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The α Isoform of Protein Kinase C Is Involved in Signaling the Response of Desmosomes to Wounding in Cultured Epithelial Cells

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Initiation of reepithelialization upon wounding is still poorly understood. To enhance this understanding, we focus here on changes in the adhesive state of desmosomes of cultured Madin-Darby canine kidney cells in response to wounding of confluent cell sheets. Previous results show that desmosomal adhesion in Madin-Darby canine kidney cells changes from a calcium-dependent state to calcium independence in confluent cell sheets. We show that this change, which requires culture confluence to develop, is rapidly reversed upon wounding of confluent cell sheets. Moreover, the change to calcium dependence in wound edge cells is propagated to cells hundreds of micrometers away from the wound edge. Rapid transition from calcium independence to calcium dependence also occurs when cells are treated with phorbol esters that activate PKC. PKC inhibitors, including the conventional isoform inhibitor Gö6976, cause rapid transition from calcium dependence to calcium independence, even in subconfluent cells. The cellular location of the α isoform of PKC correlates with the calcium dependence of desmosomes. Upon monolayer wounding, PKCα translocates rapidly to the cell periphery, becomes Triton X-100 insoluble, and also becomes concentrated in lamellipodia. The PKCα translocation upon wounding precedes both the increase in PKC activity in the membrane fraction and the reversion of desmosomes to calcium dependence. Specific depletion of PKCα with an antisense oligonucleotide increases the number of cells with calcium-independent desmosomes. These results show that PKCα participates in a novel signaling pathway that modulates desmosomal adhesion in response to wounding.

INTRODUCTION

When epithelia are wounded, cells commence migration to reepithelialize the wound. To do this, they must become motile and modulate their adhesions with each other and with the substratum. There is considerable interest in how this process is "kick-started" (Martin, 1997). A number of growth factors, including EGF, TGFα, heparin-binding EGF, and keratinocyte growth factor, are considered to be important in stimulating epithelial cell motility and proliferation in the wounded epidermis (Marikovsky *et al.*, 1993; Abraham and Klagsbrun, 1996; Nanney and King, 1996). Up-regulation of various proteases, including tissue plasminogen activator, urokinase plasminogen activator, and matrix metalloproteinases 1, 9, and 10, occurs in the migrating keratinocytes (Saarialho-Kere *et al.*, 1992, 1994; Salo *et al.*, 1994; Romer *et al.*, 1996). These appear to be important in releasing cells from their matrix attachments. The cytoskeleton also has an important function. In embryonic wounds, an actin cable forms rapidly around the wound edge to draw the wound together like a purse string (Brock *et al.*, 1996). Formation of this cable is prevented by inactivation of the small GTPase, Rho. The keratin cytoskeleton also appears to be important, although its role is not clear (Guo *et al.*, 1995; Paladini *et al.*, 1996). Deletion of the gene encoding bullous pemphigoid antigen 1, which links keratin filaments to the hemidesmosomal plaque, blocks epidermal reepithelialization, suggesting that this linkage is crucial for

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wound healing (Guo *et al.*, 1995). However, it appears that almost no attention has been given to the possible modulation of cell–cell junctions in response to wounding.

Desmosomes are one of the principal types of cell–cell junctions in epithelia and are particularly abundant in epidermis. These are multimolecular complexes containing, as major components, two glycoproteins, desmocollin and desmoglein, two armadillo proteins, plakoglobin and plakophilin, and the plakin family protein desmoplakin (Garrod *et al.*, 1999). The glycoproteins are involved in desmosomal adhesion, probably by heterophilic interaction between them (Chitaev and Troyanovsky, 1997; Marcozzi *et al.*, 1998; Tselepis *et al.*, 1998). Desmoplakin provides linkage between the desmosomal plaque and the keratin intermediate filament cytoskeleton (Stappenbeck and Green, 1992; Kouklis *et al.*, 1994; Bornslaeger *et al.*, 1996; Kowalczyk *et al.*, 1997; Smith and Fuchs, 1998). Plakoglobin is essential for the adhesive function of the glycoproteins and in linking the glycoproteins to desmoplakin (Bierkamp *et al.*, 1996; Ruiz *et al.*, 1996; Kowalczyk *et al.*, 1997). A similar role to that of plakoglobin may be inferred for plakophilin from studies of human mutations leading to an epidermal dysplasia/skin fragility syndrome (McGrath *et al.*, 1997). Mutation of desmoglein 1 and haploinsufficiency of desmoplakin also give rise to epidermal disease (Armstrong *et al.*, 1999; Rickman *et al.*, 1999).

The desmosomes of epithelial cells in confluent cell sheets differ in adhesive properties from those in subconfluent cultures. In the latter, desmosomes are calcium dependent, because their formation may be induced by increasing, and their disruption may be induced by decreasing, the extracellular calcium concentration (Kartenbeck *et al.*, 1982; Hennings and Holbrook, 1983; Watt *et al.*, 1984; Mattey and Garrod, 1986a,b; Duden and Franke, 1988). This calcium-dependent phenotype is expected, because desmocollin and desmoglein are members of the cadherin family of calcium-dependent adhesion molecules. By contrast, the desmosomes of cells in confluent culture become resistant to disruption even by divalent cation chelation. This has been demonstrated for keratinocytes, Madin-Darby canine kidney (MDCK) cells, and two colorectal carcinoma cell lines (Watt *et al.*, 1984; Mattey and Garrod, 1986b; Collins *et al.*, 1990).

