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Cytoskeletal involvement in the modulation of cell-cell junctions by the protein kinase inhibitor H-7

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SUMMARY

The protein kinase inhibitor H-7 has been shown to block junction dissociation induced by low extracellular calcium in Madin Darby canine kidney epithelial cells (S. Citi, *J. Cell Biol.* (1992) 117, 169-178). To understand the basis of this effect, we have examined how H-7 affects the organization of junctions and the actin cytoskeleton in different types of epithelial cells in culture. Immunofluorescence microscopy showed that H-7 confers Ca^{2+} independence on cultured epithelial lens cells, which lack tight junctions and desmosomes but have microfilament-associated adherens junctions. In these cells, H-7 did not protect N-cadherin from trypsin digestion at low extracellular calcium, suggesting that H-7 does not stabilize the 'active' cadherin conformation. In cultured Madin Darby canine kidney cells, H-7 partially prevented the fall in transepithelial resistance induced by cytochalasin D, either alone or in conjunction with calcium chelators. Double-immunofluorescence microscopy showed that H-7 inhibits both the fragmentation of labeling for the tight junction protein cingulin and the condensation of actin into cytoplasmic foci induced by cytochalasin D. Taken together, these observations indicate that H-7 inhibits junction dissociation by affecting the contractility of the adherens junction-associated microfilaments following treatment with calcium chelators or cytochalasin D.

Key words: H-7, cytoskeleton, junction, calcium

INTRODUCTION

Interaction between epithelial cells occurs primarily via a subapical junctional complex consisting of several structurally and functionally distinct adhesion sites (Farquhar and Palade, 1963; Staehelin, 1974). The most apical of these is the tight junction (TJ), which consists of tightly attached membranes that form a complex network of branching strands and complementary grooves (Goodenough and Revel, 1970). The TJ efficiently seals epithelial sheets and is responsible for the permeability barrier function of epithelia as well as the maintenance of apical versus basolateral polarity (Gumbiner, 1987). At the molecular level several proteins have been assigned to the cytoplasmic aspect of the TJ, namely ZO-1 (Stevenson et al., 1986), cingulin (Citi et al., 1988), ZO-2 (Gumbiner et al., 1991) and 7H6 (Zhong et al., 1993; see also Citi, 1993).

The junctional structure neighboring the TJ is the microfilament-associated *zonula adhaerens*, which is the epithelial form of adherens junction (AJ) (Geiger et al., 1981; Geiger, 1983). Recent studies indicate that the AJ contain cadherins as their major 'contact receptors' (Behrens et al., 1985; Boller et al., 1985; Vestweber et al., 1985; Geiger and Ayalon, 1992). The interaction between the microfilament system and the membrane at the AJ is mediated through an electron-dense plaque structure, which consists of a variety of proteins including vinculin (Geiger et al., 1980), plakoglobin (Cowin et al., 1986) and catenins (Ozawa et al., 1989). Another class of cytoskeleton-associated epithelial junctions is the desmosomes, which are scattered throughout the basolateral membranes (Farquhar and Palade, 1963; Staehelin, 1974; Schwarz et al., 1990). Desmosome membranes contain desmogleins and desmocollins, which belong to the cadherin super-family (Koch et al., 1990; Collins et al., 1991; Mechanic et al., 1991; Wheeler et al., 1991) and are associated with intermediate filaments, mostly cytokeratins (Franke et al., 1981b; Geiger et al., 1983), through a submembrane plaque that contains several proteins, including desmoplakins and plakoglobin (Franke et al., 1981a; Geiger et al., 1983; Mueller and Franke, 1983; Cowin et al., 1986).

Several studies have demonstrated that the junctional complex of epithelia is highly calcium-dependent and that depletion of extracellular calcium ions leads to a rapid destruction of the entire junctional complex (Kartenbeck et al., 1982; Mattey and Garrod, 1986; Volberg et al., 1986; Green et al., 1987; Siliciano and Goodenough, 1988; Kartenbeck et al., 1991) and to endocytosis of some of its components (Kartenbeck et al., 1982, 1991). The requirement for calcium is usually attributed to the Ca^{2+} dependence of the interaction mediated by the cadherin superfamily proteins (Hyafil et al., 1981; Takeichi, 1988, 1990; Geiger and Ayalon, 1992). This is es-

pecially relevant for the AJ, which is associated with the contractile microfilament system (Bretscher and Weber, 1978; Geiger et al., 1981; Owaribe et al., 1981; Hirokawa et al., 1983; Drenckhahn and Dermietzel, 1988). However, the mechanism responsible for the Ca^{2+}-mediated potentiation of cadherin activity is still not entirely clear. On the one hand, putative Ca^{2+}-binding sites were identified on the extracellular domain of cadherins, suggesting that the presence of calcium is needed for the homophilic binding (Ozawa et al., 1990). On the other hand, it was shown that pretreatment of MDCK epithelial cells with the protein kinase inhibitor H-7 stabilizes the junctional complex and renders it Ca^{2+}-independent (Citi, 1992). The identity of the direct and indirect cellular targets of H-7, namely the affected kinases and their substrates, is still unclear.

In the present study we address this issue, showing that AJs are the primary junctional element affected by H-7 treatment and that the main subcellular target is the microfilament system.

MATERIALS AND METHODS

Antibodies

Rabbit anti-cingulin antiserum was prepared against purified chicken cingulin (Citi et al., 1988). Previous studies have described the preparation of the antibodies against vinculin (Geiger, 1979), desmoplakin (Franke et al., 1981a), and N-cadherin (Volk and Geiger, 1984). FITC-phalloidin was purchased from Sigma (St Louis, MO). Immunoblotting was carried out as described (Volk et al., 1990).

