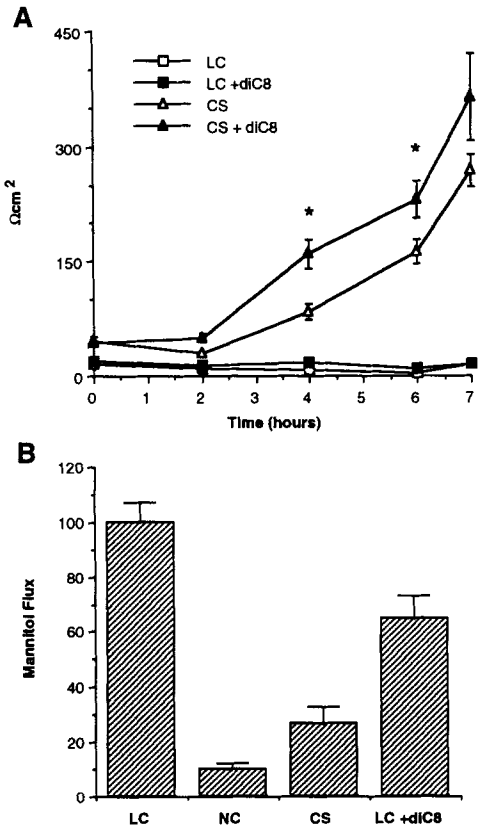



*Figure 3.* diC8 leads to the appearance of tight junction fibrils. Freeze-fracture replicas of MDCK cells in: LC + diC8 for 15 min (*A*), and LC (*B*). Notice that in *B* an occasional knot of filaments can be observed (*arrows*). Bar, 0.1 μm.




ZO-1 without an obvious accumulation of E-cadherin in the lateral plasma membrane.

Translocation of ZO-1 to the plasma membrane during the Ca-switch can be blocked by pretreatment with anti-E-cadherin antibodies (Gumbiner et al., 1988). To confirm that the diC8 effect on the assembly of TJ is downstream of the E-cadherin step we studied whether, in the presence of anti-E-cadherin antibodies, diC8 treatment still induces translocation of ZO-1 to the plasma membrane. Fig. 6 *A* (ZO-1) and *C* (f-actin) confirms the inhibitory effects on TJ formation of anti-E-cadherin antibodies if applied before and during the Ca-switch. Staining for ZO-1 at sites of cell-cell contacts was strongly reduced but not completely abolished in the presence of anti-E-cadherin antibodies. If

*Figure 4.* Effect of diC8 on the development of TER and on the permeability for [$^3$H]mannitol. (*A*) Kinetics of the development of TER in LC (-□-), LC + diC8 (-■-), Ca-switch (CS, -△-) or CS + diC8 (-▲-). Preincubation with diC8 for 2 h was performed before the CS. Long incubations in diC8 were in the presence of an inhibitor of its metabolism, EGD, as described before (Balda et al., 1991). Shown are mean values ± SE of six determinations. $*p < 0.05$. (*B*) Permeability for [$^3$H]mannitol of MDCK cell monolayers incubated in LC, NC, CS, or LC + diC8, determined at the end of the TER determinations showed in *A*. Values are expressed as percent of flux measured in monolayers in LC. Shown are mean values ± SE of six determinations. diC8 in LC significantly reduced ($p < 0.05$) the permeability for mannitol.

