Different effects of protein kinase inhibitors on the localization of junctional proteins at cell-cell contact sites

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SUMMARY

The protein kinase inhibitor H-7 prevents the assembly of tight junctions in cultured Madin Darby Canine Kidney (MDCK) epithelial cells (Balda et al. (1991) J. Membr. Biol. 122, 193-202; Nigam et al. (1991) Biochem. Biophys. Res. Commun. 181, 548-553); however, its mechanism of action is unknown. To understand the basis of the activity of H-7 and other inhibitors we compared the effect of H-7 on the localization of proteins belonging to tight junctions and adherens-type junctions (zonula adhaerens and desmosome), and on the organization of actin microfilaments. Junction assembly was induced in MDCK cells either by the ‘Ca\textsuperscript{2+} switch’ procedure or by incubating trypsinized cells at normal extracellular Ca\textsuperscript{2+}. The cells were then immunofluorescently labeled with antibodies against cingulin, ZO-1, E-cadherin and desmoplakin, and with FITC-phalloidin. Here we show by measuring the transepithelial resistance that, in addition to H-7, H-8 and staurosporine can also significantly block the assembly of tight junctions, whereas HA1004 is poorly active. H-7 inhibited the accumulation of cingulin and ZO-1 in junctional areas most effectively when added during assembly at normal extracellular Ca\textsuperscript{2+}. On the other hand, H-7 did not have major effects on the accumulation of E-cadherin and desmoplakin in the regions of cell-cell contact using either assembly protocol. Electron microscopy confirmed that H-7 does not abolish the formation of adherens-type junctions, suggesting that phosphorylation plays a different role in the assembly of tight junctions versus adherens-type junctions. Finally, in both protocols of junction assembly H-7 caused a major disorganization of actin microfilaments, suggesting that H-7 may prevent TJ assembly through its effect on the cytoskeleton.

Key words: junction, phosphorylation, epithelial cell

INTRODUCTION

Polarized epithelial cells are held together by a junctional complex, including a tight junction (TJ), a zonula adhaerens (ZA) and a desmosome (Farquhar and Palade, 1963; Staehelin, 1974). Each of these junctions displays a characteristic morphology, molecular composition and function. The TJ is the most apical element of the complex and forms a barrier to the passage of solutes along the paracellular pathway (reviewed by Gumbiner, 1987; Schneeberger and Lynch, 1992). Several protein components associated with the cytoplasmic faces of epithelial TJs have been characterized (reviewed by Citi, 1993). These include the ZO-1/220 kDa protein (Itoh et al., 1991, 1993; Stevenson et al., 1986), cingulin (Citi et al., 1988), ZO-2 (Gumbiner et al., 1991) and 7H6 (Zhong et al., 1993).

The ZA is located below the TJ and is associated with actin microfilaments (Geiger et al., 1983). The cell adhesion molecule uvomorulin/E-cadherin (Peyriras et al., 1983; Takeichi, 1991) is distributed along the lateral plasma-membrane of polarized epithelial cells (Gumbiner et al., 1988), and is thought to be the adhesion receptor concentrated at the ZA (Boller et al., 1985; Geiger and Ayalon, 1992). Desmosomes contribute to cell-cell adhesion and the mechanical integrity of tissues, and have been characterized in detail at the biochemical level (Schwarz et al., 1990). The desmosomal transmembrane adhesion proteins are members of the cadherin superfamily (Collins et al., 1991; Koch et al., 1990; Mechanic et al., 1991; Wheeler et al., 1991). The cytoplasmic ‘plaque’ domain of desmosomes links the membrane domain to intermediate filaments and contains several proteins, including desmoplakins (Franke et al., 1982).

