Protein Kinase Inhibitors Prevent Junction Dissociation Induced by Low Extracellular Calcium in MDCK Epithelial Cells

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Abstract. When epithelial cell cultures are transferred from a medium with a normal extracellular calcium concentration (1–2 mM) to a medium with a low extracellular calcium concentration (LC, <50 μM free Ca^{2+}) cell–cell contacts are disrupted, and the tight junction–dependent transepithelial resistance drops. In this study, I used MDCK epithelial cells to investigate the effects of LC on the localization of the tight junction protein cingulin, and the role of protein kinases in the events induced by LC. Immunofluorescence analysis showed that within 15 min of incubation of confluent monolayers in LC, cingulin labeling was dislocated from the cell periphery, as an array of granules forming a ring-like structure. At later times after calcium removal, cingulin labeling appeared mostly cytoplasmic, in a diffuse and granular pattern, and cells appeared rounded and smaller. These events were not influenced by lack of serum, or by preincubation with 10 mM sodium azide or 6 mg/ml of cycloheximide. However, the disruption of cell–cell contacts, the cell shape changes, and the redistribution of cingulin and other junctional proteins induced by LC were inhibited when cells were pretreated with the protein kinase inhibitor H-7 (≥30 μM). The inhibitors H-8 and, to a lesser degree, staurosporine were also effective, whereas HA-1004 and ML-7 showed essentially no activity, suggesting a specificity of action of different inhibitors. Measurement of the transepithelial resistance showed that the kinase inhibitors that could prevent junction disassembly could also reduce the drop in transepithelial resistance induced by LC. Dose–response curves demonstrated that H-7 is the most effective among the inhibitors, and the transepithelial resistance was 70% of control up to 1 h after calcium removal. These results suggest that low extracellular calcium modulates junctional integrity and cytoskeletal organization through an effector system involving protein kinases.

In metazoan tissues cell–cell interaction and communication are largely mediated by specialized intercellular junctions. In epithelia, the apicolateral membranes of polarized cells are held together by *zonula occludens* (tight junction), *zonula adhaerens* (ZA)[1], and desmosome, which are ultrastructurally, biochemically, and functionally distinct (Farquhar and Palade, 1963; Geiger et al., 1983; Schwarz et al., 1990; Staehelin, 1974). Adherens-type junctions (ZA and desmosomes) are important in cell–cell adhesion and in the mechanical integrity of tissues, whereas the primary role of tight junctions is to form a selective permeability barrier between apical and basolateral compartments of the extracellular space (Gumbiner, 1987; Madara and Hecht, 1989). It has been proposed (Geiger et al., 1983) that adherens-type junctions are organized into major subdomains: an extracellular membrane domain, containing specific cell–cell adhesion molecules involved in homophilic interactions (Kemler et al., 1989; Takeichi, 1991, 1988), and a cytoplasmic "plaque" domain, anchored to the actin cytoskeleton (ZA), or to intermediate filaments (desmosomes). In tight junctions, the membrane domains appear as areas of close contact between adjoining cells, and produce a complementary pattern of grooves and fibrils when examined by freeze-fracture EM (Staehelin, 1974). Two specific proteins of tight junctions, ZO-1 (Stevenson et al., 1986) and cingulin (Citi et al., 1988, 1989), have been characterized, and both appear to belong to the cytoplasmic "plaque" of tight junctions, based on immunolocalization and extractability experiments.

One key question in studying the assembly and function of junctions is how components of the extracellular membrane domain can transduce signals to components of the cytoplasmic plaque domain (and vice versa), resulting in changes in cell–cell interactions and cytoskeletal organization. For example, a number of studies have shown that extracellular calcium can modulate junction assembly and affect the conformation and function of cell adhesion molecules. Depletion of extracellular calcium ions using chelating agents or low calcium (LC) medium causes junctional splitting in gastric glandular epithelium (Sedar and Forte, 1964), disassembly of tight junctions in pancreatic tissue

1. *Abbreviations used in this paper*: LC, low calcium; TER, transepithelial resistance; ZA, *zonula adhaerens*.

(Meldolesi et al., 1978), endocytic internalization of membrane and cytoplasmic components of adherens junctions in MDBK cells (Kartenbeck et al., 1982, 1991; Mattey and Garrod, 1986; Volberg et al., 1986), dislocation of desmoplakin from desmosomal plaques in myocytes (Kartenbeck et al., 1983), and redistribution and solubilization of fodrin and desmoplakin in MDCK cells (Nelson and Veshnock, 1987; Pasdar and Nelson, 1988). Tight junction dissociation induced by LC is followed by a dramatic drop in the transepithelial permeability of MDCK monolayers grown on semi-permeable supports (Martinez-Palomo et al., 1980; Meza et al., 1980).

Although removal of extracellular calcium has been widely used as an experimental model to investigate the dynamics of membrane–cytoskeleton interactions, the signal transduction pathway(s) by which LC induces disruption of epithelial junctions and cytoskeletal rearrangements is unknown. Using the immunofluorescence technique and measurement of the transepithelial resistance, I now show that the effects of LC on cell–cell contact, distribution of junctional proteins, and paracellular permeability in epithelial MDCK cells can be prevented by specific protein kinase inhibitors. These observations suggest a role for protein kinases in the cascade of events stimulated by removal of extracellular calcium in epithelial cells.

Materials and Methods

Materials

Cell culture media were from Gibco Laboratories (Grand Island, NY). FBS was from Hyclone Labs (Salt Lake City, UT). Plasticware was from Gibco Laboratories and Costar (Cambridge, MA). Tissue-culture grade reagents were from Sigma Chemical Co. (St. Louis, MO), unless otherwise stated. Protein kinase inhibitors were purchased from Seikagaku America Inc. (St. Petersburg, FL), except for H-7 (Sigma Chemical Co.) and staurosporine Calbiochem-Behring Corp., San Diego, CA).

Antibodies

Rabbit anti-cingulin polyclonal antiserum was prepared against purified chicken cingulin (Citi et al., 1988), and has been shown to cross react with mammalian cingulin (Citi et al., 1991). For immunofluorescence, the antiserum was diluted 1:750 in PBS (150 mM NaCl, 25 mM sodium phosphate, pH 7.0). Rat mAb (culture supernatant) against ZO-1 was a kind gift of Dr. D. Goodenough (Harvard University, Cambridge MA), and was used undiluted. Mouse mAbs against desmoplakin (Boehringer-Mannheim Biochemicals, Indianapolis, IN) and uvomorulin/E-cadherin/L-CAM (Sigma Chemical Co.) were used following the directions of the manufacturers. Fluorescently labeled secondary antibodies were obtained from Jackson Immuno Research Laboratories (West Grove, PA).

