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Phosphorylation of the tight junction protein cingulin and the effects of protein kinase inhibitors and activators in MDCK epithelial cells

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SUMMARY

In previous studies we have shown that protein kinase inhibitors and extracellular calcium can affect dramatically the assembly of tight junctions (TJ) and the localization of the TJ protein cingulin at sites of cell-cell contact in renal epithelial (MDCK) cells. To characterize in more detail the relationships between kinase activity and junction organization, we have studied the effects of the protein kinase C agonist phorbol myristate acetate (PMA) on the intracellular localization of cingulin, E-cadherin, desmoplakin and actin microfilaments in confluent MDCK monolayers. To study cingulin phosphorylation, MDCK cells were metabolically labelled with [^{32}P]orthophosphate and immunoprecipitates were prepared with anti-cingulin antiserum. We show here that cingulin is phosphorylated in vivo on serine, and its specific phosphorylation is not significantly changed by treatment of confluent MDCK monolayers with PMA, with the protein kinase inhibitor H-7, or with the calcium chelator EGTA. Metabolic labeling with a pulse of [^{35}S]methionine/cysteine showed that at normal extracellular calcium net cingulin biosynthesis was not affected by PMA or H-7. During junction assembly by calcium switch, H-7 did not change the specific phosphorylation of the immunoprecipitated cingulin, however, it prevented the increase in the amount of cingulin in the immunoprecipitates, suggesting that H-7 may block tight junction assembly by interfering with cellular processes that lead to the accumulation and stabilization of TJ proteins at sites of cell-cell contact.

Key words: cingulin, tight junction, phosphorylation, H-7, PMA, MDCK, adherens junction, cytoskeleton

INTRODUCTION

Tight junctions (TJ) are the apical component of the junctional complex connecting adjacent cells in vertebrate epithelial tissues, and form a semipermeable barrier that prevents the free diffusion of solutes between lumenal and interstitial compartments (Gumbiner, 1987; Madara and Hecht, 1989; Schneeberger and Lynch, 1992). In addition to maintaining distinct tissue compartments, TJ also act as fences that separate apical and basolateral domains of the epithelial plasma membrane. In recent years, much progress has been made in clarifying the molecular structure of TJ (for reviews see Citi, 1993, 1994; Gumbiner, 1993). The membrane domain of TJ contains the protein occludin, which shows four membrane-spanning regions and a C-terminal cytoplasmic tail (Furuse et al., 1993). The cytoplasmic plaque domain of TJ contains ZO-1 (Stevenson et al., 1986; Itoh et al., 1991, 1993; Willott et al., 1993), ZO-2 (Gumbiner et al., 1991; Jesaitis and Goodenough, 1994), cingulin (Citi et al., 1988), and 7H6 (Zhong et al., 1993). Two members of the rab family of monomeric GTPases, rab13 (Zahraoui et al., 1994) and rab3B (Weber et al., 1994) have also been localized at the level of TJ in epithelial cells, and may act as regulators of vesicle traffic to the apical membrane domain.

The structure of TJ, as determined by electron microscopy or immunocytochemistry, and their function, as determined by measurement of transepithelial resistance and ion fluxes, can dynamically change in response to a number of physiological, pathological, and experimental agents (reviewed by Madara and Hecht, 1989; Schneeberger, 1994). The modulation of TJ structure and function must be due to specific regulatory mechanisms, however, little is known about their molecular nature. Several lines of evidence indicate that phosphorylation may be an important signal in establishing and maintaining TJ integrity. For example, protein kinase inhibitors prevent the assembly of TJ in experimental models of cultured epithelial cells (Balda et al., 1991; Nigam et al., 1991), whereas they have less effect on adherens-type junctions (AJ) (Denisenko et al., 1994). Treatment of epithelial monolayers with protein kinase C agonists abolishes transepithelial resistance at normal extracellular calcium (Mullin and O'Brien, 1986; Ojakian, 1981), and promotes TJ assembly at low extracellular calcium (Balda et al., 1993). Finally, protein kinase inhibitors prevent or reduce the structural and functional disruption of TJ and other junctions after removal of extracellular calcium (Citi, 1992), or after teatment with cytochalasin D (Citi et al., 1994).

To determine precisely how phosphorylation and other signals may affect the function of TJ proteins it is essential to

study the effects of the drugs and experimental manipulations on the phosphorylation and molecular interactions of these proteins. Direct evidence that changes in TJ protein phosphorylation correlate with changes in TJ function or assembly is scarce. So far, the only TJ proteins that have been shown to be phosphorylated are ZO-1 (Anderson et al., 1988) and ZO-2 (Balda et al., 1993). ZO-1 phosphorylation appeared to correlate with permeability properties of MDCK strains (Stevenson et al., 1989), but its phosphorylation was not significantly affected by protein kinase C agonists (Balda et al., 1993) and dexamethasone (Singer et al., 1994). The phosphorylation of ZO-1 under low calcium (LC) conditions was reported to be not significantly different from that in normal calcium (NC) in one study (Balda et al., 1993), and lower in another study (Howarth et al., 1994).

In the present paper, we show that cingulin is phosphorylated on serine. Using immunofluorescence techniques, we have investigated the effect of the protein kinase C agonist PMA on the localization of proteins of TJ and AJ, and on the spatial relationships between cingulin and actin in confluent MDCK cultures. To investigate the relationships between cingulin phosphorylation and TJ integrity, we have examined the effect of PMA, H-7 and EGTA on the phosphorylation and biosynthesis of cingulin in confluent MDCK monolayers and during junction assembly. The results indicate the specific phosphorylation of cingulin is not significantly altered by any of these treatments, but H-7 prevents the accumulation of phosphorylated cingulin during junction assembly by the calcium switch procedure.