To understand the biogenesis and functions of the junctional complex it is critical to clarify the cellular mechanisms that control the assembly of each junction. For example, is the formation of different junctions controlled by the same signals, and how does the assembly of one type of junction influence other junctions? A number of model systems and drugs have been used to dissect putative signaling pathways controlling junction assembly. Using protein kinase inhibitors and activators, it was shown that the activity of protein kinase C influences the assembly of cell-cell junctions in several cell types (Sheu et al., 1989; Winkel et al., 1990). We have shown that in MDCK cells protein kinase inhibitors prevent junction disassembly induced by removal of extracellular calcium (Citi, 1992), and recently we identified the zonula adhaerens-associated actin cytoskeleton as a major target of the inhibitor
H-7 in this experimental system (Citi et al., 1994). Cereijido’s
and our group have reported that in the ‘calcium switch’
model in MDCK cells H-7 prevents the assembly of TJ
defects (Balda et al., 1991; Nigam et al., 1991). However, the precise
mechanism of action of H-7 was unclear, and the activity of
other protein kinase inhibitors was not investigated. Since it
was previously shown that blocking the formation of E-
cadherin-based cell-cell contacts prevented the formation of
TJs (Behrens et al., 1985; Gumbiner and Simons, 1986;
Gumbiner et al., 1988), it is reasonable to suggest that the
effect of H-7 on TJ assembly was indirect, e.g. due to its action
on cadherin-based junctions. Secondly, since H-7 has been
shown to alter the organization of the actin cytoskeleton
(Birrell et al., 1989; Citi et al., 1994), it is important to study
the effect of H-7 on the spatial relationship of actin and TJ
proteins during junction assembly.

In this study we addressed these questions, by comparing the
effects of H-7 on the localization of proteins belonging to TJs
and adherens-type junctions at sites of cell-cell contact, and
examining the organization of actin microfilaments during
junction assembly. To induce assembly, cells were grown at
low extracellular Ca²⁺ concentration (<50 µM free Ca²⁺, LC)
for 20 hours and then switched to normal Ca²⁺ concentration
(1.8 mM, NC) for 2-6 hours (Ca²⁺ ‘switch’) (Cereijido et al.,
1978, 1981; Contreras et al., 1989; Gonzalez-Mariscal et al.,
1985, 1990). Alternatively, trypsinized cells were plated at
normal extracellular [Ca²⁺] and incubated for 16-20 hours. To
monitor the assembly of TJs, we examined the transepithelial
resistance (TER) of MDCK monolayers and we examined the
distributions of cingulin and ZO-1 by indirect immunofluores-
cence. Immunofluorescence was also used to assess the local-
ization of E-cadherin and desmoplakin at junctional sites, and
the ultrastructure of cell-cell contacts was examined by trans-
mission electron microscopy. Finally, FITC-phalloidin was
used to study the organization of actin microfilaments. The
results indicate that H-7 may prevent TJ assembly through its
effects on the organization of the actin cytoskeleton, rather that
by preventing the formation of cadherin-containing cell-cell
contacts.

MATERIALS AND METHODS

Reagents and antibodies
Cell culture reagents were purchased from Gibco Laboratories (Grand
Island, NY). Chemicals were from Sigma (St Louis, MO), unless
otherwise specified.

Polyclonal rabbit antisera against cingulin was obtained as
described previously (Citi et al., 1988), and used at 1:1000 for
immunofluorescence. Anti-ZO-1 rat monoclonal antibody (culture
supernatant R26.4C, a kind gift from Dr D. Goodenough, Harvard
University) was used undiluted. Mouse monoclonal antibodies against
desmoplakin (Boehringer, Indianapolis, IN) and uvomorulin/E-
cadherin (Sigma, St Louis, MO) were used following the directions
of the manufacturers. Fluorescently labeled secondary antibodies
were obtained from Jackson Immuno Research Laboratories (West
Grove, PA). FITC-phalloidin was obtained from Sigma and used at
1:10 dilution.

Protein kinase inhibitors
H-7, H-8 and HA1004 are isoquinolinesulfonamide derivatives and
are structurally similar, and H-7 and HA1004 are, respectively, the
most and least effective inhibitors of protein kinase C (Hidaka et al.,
1984). Staurosporine (Tamaoki et al., 1986) is the most potent
inhibitor of protein kinase C among the ones used here. As recom-
manded by the manufacturers, inhibitors were used at concentrations
of about 10⁻⁶ and 100⁻⁶ the lowest in vitro measured Kᵢₑ, and their
effects were reversible and not toxic at the concentrations used here.
Stock solutions of protein kinase inhibitors were prepared as
described previously (Citi, 1992), and diluted into culture medium to
obtain the desired final concentrations (30-300 µM for H-7; 12-120
µM for H-8 and HA1004; 10-100 nM for staurosporine).