Epithelial cells in vivo exist as confluent cell sheets. However, confluence is disrupted if the sheets are wounded, if the cells are undergoing morphogenetic movements during development, or if the cells are taking part in invasion and metastasis. It is likely that modulation of desmosomal adhesion is required to facilitate epithelial cell motility in these and other situations. Therefore, we have investigated the regulation of desmosomal calcium independence in more detail with the use of MDCK cells as a model. We find that the development of calcium independence requires monolayer confluence and is rapidly reversed upon wounding. Furthermore, the signal that modulates calcium dependence involves the α isoform of PKC, which may also signal increased motility. These observations may provide a novel insight into the mechanisms that regulate epithelial cell–cell adhesion in normal and disease processes.

MATERIALS AND METHODS

Cell Culture

MDCK II cells were cultured in standard medium (SM) consisting of DMEM plus 10% FBS (Life Technologies, Paisley, Scotland) on sterile 13-mm glass coverslips or Costar filters (0.4-μm pore size; Corning-Costar, High Wycombe, United Kingdom) with medium replenishment every 2 d during continuous culture. Cells were seeded at 1.35×10^5 cells/cm^2 for confluent density or at 5 cells/cm^2 so as not to achieve confluence by 6 d.

Monolayer Wounding

Wounds were created by scoring the monolayer with the use of a scalpel blade, following a predrawn rectangular grid. A cell scraper was used to remove cells between alternate score lines to create cell islands.

Assay for Calcium Sensitivity of Desmosomes

Cells were washed three times with HBSS without calcium or magnesium and then incubated in low-calcium medium (LCM) consisting of calcium-free DMEM, 3 mM EGTA, and 10% chelated FBS for 1 h at 37°C. Cells were then fixed in ice-cold methanol for 10 min and stained for desmoplakin by immunofluorescence. Cells were scored for calcium-independent desmosomes by selecting fields of view at random and counting the number of cells that still remained attached by at least one desmoplakin-staining projection after LCM treatment. This number was then expressed as a proportion of the total number of cells in the fields. At least 10 fields of view in each culture were counted on a minimum of three duplicate cultures for each point.

Drug Treatment of Cells

Forskolin, dibutyryl-cAMP, 3-isobutyryl-1-methylxanthine, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), CD, mitomycin C, phorbol-12,13-didecanoate, and cycloheximide were obtained from Sigma (Poole, United Kingdom). Chelerythrine, Gö6976, and okadaic acid were from Calbiochem (Nottingham, United Kingdom).

Growth Factor Treatment of Cells

EGF, TGFα, aFGF, and bFGF were obtained from RD Systems (Minneapolis, MN). Medium conditioned by a ras-transformed NIH 3T3 cell line was used as a source of HGF/SF. The scattering activity of the medium was determined with the use of a scatter assay on subconfluent MDCK cells (Stoker *et al.*, 1987) and the cyst-branching assay (Montesano *et al.*, 1991). Growth factors were applied to both apical and basal aspects of MDCK cells grown on filters. During long-term exposure (i.e., during 6 d of confluent culture), growth factors were replenished every day. During this long-term exposure, cells were shown to undergo proliferation in response to hepatocyte growth factor/scatter factor (HGF/SF) and EGF, but no response to the other factors was detected. The concentrations used were: HGF/SF, 1:32 dilution of NIH 3T3 ras-conditioned medium; EGF, 10 ng/ml; TGFα, 10 ng/ml; insulin/insulin-like growth factor-1, 10 μg/ml; aFGF, 10 ng/ml; bFGF, 5 ng/ml; PDGF, 5 ng/ml.

Antibodies

Mouse mAb to desmoplakin I and II (11-5F) (Parrish *et al.*, 1987) was used to stain desmosomes. Rat mAbs to ZO-1 (R40) (Stevenson *et al.*, 1986) and E-cadherin (DECMA-1 [Vestweber and Kemler, 1985] or ECCD-2 [Shirayoshi *et al.*, 1986]) were used to stain tight junctions and adherens junctions, respectively. Anti-PKCα, -β1, and -ζ (Sigma), anti-PKCζ (Transduction Laboratories, Cowley, United Kingdom), and anti-PKCβ1, -ζ, and -ϵ (N. Groome, Oxford Brookes University, Oxford, United Kingdom) (Gott *et al.*, 1994) were used for staining and Western blotting of PKC isozymes.

Immunofluorescence

Cells were fixed in ice-cold methanol for 10 min or in 3.5% paraformaldehyde at 4°C for 5 min, followed by 0.5% Triton X-100 in PBS for 20 min at room temperature. They were incubated in primary

antibody for 1 h at room temperature, washed three times for 5 min each in PBS, incubated in FITC- or TRITC-conjugated secondary antibodies (Jackson, Luton, United Kingdom) for 30 min, and washed three times for 5 min each in PBS before mounting in gelvatol. Cells were viewed with a Zeiss (Welwyn Garden City, United Kingdom) Photomicroscope III or Axioplan by epifluorescence.