MATERIALS AND METHODS

Reagents and media

Phorbol 12-myristate 13-acetate (PMA), 4-alpha-phorbol 12,13-didecanoate (4-alpha-PDD), 1-(5-isoquinolinylsulfonyl)-2-methyl-piperazine hydrochloride (H-7) and miscellaneous chemicals were obtained from Sigma (St Louis, MO). Radiochemicals were obtained from New England Nuclear (Boston, MA). Antibodies and fluorescence reagents were as described (Denisenko et al., 1994).

Fetal bovine serum was obtained from Hyclone labs (Logan, UT). Tissue culture reagents were purchased from Gibco Labs (Grand Island, NY). For calcium switch experiments, cells were incubated in low calcium (LC) medium (S-MEM, Gibco Cat #072-02300A) containing 0.2 g/liter of sodium bicarbonate, 20 mM Hepes, pH 7.2, and 5% dialysed FBS (dialysis was carried out against one change of 0.15 M NaCl, 0.2 mM EDTA, and three changes of 0.15 M NaCl). Phosphate-free medium was obtained from Sigma (St Louis, MO) (Cat. # D3656), and was supplemented with 5% dialysed fetal bovine serum and 20 mM Hepes, pH 7.2. Medium lacking cysteine and methionine was prepared with MEM Select Amine kit (Gibco Cat. # 300-9050AV). Specially formulated medium (without calcium chloride and without phosphate) for metabolic labeling of cells with radioactive phosphate in low calcium conditions was obtained as a special order from Gibco (Grand Island, NY).

Cell culture, assembly protocols and treatment with drugs

MDCK cells were cultured as described (Citi, 1992; Denisenko et al., 1994), and were plated on glass coverslips for immunofluorescence analysis and onto polycarbonate filters (4.7 cm^2 Transwell cat# 3412, Costar Inc. Cambridge, MA) for metabolic labeling experiments (2×10^6 cells per filter). For experiments on confluent cultures, cells were grown for 2 days after plating. For calcium switch (CS) experiments, freshly trypsinized cells were incubated in normal calcium medium (NC) for 90 minutes, then incubated in low calcium medium (LC) for 20 hours, and then switched to NC for 2 hours.

Cells were treated with the protein kinase inhibitor H-7 by adding stock solution (10 mM, stored at 4°C) to the media, to obtain a final concentration of 300 μM. The protein kinase activator PMA (stock 1.6 mM in DMSO at −20°C) was added to obtain a final concentration of 16 nM. EGTA (stock 0.2 M, pH.7.5) was added to obtain a final concentration of 4 mM. In metabolic labeling experiments, drugs were typically added to the media 30-60 minutes before labeling and throughout the labeling period. Control experiments consisted of adding the drug solvent(s) to the medium. As a control in PMA experiments, cells were also treated with the inactive phorbol, 4-alpha-PDD (stock 1.6 mM in DMSO at −20°C) to obtain a final concentration of 160 nM. The results obtained with 4-alpha-PDD were essentially identical to those obtained with solvent alone and are therefore shown as 'control' in the figures.

Metabolic labeling

Metabolic labeling with [^{32}P]orthophosphate ([^{32}P]P_i) was carried out by preincubating cells on filters in phosphate free-medium for 3 hours, replacing with phosphate-free medium containing [^{32}P]P_i (final specific activity 0.2-0.3 mCi/ml), and incubating for 3 hours. In the calcium switch/metabolic labeling experiments cells were plated in normal medium and grown in low calcium as described previously (Denisenko et al., 1994), then they were incubated in special calcium-and-phosphate-free medium for 3 hours, and then in the same medium containing [^{32}P]P_i. The switch was carried out by adding $CaCl_2$ (stock 1 M) to the medium (final concentration 2 mM) 1 hour after [^{32}P]P_i, and incubating for a further 2 hours before lysis. For calcium removal experiments EGTA was added 2 hours after [^{32}P]P_i, and incubated for a further 1-2 hours.

For [^{35}S] labeling, cells were incubated in medium lacking cysteine and methionine for 15-30 minutes, and pulsed for 30 minutes with Express[^{35}S](a mixture of [^{35}S]methionine and [^{35}S]cysteine, specific activity 1 mCi/ml) by placing a small volume (150-200 μl) of label on the bottom (basolateral) surface of upside-down filter supports in a humidified chamber at 37°C.

Immunoprecipitation

Filters were washed twice with 2 ml of medium and twice with PBS at room temperature. They were then excised from the plastic support with a razor blade and placed in an Eppendorf tube containing 1 ml of lysis buffer (LB: 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.2% BSA, 1% Triton X-100, 25 μg/ml leupeptin, 25 μg/ml pepstatin, 25 μg/ml antipain, 200 μM sodium orthovanadate and 20 μM phenylarsine). Cell were lysed for 1 hour at 4°C by gentle rotation of tubes. The filters were removed and the lysates were preabsorbed with 100 μl/sample of resuspended *Staphylococcus A* (Pansorbin cells 10%, Calbiochem, LaJolla, CA) for 30 minutes at 4°C. After centrifugation (1 minute at 10,000 ***g***) the lysates were incubated for 12-16 hours at 4°C with Protein A-Sepharose conjugated to anti-cingulin antibodies (previously prepared by incubating 10 mg of Protein A-Sepharose per sample with 2 μl of rabbit antiserum diluted in 1 ml LB for 6-16 hours at 4°C). Immunoprecipitates were washed 3× with LB, 3× with LB containing 0.1% SDS, and 3× with LB containing 0.5 M NaCl prior to SDS-PAGE.