Protein Kinase Inhibitors

Stock solutions of H-7 (1-[5-isoquinolinylsulfonyl]-2-methyl-piperazine dihydrochloride), H-8 (*N*-2-[methylaminoethyl]-5-isoquinolinesulfonamide dihydrochloride), HA1004 (N-[2-guanidinoethyl]-5-isoquinolinesulfonamide hydrochloride), and ML-7 (1-[5-iodonaphtalene-1-sulfonyl]-1H-hexahydro-1,4-diazepine hydrochloride) were made in sterile water, at a concentration of 10 mM, except for H-7, which was 30 mM. The stock solution for staurosporine was 1 mg/ml in DMSO. The stock solutions were stored at 4°C, further diluted if necessary, and added directly to the culture medium, to obtain final concentrations that were approximately 1×, 10×, and 100× the lowest inhibitor constant (K_i) for each drug (Table I).

Table I. Inhibition Constants of Protein Kinase Inhibitors Used in This Study

	K_i-pKC	K_i-pKA	K_iMLCK	K_ipKG
H-7	6.0 μM	3.0 μM	97 μM	5.8 μM
H-8	15 μM	1.2 μM	68 μM	0.48 μM
HA1004	40 μM	2.3 μM	150 μM	1.3 μM
ML-7	42 μM	21 μM	0.3 μM	NA
staurosporine	0.7 nM	7 nM	NA	8.5 nM

The K_i values of each drug against protein kinase C (pKC), cAMP-dependent protein kinase (pKA), myosin light chain kinase (MLCK), and cGMP-dependent protein kinase (pKG) are shown (values obtained from Seikagaku America., Inc., and Calbiochem). NA, not available.

Cell Culture and Calcium Removal

MDCK cells (a gift from Dr. E. Rodriguez-Boulan, Cornell University Medical College, New York, NY) were cultured in DMEM + 5% FBS, in a humidified 5% CO_2 incubator at 37°C, and passaged by trypsin-EDTA treatment.

To remove calcium, EGTA (stock 0.2 M, pH 8.0) or BAPTA (Molecular Probes, Eugene, OR) (stock 0.5 M) were added to the medium. When EGTA was used, the pH was adjusted by addition of 0.1 M NaOH to give 4 mM final concentration. Alternatively, to remove extracellular calcium cells were rinsed six times with low calcium medium (S-MEM) and incubated with S-MEM containing 1% calcium-free FBS and, if necessary, appropriate concentrations of protein kinase inhibitors. Thus, drugs and protein kinase inhibitors were added before calcium removal, and maintained in the medium after addition of chelators or S-MEM.

To remove serum, cultures were rinsed four times and incubated in serum-free medium before addition of drugs and chelators. To prevent protein synthesis, a fresh stock solution of cycloheximide (100 mg/ml) was added to the culture medium (final concentration 6 mg/ml) for 2 h before calcium removal. In another experiment, sodium azide (stock 1 M) was added to a final concentration of 10 mM and incubated for 30 min before calcium removal.

Transepithelial Resistance

To measure the transepithelial resistance, cells were plated onto polycarbonate filters (Transwell, Costar), and the resistance of the monolayers was determined with a Millicell ERS Voltohmeter (Millipore, Bedford, MA). Values were normalized for the area of the filter (4.7 cm^2), after subtracting the background resistance from a filter with medium alone, and were expressed as percent of the resistance at time 0 (just before removing calcium). The resistance of cultures grown for 3–5 d was typically 200–300 $\Omega \cdot cm^2$. Inhibitors and calcium chelators were added to the medium in both the apical and basolateral compartments of the Transwells.

Immunofluorescence Microscopy

For immunofluorescence, cells were grown on glass coverslips in 24-well plates. To fix and permeabilize cells, coverslips were transferred into methanol at −20°C for 30 min, and then cells were rehydrated in PBS and incubated with diluted first antibody for 1 h at room temperature. Coverslips were washed three times for 5 min in PBS, and then incubated for 1 h at room temperature with TRITC-labeled goat anti-rabbit or TRITC-labeled rabbit anti-mouse antibodies (stock 1 mg/ml in water, diluted 1:75 in PBS). Finally, coverslips were washed and mounted with 20% polyvinyl alcohol and 2% 1-4 diazabicyclo[2,2,2,]octane as an anti-bleach agent. Slides were examined with a Leitz Ortholux II epifluorescence microscope, fitted with a 63× lens (1 NA). Photographs were taken using Tmax 100 ASA film (Eastman Kodak Co., Rochester, NY).

Results

Redistribution of Cingulin following Removal of Extracellular Calcium

The localization of cingulin in monolayers of kidney epithelial (MDCK) cells was studied by indirect immunofluorescence with a polyclonal antiserum (Citi et al., 1988). In control cultures grown to confluence, cingulin labeling in the