Cell culture

Chicken embryonic lens cells were cultured and treated with trypsin-EGTA as previously described (Volk and Geiger, 1986; Volk et al., 1990).

Madin-Darby canine kidney (MDCK) cells were cultured on glass coverlips for immunofluorescence analysis, and on Transwell filters (Costar, Cambridge, MA) for measurement of transepithelial resistance (Citi, 1992). Calcium removal, treatment with H-7 and HA1004, and measurement of transepithelial resistance were as described (Citi, 1992). H-7 was routinely added to obtain a final concentration of 300 μM. However, experiments performed with lower concentrations of H-7 (30-100 μM) gave nearly identical results (see also Citi, 1992). Cytochalasin D (Sigma, St Louis, MO) was added from a concentrated (10 mM) stock solution to the culture medium, in order to obtain a final concentration of 10 μM.

Other cell lines were obtained from the American Tissue Culture Collection and were cultured in DMEM containing 10% fetal calf serum (FCS). Treatment with EGTA and H-7 was carried out as described for MDCK cells.

Immunofluorescence microscopy

Immunofluorescence was carried out as described previously (Citi, 1992), except that permeabilization and fixation were done with 0.5% Triton X-100 mixed with 2-3% paraformaldehyde. Double labeling for cingulin and actin was carried out using rabbit anti-cingulin and TRITC-labelled anti-rabbit antibodies (Jackson Laboratories, West Grove, PA) in conjunction with FITC-labelled phalloidin.

Specimens were observed in 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).

Electron microscopy

MDCK cells were fixed in 2% glutaraldehyde and postfixed in 1% osmium tetroxide. After extensive rinses, cells were flat-embedded in Polybed 812, (Polysciences, Warrington, PA), and ultrathin sections were cut in a Reichert-Jung ultramicrotome. Sections were stained with lead nitrate and uranyl acetate and examined in a CM12 transmission electron microscope (Phillips, Eindhoven, Holland), operated at 80 kV.

RESULTS

Effect of the protein kinase inhibitor H-7 on the Ca^{2+} dependence of cell-cell junctions

In MDCK epithelial cells the junctional complex consists of tight junctions (TJ), adherens junctions (AJ) and desmosomes, which can be immunolocalized using antibodies to their constituents including cingulin (Fig. 1a), vinculin (Fig. 1b) or desmoplakin (Fig. 1c), respectively. Upon depletion of Ca^{2+} all three junctions underwent coordinated deterioration accompanied by massive cell retraction (Fig. 1d, e and f, respectively). Pretreatment of the cells for 30 minutes with 300 μM H-7 had no apparent effect on the gross morphology of the cells (not shown), yet it rendered all three junctions Ca^{2+}-independent, as shown in Fig. 1 (g, h and i). Electron microscopic examination of these cells confirmed that H-7 does not affect the morphology of cell-cell junctions (Fig. 2c), whereas it prevents the EGTA-induced cell contraction and separation (Fig. 2d).

To establish the generality of the H-7 effect and to identify the specific junction that becomes Ca^{2+}-independent after treatment with this drug, we have exposed a battery of cultured cells, including primary human endothelial cells, human breast adenocarcinoma cells (MCF 7), Madin Darby bovine kidney cells (MDBK) and chicken lens cells, to EGTA or BAPTA with or without pretreatment with H-7, and examined their organization. All these cells responded in a manner very similar to that of MDCK cells. It is noteworthy that cultured embryonic chicken lens cells, which contain neither TJ nor desmosomes but have a prominent AJ, became Ca^{2+}-independent following short incubation with H-7. This effect is depicted in Fig. 3, showing that the organization of the AJ molecule N-cadherin, which normally deteriorates after a short incubation with EGTA (Fig. 3b, compare to 3a), is protected by pretreatment with 300 μM H-7 (Fig. 3c).

Previous studies have shown that cadherins, the adhesion molecules present in AJ, are trypsin resistant in the presence of Ca^{2+} and become highly sensitive to this protease upon Ca^{2+} depletion, suggesting that the 'active conformation' of cadherins (in normal Ca^{2+}) can be distinguished from the inactive state (in low Ca^{2+}) by the proteolytic assay (Takeichi et al., 1981; Volk et al., 1990). To determine whether H-7 affects cadherins by inducing transition to the active conformation even in low Ca^{2+} concentrations we have examined the effect of H-7 and EGTA, either alone or in conjunction, on the trypsin sensitivity of cadherins. We examined this effect on cultured lens cells, in which cadherin proteolysis was studied in the past (Volk et al., 1990). Cultured chick lens cells, which apparently contain only N-cadherin (Volk and Geiger, 1984; Volk and Geiger, 1986), were incubated with 300 μM H-7, and then treated with trypsin, either alone or in conjunction with EGTA. The results, shown in Fig. 4, indicate that in spite of the apparent stability of the junctions of H-7-treated cells to EGTA, N-cadherin in such cells was fully sensitive to trypsin. This finding suggests that H-7 does not induce conformational