SDS-polyacrylamide gel electrophoresis, immunoblotting and measurement of label incorporation and amount of protein

For SDS-PAGE, immunoprecipitates were solubilized in 10 μl of 10% SDS and 90 μl SDS sample buffer, boiled and electrophoresed onto 8.25% polyacrylamide-SDS gels at 250 V for 3 hours. Gels were calibrated for determination of apparent molecular size using a prestained kit (MWSDSBLUE, Sigma). For immunoblotting, gels were transferred onto nitrocellulose (0.1 μm pore size, Schleicher and

Schuell, Keene, NH) for 16 hours at 4°C. The nitrocellulose was blocked in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) containing 10% non-fat milk and 0.05% Tween-20 (TBS-milk), then incubated with primary anti-cingulin antiserum (1:5,000 in TBS-milk). Blots were washed 3× in TBS, incubated with alkaline phosphatase-labelled anti-rabbit antibodies diluted 1:7,500 in TBS-milk (Promega, Madison WI), washed and developed with the bromochloroindolyl phosphate/nitro blue tetrazolium substrate.

For autoradiography gels were exposed to XAR-5 film overnight. The incorporation of [^{32}P] and [^{35}S] into the immunoprecipitated cingulin polypeptide was measured by excision of the bands from the gel, followed by counting in a scintillation spectrometer, or by densitometry of autoradiograms, using a Bio-Rad Model GS 670 Video Densitometer. The amount of cingulin present in each immunoprecipitate was determined semiquantitatively by densitometric analysis of blots. The amounts of protein detected by these methods fell within the linear range of signal, calibrated using purified cingulin as a standard. Specific cingulin phosphorylation was determined by ratioing the [^{32}P]P_i incorporation with the amount of cingulin protein. Statistical Student's *t*-test analysis of data was performed with Statworks 1.2 (Cricket Software Inc., Philadelphia, PA, USA) on a Macintosh IISi computer. Results were considered statistically significant when $P<0.05$.

Phosphoaminoacid analysis

The following modification (Hunter and Sefton, 1980) was used for phosphoaminoacid analysis. The phosphorylated cingulin 140 kDa band was excised from SDS-polyacrylamide gels, and solubilised in 50 mM ammonium bicarbonate, pH 7.3, 5% β-mercaptoethanol, 1% SDS, for 16 hours at 37°C. The solubilized protein was precipitated by addition of 20 μg carrier protein (heat-treated RNAse A) and trichloroacetic acid to 20%. The pellet was washed with cold acetone, dried, and hydrolyzed in 50 μl constant boiling 5.7 M HCl (Pierce Chemical Co., Rockford, IL) at 106°C for 90 minutes. The hydrolysate was lyophylized, resuspended in 10 μl of pH 1.9 buffer (2.2% formic acid, 7.8% acetic acid, pH 1.9) containing 1 μg each of cold phosphoaminoacid standard, and applied to TLC cellulose plates (EM Science, Cherry Hill, NJ). Two-dimensional electrophoresis was carried out on a HTLE-7000 apparatus (CBS Scientific Co., Del Mar, CA), using pH 1.9 buffer for the first dimension, and pH 3.5 buffer (5% acetic acid, 0.5% pyridine, 0.5 mM EDTA, pH 3.5) for the second dimension. The TLC plate was sprayed with ninhydrin to visualize phosphoaminoacid standards, and exposed for 12 hours at −70°C in a cassette fitted with an intensifying screen.

Immunofluorescence microscopy

Cells were immunofluorescently labelled and observed with the conventional epifluorescence microscope as described previously (Citi, 1992; Denisenko et al., 1994). Double immunofluorescence was carried out with FITC-phalloidin and anti-cingulin antiserum followed by Texas Red-conjugated anti-rabbit antibodies. Confocal microscopy was carried out using a Sarastro confocal microscope.

RESULTS

Cingulin is phosphorylated on serine residues in vivo

Confluent MDCK cells were metabolically labelled with [^{32}P]orthophosphate ([^{32}P]P_i) and cell lysates were immunoprecipitated with anti-cingulin polyclonal antiserum. The immunoprecipitates were analyzed by SDS gel electrophoresis, followed by autoradiography (Fig. 1A, lane 1), or immunoblotting with anti-cingulin (Fig. 1A, lane 2). A polypeptide of 140 kDa was phosphorylated (Fig. 1A, lane 1), and specifically labelled by the antiserum (Fig. 1A, lane 2), indicating that cingulin is phosphorylated in vivo. Using the same procedure, cingulin phosphorylation was also observed in human intestinal (CaCo-2) cultured cells (not shown).

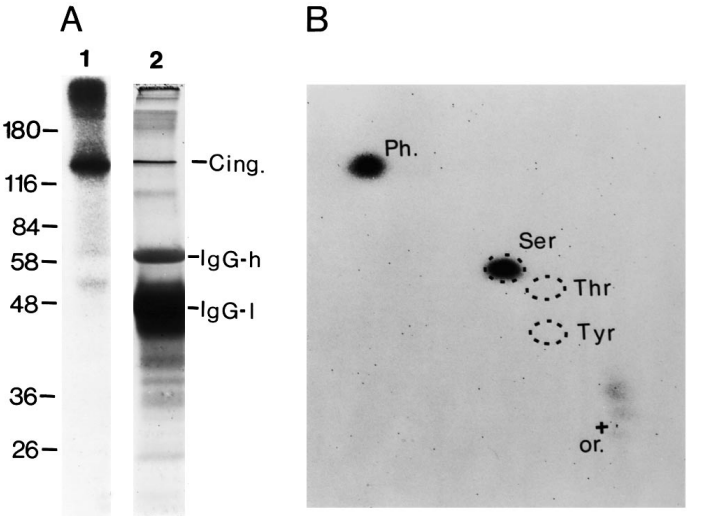

Fig. 1. Immunoprecipitation (A) and phosphoaminoacid analysis (B) of cingulin in MDCK cells. Lane A1, autoradiography of an SDS-polyacrylamide gel, showing a cingulin immunoprecipitate from ^{32}P-labelled MDCK cells. Lane A2, immunoblot of a parallel sample with anti-cingulin antiserum followed by alkaline-phosphatase labelled secondary antibody. Numbers on the left indicate apparent molecular masses, determined on the basis of migration of prestained marker proteins. The 140 kDa cingulin polypeptide is indicated on the right (cing.). IgG-h=immunoglobulin heavy chain; IgG-l=immunoglobulin light chain (the smeared band may indicate the presence of different populations of heavy chains). No 140 kDa polypeptide was immunoprecipitated with a non-immune rabbit serum or when no serum was used. For phosphoaminoacid analysis (B), partial hydrolisates of phosphorylated cingulin were applied to TLC cellulose plates at the origin (or.). Dotted ovals indicate the migration of standard phosphoaminoacids, identified by ninhydrin staining: phosphotyrosine (Tyr), phosphothreonine (Thr), and phosphoserine (Ser). Ph.= free phosphate. The TLC plate was autoradiographed for 40 hours at –80°C.