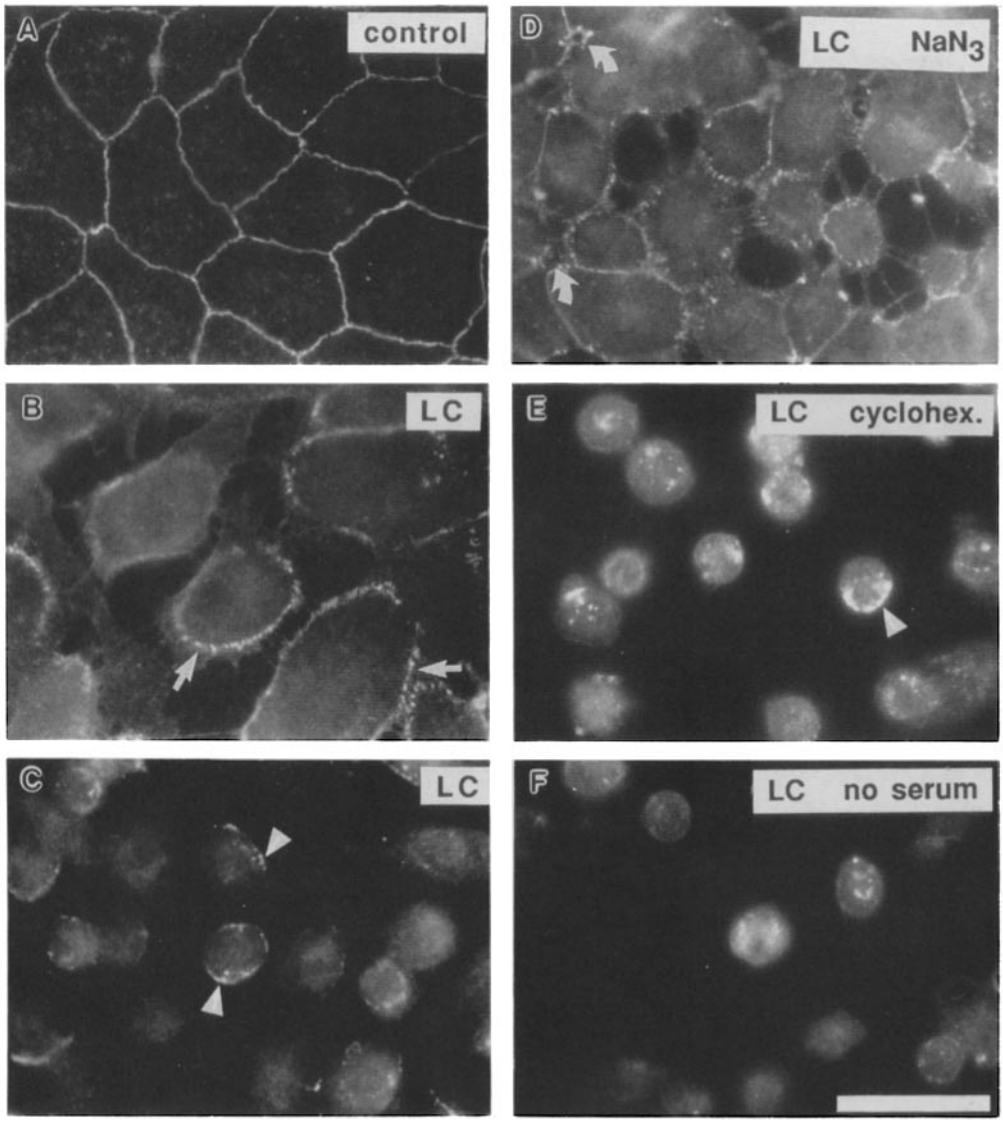

Figure 1. Effect of removal of extracellular calcium on the immunofluorescent localization of cingulin in MDCK cells. Monolayers were incubated in normal medium (*A*) or in low calcium (*LC*) medium for 15 min (*B*) or 1 h (*C–F*), and immunofluorescently labeled with anti-cingulin antiserum followed by TRITC anti-rabbit. Cells in *D* were incubated with 10 mM NaN_3 for 30 min before LC, cells in *E* with 6 mg/ml cycloheximide for 2 h, and cells in *F* were deprived of serum for 1 h before LC. Arrows in *B* point to disgregated cingulin labeling, forming a subcortical ringlike structure. Arrowheads in *C* and *E* indicate granular cingulin labeling apparently associated with the cell cortex 1 h after LC. Curved arrows in *D* point to early changes in cingulin distribution at points of intersection between more cells. Removal of calcium was obtained by addition of chelators or by changing the medium with S-MEM (see Materials and Methods). Bar, 10 μm.

tight junction is distributed uniformly along the regions of cell–cell contact (Fig. 1 *A*). The protein is localized in the cytoplasmic plaque domain of the junction, ~40 nm from the midline of the junctional membrane (Citi et al., 1988).

Removal of extracellular calcium caused dissociation of cell–cell contacts, changes in cell shape, and redistribution of cingulin (Fig. 1, *B–F*). The effects were similar when calcium was removed either with the calcium chelators EGTA or BAPTA (Marks and Maxfield, 1991), or by replacement of the medium with low-calcium medium (S-MEM) (see also Fig. 3). At early stages (e.g., 15 min) after calcium removal, most cells showed a relatively normal size and shape, however evident gaps due to splitting of junctions were apparent in the monolayer, and stretched, thin cellular processes were often visible between neighboring cells (Fig. 1 *B*). Cingulin labeling appeared partially or completely dislocated from the cell periphery and internalized in the cytoplasm, in the form of arrays of punctate or granular staining, forming a ring like structure below the cell membrane (Fig. 1 *B*). Junctional areas bordering the contacts between three or four cells appeared to be the most susceptible to the effects of LC, since they were the first to display changes in cingulin localization (see for example Fig. 1 *D*). Labeling could occasionally be seen in the thin projections connecting adjacent cells (not shown for cingulin, but see ZO-1 labeling in Fig. 4 *D*), indicating a tighter association of the protein with the cell membranes. One hour after calcium removal, as disruption of cell–cell contacts became more complete, cells appeared smaller (<5 μm in diameter) and rounded in shape (Fig. 1, *C*, *E*, and *F*). Cingulin labeling was then in the form of diffuse staining and intensely labeled granules, which were isolated or in irregular aggregates. Focusing up and down these cells revealed that most of the labeling was cytoplasmic, but it was also detected in a submembranous localization (arrowheads in Fig. 1, *C* and *E*), suggesting that 1 h after calcium removal some cingulin was localized within the cell cortex. It has been shown that in MDBK cells a peripheral ring of actin filaments becomes increasingly visible at the cell periphery about 60 min after EGTA addition (Kartenbeck et al., 1991), and the cortical cingulin labeling may imply its association

with the cortical actin cytoskeleton. Finally, intense labeling was observed in some areas of cell–cell contact (Fig. 2 *B*), suggesting that in these areas the organization of cingulin and the integrity of the junctions were similar to those of normal cells. In summary, the redistribution of cingulin labeling in LC appeared to consist of a series of events: (*a*) detachment from the junctional membrane as a "belt"; (*b*) disgregation of the belt into diffuse and granular labeling; and (*c*) stabilization of the cytoplasmic labeling and partial reorganization within the cell cortex.

The effects of LC on cingulin distribution did not appear to depend on oxidative phosphorylation, protein synthesis, or serum factors. When exposure to LC was carried out on cells incubated for 30 min in 10 mM NaN_3 (Fig. 1 *D*), or for 2 h in 6 mg/ml cycloheximide (Fig. 1 *E*) or in serum-free medium (Fig. 1 *F*), the results were very similar to those obtained in the absence of the drugs. However, preincubation at 4°C for 30 min blocked the redistribution of cingulin (not shown). These results confirm and extend previous observations (Martinez-Palomo et al., 1980), suggesting that the drop in tight junction–dependent transepithelial resistance caused by LC in MDCK monolayers is not influenced by protein synthesis and serum factors, but is reduced by low temperature.

The Protein Kinase Inhibitor H-7 Prevents the Redistribution of Cingulin and Other Junctional Proteins Caused by LC

A concentrated stock solution of the protein kinase inhibitor H-7 (1-[5-isoquinolinylsulfonyl]-2-methyl-piperazine dihydrochloride) (Hidaka et al., 1990) was added to confluent MDCK monolayers (final concentration 300 μM), and incubated for 1 h. Extracellular calcium was then removed by ad-

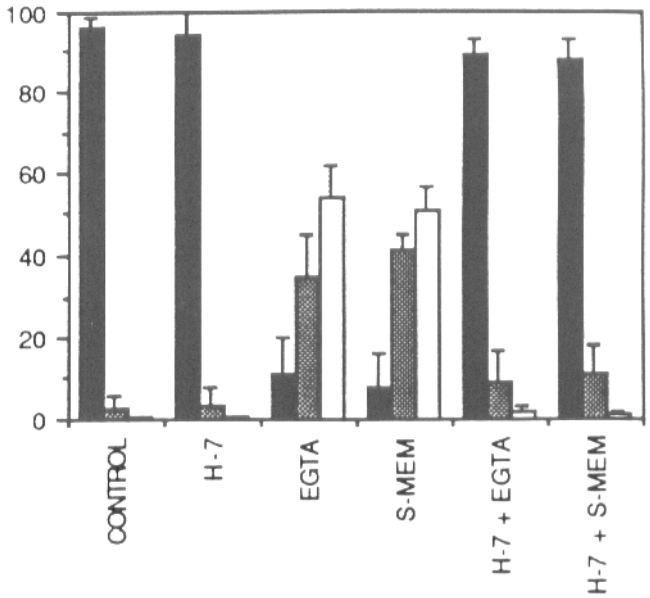

Figure 3. Semiquantitative morphological analysis of junction dissociation under low extracellular calcium conditions. The histogram shows the percent of cells with complete junctions (normal, ■), partial junctional contacts (partial, ▩), and total disruption of junctional contacts (total, □) under the various experimental conditions tested. Error bars represent standard errors for three separate experiments. At least 100 cells per experiment were scored. Cells were scored as "partials" when >50% of their junctional perimeter was dissociated from neighboring cells and, in cells treated with EGTA, cingulin labeling was redistributed. Cells were scored as "total," when no cingulin-containing intercellular contacts were detected, as "normal" when all the junctional perimeter displayed continuous cingulin labeling.