Western Blotting

Cells were lysed in a buffer consisting of 20 mM Tris, pH 7.5, 5 mM EGTA, 50 mM β-mercaptoethanol, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin (all from Sigma), 50 mM NaF, 1 μM okadaic acid (Calbiochem), and 25 μM calpain inhibitor 1 (Boehringer Mannheim, Lewes, United Kingdom). Insoluble material was pelleted by spinning at 13,500 × *g*, and this was resuspended in the above buffer with 0.5% Triton X-100 to extract detergent-soluble protein. After spinning as before, the remaining soluble material was resuspended in Laemmli (1970) sample buffer. Protein concentration was estimated with the use of the Bio-Rad (Richmond, CA) detergent-compatible assay. Equal amounts of protein were then separated on a polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore, Walford, United Kingdom). The membrane was blocked in 2% nonfat milk, 2% goat serum in Tris/Tween-buffered saline (TTBS) for 20 min, then incubated in primary antibody diluted in TTBS for 1 h. Three washes in TTBS were followed by incubation in the appropriate alkaline phosphatase–conjugated secondary antibody (Amersham, Little Chalfont, United Kingdom) for 1 h. Membranes were washed as before, and bound antibody was visualized by ECL (Amersham).

Measurement of PKC Activity

Total PKC activity was determined with the use of a kit (Biotrak, Amersham). This assay depends on the transfer of phosphate from [^{32}P]ATP to a specific PKC substrate. Full details are given by the manufacturers. Four 9-cm dishes of MDCK cells were used for each sample. Samples were washed two times in HBSS, and the cells were then scraped into a total of 1 ml of ice-cold cytoplasmic buffer (20 mM Tris-HC1, pH 7.5, 5 mM EGTA, 3 mM EDTA, 50 mM β-mercaptoethanol, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin, 2 μg/ml leupeptin, 50 mM NaF, 1 μM okadaic acid, 25 μM calpain inhibitor 1) and sonicated. Samples were then centrifuged at 13,000 × *g* for 15 min at 4°C, and the supernatant (cytosolic fraction) and the pellet (membrane fraction) were separated. The latter was resuspended in cytoplasmic buffer containing 0.5% Triton X-100 and 0.5% SDS. Partial purification of PKC on DEAE-cellulose was done to reduce background (Golpalakrishna *et al.*, 1986; Shea *et al.*, 1994). Twenty-five microliters of partially purified sample was used for assay.

PCR and DNA Sequencing

Degenerate primers (Genosys, Cambridge, United Kingdom) were designed to amplify the 5′ end of the canine PKCα gene from MDCK cDNA. PKCα sequences were found for the human, rat, and rabbit genes with the use of PCgene (Intelligentics, Geel, Belgium). The 3′ primer was designed against an 18-base pair (bp) sequence 129 bp from the start codon that was completely homologous among the three species. The 5′ primer was designed against the first 18 bases from and including the ATG start codon of the PKCα cDNA. The primer was degenerate and included all combinations of base differences among the different species.

Primers were as follows: 5′ ATGGCTGAC(G/C)(T/C)(T/G)(T/C/A)(C/T)(C/G)CG and 3′ AGGTGGGCTGCTTGAAGA. These two primers yielded a 147-bp product on amplification of rat, human, and dog PKCα cDNA. The forward primer was used at 500 pmol/μl, and the reverse primer was used at 25 pmol/μl. PCR conditions were 1 cycle at 96°C for 5 min and 68°C for 1 min, 35 cycles at 96°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final cycle at 96°C for 30 s, 56°C for 30 s, and 72°C for 10 min. Products were separated on a 1% agarose gel. The PCR product was excised from the gel, spun through sterile glass wool to elute it, and then extracted with phenol:chloroform. The DNA pellet was resuspended in 10 μl of sterile dH_2O. The PCR