Cell culture
MDCK cells were grown in DMEM supplemented with 5% fetal
bovine serum (FBS) (Hyclone Laboratories, Logan, UT). Cells
(passage 4-12) were plated onto glass coverslips, for immunofluores-
cence, or on polycarbonate filters (4.7 cm² Transwell, Costar Inc.,
Cambridge MA), for measurement of transepithelial resistance. Low
Ca²⁺ medium was prepared from powdered medium (S-MEM, Gibco)
and supplied with 1% dialyzed FBS (dialyzed against 3 changes of
phosphate buffered saline (PBS: 150 mM NaCl, 50 mM sodium
phosphate, pH 7.2) over 3 days.

Ca²⁺ switch
For the Ca²⁺ switch, confluent monolayers were trypsinized and
seeded onto glass coverslips or Transwell filters in normal medium.
Ninety minutes after plating, cells were rinsed 5 times with low Ca²⁺
medium (LC), and incubated further in LC (containing 1% dialyzed
FBS) for about 20 hours. The switch was initiated by removing the
LC medium, and replacing it with normal medium (Gonzalez-
Mariscal et al., 1985). When protein kinase inhibitors were used, con-
centrated stock solutions were added directly to the LC medium for
30-60 minutes before the switch, and to the medium with normal Ca²⁺
after the switch.

Assembly at normal extracellular Ca²⁺
Confluent monolayers were trypsinized and plated onto glass coverslips
or Transwell filters in normal medium, and processed for immuno-
forescence or electron microscopy 16-20 hours after plating. When
protein kinase inhibitors were used, concentrated stock solutions were
added directly to the normal medium at the time of plating.

Transepithelial resistance
Transepithelial resistance (TER) was measured with a Millicell ERS
System (Millipore Co., Bedford, MA). The electrical resistance of a
MDCK cell monolayer, minus the background resistance from a blank
filter, was typically around 300-400 Ω.cm². In Ca²⁺-switch experi-
ments, control filters included cultures maintained at NC or in LC
throughout the experiment.

Immunofluorescence microscopy
Immunofluorescence microscopy was carried out as described previ-
sely, by permeabilization and fixation in cold methanol (Citi, 1992).
For FITC-phalloidin staining, permeabilization was in 0.1% Triton X-
100 for 5 minutes, and fixation in 2% paraformaldehyde for 30
minutes. Note that the term ‘cytoplasmic’ labeling is used in this paper
to distinguish non-junction-associated from junction-associated
labeling.

Electron microscopy
MDCK cells grown on filters were fixed in 2% glutaraldehyde (in 0.15
M sodium cacodylate, pH 7.2, 4% sucrose) for 30 minutes, exten-
sively rinsed in the same buffer, postfixed in 1% osmium tetroxide in
0.15 M cacodylate buffer, pH 7.2, dehydrated in ethanol, and
embedded in glycideter (Serva Feinbiochemica, Germany). Sections
were cut on a LKB Ultrotome IV, and stained with uranyl acetate and
lead citrate. Specimens were observed in a Hitachi H-600 electron
microscope, operated at 100 kV.
RESULTS

The effect of different protein kinase inhibitors on the development of transepithelial resistance in MDCK monolayers

H-7 is a member of the isoquinolinesulfonamide group of protein kinase inhibitors, which includes molecules with different Ki against different types of kinases (Hidaka et al., 1984). To characterize further the specificity of the kinase(s) involved in TJ assembly, we have examined the effects of different concentrations of a battery of inhibitors on the development of transepithelial resistance (TER) in MDCK cells during the Ca^{2+} switch (Fig. 1). Cells maintained in low Ca^{2+} medium (LC) displayed a very low TER (Fig. 1A). When monolayers were switched to medium containing normal (1.8 mM) Ca^{2+} concentration (NC) TER increased rapidly, and reached a plateau level about 6 hours after the switch (Fig. 1B).

In the presence of 30-300 µM H-7 (Fig. 1C), 10-100 nM staurosporine (Fig. 1D), and 12-120 µM H-8 (Fig. 1E), the TER remained at very low levels (<20% of control cultures) 6 hours after the switch. The effect of HA1004 (Fig. 1F) was very weak even at the highest concentration, suggesting that protein kinase C may be involved in TJ assembly. The effect of the two most potent inhibitors, H-7 and staurosporine, was further tested by immunofluorescence (Figs 2-5).