The identity of the phosphorylated aminoacid was established by partial hydrolysis and two-dimensional electrophoresis of the [^{32}P]labelled 140 kDa polypeptide. Autoradiography showed phosphorylation only on serine residue(s) (Fig. 1B).

Treatment of MDCK cells with phorbol esters alters cell shape and junctional protein and actin distribution

To study the correlation between cingulin phosphorylation and localization in cells treated with PMA, we first wanted to determine the effect of PMA on the localization of cingulin in MDCK cells.

Confluent cells treated with PMA were immunofluorescently labelled with antibodies against cingulin, E-cadherin and desmoplakin, which can be used a markers for tight junctions, *zonula adhaerens* and desmosomes, respectively (Fig. 2). PMA-treated cells lost their polygonal shape, the cell borders became irregular, and cell-cell contacts were often interrupted (Fig. 2B,D,F). In control cells, cingulin labeling

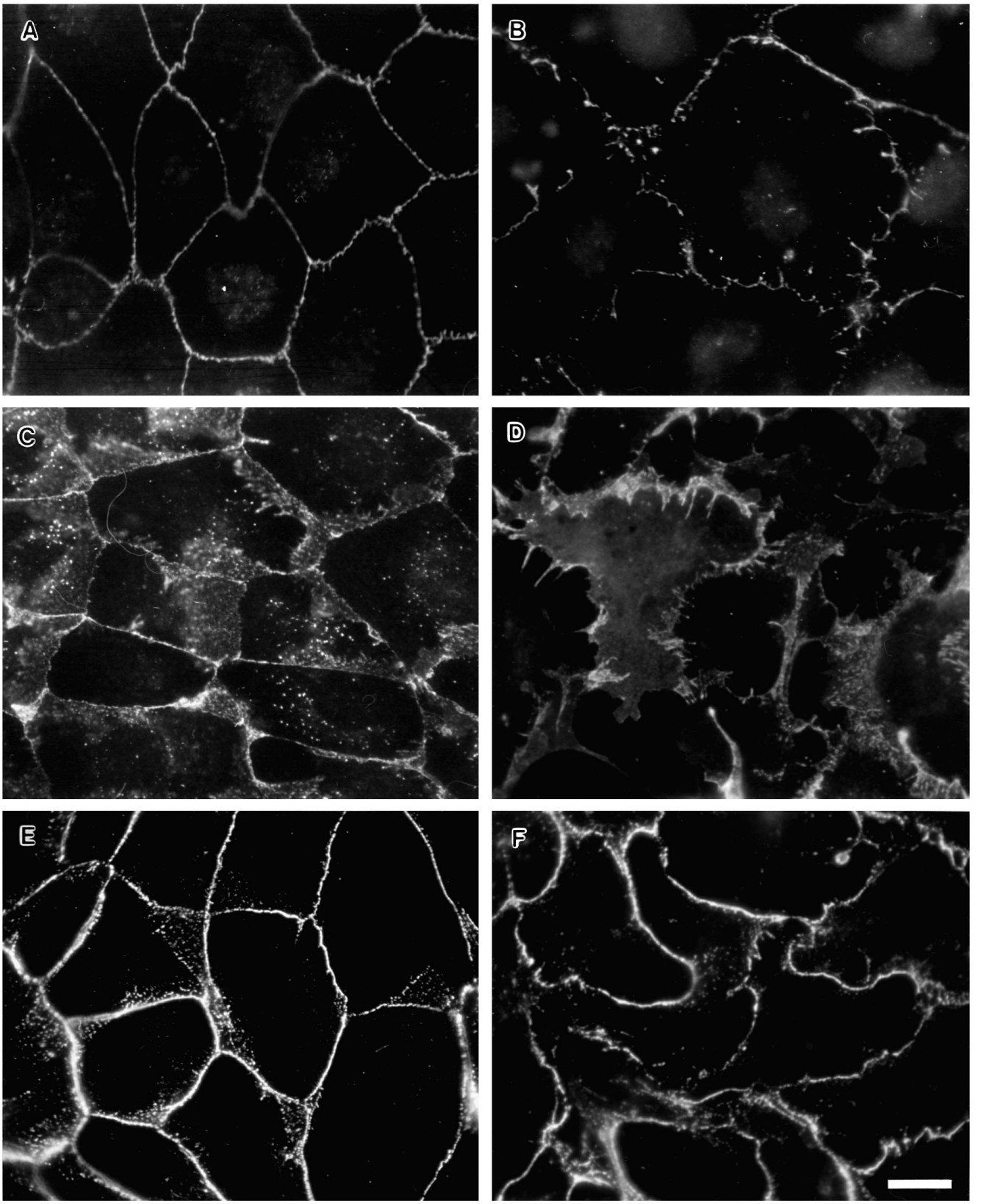

Fig. 2. The effect of PMA on the localization of junctional proteins in MDCK cells. Confluent monolayers grown on glass coverslips were either treated with 16 nM PMA for 3 hours (B,D,F), or control (DMSO or 160 nM 4-alpha-PDD) (A,C,E). Cells were then fixed and immunofluorescently labelled with antibodies against the TJ protein cingulin (A,B), the protein E-cadherin (C,D), and the desmosomal protein desmoplakin (E,F). Secondary antibodies were Texas Red-conjugated anti-rabbit (cingulin) or anti-mouse (E-cadherin and desmoplakin). Bar, 5 μm.