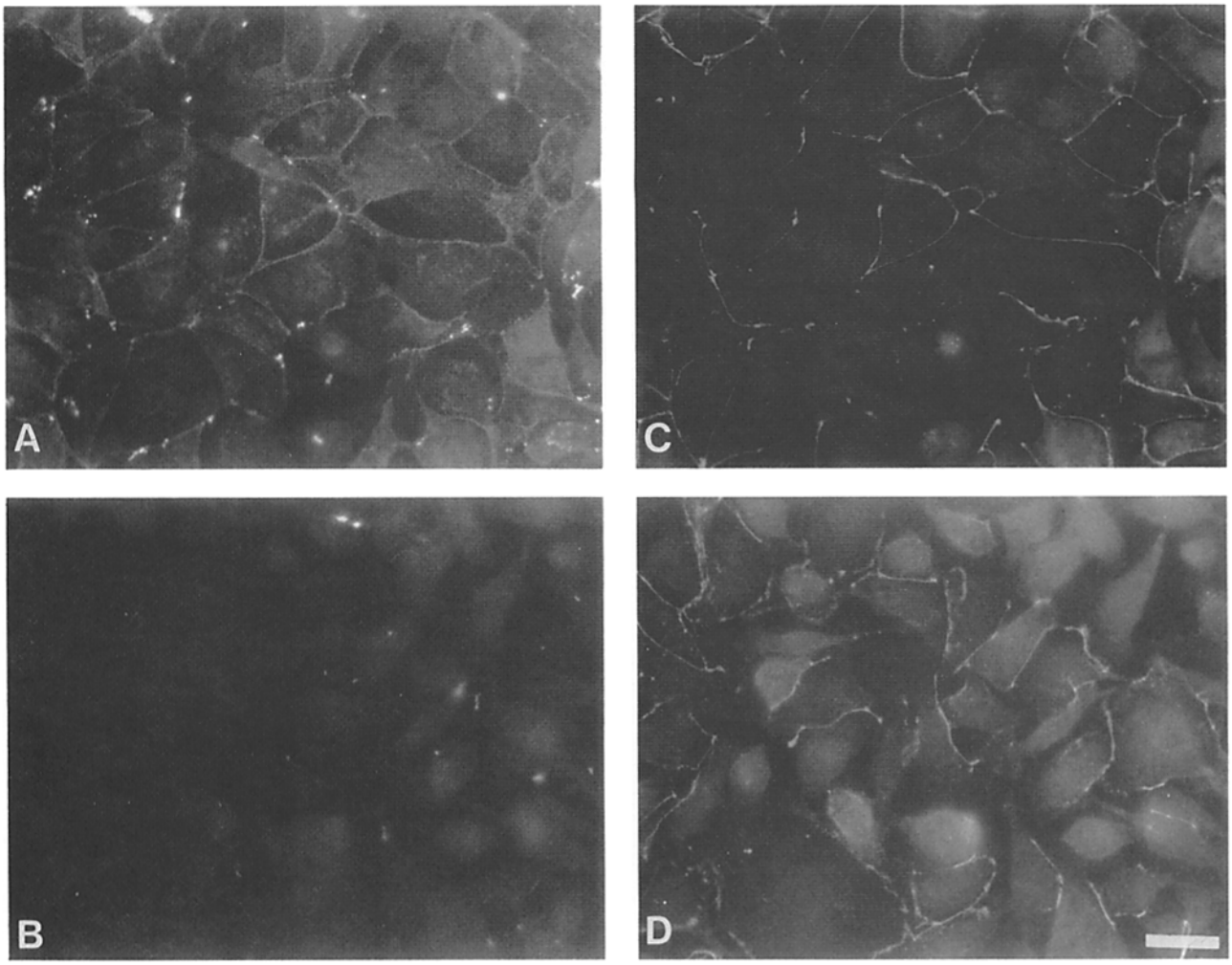



*Figure 5.* diC8 does not induce redistribution of E-cadherin. MDCK cells were incubated in LC for 20 h and then transferred to NC for 2 h (*A* and *C*) or LC + diC8 for 2 h (*B* and *D*). Shown are double-labeled immunofluorescence stainings for E-cadherin (*A* and *B*) and ZO-1 (*C* and *D*). Bar, 6 μm.

diC8 was added together with $Ca^{2+}$ to cells pretreated with anti-E-cadherin antibodies, ZO-1 staining revealed a typical reticular pattern (Fig. 6 *B*) and f-actin could be detected along the lateral plasma membrane (Fig. 6 *D*) to a similar degree as in a control monolayer (Fig. 1 *B*: ZO-1; Fig. 2 *B*: f-actin).

To quantify the inhibitory effect on TJ formation by anti-E-cadherin antibodies and the counteraction by diC8, we measured mannitol fluxes (Table I). The presence of anti-E-cadherin antibodies partially inhibited the decrease of mannitol flux during the Ca-switch. diC8 completely counteracted this inhibitory effect. These data indicate that diC8 activates a pathway downstream or independent of E-cadherin. We also found that anti-E-cadherin antibodies did not inhibit the diC8 induced translocation of ZO-1 and actin reorganization in LC, an observation that further supports this conclusion (data not shown).

### *Phosphorylation of ZO-1 Does Not Change during the Ca-switch or after diC8 Treatment*

ZO-1 is a phosphoprotein (Anderson et al., 1988), and contains twice as much phosphate in low-resistance MDCK cells as in the high-resistance strain (Stevenson et al., 1989*b*). To test whether phosphorylation of ZO-1 is altered by the Ca-switch or diC8 treatment, we performed immunoprecipitation studies of ZO-1. MDCK cells were incubated for 20 h in NC or LC. After phosphate depletion, cells were metabolically labeled with [$^{32}$P]orthophosphate for 3 h in NC or LC, and in the absence or presence of diC8. Cell extracts were immunoprecipitated with an anti-ZO-1 antibody (7445; Willott et al., 1992). Upon analysis by SDS-PAGE and immunoblotting, the ratio $^{32}$P-ZO-1/total ZO-1 protein was determined. The data in Table II indicate that neither the addition of $Ca^{2+}$ nor diC8 significantly changed the phosphorylation of ZO-1. The antibody precipitated similar amounts of protein under all the different conditions and the same results were obtained if cells were extracted by boiling in SDS (data not shown). Additionally, similar results were obtained when the cells were directly transferred to LC medium after trypsinization, instead of 1 h in NC and then LC for 20 h, indicating that the 1-h attachment period in NC did not affect phosphorylation of ZO-1 (data not shown). Thus, ZO-1 does not significantly change its phosphorylation state during junction assembly induced by either $Ca^{2+}$ or diC8.