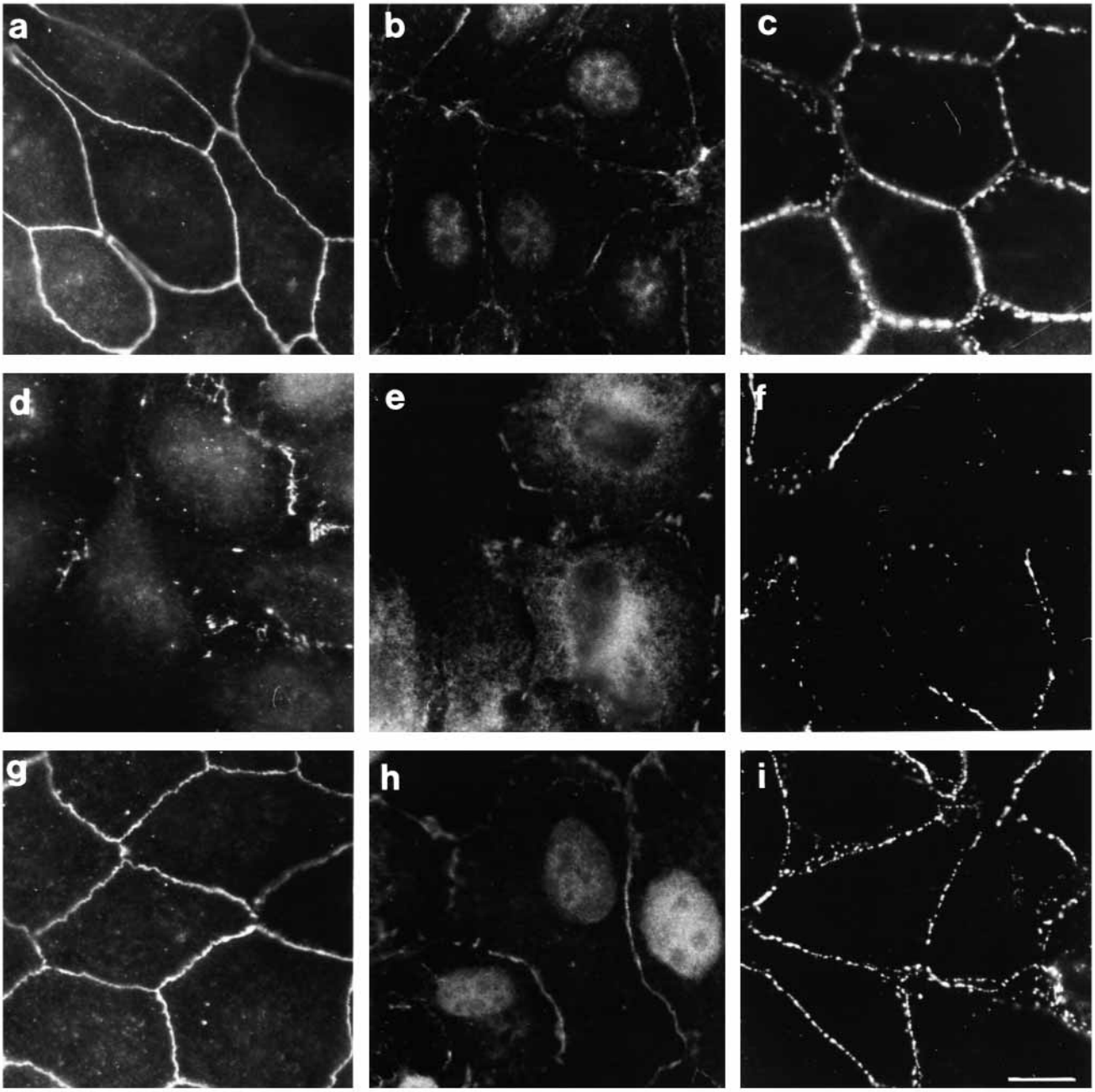

Fig. 1. Effect of H-7 on the Ca^{2+} dependence of different cell-cell junctions in MDCK cells including cingulin-containing tight junctions (a,d,g), vinculin-containing adherens junctions (b,e,h) and desmoplakin-containing desmosomes (c,f,i). The cells were either untreated (a,b,c), incubated with 4 mM EGTA for 30 minutes (d,e,f) or pretreated with 300 μM H-7 for 30 minutes and then incubated for additional 30 minutes with EGTA (g,h,i). The retraction in the cell margins induced by EGTA is best seen in (e). Note that all the elements of the subapical junctional complex are affected by EGTA and are all equally stabilized by H-7. Bar in (i), 10 μm.

changes in the cadherin molecule similar to those induced by Ca^{2+} and that the protective effect of H-7 on junctions does not depend on stabilizing the active conformation of the molecule.

Cytoskeletal involvement in the stabilization of cell junctions by H-7

An alternative mechanism that could account for the stability of the AJ following H-7 treatment is the loss of contractility of junction-associated microfilaments, rather than a direct effect on the binding properties of the cadherin molecules. To explore this possibility, we have examined the transepithelial resistance (TER) and structural integrity of junctions in MDCK cells treated with the microfilament disrupting drug cytochalasin D.

Incubation of cells with cytochalasin D, for even a short period of time, resulted in a major drop in TER to 20-25% of the initial value (see also Meza et al., 1980, 1982; Madara et al., 1986), in contrast with H-7 treatment, which had no apparent effect on TER (Citi, 1992). Pretreatment of cells with H-7 significantly reduced both the rate and extent of decline in TER, though it did not fully protect TJs from cytochalasin D