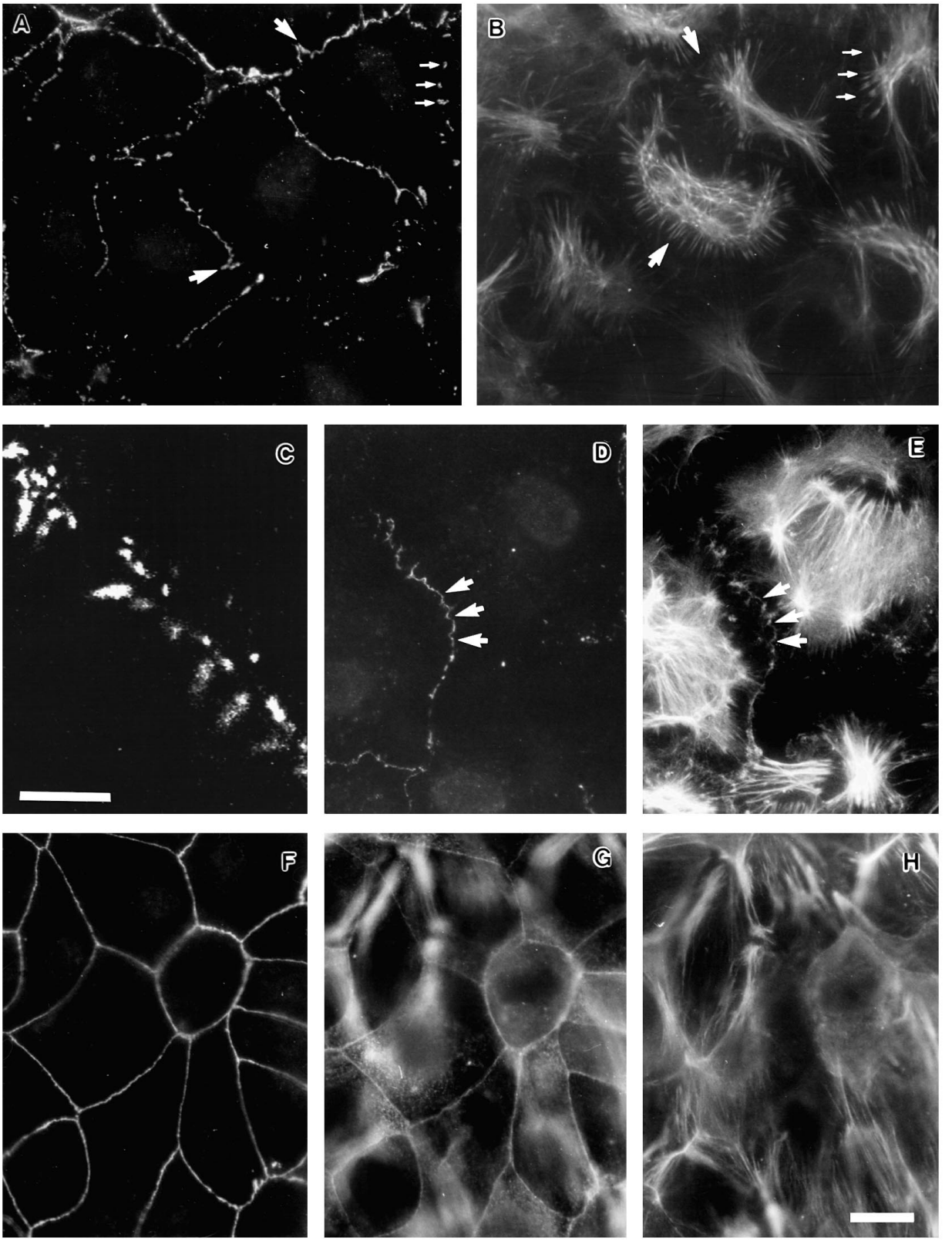

Fig. 3. The effect of PMA on the spatial relationships between cingulin and actin. Confluent MDCK monolayers were treated with PMA for 3 hours (A,B,C,D,E), or control (F,G,H). Cells were fixed and immunofluorescently labelled with anti-cingulin (A,C,D,F) or FITC-phalloidin (B,E,G,H). (A-B, D-E, and F,G,H) Images of double-labelled cells, to compare the localizations of cingulin and actin. Arrows in A indicate invagination and fragmentation of cingulin labeling (corresponding positions are marked in B). Arrows in D (corresponding arrows in E) indicate cingulin and actin labeling at sites of cell-cell contact. C shows a high magnification optical section of a cell border in a PMA-treated, cingulin-labelled specimen, obtained with the confocal microscope. Bar, 1 μm. Bar in H (also applies to A,B,D,E,F,G), 5 μm.

was distributed linearly along the sites of cell-cell contact (Fig. 2A). In PMA-treated cells cingulin labeling remained concentrated along peripheral regions, however, it was focally interrupted and stretched, reflecting the irregularity of the cell borders and the disruption of junctions (Fig. 2B, see also Fig. 3A and C). When cells were observed by either conventional or confocal microscopy, there was no apparent increase in cytoplasmic labeling for cingulin, suggesting that treatment with PMA does not induce cingulin to detach from the submembrane region of the junction. For E-cadherin most of the labeling appeared concentrated along the lateral surface (Fig. 2D). For desmoplakin, we observed linear junctional labeling where adjacent cells were in contact, and fragmented and stretched labeling elsewhere (Fig. 2F). PMA treatment for 3 hours also induced a 90% fall in transepithelial resistance in monolayers grown on filters (not shown, see Ojakian, 1981).