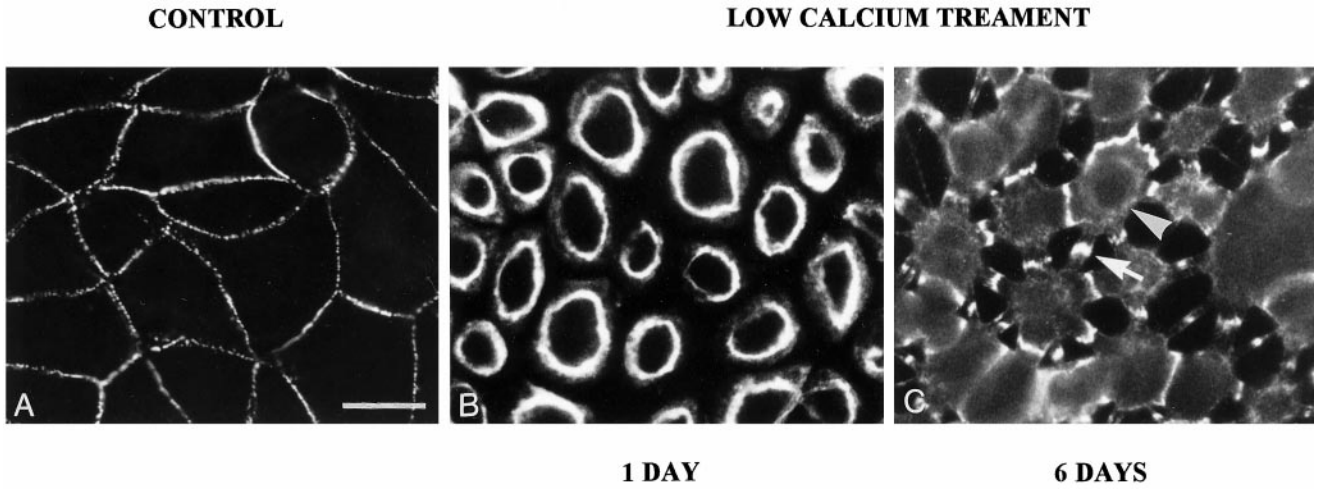

Figure 1. Desmosomes of MDCK cells can be Ca-Dep or Ca-Ind. (A) Desmosomes of MDCK cells in confluent culture stained with mAb 11-5F to desmoplakin. Note that the desmosomes are located at the cell peripheries and that the staining is generally punctate. (B) A monolayer that has been cultured at confluent density for 24 h in SM and then treated with LCM-EGTA for 90 min, showing loss of intercellular contact and of desmosomal staining from the cell peripheries. This is indicative of Ca-Dep desmosomes. (C) A 6-d-confluent monolayer treated with LCM-EGTA for 90 min, showing partial loss of intercellular contact but persistence of joining processes with intense desmosomal staining (e.g., arrow). This is indicative of Ca-Ind desmosomes. Note that the cells in C show some cytoplasmic staining (e.g., arrowhead), probably indicative of the persistence of some Ca-Dep desmosomes. Bar, 20 μm.

products were then ligated into the vector PCR2.1, and this was used to transform competent XL1-Blue *Escherichia coli*. Blue/white screening for β-galactosidase activity was used to select positive clones. DNA minipreps (Qiagen, Crawley, United Kindgom) were performed on these clones, and an *Eco*RI digest was used to excise the 147-bp fragment from the clones to confirm that it contained the correct insert. Positive clones were identified and sequenced from the M13 forward primer with the use of a kit from Perkin Elmer-Cetus (Cambridge, United Kingdom).

Antisense Transfection

Phosphorothioate-modified deoxyoligonucleotides were as follows: PKCα antisense, CGCATAAACGTCAGCCAT; PKCα sense, ATGGCTGACGTTTATGCG; nonsense, ACCTTGCACCAAAACGTG (Genosys). Oligonucleotides were transfected into newly confluent MDCK cells at 1 μM with the use of Superfect reagent (Qiagen). Oligonucleotides (10 μM) were mixed with 20 μl of Superfect reagent. The mixture was left at room temperature for 15 min to form complexes. The volume was made up to 1 ml with SM. The transfection mixture was then added to a 3-cm dish of cells (6×10^4 cells/cm^2) from which the medium had been removed. The time course of accumulation of ^{32}P-labeled oligonucleotides by the cells was determined. At various times after transfection, tissue culture medium was removed and retained, and the cells were washed several times in HBSS. The wash solution was retained and combined with the original medium, and the radioactivity was measured in a scintillation counter. The cells were scraped into a scintillation vial with the use of a rubber policeman. The dish was washed several times, wash was added to the cells, and the radioactivity was measured. The ratio of radioactivity present in the cells and in the medium gave a rough estimate of the extent of oligonucleotide uptake. Cellular radioactivity peaked at 20.9% of the total 48 h after transfection, then declined continuously. Transfection of less dense cultures (1×10^4 cells/cm^2) was attempted on three occasions, with the use of Superfect reagent as above, to study the effect of PKCα depletion on purely calcium-dependent cells. However, transfection effi-