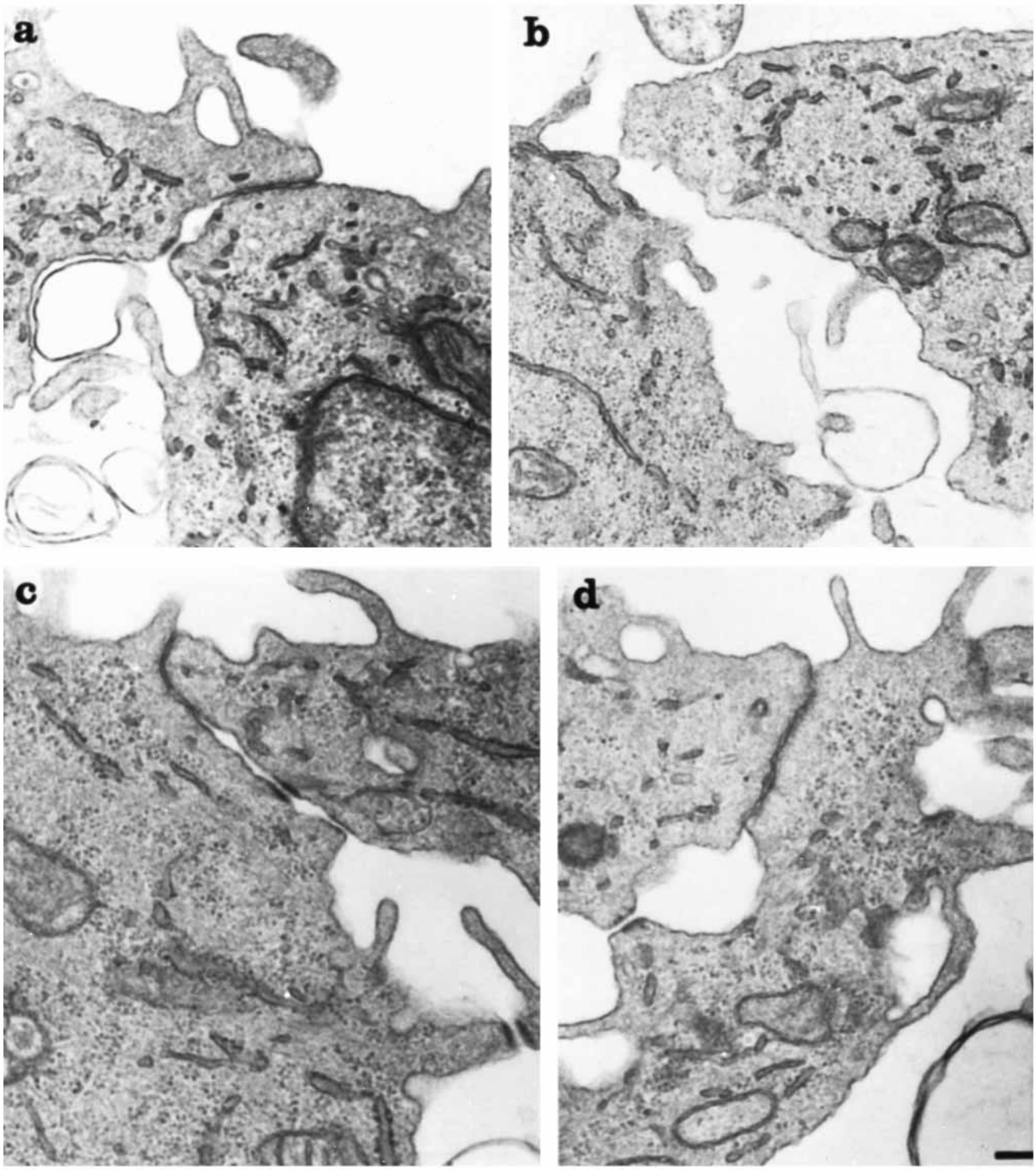

Fig. 2. Electron micrographs showing the subapical junctions in MDCK cells following various treatments: (a) untreated cells; (b) cells incubated for 10 minutes with EGTA; (c) cells incubated for 30 minutes with H-7; (d) cells preincubated (30 minutes) with H-7 and then treated for 10 minutes with EGTA. Note the complete splitting of all junctions following EGTA treatment and the stabilization of the junctions following H-7 treatment. It is noteworthy that the membrane-to-membrane distances in AJ and desmosomes in H-7-treated as well as H-7/EGTA-treated cells are indistinguishable from those found in untreated cells. Bar in (d), 0.2 μm.

(Fig. 5A). Calcium removal (by EGTA or BAPTA) alone caused a rapid and complete loss of TER, and simultaneous treatment with cytochalasin D had no apparent effect on this process (Fig. 5B). Pretreatment of cells with H-7 effectively blocked the BAPTA-induced reduction of TER, as previously reported (Citi, 1992), and partially inhibited the combined effects of cytochalasin D and BAPTA (Fig. 5B).