Next, we analysed the spatial relationship between cingulin and actin in cells treated with PMA (Fig. 3). In control cells cingulin labeling was co-localized with actin in the junctional regions when cells were observed in the apical plane of focus (compare Fig. 3F and G). In addition, punctated actin labeling in this plane of focus was probably due to apical microvilli (Fig. 3G). Actin filaments organized in stress fibers could be observed in a more basal plane of focus (Fig. 3H). Treatment with PMA induced fragmentation, invagination and stretching of cingulin labeling (arrows in Fig. 3A), which was well documented in cells observed by confocal microscopy (Fig. 3C). PMA also altered dramatically the organization of actin microfilaments, and induced the appearance of intensely labelled clusters of filaments (Fig. 3B and E) in agreement with previous observations on epithelial and non-epithelial cells (Schliwa et al., 1984; Kellie et al., 1985; Meigs and Wang, 1986; Opas and Dziak, 1990; Sobue et al., 1988). In double-labelled cells, it was difficult to detect cingulin and actin colocalization at cell-cell contact sites (compare Fig. 3A and B), unless photographs were overexposed (arrows in Fig. 3D and E). Thus, although most of the labelled actin was present in cytoplasmic aggregates, a detectable amount remained associated with the cingulin-containing submembrane junctional plaque. Pre-treatment of confluent monolayers with the protein kinase inhibitor H-7 partially prevented the effects of PMA on the integrity of junctional contacts and the distribution of actin microfilaments (not shown).

Cingulin phosphorylation and net biosynthesis in confluent MDCK cultures

To determine the effect of various drugs on cingulin phosphorylation, confluent cultures at normal extracellular calcium (NC) were labelled with [^{32}P]P_i, and during the same period of time they were treated with the drugs (see also Materials and Methods). Cingulin phosphorylation was visualized by autoradiography (Fig. 4), and the specific phosphorylation was quantitated by ratioing the [^{32}P]P_i-incorporation and the total amount of cingulin protein determined by immunoblotting (Table 1). The results showed that in cultures treated with H-7 (for different lengths of time), PMA and EGTA specific cingulin phosphorylation was not significantly different from that in control cultures (Table 1).

To study whether treatment with protein kinase activators and inhibitors may affect net cingulin biosynthesis in confluent cultures, cells were treated with the drugs and pulsed for 30 minutes with [^{35}S]Express prior to lysis. Autoradiography (Fig. 5) indicated that neither PMA nor H-7, alone or in combination, significantly altered the incorporation of [^{35}S] into cingulin, suggesting that they do not prevent or enhance net cingulin biosynthesis. Interestingly, cingulin immunoprecipitates contained a co-immunoprecipitating polypeptide of

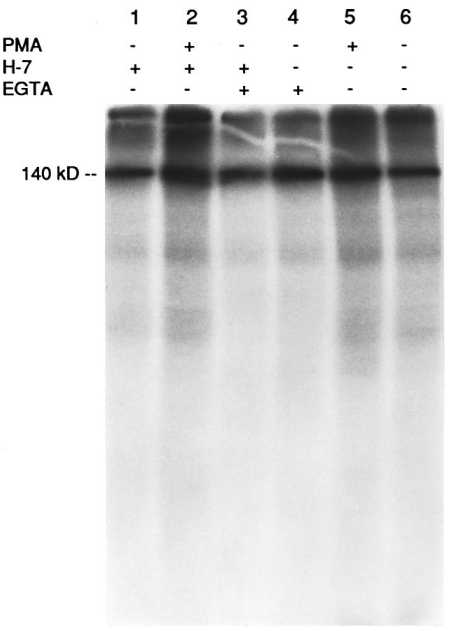

Fig. 4. Cingulin phosphorylation in confluent MDCK monolayers. Autoradiograph of an SDS-PAGE gel of cingulin immunoprecipitates, prepared from confluent MDCK monolayers treated with H-7 (Lane 1), H-7 and PMA (Lane 2), H-7 and EGTA (Lane 3), EGTA (Lane 4), PMA (Lane 5), and control (Lane 6). 140 kDa indicates the position of the cingulin polypeptide. Treatments with H-7, PMA and EGTA were for 4 hours, 3 hours, and 2 hours, respectively, prior to lysis and immunoprecipitation, and the drugs were added to the labeling medium (see Materials and Methods).

Table 1. Specific cingulin phosphorylation in confluent MDCK monolayers under different conditions

Medium	*n*	^{32}P/protein
NC	12	100±4
NC+H-7	12	96±4
NC+H-7 (12 hours)	2	94±8
NC+H-7 (24 hours)	3	91±7
NC+PMA	7	107±9
NC+PMA+H-7	6	101±7
NC+EGTA	4	103±9
NC+H-7+EGTA	2	94±8

MDCK cells were trypsinized and grown to confluence for 2 days in NC medium. They were then incubated in phosphate-free medium for 3 hours, and in medium containing [^{32}P]P_i. Typically, H-7 was added for 1 hour before addition of [^{32}P]P_i, and maintained in the medium throughout the labeling period (3 hours). In other experiments (NC+H-7, 12 hours; NC+H-7, 24 hours) H-7 was added 9 hours or 21 hours before [^{32}P]P_i. PMA was added at the same time as [^{32}P]P_i, and EGTA was added 1 hour after [^{32}P]P_i. Specific phosphorylation was determined as the ratio of phosphorylated cingulin to total cingulin protein, as described in Materials and Methods. Values were expressed as means of percentages, referring to the value at NC as 100, and plus or minus standard deviation (s.d.). *n* indicates the number of experiments performed for each experimental condition. The changes in cingulin phosphorylation were not statistically significant (P>0.05).