The effect of H-7 on the localization of cingulin, ZO-1, E-cadherin and desmoplakin in MDCK cells in the Ca^{2+} switch

To test whether protein kinase inhibitors block the formation of TJs by preventing the formation of cadherin-containing cell-cell contacts, cells before and after the Ca^{2+} switch were immunofluorescently labeled with antibodies against cingulin, ZO-1, E-cadherin and desmoplakin.

In cells maintained in LC medium, labeling for both cingulin (Fig. 2A) and ZO-1 (Fig. 2D) appeared mostly cytoplasmic and diffuse. However, few junctional regions were intensely stained, suggesting that TJs can persist even in LC (see also Citi, 1992; Siliciano and Goodenough, 1988; Stevenson and Goodenough, 1984). The same regions did not display E-cadherin labeling in double-labeled cells (not shown), indicating that TJs are less Ca^{2+}-dependent than adherens-type junctions. In LC medium staining for E-cadherin was detected diffusely throughout the cell and was largely absent from the cell peripheries, except for a few faintly stained areas (Fig. 2G) (see also Gumbiner et al., 1988). Similarly, in LC desmoplakin was distributed in a diffuse and granular cytoplasmic pattern, with no or occasional weak labeling along cell peripheries (Fig. 2J) (see also Mattey and Garrod, 1986; Pasdar and Nelson, 1988).

When cells were stained 2 hours after the Ca^{2+} switch, they all appeared completely surrounded by continuous junctions, as shown by the distribution of cingulin (Fig. 2B), ZO-1 (Fig. 2E), E-cadherin (Fig. 2H) and desmoplakin (Fig. 2K). The localization of these molecules 2 hours after the switch was essentially identical to that observed in confluent monolayers, always maintained at NC (not shown).

When H-7 was added to the culture medium 30 minutes before the switch, cingulin (Fig. 2C) and ZO-1 (Fig. 2F) were distributed mostly as interrupted segments along the regions of cell-cell contact, differently from cells switched in the absence of H-7 (Fig. 2, compare B to C and E to F). On the other hand, E-cadherin (Fig. 2I) and desmoplakin (Fig. 2L) were distributed mostly as interrupted segments along the regions of cell-cell contact, cells before and after the Ca^{2+} switch. The effect of H-7 on the localization of cingulin, ZO-1, E-cadherin and desmoplakin. In cells maintained in LC medium, labeling for both cingulin and desmoplakin, but not cingulin and ZO-1, after the Ca^{2+} switch in the presence of H-7

To analyze further the effect of H-7 on the spatial relationship between cingulin, ZO-1, E-cadherin and desmoplakin, the codistribution of these proteins in MDCK cells was examined by double immunofluorescence (Fig. 3).

Cingulin and ZO-1 were exactly colocalized in cells switched from LC to NC in the presence of H-7 (Fig. 3, top panels). Cingulin and E-cadherin showed partial colocalization; however, several cell-cell contact regions lacked cingulin but displayed clear E-cadherin labeling (arrowheads in Fig. 3,
Similarly, desmoplakin and cingulin were both detected in junctions containing cingulin (arrows in Fig. 3, bottom panels); however, certain regions of cell-cell contact appeared to contain desmoplakin but not cingulin (arrowheads in Fig. 3, bottom panels).

**Different localizations of proteins from tight and adherens-type junctions in MDCK cells after the Ca²⁺ switch in the presence of staurosporine**

Since staurosporine can block the development of TER in the Ca²⁺ switch (Fig. 1D), we investigated its effects on the dis-

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**Fig. 2.** Distribution of junctional molecules in the Ca²⁺ switch and the effect of H-7. Immunofluorescence microscopy of MDCK cells after 20 hours in LC (A,D,G,J), 2 hours after the Ca²⁺ switch (B,E,H,K), and 2 hours after the Ca²⁺ switch in the presence of H-7 (C,F,I,L), after immunostaining with antibodies against cingulin (A,B,C), ZO-1 (D,E,F), E-cadherin (G,H,I), and desmoplakin (J,K,L). The Ca²⁺ switch induced the translocation of labeling for all junctional molecules from a 'cytoplasmic' pool to the sites of cell-cell contact. Note that H-7 (300 µM) prevents the localization of cingulin and ZO-1 along continuous TJs (C,F), whereas E-cadherin and desmoplakin appear localized along all sites of cell-cell contact (I,L), as in cultures where the switch was carried out in the absence of H-7 (H,K). Bar, 10 µm.
Staurosporine induced dramatic changes in cell shape, resulting in the appearance of several filopod-like extensions, and irregular cell borders and cell-cell contacts, as shown by phase-contrast analysis (Fig. 4B). Labeling for cingulin (Fig.