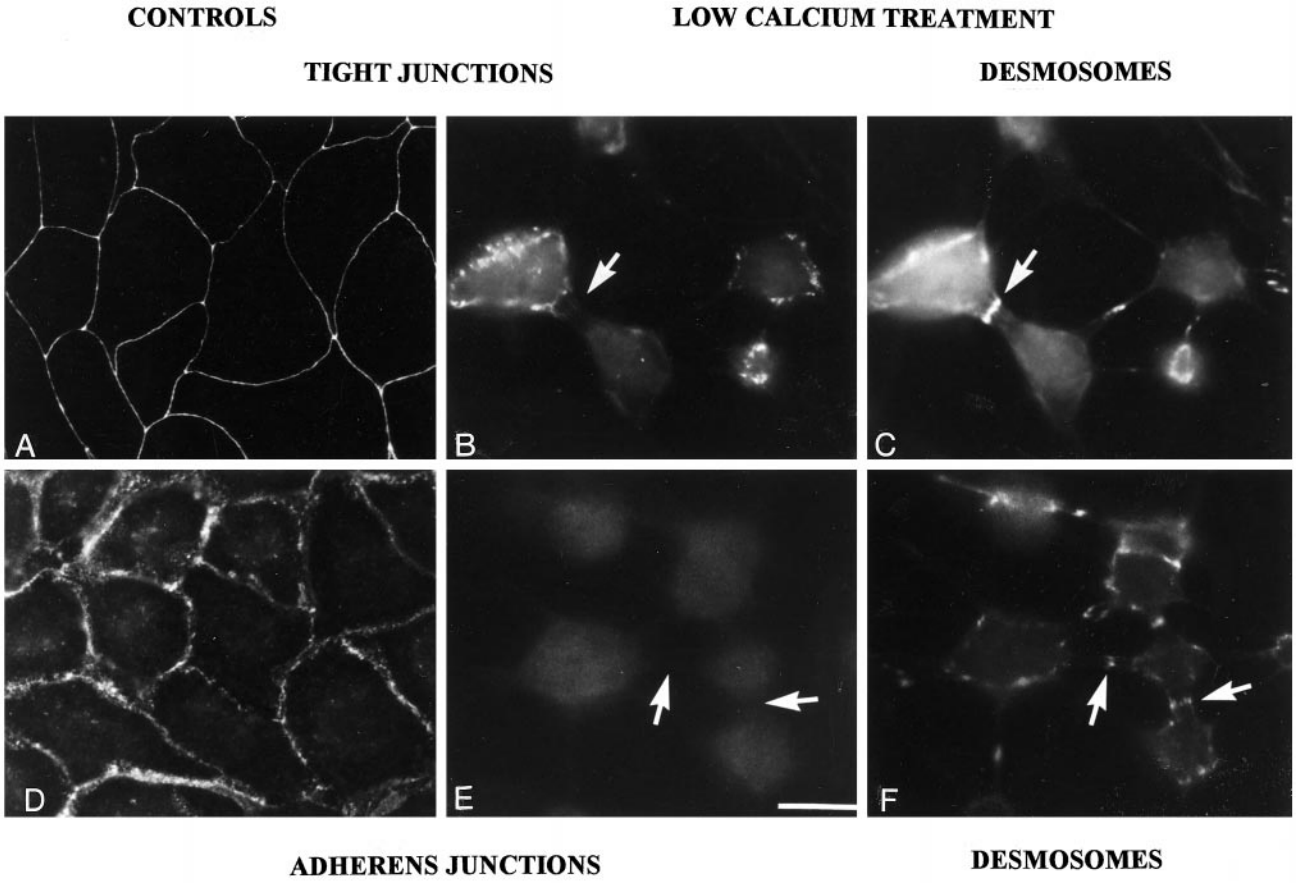

Figure 2. The change to Ca-Ind is specific for desmosomes and does not involve tight or adherens junctions. (A and D) Control cells cultured for 6 d at confluent density and stained with mAb ZO-1 to tight junctions (A) or with mAb DECMA-1 to E-cadherin (D). Note that not all E-cadherin is located in adherens junctions, but it is concentrated there, so E-cadherin staining provides a useful marker of adherens junctions. (B and C) The same field showing 6-d-confluent cells treated with LCM-EGTA for 90 min and then double stained for tight junctions (ZO-1) (B) and desmosomes (polyclonal guinea-pig antibody to desmoplakin) (C). Note the absence of ZO-1 staining in the cell processes in B (e.g., arrow) but that the same processes stain intensely for desmosomes (C, arrow). (E and F) The same field showing 6-d-confluent cells treated with LCM-EGTA for 90 min and stained for E-cadherin (E) and for desmosomes (F), as in C. Note the absence of DECMA-1 staining in the cell processes in E (arrows) but the persistence of desmosomal staining in the same processes in F. Similar results indicative of adherens junction breakdown were obtained by staining for actin and vinculin (our unpublished data). Bar, 20 μm.

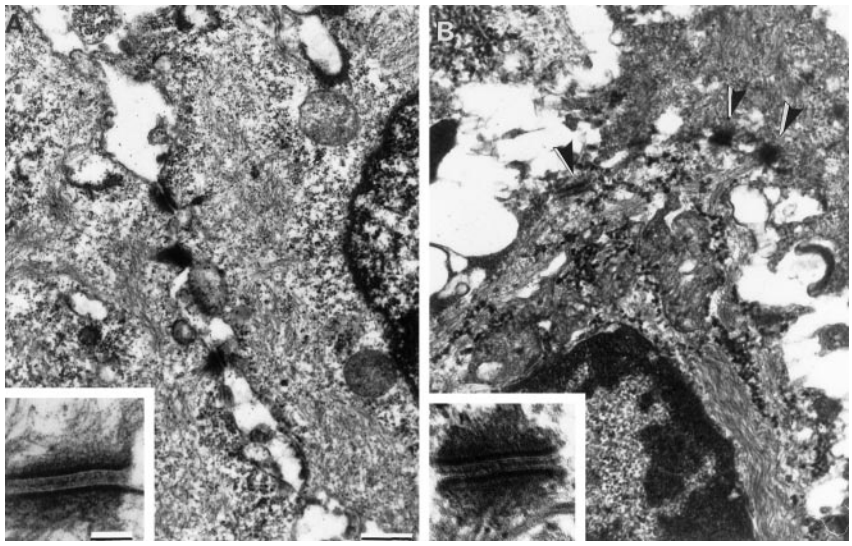

Figure 3. Desmosomes of epithelial cell sheets in vivo are generally Ca-Ind. Electron micrographs of mouse esophageal epithelium that has been incubated for 6 h at 37°C in SM (A) or LCM-EGTA (B). Note that the intercellular spaces in B are generally wider than those in A but that the desmosomes (arrowheads) are still intact. The insets show higher-magnification images of individual desmosomes showing that those incubated in LCM-EGTA retain typical desmosomal ultrastructure. Bar, 1 μm; bar in inset, 0.1 μm.

ciency was extremely poor (5% PKCα depletion was the best obtained), so cells were routinely cultured at higher density.