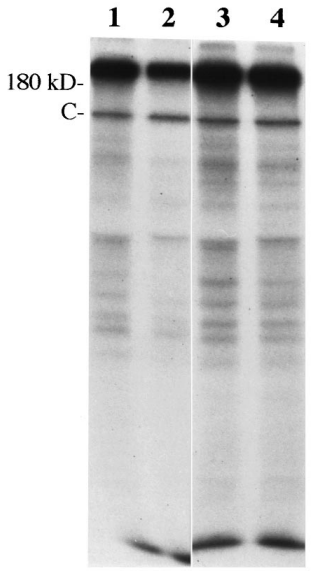

Fig. 5. Cingulin net biosynthesis in confluent MDCK monolayers. Autoradiograph showing an SDS-PAGE gel of cingulin immunoprecipitates after metabolic labeling of confluent monolayers with [^{35}S]Express. Cells were treated with H-7 + PMA (Lane 1), PMA (Lane 2), H-7 (Lane 3) and control (Lane 4). On the left, 180 kDa indicates the migration of prestained marker (alpha 2-macroglobulin), and C indicates the migration of the cingulin 140 kDa polypeptide. Note that a polypeptide of apparent size about 200 kDa coimmunoprecipitates with cingulin. Treatments with H-7 and PMA were for 4 hours and 3 hours, respectively, prior to metabolic labeling (30 minutes) and lysis, and the drugs were also added to the labeling medium.

approximately 200 kDa (Fig. 5). Preliminary immunoblotting experiments indicate that this polypeptide is not ZO-1 nor myosin heavy chain (unpublished observations).

Cingulin phosphorylation during junction assembly by calcium switch

In MDCK cells, H-7 prevents the localization of cingulin into sites of cell-cell contact during junction assembly by the calcium switch and during assembly at normal extracellular calcium (Denisenko et al., 1994; Nigam et al., 1991). To study the effects of H-7 on cingulin phosphorylation during junction assembly, cells were metabolically labelled with [^{32}P]P_i and immunoprecipitates were analyzed as before. The results (Table 2) showed that the specific phosphorylation of cingulin in LC medium, after the calcium switch (CS) in the absence or in the presence of H-7 was not significantly different from that in NC medium. Furthermore, H-7 did not affect the specific phosphorylation of cingulin in cells plated and grown in NC, e.g. under conditions where junction assembly occurs at normal extracellular calcium (not shown).

Table 2. Specific cingulin phosphorylation during junction assembly in MDCK cells

Medium	*n*	^{32}P/protein
NC	4	100±4
LC	3	92±8
CS	4	102±9
CS+H-7	4	89±7

MDCK cells were trypsinized, allowed to attach to the filters for 90 minutes in NC medium, and then incubated in NC for 22 hours (NC), in LC for 22 hours (LC), in LC for 20 hours followed by NC for 2 hours (calcium switch-CS), in LC for 20 hours followed by NC + H-7 for 2 hours (CS + H-7). H-7 was added also to the LC medium 1 hour before the switch. The changes in cingulin phosphorylation were not statistically significant (P>0.05). Labeling, determination of specific phosphorylation was as described in the legend of Table 1.

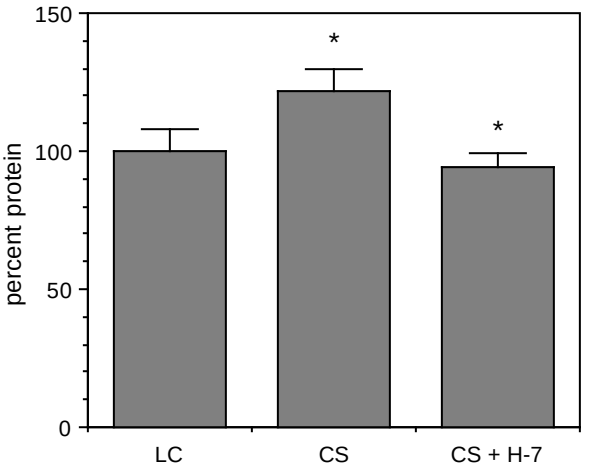

Fig. 6. The effect of H-7 on the amount of cingulin protein in immunoprecipitates after the calcium switch. Histogram showing the relative amounts of cingulin protein in cingulin immunoprecipitates prepared from cells incubated in low calcium (LC) medium, or switched to normal calcium (NC) medium in the absence of H-7 (CS) or in the presence of H-7 (CS+H-7). The amount of protein was determined by semiquantitative immunoblotting (see Materials and Methods) and the amount in the LC sample was taken as 100%. The amount of cingulin in CS was significantly more than in LC, and H-7 significantly reduced the amount of immunoprecipitated cingulin after the CS (P<0.01 in both cases, shown by asterisks).

Semiquantitative analysis of the amount of cingulin protein in the immunoprecipitates showed that 2 hours after the calcium switch the amount of cingulin was significantly increased (by about 20%, Fig. 6), indicating that the calcium switch stimulates the accumulation of phosphorylated cingulin in the cell. When the switch was carried out in the presence of H-7, the amount of cingulin was significantly lower than in the absence of H-7 (compare CS and CS+H-7 in Fig. 6), and essentially stayed at the same level measured before the switch.

DISCUSSION

The experiments described in the present paper represent a first step towards understanding the role of phosphorylation in cingulin function under physiological conditions and during modulation of junctions by a variety of agents. Our results indicate that neither PMA, H-7 nor EGTA influence net cingulin phosphorylation, however, H-7 prevents the accumulation of phosphorylated cingulin induced by the calcium switch.