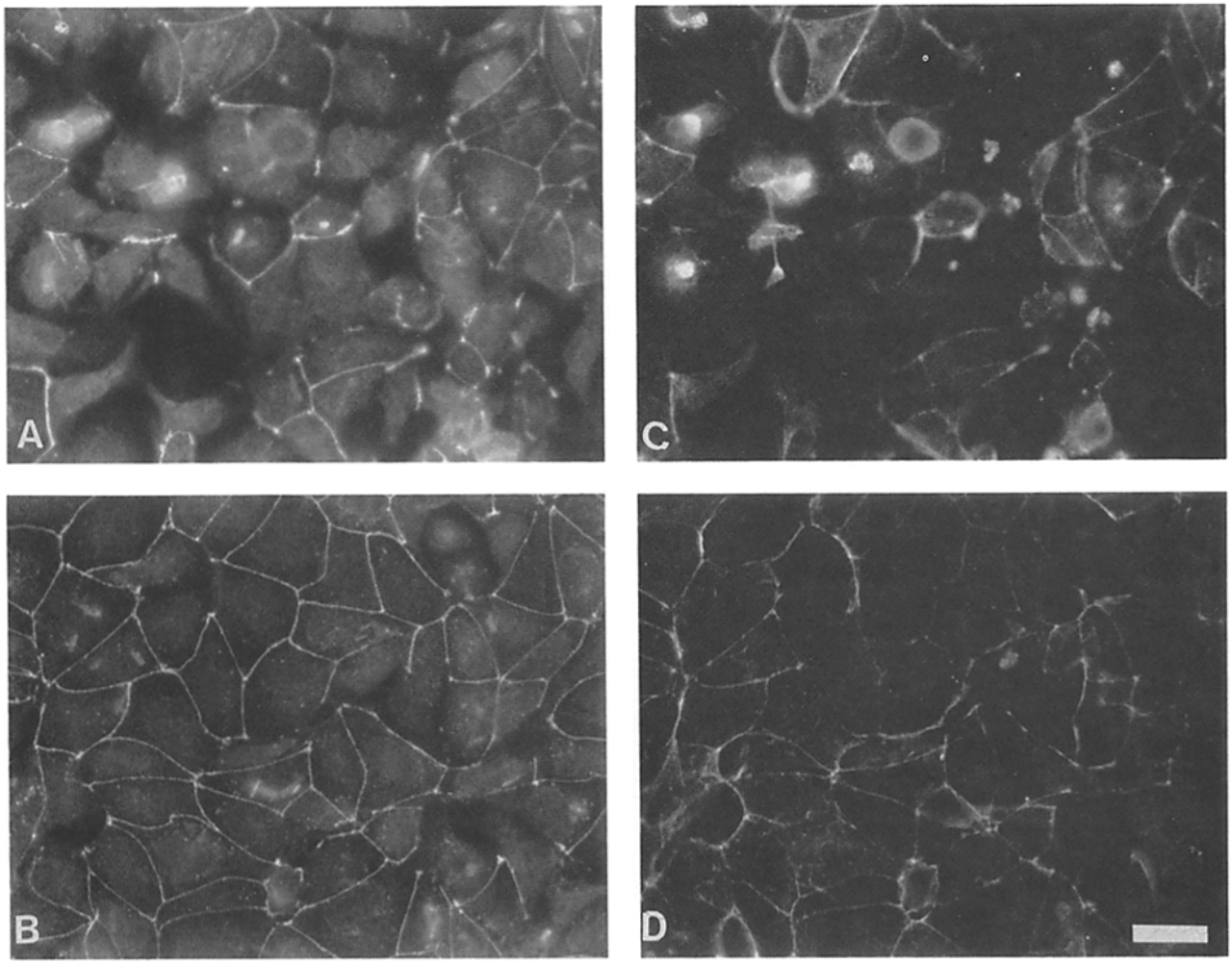



*Figure 6.* diC8 induces ZO-1 and f-actin redistribution in an E-cadherin–independent manner. Cells in LC were incubated with anti-E-cadherin antibodies (5 μl/250 μl) for 1 h, and then calcium was raised to 1.8 mM for another 2 h in the absence (*A* and *C*) or presence of 2.7 mM diC8 (*B* and *D*). Double labeling was performed for ZO-1 (*A* and *B*) by immunofluorescence and f-actin (*C* and *D*) with rhodamine–phalloidin. The inhibitory effect of anti–E-cadherin antibodies was observed with 5 to 10 μl of ascites fluid in 250 μl of medium. Bar, 6 μm.

### *Two Phosphoproteins Coimmunoprecipitate with ZO-1*

Recently, a 160-kD protein was found to coimmunoprecipitate with ZO-1 from [$^{35}$S]methionine-labeled MDCK cell extracts and was subsequently called ZO-2 (Gumbiner et al., 1991). To test whether ZO-2 is a substrate for PKC, we performed coimmunoprecipitation studies. MDCK cells were metabolically labeled with either [$^{32}$P]orthophosphate or [$^{35}$S]methionine in NC, LC, during a Ca-switch or in the presence of diC8. Cells were extracted and ZO-1 was immunoprecipitated as described by Gumbiner et al. (1991). After gel electrophoresis, transfer to nitrocellulose, and autoradi-

*Table I. Mannitol Flux in the Presence of Anti-E-Cadherin Antibodies and diC8*

| Conditions | Mannitol flux (percent of LC) |
|---|---|
| LC | 100 ± 7 |
| CS | 34 ± 6 |
| CS + α-E-cad | 57 ± 6 |
| CS + α-E-cad + diC8 | 31 ± 7 |

Cells in LC were preincubated with or without anti-E-cadherin antibodies (α-E-cad, 7/250 μl of medium) and/or diC8 (2.7 mM) for 1 h. Then cells were switched to NC including the same ingredients. After 6 h of incubation mannitol flux was measured as described in Materials and Methods. Given are mean values ± SE of six determinations. diC8 significantly counteracted ($p < 0.05$) the effect of α-E-cadherin on mannitol flux.