To assess the possible structural basis for the different

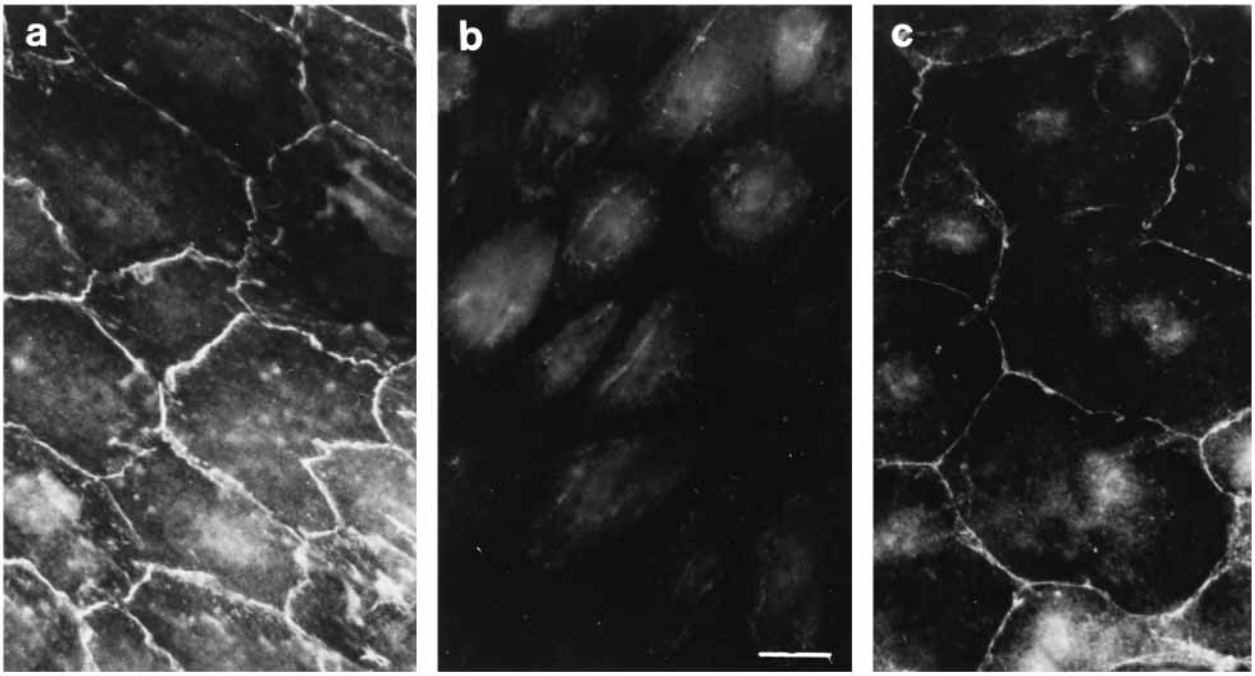

Fig. 3. Effect of H-7 on the Ca^{2+} dependence of AJ in cultured chicken lens cells. Cells were either untreated (a), treated for 30 minutes with EGTA (b) or preincubated for 30 minutes with H-7 and then treated for an additional 30 minutes with EGTA (c). Following treatment cells were fixed and stained for N-cadherin. Bar in (b), 10 μm.

effects of H-7 and cytochalasin D on junction integrity, MDCK cells were exposed to BAPTA, to cytochalasin D or to both and the distributions of cingulin and actin were determined by fluorescence microscopy. As shown in Fig. 6(a and b), a 30 minute treatment with BAPTA resulted in cell-cell dissociation, accompanied by deterioration of the junctional belt of actin and apparent internalization of cingulin-containing structures (see also Fig. 1). Brief (1 minute) treatment with cytochalasin D had a major destructive effect on the microfilament system, which became fragmented into numerous foci (Fig. 6d) and a limited, yet significant effect on cingulin organization, manifested by segmental interruptions along the cell periphery (Fig. 6c). It is noteworthy that the loss of cingulin was most prominent in areas from which actin labeling was absent. Short pretreatment with cytochalasin D followed by addition of BAPTA produced an effect that was very similar to that of cytochalasin alone, mainly limited interruption of cingulin (Fig. 6e) and disruption of actin (Fig. 6f), but no gross contraction and cell separation, comparable to that obtained with BAPTA alone.

Longer (2 hour) exposure to cytochalasin D resulted in a major disruption of junctional cingulin (Fig. 7a), which became apparently discontinuous, nevertheless gross cell separation was not typically noticed. Actin was mostly associated with foci and aggregates distributed throughout the cytoplasm (Fig. 7b and high magnification inset in 7b′). Pretreatment with the protein kinase inhibitor HA1004, which had no effect on cell-cell junctions (Citi, 1992), had also no apparent effect on cytochalasin-induced changes in cingulin or actin distribution (Fig. 7c and d, respectively). On the other hand, pretreatment with H-7 prevented the fragmentation of cingulin and the appearance of cytoplasmic labeling (Fig. 7e). In addition, this treatment largely prevented the formation of cytoplasmic aggregates of actin (Fig. 7f). Instead, actin was organized as short fibers forming parallel or converging arrays, as is best shown at high magnification (Fig. 7f′). It thus appears that H-7 prevents at least in part the effects of cytochalasin D both on

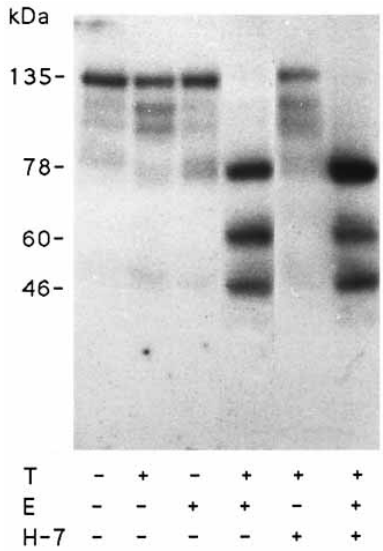

Fig. 4. Effects of Ca^{2+} and H-7 on trypsin sensitivity of N-cadherin. Chick lens cells were exposed to trypsin (0.1 mg/ml) with or without EGTA, with or without pretreatment with H-7. The cells were then washed, lysed in sample buffer and the breakdown of N-cadherin was examined by immunoblotting using anti-N-cadherin (A-CAM) antibodies (Volk et al., 1990). The typical cleavage products of N-cadherin in the presence of both EGTA (E) and trypsin (T) are 78, 60 and 46 kDa peptides, as previously described (Volk et al., 1990). Notice that in the absence of EGTA N-cadherin is largely trypsin resistant. Pretreatment of the lens cells with H-7 did not affect this pattern of trypsin sensitivity of N-cadherin.