Fig. 3. Double immunofluorescence distribution of junctional proteins in the Ca^{2+} switch in the presence of H-7. Each group of two micrographs shows MDCK cells simultaneously labeled with antibodies against cingulin (C) and ZO-1 (Z) (top panels), cingulin (C) and E-cadherin (E) (middle panels), and cingulin (C) and desmoplakin (D) (bottom panels). Matching arrows indicate sites where cingulin appears to be codistributed with either ZO-1, E-cadherin or desmoplakin. Matching arrowheads indicate areas that are stained for E-cadherin or desmoplakin, but not for cingulin. Magnification as in Fig. 4.
	ntribution of cingulin and ZO-1 versus cadherin and desmoplakin in the Ca^{2+} switch.

Staurosporine induced dramatic changes in cell shape,
4A) and ZO-1 (not shown) was co-localized and considerably reduced. Cingulin staining was detectable only as dots and rare segments (Figs 4A, C and E), whereas labeling for E-cadherin was abundant, and distributed along the irregular cell peripheries (Fig. 4D). As a result, there was only partial colocalization of the two proteins (arrow in Fig. 4C and D).

Fig. 4. The effect of staurosporine on the localization of junctional proteins in the Ca^{2+} switch. (A) and (B) The same field of MDCK cells after immunofluorescent labeling with anti-cingulin (A) or by phase-contrast microscopy (B). The matching arrow in (A) and (B) indicates a cingulin-containing site of cell-cell contact. (C-F) Double immunofluorescence labeling of MDCK cells for cingulin and E-cadherin (C,D), and cingulin and desmoplakin (E,F). Matching arrows indicate sites where cingulin appears to be codistributed with either E-cadherin or desmoplakin. Matching arrowheads indicate areas that are stained for E-cadherin or desmoplakin, but not for cingulin. Note the dramatic morphological changes (best seen in B), and the reduction in cingulin labeling (A,C,E) induced by staurosporine. Bar, 5 \mu m.
Similar observations were made when cells were double immunolabeled for cingulin and desmoplakin (Fig. 4E and F). Desmoplakin labeling was abundant, and distributed along thin cellular extensions and cell borders, and diffusely in the cytoplasm (Fig. 4F). Cingulin appeared to be absent from these thin processes (arrowheads in Fig. 4E and F), and only in rare cases was it detected along cell-cell borders as focal patches (Fig. 4E, arrowheads).

Fig. 5. The effect of H-7 on junction assembly at normal extracellular Ca$^{2+}$. Double immunofluorescence labeling of MDCK cells for cingulin and ZO-1 (A, B), cingulin and E-cadherin (C, D), and cingulin and desmoplakin (E, F). Cells were plated and incubated in NC medium containing H-7 for 18 hours. Labeling for cingulin and ZO-1 is scant and patchy (A, B, C, E), whereas labeling for E-cadherin (D) and desmoplakin (F) along the junctions is distributed along apparently all regions of cell-cell contact. Arrows in A, C, E (matched arrows in B, D, F) show corresponding positions that are labeled both for cingulin and for ZO-1, E-cadherin or desmoplakin. Arrowheads in C, E (matching arrowheads in D, F) indicate areas that are labeled for E-cadherin and desmoplakin, but not for cingulin. Bar, 5 µm.
regions of cell-cell contact was it clearly colocalized with desmoplakin (arrow in Fig. 4E and F).

**Cell-cell contacts containing E-cadherin and desmoplakin, but not cingulin and ZO-1, after junction assembly at normal extracellular Ca\(^{2+}\) in the presence of H-7**

As an alternative method to induce junction assembly, confluent MDCK monolayers were trypsinized and cells were plated and grown for 16-20 hours in NC. In the absence of H-7, junction assembly is complete within this time, as shown by measurement of TER and immunolocalization of junctional proteins (not shown).