*Table II. Phosphorylation of ZO-1 before and after Junction Assembly under Different Conditions*

| Media | n | $^{32}$P/protein |
|---|---|---|
| NC | 4 | 1 |
| LC | 4 | 0.94 ± 0.10 |
| LC + diC8 | 2 | 0.99 ± 0.20 |
| CS | 3 | 1.00 ± 0.45 |
| CS + diC8 | 2 | 0.97 ± 0.11 |

MDCK cells were plated in NC for 1 h after trypsinization and subsequently transferred to NC or LC. After 20 h, monolayers were phosphate depleted for 1 h and then metabolically labeled with [$^{32}$P]orthophosphate for 3 h, at the same time one group in LC was transferred to NC (Ca-switch). diC8 was used at 0.5 mM where indicated. The phosphorylation state of ZO-1 was determined as the ratio of $^{32}$P-ZO-1 and total ZO-1 protein, as described in Materials and Methods. Phosphorylation at 24 h in NC was taken as 1 to normalize the results of different experiments. The results are expressed as means ± SE of n experiments. There are no significant differences between the different conditions as determined by a *t* test.

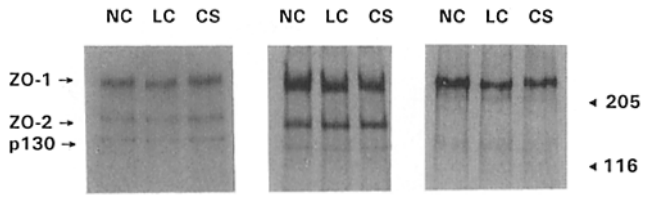



*Figure 7.* Two phosphoproteins coimmunoprecipitate with ZO-1. (*Left panel*) Autoradiograph of immunoprecipitates of $^{35}$P-labeled MDCK cells in NC, LC, or CS. (*Middle panel*) Fluorograph of immunoprecipitates of [$^{35}$S]methionine labeled MDCK cells in NC, LC, or CS. (*Right panel*) Immunoblot of the nitrocellulose membrane shown in the left panel using anti-ZO-1 antibodies. Positions of molecular weight markers are indicated on the right.

ography, three phosphoproteins were observed that migrated at ~210, ~160 and ~130 kD (Fig. 7, *left panel*). The same three bands could also be detected after [$^{35}$S]methionine labeling (Fig. 7, *middle panel*). However, the relative intensity of the 130-kD compared to ZO-1 band was lower in cells labeled with [$^{35}$S]methionine than [$^{32}$P]orthophosphate (Table III). The 210-kD protein was subsequently identified as ZO-1 by immunoblotting the same nitrocellulose membrane which had been used for autoradiography (Fig. 7, *left panel*) with the same anti-ZO-1 antibody used for the immunoprecipitation (Fig. 7, *right panel*). Since the 160- and 130-kD bands could not be detected by immunoblotting with anti-ZO-1 antibodies, it is unlikely that their coimmunoprecipitation was due to cross-reactivity of the antibody. In addition, we obtained the same gel pattern using either a monoclonal anti-ZO-1 antibody (R40.76; Anderson et al., 1988) or a polyclonal antibody generated against a recombinant antigen containing only 54 amino acids of ZO-1 (8040; Willott et al., 1992). Furthermore, only ZO-1 was immunoprecipitated when the samples were denatured by boiling in 1% SDS before immunopurification with the same antibody as used in Fig. 7 (data not shown).

The 160-kD band most likely represents ZO-2 which was not previously known to be a phosphoprotein. The 130-kD protein (p130) is strongly labeled with [$^{32}$P]orthophosphate but only weakly with [$^{35}$S]methionine. This may explain why this band has not been observed by Gumbiner et al. (1991). Preliminary characterization by immunoblotting and immunoprecipitation showed that it is not cingulin, a tight junction protein of 140 kD (data not shown, anti-cingulin antibody kindly provided by S. Citi).

Since there are no antibodies specific for ZO-2 or p130, we performed coimmunoprecipitation experiments to determine whether these proteins change their phosphorylation state upon stimulation of TJ assembly. Analysis of several experiments analogous to the one shown in Fig. 7 revealed that the ratios of ZO-2/ZO-1 and p130/ZO-1 of [$^{35}$S]methionine or [$^{32}$P]orthophosphate-labeled proteins are similar in NC and LC. Additionally, they do not significantly change after diC8 treatment (Table III). This indicates that neither ZO-2 nor the 130-kD protein change their phosphorylation state during the Ca-switch or upon diC8 treatment. We can not rule out the possibility that pools of ZO-2 or p130 exist which are not bound to ZO-1, thus can not be co-immunoprecipitated, but which change their phosphorylation state in response to $Ca^{2+}$ or diC8.