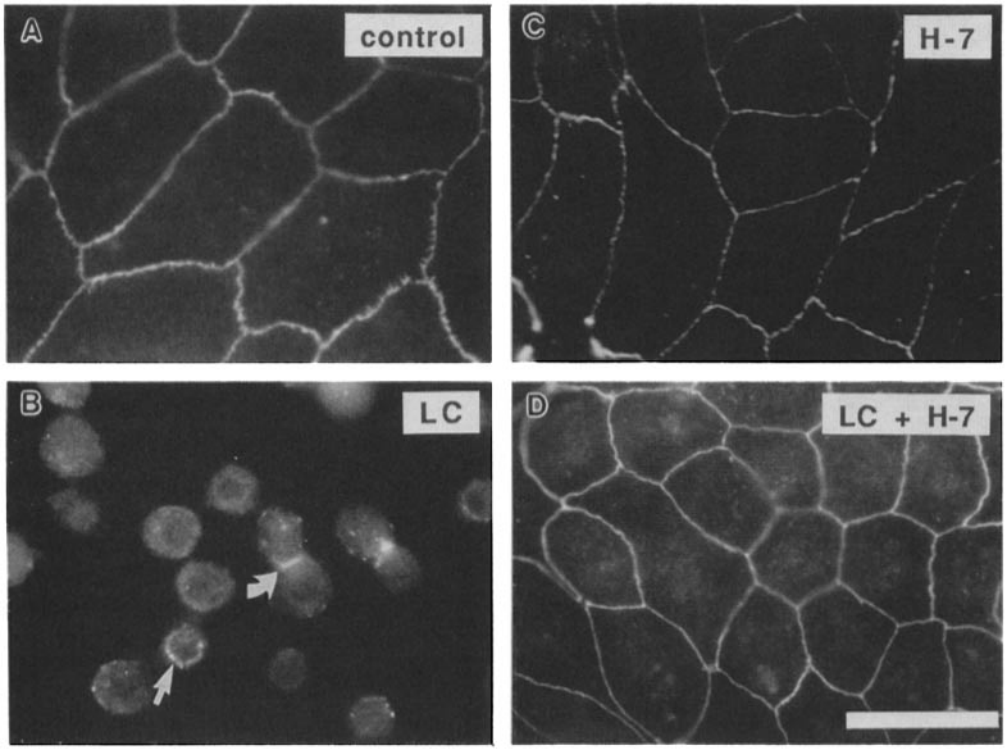

Figure 2. The protein kinase inhibitor H-7 prevents junction dissociation and cingulin redistribution in MDCK cells at low extracellular calcium. Cells were incubated in normal medium (*A* and *C*) or in LC medium (*B* and *D*) for 1 h, in the absence (*A* and *B*) or in the presence (*C* and *D*) of 300 μM H-7, and immunofluorescently labeled. Notice that in the presence of H-7 no junction dissociation or cingulin redistribution can be observed (cell perimeters in *D* are shorter than in *A* and *B* because a field with higher cell density was photographed, and not because any "apical constriction" occurs under these conditions). The arrow in *B* points to subcortical cingulin labeling (compare with Fig. 1 *B*), and the curved arrow in *B* points to junctional cingulin labeling. Bar, 10 μm.

dition of EGTA and BAPTA to 4 mM final concentration (or by addition of S-MEM containing H-7), and the cells were incubated for one additional hour, before being fixed and labeled. The same protocol was used to test the effect of other protein kinase inhibitors on cingulin distribution (see Fig. 5).

As shown in Fig. 2 *D*, in cultures preincubated with H-7 and then subjected to calcium removal, there was essentially no change in cell shape and integrity of junctional contacts. The distribution of cingulin in the vast majority of cells was identical to control cultures, e.g., uninterrupted labeling was detected exclusively along the junctions and was absent from the cytoplasm (Fig. 2 *D*). When confluent monolayers were treated with H-7 alone for 1–2 h cingulin distribution was also like in control cultures (Fig. 2, compare *C* to *A*). The ability of H-7 to prevent changes induced by LC was not dependent on cell density or the presence of a continuous monolayer, since the same result was obtained in semi-confluent cultures, where cingulin labeling is restricted to