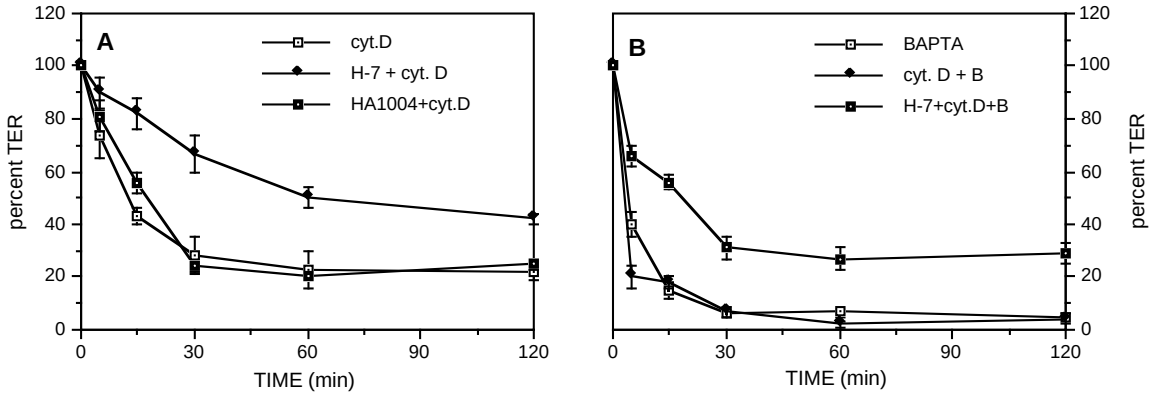

Fig. 5. Effects of H-7 and cytochalasin on the Ca^{2+} dependence of TJs in MDCK cells. The graphs show the transepithelial resistance of MDCK monolayers cultured on Transwell filters as a function of time. The TER value at time 0 was taken as 100%, and values were expressed as percentages. (A) Cells were treated for 2 hours with cytochalasin (⊡), or were preincubated for 30 minutes with H-7 (◆) or HA1004 (■), followed by treatment with cytochalasin for 2 hours. (B) Cells were treated for 2 hours with BAPTA (⊡) or with cytochalasin and BAPTA together (◆), or were preincubated for 30 minutes with H-7, followed by treatment with cytochalasin and BAPTA for 2 hours (■). Error bars indicate the standard error, based on three separate experiments.

TJ integrity and on the organization of the microfilament system.

DISCUSSION

The major objective of the present study was the identification of the specific subcellular targets for the protein kinase inhibitor H-7, which confers Ca^{2+} independence on epithelial junctions (Citi, 1992). The present results confirm and extend our previous study and suggest that the specific junctional element affected by H-7 is the microfilament-associated AJ.

The results presented here bear on the structural and functional hierarchy of cell junctions, namely that modulation of a junctional element that is intrinsically Ca^{2+}-dependent, like the AJ, can affect in a major way the entire organization of the junctional complex, including that of the TJ, which is, intrinsically, less dependent upon extracellular Ca^{2+}. In fact, TJ ribbons are resistant to EGTA treatment (Stevenson and Goodenough, 1984), and strong junctional immunolabeling for cingulin and ZO-1 can be observed in cells incubated in the absence of extracellular calcium (Siliciano and Goodenough, 1988; Citi, 1992; and Denisenko and Citi, unpublished observations). The view that the Ca^{2+} dependence of AJs plays a primary role in the overall stability of the entire epithelial junctional complex is supported by the effect of H-7 on lens cells, which contain prominent AJs but no desmosomes or TJs, by the observation that cadherin conformation and activity is Ca^{2+}-dependent (Takeichi, 1988, 1990; Geiger and Ayalon, 1992), and that AJs are tightly associated with contractile filaments (Bretscher and Weber, 1978; Owaribe et al., 1981; Geiger et al., 1981, 1983; Hirokawa et al., 1983; Drenckhahn and Dermietzel, 1988). Electron microscopy and functional studies have also suggested that contraction of the actomyosin belt present at AJs can be responsible for modulating or opening TJs (Madara, 1987; Madara et al., 1987, 1988; Meza et al., 1982). The key role of cadherins in the maintenance of the junctional complex, including TJs, has also been shown by experiments in which antibodies, rather than calcium chelators, were used to interfere with cadherin activity (Behrens et al., 1985; Gumbiner and Simons, 1986; Gumbiner et al., 1988).

A molecular description of the Ca^{2+} dependence of AJs may be that depletion of Ca^{2+} from the medium leads to a reduction in the homophilic affinity between cadherins and thus decreases the binding forces between adjacent cells. Under these conditions the contractile forces of actin filaments on the junctions are sufficient to pull the membranes centripetally and separate the cells from each other. The stabilizing effect of H-7 could, conceptually, stem from either an increased affinity of cadherins for each other, even in the absence of Ca^{2+}, or from a reduction in the pulling forces generated by the contractile system. The results presented here support the latter view; namely, that the main effect of H-7 is on the contractile system rather than on the junctional receptors. This conclusion is based on the observation that H-7 could not render cadherins trypsin-resistant, and it protected the microfilament-associated AJ from dissociation in the presence of EGTA. The morphological evidence presented here is supported by direct measurements of the ATP-induced contractions of detergent-permeabilized model systems, indicating that H-7 reduces the overall contractility of the actomyosin cytoskeleton (T. Volberg, B. Geiger, S. Citi and A. D. Bershadsky, unpublished results).

To substantiate the conclusion that disruption of the microfilament system can confer Ca^{2+} independence we have exposed cells to cytochalasin D, which caps and disrupts actin filaments. The present results indicate that the effect of cytochalasin D treatment is different from that of H-7, reflecting the different modes of action of the two drugs. Unlike H-7, cytochalasin D induces contractility and drastically changes the organization of actin bundles, leading to the formation of numerous F-actin foci throughout the cytoplasm. In MDCK cells this effect resulted in the apparent fragmentation of cell-cell junctions, which are normally stabilized by the underlying microfilaments, leading to a marked decrease in TER (Fig. 5). Pretreatment with H-7 partially inhibited the effect of cytochalasin D on TER (Fig. 5), confirming that splitting of cell