The constant [$^{32}$S]methionine ratios indicate that the stoichiometry of the immunoprecipitated complex is the same under all conditions. Thus, even in the absence of cell-cell contact, at least three of the tight junctional components are associated. This suggests that $Ca^{2+}$ induces the translocation of preexisting subcomplexes to the plasma membrane which subsequently become integrated into the forming TJ.

*Table III. Stoichiometry of the ZO-1 Co-immunoprecipitated Proteins under Different Conditions*

| Conditions | n | ZO-2/ZO-1 | p130/ZO-1 |
|---|---|---|---|
| $^{32}$P-ratios | | | |
| NC | 3 | 0.56 ± 0.10 | 0.24 ± 0.07 |
| LC | 3 | 0.56 ± 0.11 | 0.23 ± 0.07 |
| LC + diC8 | 2 | 0.64 ± 0.10 | 0.16 ± 0.04 |
| CS | 3 | 0.61 ± 0.10 | 0.26 ± 0.05 |
| CS + diC8 | 2 | 0.79 ± 0.11 | 0.32 ± 0.06 |
| $^{35}$S-ratios | | | |
| NC | 3 | 0.45 ± 0.10 | 0.12 ± 0.04 |
| LC | 3 | 0.55 ± 0.09 | 0.14 ± 0.05 |
| LC + diC8 | 3 | 0.61 ± 0.07 | 0.15 ± 0.05 |
| CS | 3 | 0.62 ± 0.04 | 0.14 ± 0.07 |
| CS + diC8 | 3 | 0.55 ± 0.05 | 0.14 ± 0.04 |

See Table I and Materials and Methods for further details.

## Discussion

In the present study, we analyzed the effect of diC8, a stimulator of PKC, on the assembly and sealing of TJs. We have demonstrated that stimulation of cells with diC8 in low $Ca^{2+}$ can induce the translocation of ZO-1 to the plasma membrane, the reorganization of actin filaments, the appearance of TJ fibrils and a partial sealing of the intercellular space. The diC8 target, presumably PKC, seems to be downstream of E-cadherin in the signaling pathway for TJ assembly, since diC8 treatment in LC does not induce E-cadherin redistribution and is able to counteract the effect of anti-E-cadherin antibodies. Furthermore, ZO-1, ZO-2, and a novel 130-kD protein seem to form a subcomplex that is present even in low $Ca^{2+}$. None of these proteins changes its phosphorylation state during the Ca-switch or after diC8 treatment.

### Regulation of Tight Junction Assembly

Previous observations on the role of PKC in regulation of TJs have been inconsistent. Activation of PKC with PMA has been reported to decrease TER of established monolayers of MDCK (Ojakian, 1981) and LLC-PK$_1$ cells (Mullin et al., 1990). However, PMA has been shown to have opposite effects on TER in different clones of LLC-PK cells (Ellis et al., 1992). We found that diC8, another stimulator of PKC, does not reduce TER in established MDCK monolayers but actually increases this parameter during the Ca-switch (Balda et al., 1991). PMA is a powerful activator of PKC and can even lead to downregulation of the enzyme (Huang et al., 1989). If we added PMA at concentrations similar to those used in the studies mentioned above (6–60 ng/ml) to MDCK cells in LC, ZO-1 translocated to the cell periphery but was found in dots, and f-actin staining revealed the induction of extensive membrane ruffling (data not shown). In contrast,

PMA at low concentrations (1.0–0.1 ng/ml) induced the same effects as diC8.

In addition to activating PKC, diC8 may act via other pathways (Hockberger et al., 1989; Shariff and Luna, 1992). It was recently shown by cDNA sequence homology and biochemical observations that diacylglycerol binds non-PKC proteins involved in signal transduction, such as diacylglycerol kinase of *Drosophila* and the unc-13 gene product of *C. elegans* (Masai et al., 1992; Maruyama and Brenner, 1991). Furthermore, the unc-13 gene product and the human brain n-chimaerin were identified as PMA receptors (Ahmed et al., 1990, 1992). Thus, there are neither specific PKC agonists nor inhibitors. Therefore, it is not possible to make final conclusions about the mechanisms by which diC8 stimulates assembly of TJ.

The effect of $Ca^{2+}$ on the assembly and sealing of TJ could only be partially mimicked by diC8. Even though addition of the drug led to numerous structural changes and a reduction of paracellular diffusion of mannitol, it did not induce the formation of an electrically tight monolayer. One possible reason for this might be that the stimulation of some PKC isoforms by diacylglycerol depends on $Ca^{2+}$ (Kikkawa et al., 1989) and intracellular $Ca^{2+}$ in MDCK cells plated in LC is only 20 nM, while in NC it is 100 nM (Gonzalez-Mariscal et al., 1990). Our results suggest that additional intracellular signaling pathways, for example, calmodulin-mediated effects (Balda et al., 1991) may be involved in the assembly and sealing of TJs. In addition, extracellular components of the TJ may also require $Ca^{2+}$ to create a tight paracellular seal.