RESULTS

The Calcium Dependence of Desmosomes Changes with Time in Cells Cultured at Confluent Density and Responds Rapidly to Wounding

Desmosomes in newly seeded MDCK cells were calcium dependent (Ca-Dep). (From here on, Ca-Dep will be used to indicate calcium-dependent/dependence and Ca-Ind will be used to indicate calcium independent/independence.) Thus, when such cells were placed in LCM plus 3 mM EGTA (LCM-EGTA), they rounded-up, lost all cell–cell contacts, and internalized desmosomal proteins (Figure 1, A and B) (Mattey and Garrod, 1986b). However, MDCK cells maintained at confluent density for several days had Ca-Ind desmosomes. When these cells were placed in LCM-EGTA, the cells rounded up but remained attached at discrete points that stained for desmosomal proteins such as desmoplakin (Figure 1C) (Mattey and Garrod, 1986b). The change to Ca-Ind was found to be specific to desmosomes (Figure 2, C and F): tight junctions (Figure 2, A and B) and adherens junctions (Figure 2, D and E) remained susceptible to disruption by LCM after the cells had been cultured for 6 d at confluent density.

The phenomenon of Ca-Ind is not unique to MDCK cells; it has also been reported in human keratinocytes and in colon carcinoma cell lines (Watt *et al.*, 1984; Collins *et al.*, 1990). In the present study, Ca-Ind was also found in Caco-2 (colon), A549 (airway), and SVJD (keratinocyte) cells (our unpublished data). Desmosomes in tissues were also found to be Ca-Ind. Thus, exposure of small pieces of mouse epidermis, trachea, esophagus, tongue, liver, and cardiac muscle to LCM-EGTA for up to 6 h did not disrupt desmosome adhesion, as shown by transmission electron microscopy, even though separation of the cell membranes and vacuolation of cells indicated that the tissues were clearly affected by calcium removal (Figure 3). Parallel immunofluorescence studies indicated that E-cadherin in tissues was internalized during the exposure to LCM-EGTA (our unpublished results), and 3-(4,5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide assay (Ealey *et al.*, 1988) showed that the tissues remained viable after this treatment.

The proportion of cultured MDCK cells with Ca-Ind desmosomes could be scored by counting those that remained connected by desmoplakin-staining projections and expressing this count as a proportion of the total number of cells. Using this procedure, we studied the acquisition of Ca-Ind desmosomes in greater detail by seeding cells at confluent density and counting the proportion of cells that had acquired Ca-Ind desmosomes on each day of subsequent culture. The change to Ca-Ind desmosome adhesion in the cell population was found to be gradual (Figure 4A). Different batches of cells showed variation in the time taken to reach >90% Ca-Ind. However, replicate cultures in individual experiments were found to acquire Ca-Ind desmosomes at similar rates. Confluence of the epithelial cell layer was essential for the acquisition of Ca-Ind desmosomes in MDCK cells, because prolonged culture at subconfluent density did not produce this change (Figure 4, A–C).

In view of these results, we determined the effect of wounding 6-d-confluent epithelial sheets on the state of desmosomal adhesion. It was found that disruption of confluence by wounding of the monolayer initiated reversion to Ca-Dep desmosome adhesion (Figure 5A). The change to Ca-Dep adhesion occurred rapidly (within 1 h) at the wound edge and, interestingly, was propagated away from the edge so that, eventually, cells within the monolayer, hundreds of micrometers remote from the edge, acquired Ca-Dep desmosomes (Figure 5B). Reversion to Ca-Dep adhesion was not dependent on protein synthesis because it was still propagated after cycloheximide (20 μg/ml) treatment. It was also not dependent on reentry into the cell cycle because it still occurred in cells after treatment with mitomycin C (25 ng/ml) or after serum starvation for up to 2 d before wounding. It also did not require cell motility because it still occurred in the