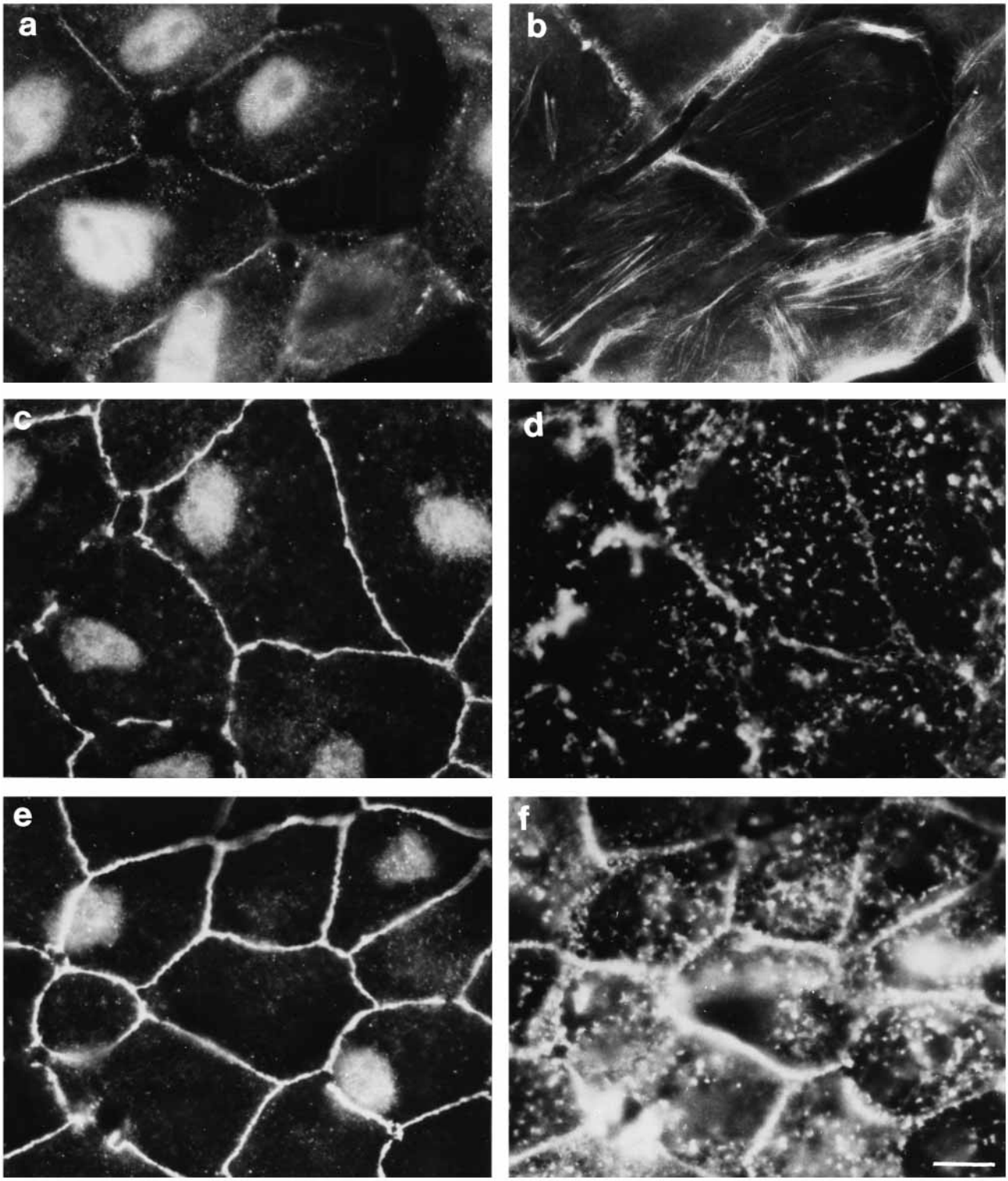

Fig. 6. Effects of Ca^{2+} and cytochalasin on tight junctions and actin microfilaments in MDCK cells. Cells were treated for 30 minutes with BAPTA (a,b) or incubated for 1 minute with cytochalasin followed by normal medium for 30 minutes (c,d) or incubated for 1 minute with cytochalasin followed by treatment with BAPTA for 30 minutes (e,f). Following treatment cells were fixed and stained for cingulin (a,c,e) or actin (b,d,f). Nuclear labeling in (a,c,e) is due to non-specific reactivity in some batches of the rabbit anti-cingulin antiserum. Bar in (f), 10 μm.

junctions requires an active cytoskeletal contraction and that the two drugs affect actin organization via different mechanisms. Interestingly, examination of cytochalasin D-treated cells indicated that those cell-cell contacts left intact after the treatment were affected to only a limited extent by the addition of EGTA or BAPTA (Fig. 6). This dual effect of cytochalasin, namely the initial fragmentation of junctions and the conversion of the residual cell-cell adhesions to Ca^{2+}-independence,