E-cadherin, a Ca-dependent cell–cell adhesion protein, seems to play a central role in the assembly of epithelial cell junctions. The binding of antibodies specific for E-cadherin to MDCK cells before a Ca-switch not only blocks the formation of adherens junctions but also the assembly of TJ, desmosomes, and gap junctions (Behrens et al., 1985; Gumbiner et al., 1988; for review see Gumbiner, 1991). When we stimulated MDCK cells in LC with diC8, E-cadherin did not change its distribution, suggesting that diC8 can bypass the Ca-induced formation of E-cadherin–dependent cell–cell adhesion. It has previously been demonstrated that $Ca^{2+}$ entry during the Ca-switch is not required in order to induce the formation of a tight monolayer (Contreras et al., 1992). Thus, we propose that the Ca-induced cell–cell adhesion acting initially at an extracellular site leads to the activation of PKC which in turn activates the assembly of TJ. Consistent with this hypothesis, the inhibitory effect of anti–E-cadherin antibodies on TJ formation could be counteracted with diC8 during a Ca-switch, and diC8-induced assembly in low extracellular $Ca^{2+}$ was not inhibited by anti–E-cadherin antibodies. ZO-1 movement to the plasma membrane requires cell–cell contact since neither $Ca^{2+}$ nor diC8 clearly induced this translocation in single cells (data not shown). Since fibril formation is a rapid event during assembly it is also possible that cell–cell contact via TJ transmembrane components is required for localization of ZO-1 at the plasma membrane.

The actin cytoskeleton is required for TJ structure and function. Some actin microfilaments terminate at or near TJs and disruption of actin filaments causes a decrease in TER (Meza et al., 1980, 1982; review by Madara, 1991). We observed that diC8 induced reorganization of the actin cytoskeleton in the absence of Ca-mediated cell–cell adhesion and, as observed previously (Gumbiner et al., 1988), anti–E-cadherin antibodies inhibited the reorganization of actin filaments along the lateral membrane upon the addition of $Ca^{2+}$. The inhibition of actin and ZO-1 redistribution as well as barrier formation could be counteracted by stimulation with diC8. Thus, the reorganization of the actin cytoskeleton upon induction of Ca-dependent cell–cell contact may be regulated by PKC. If one considers the importance of the actin-based cytoskeleton for epithelial cell polarity (Nelson, 1992), it is tempting to speculate that PKC is an important regulator of the Ca-induced polarization of MDCK cells. It will be interesting to examine the regulatory effect of PKC on the distribution of other proteins associated with the actin cytoskeleton and intercellular junctions (Luna and Hitt, 1992).

It is unclear how diC8 stimulates the assembly of TJs. We analyzed the phosphorylation state of two proteins known to be associated with TJs as possible substrates for PKC. In addition, a third phosphoprotein was studied which was found to co-immunoprecipitate with ZO-1 and is, therefore, likely to be associated with TJs. The incorporation of [$^{32}$P]phosphate into these proteins, however, was not affected by stimulation of PKC or by the addition of $Ca^{2+}$. Thus, none of these phosphoproteins appears to be a target of PKC.

### *Structure and Assembly of Tight Junctions*

In addition to ZO-2, a previously unknown 130-kD protein coimmunoprecipitated with ZO-1. Since ZO-2 and the 130-kD protein were found with ZO-1 in immunoprecipitates generated with different antibodies, and only when the samples had not been denatured by boiling in SDS, the co-immunopurification of these three proteins most likely represents native protein–protein interaction and not antibody cross-reactivity. It is unlikely that the 130-kD band arises by proteolysis of either ZO-1 or ZO-2 since a cocktail of inhibitors was used, specific for a wide variety of proteases. Additionally, the ratios of p130 to ZO-1 and ZO-2 did not change in the absence of protease inhibitors. It has been previously suggested that the absence of labeled ZO-2 in immunoprecipitates after a short pulse-labeling period indicates that its co-immunoprecipitation with ZO-1 does not result from degradation of ZO-1 or artificial binding during its isolation. The appearance of labeled ZO-2 in an immunoprecipitable pool over time probably reflects the kinetics of its intracellular association with ZO-1 (Gumbiner et al., 1991). Preliminary pulse–chase labeling experiments showed that labeled p130 can be detected with ZO-1 after 60 min of chase but not at earlier times (our own unpublished data). In contrast, labeled ZO-2 was found only after 120 min of chase, as described previously (Gumbiner et al., 1991). These results suggest that p130 is not a degradation product of ZO-1 or ZO-2, and the appearance of p130 in immunoprecipitates may result from its association with ZO-1. In addition, immunoblotting and immunoprecipitation experiments indicated that the 130-kD protein is not cingulin (our own unpublished data), a TJ protein of 140 kD (Citi et al., 1988) and may represent a novel TJ-associated protein.

Co-immunoprecipitates derived from [$^{35}$S]methionine-labeled cells revealed that the stoichiometry of the ZO-1/ZO-2/p130 complex was the same regardless of whether cells

were in low or normal $Ca^{2+}$, or whether assembly of TJ was stimulated by the addition of $Ca^{2+}$ or diC8. This indicates that the stimulation of TJ formation in the Ca-switch model appears to induce the translocation and assembly of pre-existing junctional subcomplexes.