In the presence of H-7, the amount of cingulin and ZO-1 labeling was remarkably low, and distributed as a few dots and segments along the cell periphery (Fig. 5A and B). The decrease in immunofluorescent staining for TJ proteins appeared to correlate with the decrease in ultrastructurally defined TJs in the presence of H-7, as determined by electron microscopy (Fig. 6C and D). In contrast to the staining for cingulin and ZO-1, essentially all regions of cell-cell contact displayed E-cadherin (Fig. 5D) and desmoplakin (Fig. 5F). Thus, in double-labeled cells, several cell-cell contact areas displayed prominent E-cadherin and no cingulin (arrowheads in Fig. 5D and C), or clear desmoplakin and no cingulin (arrowheads in Fig. 5F and E). As observed in the Ca\(^{2+}\) switch, the few junctional areas displaying cingulin labeling were also stained for E-cadherin and desmoplakin (matching arrows in Fig. 5C-D and E-F).

**The effect of H-7 on the ultrastructure of cell-cell contacts**

To support the conclusion that H-7 does not significantly inhibit the assembly of cadherin-containing junctional structures, trypsinized cells were incubated at NC in the absence or in the presence of H-7, and examined by transmission electron microscopy (Fig. 6).

In the absence of H-7, TJ and desmosomes were clearly detected in the apical region of the cells (Fig. 6A), the former being characterized by the close apposition and occasional apparent ‘fusion’ of confronting plasma membranes (tj in Fig. 6A), the latter being characterized by a 25-30 nm wide fibril-containing intercellular space, and the accumulation of electron-dense filamentous material in the cytoplasmic plaque (d in Fig. 6A). The 

\[\text{zonula adhaerens} \] (za in Fig. 6A) was localized between the TJ and the desmosome, and was identified in control cells as a region of contact with a 15-20 nm wide intercellular space, and a faint accumulation of electron-dense material on the cytoplasmic face, presumably corresponding to actin filaments and associated proteins (Geiger et al., 1985).

When junction assembly occurred in the presence of H-7, at low magnification cells appeared polarized and formed extensive contacts along their lateral surfaces (not shown). Occasionally, junctional complexes including all three elements, TJ, ZA and desmosomes were detected (Fig. 6B). In most instances, however, the regions of cell-cell contact lacked TJs, whereas they displayed clear desmosomes (Fig. 6C and D). ZAs were also detected in the presence of H-7; however, they were less clearly identifiable than in control cells, probably as a consequence of the effect of H-7 on actin microfilaments (see below).

**The effect of H-7 on the distribution of actin during junction assembly in MDCK cells**

To study the effect of H-7 on the spatial relationship between TJ and the microfilament cytoskeleton, cells were double immunofluorescently labeled with anti-cingulin antibodies and FITC-phalloidin (Figs 7, 8). Phalloidin binds to polymeric, but not to monomeric, actin and can be therefore used to detect actin-containing microfilaments.

In cells maintained in NC, actin microfilaments were detected throughout the cell as stress fibers (arrow in Fig. 7B), and were also concentrated along the free borders of the cells (open arrow in Fig. 7B), in areas that typically lacked cingulin labeling. After the Ca\(^{2+}\) switch, cytoplasmic microfilaments (arrow in Fig. 7D) and homogeneous junctional labeling, which was apparently codistributed with cingulin, could be observed (Fig. 7C and D). In cells switched in the presence of H-7, junctional actin labeling was mostly colocalized with cingulin, although some peripheral areas appeared to contain actin but not cingulin (arrowheads in Fig. 7F and E). Interestingly, actin labeling in the cytoplasm was diffuse, and not organized in stress fibers (Fig. 7F).

The effect of H-7 on the spatial relationship between actin and cingulin was studied also during junction assembly at NC. Phase-contrast analysis showed that in the presence of H-7 cells there were gaps in the monolayer, although most cells were in contact with others (Fig. 8A and B). Cingulin appeared to be absent from most regions of cell-cell contact, except for occasional segments and dots (Fig. 8C and D). Conversely, actin was abundant and distributed along apparently all sites of cell-cell contact, thus most cell borders displayed actin but no cingulin (small arrows in Fig. 8, compare C with E and D with F). It is noteworthy that all actin labeling appeared diffuse, and no cytoplasmic stress fibers could be detected (Fig. 8E and F).