The observation that cingulin is phosphorylated on serine residues raises questions and hypotheses. First, what is the role of cingulin phosphorylation? Cingulin shows biochemical and structural properties typical of alpha-helical, coiled-coil proteins (Citi, 1994; Citi et al., 1988, 1989), and in several of these proteins phosphorylation of the coiled-coil region controls critical functions such as self-assembly or interactions with other proteins (see for example nonmuscle myosins: Kuczmarski and Spudich, 1980; Collins et al., 1982). Since purified chicken cingulin can form filamentous aggregates in vitro (S. Citi and J. Kendrick-Jones, unpublished observations), studies are in progress to test the role of phosphoryla-

tion in cingulin assembly in vitro and association with the membrane cytoskeleton in cultured cells. A second question that we are currently addressing is what kinase phosphorylates cingulin and what are the phosphorylation site(s). The derived amino acid sequence of a chicken cingulin cDNA indicates a number of potential phosphorylation sites by protein kinases C and A (S. Citi et al., unpublished results). Preliminary studies in vitro with native and recombinant cingulin show that cingulin can be phosphorylated by protein kinase C but not protein kinase A (Rabino et al., 1993). Site-directed mutagenesis followed by studies in vitro and in vivo will be necessary to investigate the role of cingulin phosphorylation at the molecular level.

Treatment of MDCK cells with protein kinase C activators has dramatic effects on TJ permeability and distribution of TJ proteins. PMA increases TJ-dependent permeability (Ojakian, 1981; Mullin and O'Brien, 1986) and alters cingulin localization in confluent monolayers (Figs 2 and 3). Yet, protein kinase C agonists do not affect significantly the phosphorylation of ZO-1 (Balda et al., 1993) and cingulin (present paper). Although the effects of PMA on the phosphorylation of other TJ proteins remains to be established, these observations suggest that PMA does not act on TJ directly, by increasing the phosphorylation of TJ proteins, but indirectly, through its action on other proteins. Among these, microfilament-associated proteins may play a key role. In fact, in epithelial cells actin filaments have been detected at the level of TJ (Madara, 1987; Drenckhahn and Dermietzel, 1988), and are concentrated in the neighbouring *zonula adhaerens* (Geiger et al., 1985). The contraction of the circumferential actin belt in the *zonula adhaerens* may affect TJ permeability and structure (Madara et al., 1987, 1988). Since PMA causes the collapse of cytoplasmic microfilaments (Schliwa et al., 1984; Kellie et al., 1985; Sobue et al., 1988) (see also Fig. 3), it can be postulated that TJ are opened by the pulling action of such microfilaments on junctional membranes. The importance of the actin cytoskeleton in modulating TJ organization has prompted us to pay special attention to the spatial relationships between cingulin and actin in cells treated with PMA. Our observations (Fig. 3) confirm the close association of cingulin and actin at sites where junctional contacts remain intact, and suggest that interruptions and alterations in cingulin distribution are caused primarily by the PMA-induced reorganization of the actin cytoskeleton at the cytoplasmic surfaces of junctions.

Removal of extracellular calcium opens TJ and causes redistribution of TJ proteins (Siliciano and Goodenough, 1988; Citi, 1992). H-7 prevents these effects, suggesting that the activity of kinase(s) is required in order to obtain junction disruption following calcium removal (Citi, 1992). H-7 also prevents TJ assembly when cells are transferred from LC to NC (calcium switch) (Balda et al., 1991; Nigam et al., 1991; Denisenko et al., 1994). How do H-7 and extracellular calcium modulate junction assembly and disassembly, and are the effects of H-7 and EGTA due to changes in junctional protein phosphorylation? Our results show that addition of EGTA or H-7 to cells in NC does not induce significant changes in net cingulin phosphorylation (Table 1). Other laboratories reported no significant change (Balda et al., 1993), or a small decrease (Howarth et al., 1994) in ZO-1 phosphorylation under LC conditions. In summary, it appears that modulation of TJ by extracellular calcium is not correlated to major changes in the net phosphorylation of these TJ proteins. However, since net phosphorylation may be the sum of phosphorylation at distinct sites with different regulatory roles, we cannot exclude in principle the possibility that extracellular calcium affects TJ structure and function by influencing the phosphorylation of cingulin, ZO-1 or other TJ proteins.

In our view, it appears more likely that the effects of EGTA on TJ are a consequence of its effects on calcium-dependent adhesion molecules concentrated at adherens junctions (Gumbiner and Simons, 1986; Gumbiner et al., 1988), through the 'contraction' of the junction associated microfilament cytoskeleton (see before). The effects of H-7 can also be explained, at least in part, on the basis of its effects on the actin cytoskeleton. In fact, H-7 has been shown to induce a reorganization of microfilaments, with the destruction of stress fibers (Birrell et al., 1989; Denisenko et al., 1994; Volberg et al., 1994) and the reduction in ATP-induced actomyosin-dependent contractility (Volberg et al., 1994). Thus, the effects of H-7 on junction assembly/disassembly may depend primarily on its inhibition of kinases that regulate actomyosin-dependent motility, such as myosin light chain kinase (Volberg et al., 1994).

The observation that H-7 prevents the accumulation of phosphorylated cingulin that occurs after the calcium switch (Fig. 6) is consistent with the observation that H-7 reduces the accumulation in immunofluorescent cingulin labeling along cell-cell contacts (Nigam et al., 1991; Denisenko et al., 1994). However, H-7 does not appear to influence specific cingulin phosphorylation (Table 2), suggesting that it does not block the overall phosphorylation of cingulin present under LC conditions. Previous studies indicate that in LC junctional proteins are synthesized from a relatively large pool of mRNA, and that the calcium switch is associated with an increase in the amount of synthesized protein and a relative decrease in mRNA (Anderson et al., 1989; Gonzalez-Mariscal, 1992). Taking these observations together, one reasonable hypothesis is that H-7 inhibits a kinase(s) that is active on the actin microfilament system and is required for the transport and/or stabilization of newly synthesized junctional proteins at cell-cell contact sites. In this scheme, both cingulin phosphorylation and the integrity of actin microfilaments would be important in ensuring the stable association of cingulin with other junctional/cytoskeletal elements at cell-cell contacts.

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