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### *References*

Ahmed, S., R. Kozma, C. Monfries, C. Hall, H. H. Lim, P. Smith, and L. Lim. 1990. Human brain n-chimaerin cDNA encodes a novel phorbol ester receptor. *Biochem. J.* 272:767–773.

Ahmed, S., I. N. Maruyama, R. Kozma, J. Lee, S. Brenner, and L. Lim. 1992. The *Caenorhabditis elegans unc-13* gene product is a phospholipid-dependent high-affinity phorbol ester receptor. *Biochem. J.* 287:995–999.

Anderson, J. M., B. R. Stevenson, L. A. Jesaitis, D. A. Goodenough, and M. Mooseker. 1988. Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.* 106:1141–1149.

Balda, M. S., and J. M. Anderson. 1993. Two classes of tight junctions are revealed by ZO-1 isoforms. *Am. J. Physiol.* 264:C918–924.

Balda, M. S., L. Gonzalez-Mariscal, R. G. Contreras, M. Macias-Silva, M. E. Torres-Marquez, J. A. Garcia-Sainz, and M. Cereijido. 1991. Assembly and sealing of tight junctions: possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. *J. Membr. Biol.* 122:193–202.

Behems, J., S. L. Birchmeier, S. L. Goodman, and B. A. Imhof. 1985. Dissociation of Madin-Darby canine kidney cells by monoclonal antibody anti arc-1: mechanistic aspects and identification of antigen as a component related to uvomorulin. *J. Cell Biol.* 101:1307–1315.

Bentzel, C. J., C. E. Palant, and M. Fromm. 1991. Physiological and Pathological factors affecting the tight junction. M. Cereijido, editor. CRC Press, Boca Raton, FL. 151–173.

Cereijido, M., L. Gonzalez-Mariscal, G. Avila, and R. G. Contreras. 1988. Tight junctions. *In* CRC Critical Reviews in Anatomical Sciences. Vol. 1, Issue 2. CRC Press, Inc., Boca Raton, FL. 171–192.

Cereijido, M., A. Ponce, and L. Gonzalez-Mariscal. 1989. Tight junctions and apical/basolateral polarity. *J. Membr. Biol.* 110:1–9.

Chapman, L. M., and E. M. Eddy. 1989. A protein associated with the mouse and rat hepatocyte junctional complex. *Cell Tissue Res.* 257:333–341.

Citi, S., H. Sabanay, R. Jakes, and B. Geiger. 1988. Cingulin, a new peripheral component of tight junctions. *Nature (Lond.).* 333:272–275.

Citi, S. 1992. Protein kinase inhibitors prevent junction dissociation induced by low extracellular calcium in MDCK epithelial cells. *J. Cell Biol.* 117: 169–178.

Contreras, R. G., J. H. Miller, M. Zamora, L. Gonzalez-Mariscal, and M. Cereijido. 1992. Interaction of calcium with the plasma membrane of epithelial (MDCK) cells during junction formation. *Am. J. Physiol.* 263:C313–318.

Ellis, B., E. E. Schneeberger, and C. A. Rabito. 1992. Cellular variability in the development of tight junctions after activation of protein kinase C. *Am. J. Physiol.* 263:F293–300.

Gonzalez-Mariscal, L., B. Chavez de Ramirez, and M. Cereijido. 1985. Tight junction formation in cultured epithelial cells (MDCK). *J. Membr. Biol.* 86:113–125.

Gonzalez-Mariscal, L., R. G. Contreras, J. J. Bolivar, A. Ponce, B. Chavez de Ramirez, and M. Cereijido. 1990. Role of calcium in tight junction formation between epithelial cells. *Am. J. Physiol.* 259:C978–C986.

Gumbiner, B. 1991. Cell adhesion molecules in epithelia. In Tight Junction. M. Cereijido, editor. CRC Press, Boca Raton, FL. 91–104.

Gumbiner, B., B. R. Stevenson, and A. Grimaldi. 1988. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of epithelial junctional complex. *J. Cell Biol.* 107:1575–1587.

Gumbiner, B., T. Lowenkopf, and D. Apatira. 1991. Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc. Natl. Acad. Sci. USA.* 88:3460–3464.

Hockberger, P., M. Toselli, D. Swandulla, and H. D. Lux. 1989. A diacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation. *Nature (Lond.).* 338:340–342.

Huang, F. L., Y. Yoshida, J. R. Cunha-Melo, M. A. Beaven, and K.-P. Huang. 1989. Differential down-regulation of protein kinase C isozymes. *J. Biol. Chem.* 264:4238–4243.

Kikkawa, U., A. Kishimoto, and Y. Nishizuka. 1989. The protein kinase C family: heterogeneity and its implications. *Annu. Rev. Biochem.* 58:31–44.