**DISCUSSION**

The primary goal of this study was to understand the basis for the effect of H-7 on TJ assembly in MDCK cells, by comparing its effect on the assembly of TJs versus the organization of cadherin-based cell–cell contacts and actin microfilaments.

Kinase inhibitors, which have been reported to be most active against protein kinase C (H-7, staurosporine and H-8), strongly prevented the assembly of TJs, since the transepithelial resistance in the Ca\(^{2+}\) switch assay was much below control at both concentrations used (Fig. 1). H-7 and staurosporine also reduced dramatically the accumulation of cingulin and ZO-1 labeling in the regions of cell-cell contact or the cell periphery (Figs 2, 3 and 4). Although the effects of staurosporine and H-7 on the morphology of MDCK cells were quite different (compare Figs 3 and 4), taken together these results suggest that protein kinase C plays a key role in the assembly of TJ proteins at cell-cell contact sites.

In contrast to what was observed for TJ proteins, H-7 did not appear to significantly block the junctional accumulation of E-cadherin and desmoplakin, which are normally concentrated in the ZA and in desmosomes, respectively. This conclusion is based on the immunofluorescent labeling of cells after junction assembly at normal [Ca\(^{2+}\)] (Fig. 5) or by Ca\(^{2+}\) switch (Figs 2 and 3). Significantly, labeling for E-cadherin and desmoplakin was observed even in those junctional areas
that were devoid of ZO-1 and cingulin (Fig. 3), underlining the distinct behavior of TJ versus adherens-type junction proteins. Ultrastructural analysis confirmed that desmosomes and ZAs were present in cells incubated in the presence of H-7, whereas TJs were overall greatly reduced (Fig. 6C and D).

One conclusion suggested by the above observations is that
**Fig. 7.** The relationship between cingulin and actin in the Ca\(^{2+}\) switch and the effect of H-7. Double immunofluorescence analysis of MDCK cells with anti-cingulin antibodies (A,C,E) and FITC-phalloidin (B,D,F) in LC (A and B), after the Ca\(^{2+}\) switch in the absence of H-7 (C and D), and after the Ca\(^{2+}\) switch in the presence of H-7 (E and F). The open arrow in B indicates an intensely labeled bundle of actin filaments, near the cell edge. Arrows in B, D indicate cytoplasmic actin stress fibers. Matching arrowheads in E and F indicate sites at the cell margins that are labeled by phalloidin (F), but not by anti-cingulin (E) antibodies. Note the diffuse cytoplasmic actin labeling in the presence of H-7 (F). Bar, 5 \(\mu\)m.

**Fig. 8.** The effect of H-7 on actin and cingulin distribution after assembly at normal extracellular Ca\(^{2+}\). Each of two sets of micrographs (A, C, E and B, D, F) shows a phase-contrast analysis (A,B) and double immunofluorescence analysis of MDCK cells stained for cingulin (C,D) and actin (E,F). Cells were trypsinized and incubated for 20 hours in the presence of 300 \(\mu\)M H-7. Note the discontinuities in the monolayer (open arrowhead in B, and corresponding open arrowheads in D, F), the scant cingulin labeling (C,D), and the abundant cytoplasmic and junctional labeling for actin (E,F). Large arrows in C and D (corresponding arrows in E and F) point to areas where cingulin and actin labeling are colocalized. Small arrows in C and D (corresponding arrows in E and F, respectively) point to junctional contacts stained for actin but not for cingulin. Bar, 5 \(\mu\)m.
Junction assembly with protein kinase inhibitors
the inhibition of TJ assembly by H-7 occurs by a mechanism different from that observed when extracellular [Ca\textsuperscript{2+}] is low, or when anti-cadherin antibodies are present in the medium before assembly (Gumbiner et al., 1988). Under those experimental conditions, the accumulation of E-cadherin at cell-cell contact sites and its physical interaction with E-cadherins on adjacent cells is inhibited. Conversely, the accumulation of E-cadherin and desmoplakin on neighboring surfaces at sites of cell-cell contact is believed to imply their functional involvement in adhesion (Gumbiner et al., 1988). We have shown that H-7 neither abolishes the accumulation of E-cadherin and desmoplakin at junctional sites (Figs 2, 3 and 5), nor influences the trypsin-sensitivity (e.g. ‘active’ conformation) of N-cadherin (Citi et al., 1994). Thus, our results suggest that: (a) the accumulation of E-cadherin at the sites of cell-cell contact may be necessary (Gumbiner et al., 1988), but is not sufficient by itself to induce the formation of TJs; and (b) blocking the formation of TJs does not prevent the accumulation of E-cadherin and desmoplakin in the regions of cell-cell contact. In summary, in a hypothetical hierarchy of junction assembly, the accumulation of these two proteins appears not to require the prior accumulation of cingulin and ZO-1 at cell-cell contact sites. The higher susceptibility of TJ proteins to the effects of H-7 suggests that specific kinases are selectively involved in the signaling pathway that controls TJ, but not adherens-type junctions assembly.