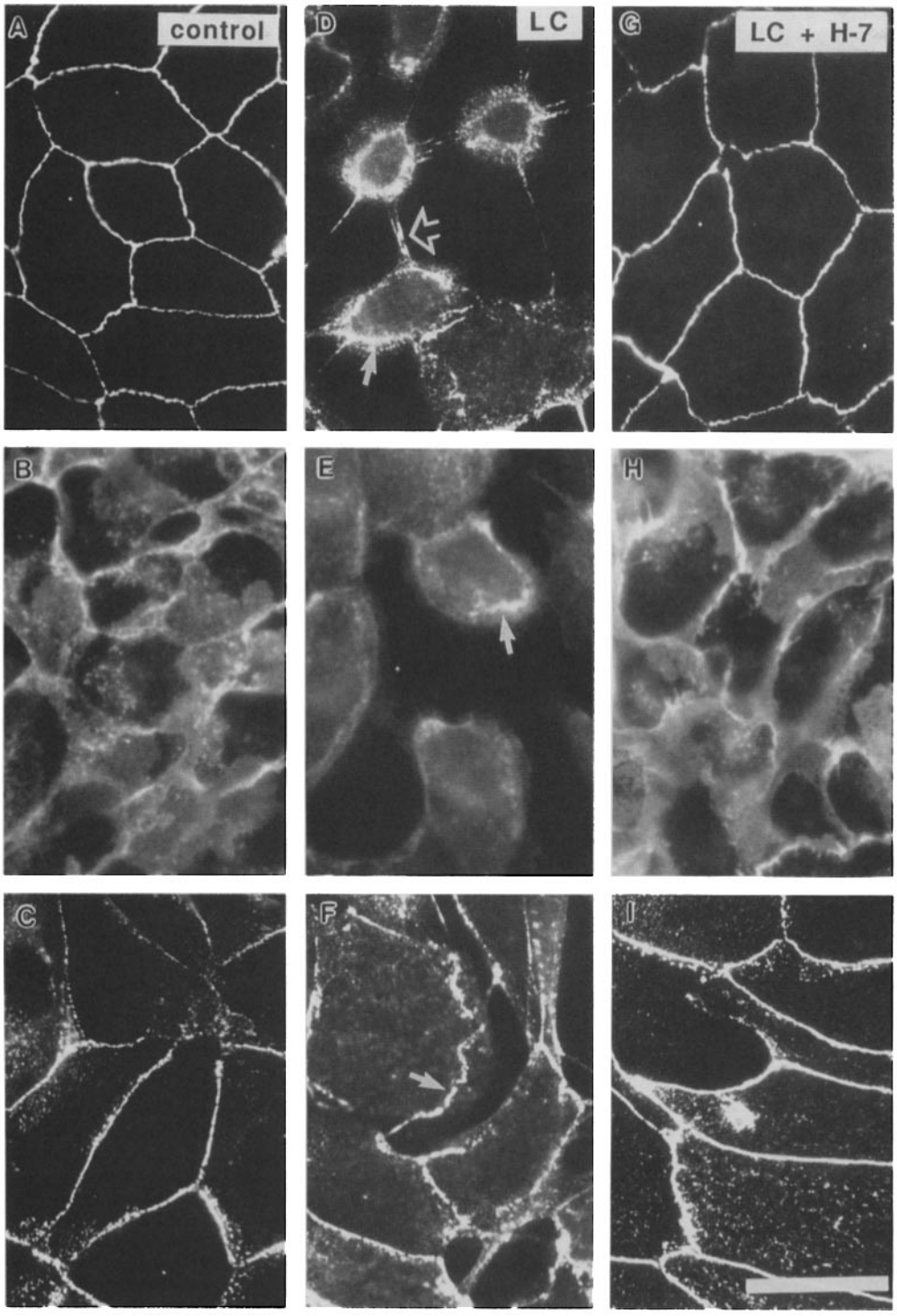

Figure 4. H-7 prevents the redistribution of ZO-1, E-cadherin, and desmoplakin in MDCK cells after calcium removal. Cells were incubated in normal medium (*A*, *B*, and *C*) or in LC medium (*D–I*) for 1 h, in the absence (*A–F*) or in the presence (*G–I*) of 300 μM H-7, and immunofluorescently labeled with antibodies against ZO-1 (*A*, *D*, and *G*), E-cadherin (*B*, *E*, and *H*) and desmoplakin (*C*, *F*, and *I*). Note that H-7 prevents the redistribution of these proteins in LC, as for cingulin. The empty arrow in *D* indicates ZO-1 labeling in the thin projections bridging two cells. Smaller arrows in *D*, *E*, and *F* point to labeling dislocated from the cell periphery in LC (see also references in text). Bar, 10 μm.

the regions of the cells that are forming junctions with neighboring cells (not shown). Although cultures were pre-treated routinely with 300 μM H-7 for 1 h, lower concentrations (30 μM) or shorter treatments (e.g., 5 min) were sufficient to observe the protective effects of H-7. When H-7 was added together with calcium chelators or shortly after, it had no activity, indicating that the mechanism by which calcium removal disrupts junctional integrity is very rapid and H-7 acts on an early step of the mechanism.

To quantify the effects of calcium chelators, S-MEM and H-7 on the separation of lateral contacts, the number of cells with complete or partial disruption of junctions was counted (Fig. 3). In control cultures >95% of the cells were completely surrounded by continuous, junctional cingulin labeling, whereas in LC ~50% of the cells were isolated and showed diffuse or granular cingulin labeling, and ~40% of the cells showed partial junction dissociation (Fig. 3). When cells were treated with H-7 before calcium removal, most cells showed intact junctions, and there was only a slight increase in the number of cells with partial junction splitting (Fig. 3). Thus, H-7 effectively prevents the changes in cell shape, the dissociation of junctional contacts and the redistribution of junctional proteins induced by LC.

The effect of H-7 was not due to an interference with the chelating properties of EGTA or BAPTA, nor to an indirect increase of the extracellular calcium concentration. Using a calcium-sensitive electrode to measure the concentration of free calcium in the spent culture media, values below 5 μM were obtained for all experimental low calcium conditions, with or without protein kinase inhibitors. This concentration is sufficient to split desmosomes and *zonulae adhaerentes* (Mattey and Garrod, 1986; Volberg et al., 1986).

To determine whether the effect of H-7 is restricted to cingulin or applies to other junctional proteins, cells were labeled with antibodies against the tight junction protein ZO-1, the adherens junction protein E-cadherin, and the desmosomal protein desmoplakin (Fig. 4). The three proteins are distributed along the junctional contacts (Fig. 4, *A*, *B*, and *C*), and 1-h treatment with EGTA induced a dislocation of the labeling from the cell periphery towards the cytoplasm (arrows in Fig. 4, *D*, *E*, and *F*), as previously shown (Kartenbeck et al., 1991, 1982; Siliciano and Goodenough, 1988; Stevenson et al., 1988). When cells were treated with H-7 before calcium removal, no redistribution could be observed (Fig. 4, *G*, *H*, and *I*), indicating that the effect of H-7 applies not only to tight junctions, but also to adherens-type junctions.

Specificity of Protein Kinase Inhibitors

To investigate the specificity of action of protein kinase inhibitors, the effect of four additional inhibitors, displaying different inhibition constants against four types of protein kinases (Table I), were tested.

As shown in Fig. 5 *A*, the inhibitor H-8, when used at a concentration of 120 μM, was as effective as H-7 in preventing junction dissociation and cingulin redistribution induced by LC, since the labeling was essentially identical to control cultures. At lower concentrations of H-8, increasing numbers of junctions were dissociated, and cingulin labeling was dislocated from the cell periphery (not shown). When control cultures at normal extracellular calcium concentrations were incubated for 1–2 h with the same amounts of H-8, no effects were detectable on cingulin distribution.

The myosin light chain kinase inhibitor ML-7 did not prevent junction dissociation, cell rounding or cingulin redistribution (Fig. 5 *B*), even when used at concentrations ~100× its lowest K_i. Interestingly, cingulin labeling appeared con-