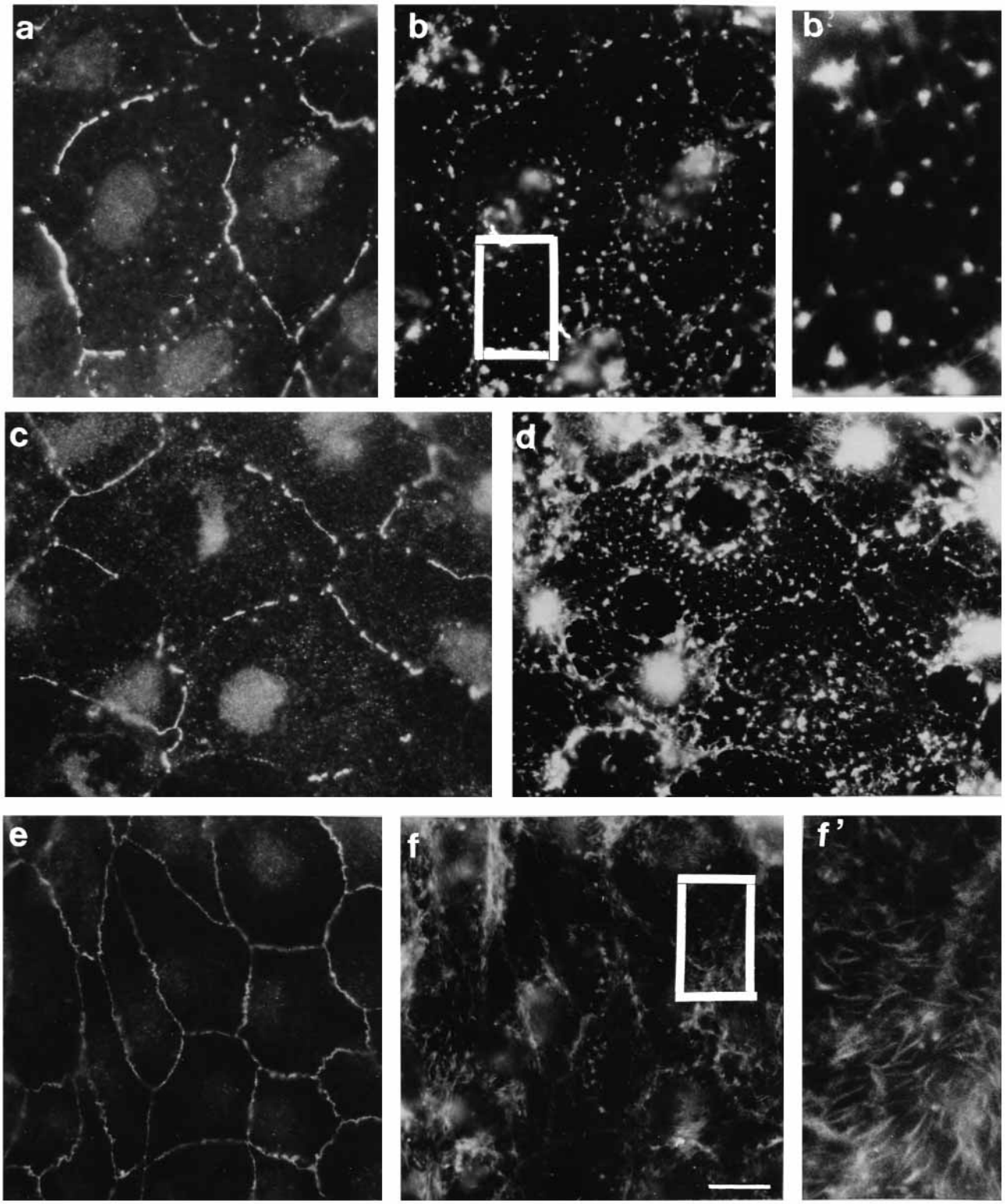

Fig. 7. Effect of H-7 on the cytochalasin-induced changes in tight junctions and actin microfilaments in MDCK cells. Cells were treated for 2 hours with cytochalasin (a,b,b′) or preincubated for 30 minutes with HA1004 and then treated with cytochalasin for an additional 2 hours (c,d), or preincubated for 30 minutes with H-7 and then treated with cytochalasin for an additional 2 hours (e,f,f′). Following treatment cells were fixed and stained for cingulin (a,c,e) or actin (b,d,f). Insets in (b) and (f) are shown at 4× higher magnification in (b′) and (f′), respectively. Notice that H-7 induces the formation of arrays of thin and short actin fibers after cytochalasin treatment (f′), instead of condensed foci (b′). Bar in (f), 10 μm.

points to the dual role of the microfilament system, which is involved in both the generation of tension and the stabilization of cell adhesion.

Our interpretation of the cytochalasin D effects is based on the current ideas about the cellular mechanisms of the action of this drug. Cytochalasins prevent actin polymerization, cap the barbed ends of actin filaments and bind to monomeric actin in vitro (for review, see Cooper, 1987). These primary effects lead to a decrease in the average actin filament length and to a rapid fall in the rigidity of actin gels. This may also lead to the transient predominance of contractile forces over the resistance to these forces and finally to partial contraction both in artificial model systems (Janson et al., 1991) and in living cells (Kolega et al., 1991). This contraction may be responsible for the partial destruction of junctions between epithelial cells following cytochalasin D treatment. Increase in contractility under the influence of cytochalasin D is a transitory event. Cytochalasin-induced contraction is a 'self-destructive' process (Kolega et al., 1991), leading to disorganization of the contractile system itself and finally to the general loss of cell contractility. Therefore further weakening of cell-cell contacts of cytochalasin-treated cells by EGTA or BAPTA does not induce additional contraction and cell separation. We suggest that pretreatment of the cells with H-7 prevents both the contraction induced following addition of calcium chelators and the short-term contraction induced by cytochalasin D, thus preventing (or reducing) junction dissociation and consequently the redistribution of junctional proteins. The thin actin fibers obvserved in cells treated with H-7 and cytochalasin D (Fig. 7) may thus represent short, capped actin filaments that cannot undergo contraction. The altered organization of the actin cytoskeleton caused by cytochalasin D even in the presence of H-7 may be responsible for the partial decrease in the transepithelial resistance observed under these conditions (Fig. 5).

In summary, the results presented in this paper suggest that H-7 prevents junction dissociation induced by low extracellular calcium by acting on the AJ-associated microfilament system. This conclusion does not rule out the possibility that calcium chelation and H-7 may have additional effects; for example, at tight junctions and desmosomes. The major points for further investigation are the nature of the kinase (or kinases) that are involved in the cellular phenomena described here and the specific components of the microfilament system that are modified by them. Both issues are currently being addressed in a separate study.

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