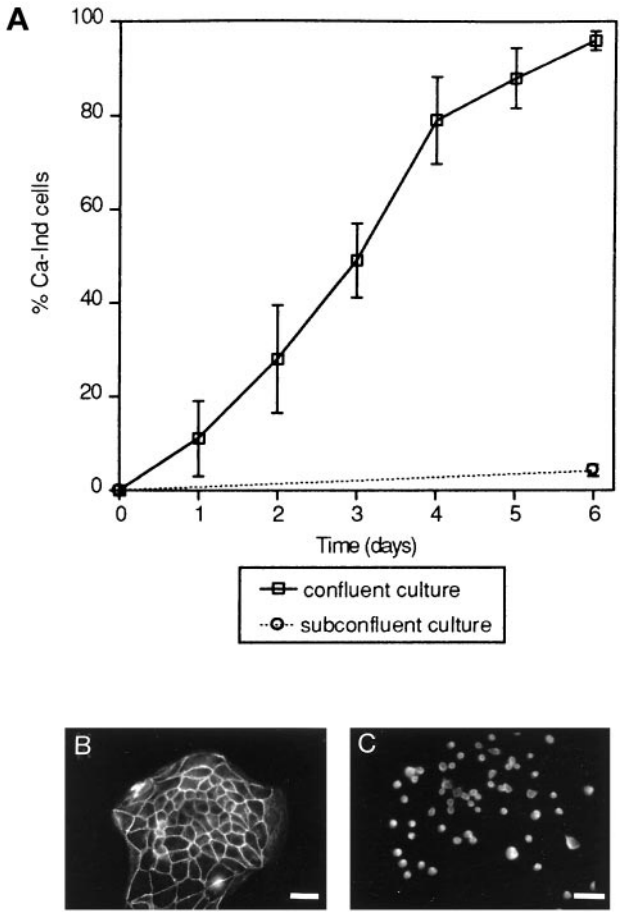

Figure 4. Development of desmosomal Ca-Ind occurs gradually in confluent cell culture but does not occur in subconfluent culture. (A) Quantification of acquisition of Ca-Ind. The solid line shows the percentage cells with Ca-Ind desmosomes in monolayers seeded at confluent density and maintained in SM for the times shown. The dotted line indicates lack of formation of Ca-Ind desmosomes, even after 6 d, by cells maintained at subconfluent density, as shown in B and C. (B) Island of cells after 6 d in SM stained for desmoplakin with mAb 11-5F. (C) Cell island similar to that shown in B after treatment with LCM-EGTA for 90 min. Note the complete loss of intercellular contact and desmosomal adhesion between cells in C.

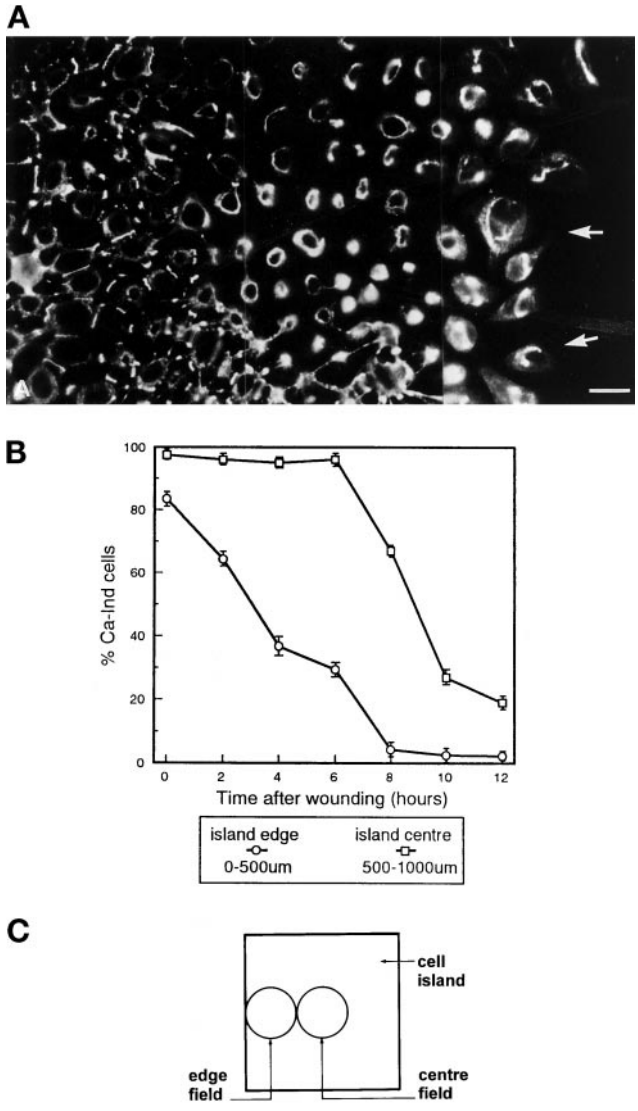

Figure 5. Wounding restores Ca-Dep in edge cells, and this is propagated through the monolayer. (A) Edge of a wound (arrows) made in a 6-d-confluent monolayer. After wounding, cells were cultured for 1 h in SM followed by 1 h in LCM-EGTA before fixation and staining for desmoplakin with mAb 11-5F. Note that the cells at and adjacent to the wound edge show Ca-Dep desmosomes, whereas cells deeper in the monolayer (left side) remain Ca-Ind. (B) Quantification of Ca-Ind after wounding in two adjacent microscope fields at (0–500 μm) and remote from (500–1000 μm) the wound edges (C). Monolayers were wounded in a grid pattern to create cell islands and then incubated for the times shown in SM. Then they were treated with LCM-EGTA for 1 h, followed by fixation and staining for desmosomes. Note that the return of Ca-Dep in cells at the island edge precedes that at the island center.

presence of the actin filament–depolymerizing agent CD at 1 μM, the lowest concentration that completely blocked motility of MDCK cells. Furthermore, microtubules were not required for initiation or propagation because it occurred in the presence of the microtubule-disrupting agent nocodazole (10 μg/ml).