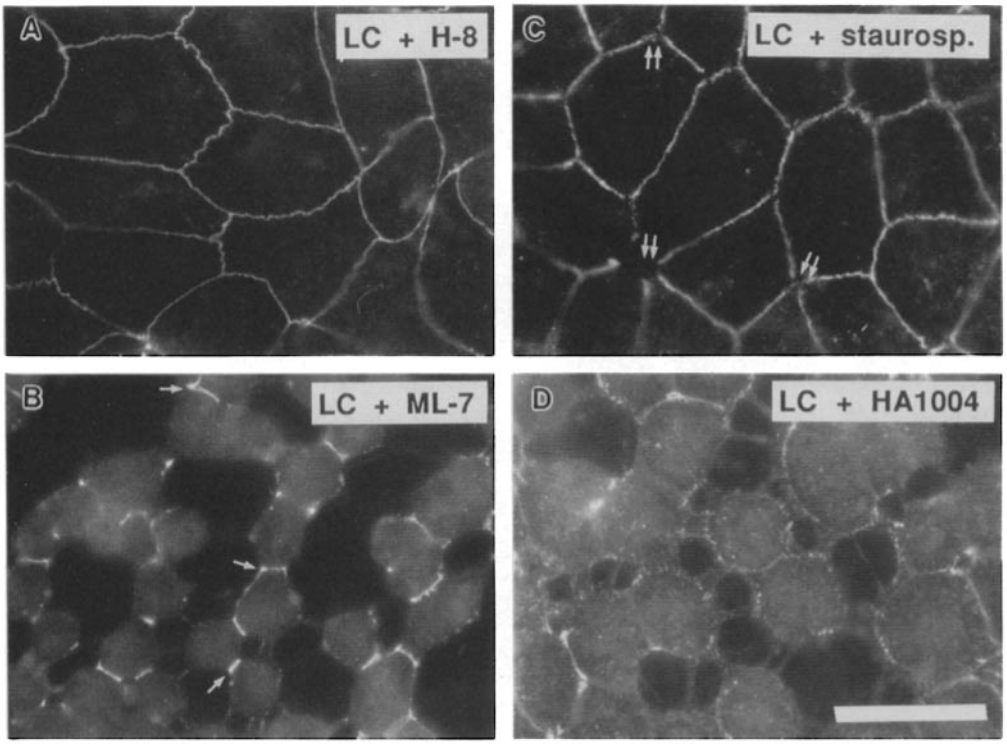

Figure 5. Differential effects of protein kinase inhibitors on junction integrity and cingulin redistribution following calcium removal. All cells shown here were immunofluorescently labeled with anti-cingulin antiserum, and were incubated for 1 h with 120 μM H-8 (*A*), 30 μM ML-7 (*B*), 100 nM staurosporine (*C*), or 120 μM HA1004 (*D*) before calcium removal. Note that H-8 prevents completely junction dissociation and cingulin internalization, whereas ML-7 and HA1004 have virtually no effect. Small arrows in *B* indicate areas of cell–cell contact that are intensely stained for cingulin. Double arrows in *C* point to areas of intersection between cells, where cingulin labeling is disgregated when calcium is removed in cells treated with staurosporine. Bar, 10 μm.

centrated in intensely labeled bars in areas of cell–cell contact (arrows in Fig. 5 *B*), rather than being predominantly distributed in diffuse or granular cytoplasmic pattern. These observations suggested that myosin light chain kinase activity is not important per se in junction dissociation, but may be required for complete disassembly and/or internalization of cingulin. When control cultures at normal extracellular calcium concentrations were incubated for 1–2 h with the same amounts of ML-7, no effects were detectable on cingulin distribution.

Staurosporine, a potent inhibitor of protein kinase C (Table I) (Tamaoki et al., 1986), could prevent junction dissociation and cell rounding in LC, but cingulin labeling appeared occasionally disgregated, especially at areas of intersection between three or more cells (double arrows in Fig. 5 *C*). Finally, the protein kinase inhibitor HA1004, which is the weakest protein kinase C inhibitor among the isoquinolinesulfonamide derivatives (Table I), did not prevent junction dissociation and cingulin distribution under LC conditions (Fig. 5 *D*). HA1004 did not produce any effect on the distribution on cingulin in control cultures at normal extracellular calcium concentrations.

Effect of Protein Kinase Inhibitors on the Fall in Transepithelial Resistance Caused by Calcium Removal: Correlation with Cingulin Distribution

To study by a functional approach the effects of low extracellular calcium and protein kinase inhibitors on the integrity of tight junctions, I next determined the transepithelial resistance (TER) of MDCK monolayers grown on semi-permeable filters. In epithelial monolayers, the resistance is a measure of the permeability to ions of the paracellular pathway, which depends largely on the efficiency and selectivity of the tight junction barrier.

Removal of extracellular calcium ions resulted in a decrease in TER, consisting of a rapid phase (0–5 min), followed by a slower decrease, reaching values close to baseline within 30 min (Fig. 6 *A*) (see also Martinez-Palomo et al., 1980). The kinetics of the decrease in TER were similar when calcium chelators (Fig. 6 *A*) or S-MEM were used (not shown).

Protein kinase inhibitors were added to the cultures at three different concentrations, roughly corresponding to 100×, 10×, and 1× their K_i values (Table I), and the TER