An observation that may have some bearing on the mechanism of action of protein kinase inhibitors was that H-7 caused a more marked decrease in the accumulation of cingulin and ZO-1 labeling at junctional contacts when junction assembly was carried out at NC, rather than by the Ca\textsuperscript{2+} switch (compare Fig. 2 to Figs 5 and 8). Previous studies indicate that assembly by Ca\textsuperscript{2+} switch is rapid because junctional components have been synthesized during the incubation at LC. Conversely, assembly at NC requires longer (16-20 hours), because new synthesis of junctional proteins must occur (Gonzalez-Mariscal et al., 1985, 1990). Thus, it is conceivable that the greater effect of H-7 during assembly at NC may be due to its effect on kinases that are active during the early steps of junction biogenesis, for example the synthesis, post-translational processing, transport of junctional proteins, and/or their stabilization by interactions with other molecules or the cytoskeleton (see below). This idea is supported by preliminary experiments indicating that H-7 decreases net cingulin biosynthesis during junctional assembly (Denisenko and Citi, unpublished observations). Therefore we envisage that cingulin labeling after assembly at NC the presence of H-7 (Fig. 5) is due to protein remaining in junctions that have been incompletely dissociated by trypsinization, rather than to newly synthesized protein. Conversely, the greater amount of cingulin and ZO-1 labeling after junction formation by Ca\textsuperscript{2+} switch in the presence of H-7 can be explained, assuming that protein synthesis occurs during incubation in LC (Gonzalez-Mariscal et al., 1985, 1990), and the assembly of this new pool of protein is not (or less) affected by H-7.

The observation that H-7 disrupts the organization of actin microfilaments both in the Ca\textsuperscript{2+} switch and during assembly at NC suggests that H-7 may block TJ assembly by interfering with the organization and function of the actin cytoskeleton. A similar mechanism of action was proposed recently to explain the ability of H-7 to prevent junction dissociation induced by removal of extracellular Ca\textsuperscript{2+} (Citi et al., 1994). Actin microfilaments may play a direct role in the structure and function of mature TJs, since F-actin has been detected in the cytoplasmic domain of TJs (Drenckhahn and Dermietzel, 1988; Madara, 1987). Secondly, experiments with microfilament-active drugs suggest that ZA-associated microfilaments may indirectly regulate TJ organization and function (Meza et al., 1980; Madara et al., 1986, 1987, 1988; Citi et al., 1994). It was also proposed that the formation of the apical domain in MDCK cells is the result of exocytotic fusion of actin-associated vacuoles, and that the cytoskeleton might drive the fusion between vacuoles and cell surface (Vega-Salas et al., 1988). Thus, one mechanism by which H-7 could prevent TJ assembly through its effect on actin could be by preventing the biogenesis, redistribution and transport of actin-associated membrane vesicles, which carry TJ proteins to the apical surface. It is interesting that although ZAs are linked to actin, H-7 did not appear to affect the junctional localization of a membrane protein component (E-cadherin) of the ZA. Desmosome assembly appears independent of the effects of H-7 on the actin cytoskeleton, as shown here, and of microtubules (Pasdar et al., 1992). It is likely that further studies with protein kinase inhibitors will help to clarify the role of phosphorylation in the complex relationships between membrane traffic, junction organization and the cytoskeleton.

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