Kurihara, H., J. M. Anderson, and M. G. Farquhar. 1992. Diversity among tight junctions in rat kidney: glomerular slits diaphragms and endothelial junctions expression only one of the two isoforms of the tight junction protein ZO-1. *Proc. Natl. Acad. Sci. USA.* 89:7075–7079.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

Linder, D., M. Gschwndi, and F. Marcks. 1992. Phorbol ester-induced down-regulation of the 80-kDa myristoylated alanine-rich c-kinase substrate-related protein in swiss 3T3 fibroblasts: inhibition by staurosporine. *J. Biol. Chem.* 267:24–26.

Luna, E. J., and A. L. Hitt. 1992. Cytoskeleton-plasma membrane interactions. *Science (Wash. DC).* 258:955–964.

Madara, J. L. 1991. Relationships between the tight junction and the cytoskeleton. M. Cereijido, editor. CRC Press, Boca Raton, FL. 105–119.

Masai, I., T. Hosoya, S.-I. Kojima, and Y. Hotta. 1992. Molecular cloning of a Drosophila diacylglycerol kinase gene that is expressed in the nervous system and muscle. *Proc. Natl. Acad. Sci. USA.* 89:6030–6034.

Maruyama, I. N., and S. Brenner. 1991. A phorbol ester/diacylglycerol-binding protein encoded by the unc-13 gene of *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA.* 88:5729–5733.

Matter, K., and H.-P. Hauri. 1991. Intracellular transport and conformational maturation of intestinal brush border hydrolases. *Biochemistry.* 30:1916–1923.

Meza, I., G. Ibarra, M. Sabanero, A. Martinez-Palomo, and M. Cereijido. 1980. Occluding junctions and cytoskeletal components in a cultured transporting epithelium. *J. Cell Biol.* 87:746–754.

Meza, I., M. Sabanero, E. Stefani, and M. Cereijido. 1982. Occluding junctions in MDCK cells: modulation of transepithelial permeability by the cytoskeleton. *J. Cell Biochem.* 18:407–421.

Mullin, J. M., M. T. McGinn, K. M. Snock, and S. Imaizumi. 1990. The effects of teleocidin and aplysiatoxin tumor promoters on epithelial tight junctions and transepithelial permeability: comparison to phorbol esters. Carcinogenesis 11:377–385.

Nelson, W. J. 1992. Regulation of cell surface polarity from bacteria to mammals. *Science (Wash. DC).* 258:948–955.

Ojakian, G. 1981. Tumor promoted-induced changes in the permeability of epithelial cell tight junctions. *Cell.* 23:95–103.

Rochat, T., J. Casale, G. W. Hunninghake, and M. W. Peterson. 1988. Neutrophil cathepsin G increases permeability of cultured type II pneumocytes. *Am. J. Physiol.* 255:C603–611.

Schneeberger, E. E., and R. D. Lynch. Structure, function, and regulation of cellular tight junctions. *Am. J. Physiol.* 262:L647–661.

Shariff, A., and J. E. Luna. 1992. Diacylglycerol-stimulated formation of actin nucleation sites at plasma membranes. *Science (Wash. DC).* 256:245–247.

Sheu, H., Y. Kitajima, and H. Yaoita. 1989. Involvement of protein kinase C in translocation of desmoplakins from cytosol to plasma membrane during desmosome formation in human squamous cell carcinoma cells grown in low calcium concentration. *Exp. Cell Res.* 185:176–190.

Siliciano, J. D., and D. A. Goodenough. 1988. Localization of the tight junction protein, ZO-1 is modulated by extracellular calcium and cell–cell contact in Madin-Darby Canine Kidney cells. *J. Cell Biol.* 107:2389–2399.

Stevenson, B. R., J. D. Siliciano, M. S. Mooseker, and D. A. Goodenough. 1986. Identification of ZO-1; a high molecular weight polypeptide associated with the tight junction (zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103:755–766.

Stevenson, B. R., J. M. Anderson, and S. Bullivant. 1988. The epithelial tight junction: structure, function and preliminary biochemical characterization. *Mol. Cell Biochem.* 83:129–145.

Stevenson, B. R., M. B. Heintzelman, J. M. Anderson, S. Citi, and M. Mooseker. 1989*a*. ZO-1 and cingulin: tight junction proteins with distinct identities and localizations. *Am. J. Physiol.* 257:C621–C628.

Stevenson, B. R., J. M. Anderson, D. Braun, and M. S. Mooseker. 1989*b*. Phosphorylation of the tight junction protein ZO-1 in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. *Biochem. J.* 263:597–599.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of protein from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350–4354.

Vestweber, D., and R. Kemler. 1985. Identification of a putative cell adhesion domain of uvomorulin. *EMBO (Eur. Mol. Biol. Organ.) J.* 139:3393–3398.

Willott, E., M. S. Balda, M. Heintzelman, B. Jameson, and J. M. Anderson. 1992. Localization and differential expression of two isoforms of the tight junction protein ZO-1. *Am. J. Physiol.* 262:C1119–C1124.

Zhong, Y., T. Saitoh, T. Minase, N. Sawada, K. Enomoto, and M. Mori. 1993. Monoclonal antibody 7H6 reacts with a novel tight junction-associated protein distinct from ZO-1, cingulin and ZO-2. *J. Cell Biol.* 120:477–483.