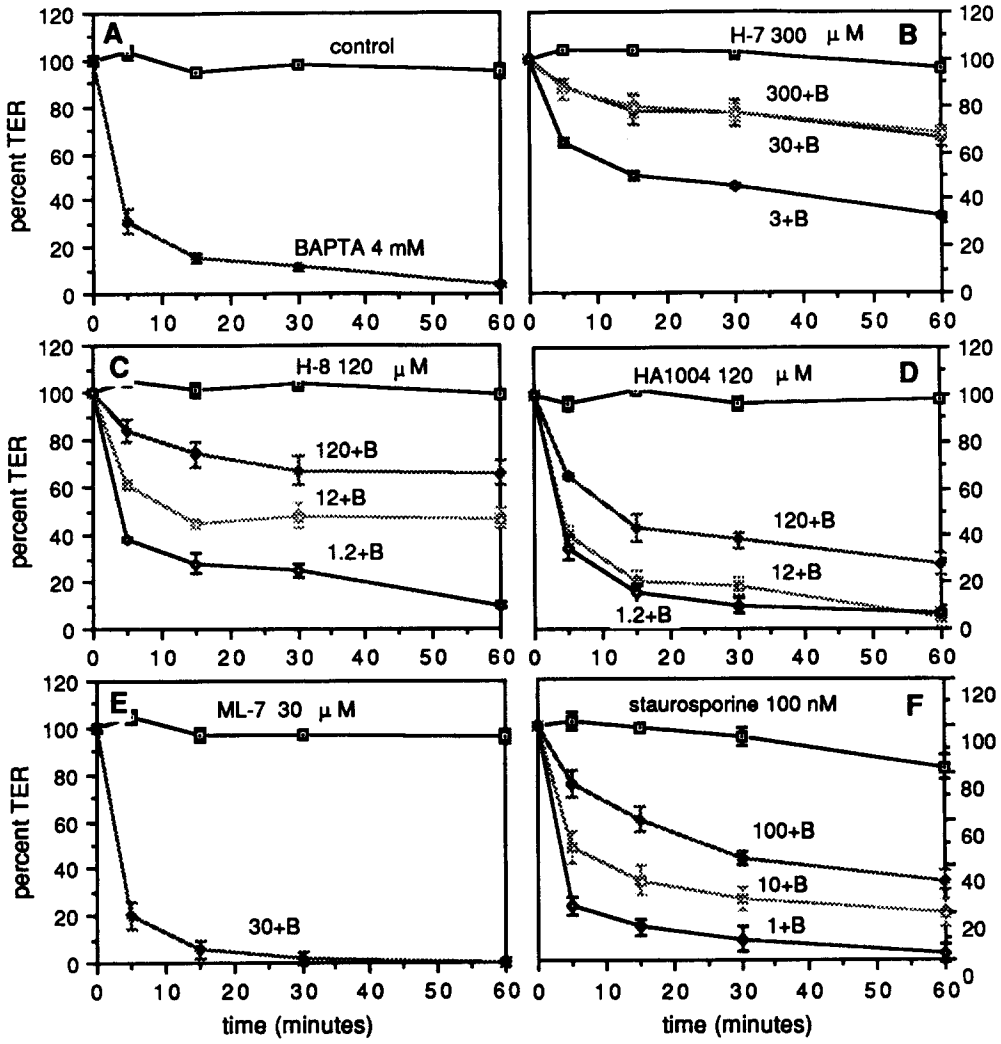

Figure 6. Time course of the effect of low extracellular calcium and protein kinase inhibitors on the TER of MDCK cells. The percent of TER value is shown, taking the value at time 0 (just before addition of BAPTA) as 100. Number and letters placed near the curves in the figure illustrate the experimental conditions for the respective curves, for example 300 + B = 300 μM H-7 + 4 mM BAPTA. (*A*) TER of control cells and cells treated with 4 mM BAPTA. (*B*) Effect of H-7. (*C*) Effect of H-8. (*D*) Effect of HA1004. (*E*) Effect of staurosporine. (*F*) Effect of ML-7. Error bars indicate standard errors for at least three separate experiments.

of the monolayers was measured (Fig. 6, *B–F*). In the presence of 300 or 30 μM H-7, the drop in TER caused by BAPTA was small. In the first 15 min the TER dropped by 10–20%, and then it slowly decreased, so that 1 h after calcium removal it was ~70% of the initial value (Fig. 6 *B*). Even at low concentrations of H-7 (3 μM), the resistance 1 h after calcium removal was ~30% of control (Fig. 6 *B*), considerably higher than with BAPTA alone (Fig. 6 *A*). The 30% drop in transepithelial resistance observed in the presence of 30–300 μM H-7 may be due to the partial opening of a few junctions, for example at susceptible points where three or more cells come together (see also Fig. 3), since minor perturbations in junctional permeability can produce major alterations in resistance (Madara and Hecht, 1989).

Similar dose-response curves indicated that 120 μM H-8 was as effective as H-7 in preventing the drop in TER, whereas at lower concentrations (12 and 1.2 μM), H-8 was less active than H-7 (Fig. 6 *C*). On the other hand, HA1004, which is similar to H-7 except for its higher K_i for protein kinase C (Table I), was only weakly effective at high concentrations (120 μM), since after 1 h of calcium removal the resistance was <30% its original value (Fig. 6 *D*). The myosin light chain kinase inhibitor ML-7 at concentrations of 30 μM or lower (not shown) did not prevent the drop in TER induced by calcium chelators (Fig. 6 *E*).

When confluent monolayers were incubated with 100 nM staurosporine alone for 1 h, the transepithelial resistance fell to ~90% its initial value (upper trace in Fig. 6 *F*). Addition of the calcium chelators further decreased the resistance by ~50% (Fig. 6 *F*), confirming that staurosporine prevents at least partially the opening of tight junctions (see also Fig. 5 *C*). At lower staurosporine concentrations (10 and 1 nM) the effect was much less pronounced. Finally, although in the presence of H-7 and H-8 (120 μM) the TER values appeared to stabilize at a high value after 15–30 min, if incubation in LC was prolonged for >2 h, the resistance started to decrease, and reached baseline values within 6 h (not shown), indicating that cells cannot maintain a functional tight junction in LC medium for extended periods, even in the presence of kinase inhibitors.

In summary, there was a fairly good correlation between the effects of the protein kinase inhibitors or junction morphology and cingulin localization in LC, as determined by immunofluorescence, and their effects on junction permeability, as determined by measurement of the transepithelial resistance. This correlation supports the idea that the fall in electrical resistance caused by calcium removal is due to an increase in the permeability of the paracellular "shunt" pathway, rather than that of the transcellular pathway (Martinez-Palomo et al., 1980).

Discussion

The results presented here describe a novel approach to understanding the molecular mechanism by which low extracellular calcium concentrations induce junction disassembly, redistribution of junctional proteins, and opening of the tight junction barrier in epithelial cells. The observation that these events can be prevented using specific protein kinase inhibitors suggests that protein kinases are important in a signal transduction pathway triggered by low extracellular calcium. These data also lead to the prediction that protein kinase inhibitors may prevent a variety of responses to low extracellular calcium in many cell types.

The dislocation of cingulin labeling from the tight junction membrane, followed by its internalization and breakdown, appears very similar to the process described for proteins of *zonula adhaerens* and desmosome in LC (Kartenbeck et al., 1991, 1982; Pasdar and Nelson, 1988; Volberg et al., 1986). H-7 prevents the LC-dependent redistribution not only of cingulin, but also of other adherens and tight junction proteins, such as ZO-1, E-cadherin, and desmoplakin (Fig. 4). Taken together, these observations suggest that calcium removal acts on the cytoplasmic plaques of all these types of junctions through a common mechanism, which could involve the phosphorylation of one or more proteins that control the linkage of the cytoskeletal plaques of junctions to their respective membranes. Future studies should investigate the effects of protein kinase inhibitors on the phosphorylation of junctional and cytoskeletal proteins in this model.

Although the topological relationships between cingulin, actin, and other junctional proteins in the LC-dependent rearrangement is unknown, it has been shown that for adherens junctions the effects of LC can be divided into two temporally separated steps (Volberg et al., 1986). The first step occurs within 3–5 min of calcium removal and consists in the dissociation of cell–cell contacts. This phase is probably related to the rapid phase in the drop in TER (Fig. 6 *A*). The second step begins at least 10 min after EGTA treatment, and consists in the detachment of the actin bundle from the cell periphery, followed by its centripetal retraction and breakdown (Volberg et al., 1986). The redistribution of cingulin described here and the disassembly of junctional strands observed by freeze-fracture EM (Meldolesi et al., 1978) appear to overlap temporally with this second phase. The observation that protein kinase inhibitors were shown either to block both or neither of these steps suggests that (*a*) junction dissociation and cytoskeletal protein redistribution induced by LC are closely linked processes, and (*b*) protein kinase inhibitors act on an early event in the pathway.

How do the kinase inhibitors prevent the effects of low extracellular calcium? These molecules have been shown to inhibit competitively in vitro, with different specificities (Table I), the activity of kinases, and to block the effects of kinase activators and reduce substrate phosphorylation in vivo (Hagiwara et al., 1987; Hidaka et al., 1984, 1990; Ido et al., 1986; Sheu et al., 1989; Watson et al., 1988; Denisenko and Citi, manuscript in preparation), suggesting that they act through specific inhibition of protein kinases. The possibility that these molecules act non-specifically by competing with ATP-binding proteins different from protein kinases cannot be ruled out, although it is difficult to test. One argument against this unspecific, "toxic" effect is that although the structures of H-7, H-8, and HA1004 are very similar, there were obvious differences in specific activity among them. For example, HA1004 is an analog of H-7, but was much less effective than H-7 in preventing junction dissociation and the fall in transepithelial resistance even at high concentrations. On the other hand, staurosporine, a potent protein kinase C inhibitor whose structure is different from the isoquinolinesulfonamide derivatives, was active.

Identification of the kinase(s) that may be involved in this process was not an objective of the present study, however comparison of the activity of various inhibitors (especially H-7 versus HA1004) suggests that protein kinase C may be important in mediating the effects of calcium removal. However caution is necessary, since the differences in K_is to-

wards one or the other type of kinase are relatively small (Table I), and the effect of the inhibitors on different kinase isoforms has not been characterized. It is noteworthy that direct activation of protein kinase C by phorbol esters causes a drop in TER (Ojakian, 1981), cell–cell dissociation, and redistribution of cytoskeletal proteins (Schliwa et al., 1984), including cingulin (Denisenko and Citi, manuscript in preparation), indirectly supporting the idea that protein kinases can modulate the organization of junctions and cytoskeleton. Protein kinase C has also been implicated in the activation of plasma membrane Ca^{2+} channels in the "nonspecific" phagocytic activity of macrophages, which, unlike receptor-mediated phagocytosis, is influenced by extracellular calcium (Hishikawa et al., 1991).

One interpretation of the present results is that calcium removal leads to the activation of protein kinases, and this event is mediated by cadherins, transmembrane glycoproteins responsible for Ca^{2+}-dependent cell adhesion (Kemler et al., 1989; Takeichi, 1988, 1991). It has been shown that the conformation of cadherins is dependent on calcium, since they are protected from tryptic digestion by calcium (Takeichi, 1988). Cadherins show sequences encoding putative calcium-binding sites in their extracellular domains (Ringwald et al., 1987), and single amino acid substitutions in one Ca^{2+} binding site completely abolish the adhesive function (Ozawa et al., 1990). However it should be pointed out that direct binding of Ca^{2+} to cadherins has not been demonstrated in vitro nor in vivo. The fundamental importance of cadherins in cell–cell adhesion has been demonstrated by showing that anti-cadherin antibodies disrupt preexisting junctions (Behrens et al., 1985) and prevent calcium-dependent junction assembly (Gumbiner and Simons, 1986), and that introduction of exogenous cadherin cDNA into cultured fibroblastic cells induces cell–cell adhesion (Mege et al., 1988; Nagafuchi and Takeichi, 1988). Thus, in the simplest model, cadherins would bind calcium through their extracellular domain and interact, directly or indirectly, through their cytoplasmic tail, with protein kinase(s). Removal of extracellular calcium from the Ca^{2+} binding site in the extracellular domain would transmit a conformational change to the intracellular domain, resulting in the activation of kinases. A link between cadherins and signal transduction has recently been suggested, when it was shown that the morphological response induced by N-cadherin in PC12 cells can be inhibited by pertussis toxin, and by a protein kinase inhibitor (Doherty et al., 1991). In addition, transfection of mouse sarcoma cells expressing the gap junction protein connexin43 with cadherin cDNA renders them communication competent and induces phosphorylation of connexin43 (Musil et al., 1990). The fact that cytoplasmic tails of cadherins are essential for adhesive function (Nagafuchi and Takeichi, 1988), are linked to other components of the submembrane cytoskeleton (Ozawa et al., 1989), and are phosphorylated (Cunningham et al., 1984; Parrish et al., 1990) would support the idea that this domain is involved in signal transduction.

Another interpretation of the present results is that phosphorylation of the cytoplasmic domain of cadherins may regulate cadherin conformation and function, so that in the absence of phosphorylation, the extracellular domain of cadherin may be "locked" into a stable conformation, which is not susceptible to the effects of calcium removal. A third possibility is that MDCK cells have a Ca^{2+}-independent adhesion system, which is regulated by phosphorylation, e.g., it is normally maintained in an inactive or low active state by phosphorylation, and is stimulated when kinases are inhibited. These models would predict that phosphorylation and dephosphorylation events would occur over short periods, to account for the rapid effects caused by calcium removal and for the fact that a 5-min exposure to H-7 is sufficient to inhibit junction dissociation. Experiments to test the role of cadherins in this system would include experimental manipulation of the phosphorylation and/or calcium binding site(s) of cadherins in transfected cells. Finally, another possibility is that extracellular Ca^{2+} acts on some lipid component of the plasma membrane, which in turn can control transmembrane or peripheral membrane protein components of the signal transduction machinery.

By demonstrating that junctions can remain intact even at low extracellular calcium concentrations, the present study implies that calcium does not simply act by "bridging" adhesion molecules and thus allow them to keep cells together, but can regulate a complex signal transduction pathway, involving protein kinase(s). This pathway can clearly be triggered in vitro in cultured epithelial cells, when the extracellular calcium concentration is $\geq 50\ \mu M$, and is likely to play a physiological role. Such low concentrations of calcium are not known to occur in extracellular fluids in vivo, however subtler variations in calcium concentrations may still be a significant modulatory signal. Alternatively, I speculate that under physiological conditions, this same pathway can be activated by some other factor(s), that could thus control the integrity of epithelial cell–cell interactions. This regulatory mechanism could be particularly important during development, where tissue morphogenesis may require dynamic assembly and disassembly of epithelial cell contacts, or in the generation of the invasive phenotype. Indeed, extracellular calcium and cadherins have been implicated in the control of cell differentiation (Hennings et al., 1980; Hyafil et al., 1981) and in tumor metastasis (Behrens et al., 1989; Birchmeier et al., 1991). Furthermore, the fat locus of *Drosophila*, which encodes a novel member of the cadherin superfamily, functions as a tumor suppressor gene (Mahoney et al., 1991). Other systems have been described where extracellular calcium plays important physiological roles, regardless of the possible involvement of cadherins. For example, in parathyroid and other cell types, changes in extracellular calcium modulate a variety of signaling systems, including phosphoinositide turnover, cAMP content, guanine nucleotide regulatory proteins and intracellular calcium (Brown, 1991; Rees et al., 1989). It has been suggested that extracellular calcium may act on a "receptor", but the existence, identity, and tissue distribution of such a receptor is unclear (Brown, 1991).

In conclusion, the experimental model of calcium removal may be a useful system to study a signaling pathway that can modulate several activities in epithelial cells, including the organization and function of junctions and the cytoskeleton. Further work on the role of protein kinases, cadherins, and other molecules in this pathway is likely to yield interesting results.

I would like to thank Ms. N. Denisenko for her excellent technical and photographic assistance, Dr. M. Gershengorn for stimulating discussions, Drs. F. Maxfield and W. Hendey for help with calcium measurements, Dr. D. Goodenough for gift of antibodies, and Dr. Enrique Rodriguez-Boulan for sharing the fluorescence microscope facility, gift of cells and encouragement.

This work was supported by the American Cancer Society (grant CD-

475), the Cornell Scholar Award, and the Andrew T. Mellon Teacher-Scientist Award.

Received 8 November 1991 and in revised form 30 December 1991.

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