Activation/Inhibition of PKC Modulates Desmosomal Adhesion

To investigate what signals may be involved in mediating the wounding response, we tested a variety of reagents for the ability to change desmosomes of confluent cells from Ca-Ind to Ca-Dep. We found that drugs that affect PKC activity elicit rapid changes in the state of desmosome adhesion. TPA, a potent activator of classic and novel isoforms of PKC, caused rapid (within 15 min) reversion of Ca-Ind desmosomes to Ca-Dep adhesion (Table 1). The PKC-activating phorbol esters phorbol-12,13-didecanoate and phor-

Table 1. Effect of PKC activators on the state of desmosome adhesion in MDCK cells (6-day cultures, initially Ca-Ind)

	Treatment					
Duration	Control	TPA (5 nM)[a]	Phorbol-12,13-didecanoate (50 nM)[a]	Phorbol-12,13-dibutyrate (50 nM)[a]	4α-Phorbol (50 nM)[b]	Okadaic acid (15 nM)[c]
15 min	97.6 ± 0.4	54.9 ± 4.2	–[d]	–	–	–
30 min	97.1 ± 0.8	30.2 ± 8.6	–	–	–	–
60 min	96.7 ± 0.7	9.7 ± 0.9	52 ± 1.9	60.1 ± 5.2	96.8 ± 0.2	5.0 ± 4.0

MDCK cultures were treated with drugs for the times indicated and then switched to drug-free LCM for 1 h. Values indicate percentage of Ca-Ind cells. Control column includes data for solvent vehicle treatment (e.g., 0.03% ethanol, 0.005% DMSO) because these were not significantly different from untreated controls. Data shown are means of a minimum of five replicates from each treatment ± SD after arcsin transformation.

[a] PKC-activating phorbol esters.

[b] Inactive phorbol ester.

[c] Okadaic acid treatment was for 2 h.

[d] –, not done.

bol-12,13-dibutyrate were also effective, but the inactive phorbol ester 4α-phorbol was unable to effect such a change.

The effect of PKC down-regulation on desmosome adhesion was also examined by exposing confluent cells with Ca-Dep desmosomes to TPA for prolonged periods (up to 48 h). This treatment, which down-regulates PKC after initial activation, caused cells to acquire Ca-Ind desmosomes prematurely, even in subconfluent culture.

The effects of PKC inhibitors were also studied. The broad-spectrum PKC inhibitor chelerythrine and the conventional isoform inhibitor Gö6976 were individually applied to cells with Ca-Dep desmosomes. Both inhibitors caused transition to Ca-Ind desmosome adhesion (Table 2). In addition, these inhibitors blocked transition to Ca-Dep after TPA treatment and wounding (Table 3), and cells treated with these inhibitors before wounding were unable to acquire Ca-Dep desmosomes at a wound edge. The inhibitors also cause subconfluent cells to become Ca-Ind. These results show that PKC activity modulates desmosome adhesion, with activation promoting the Ca-Dep state and inhibition promoting Ca-Ind adhesion. Furthermore, the effect of Gö6976 implicates a conventional isoform of PKC (i.e., α, β_1, β_2, or γ).

The involvement of PKC suggests that changes in protein phosphorylation may be essential for the modulation process. To provide support for this view, the effect of the inhibition of phosphatase action was studied by treating cells with okadaic acid, an inhibitor of PP1 and PP2A protein phosphatases. Okadaic acid treatment was found to cause rapid conversion of desmosomes to the Ca-Dep state (Table 1), supporting the view that changes in protein phosphorylation are involved.

By contrast with the effects of PKC activators and inhibitors, a number of other reagents had no effect. Thus, conditioned medium from multiple wounded cultures did not cause any quantitative change in the Ca-Dep of desmosomal adhesion when treated cultures were compared with untreated control cultures, nor did a variety of growth factors applied to the basolateral or the apical domain of the cells, or both. These growth factors included HGS/SF, EGF, TGFα, insulin/insulin-like growth factor-1, aFGF, bFGF, and PDGF. Furthermore, acute burn blister fluid, a rich source of wound growth factors, had no effect. Finally, reagents that affect intracellular cAMP concentration (db-cAMP, forskolin, and 3-isobutyryl-1-methylxanthine) had no effect on desmosomal adhesion.

Table 2. Effect of PKC inhibitors on the state of desmosome adhesion in MDCK cells (1-d cultures, initially Ca-Dep)

	Treatment			
Duration	Control	Chelerythrine (8.7 μM)	Gö6976 (5 nM)	Gö6976 (10 nM)
15 min	4.0 ± 0.7	84.0 ± 7.3	–[a]	–
30 min	9.1 ± 1.7	–	–	–
60 min	5.5 ± 2.9	–	74.2 ± 13.5	90.0 ± 6.9

MDCK cultures were treated with drugs for the times indicated and then switched to drug-free LCM for 1 h. Values indicate percentage of Ca-Ind cells. Control column includes data for solvent vehicle treatment (e.g., 0.03% ethanol, 0.005% DMSO) because these were not significantly different from untreated controls. Data shown are means of a minimum of five replicates from each treatment ± SD after aresin transformation.

[a] –, not done.

Table 3. Effect of PKC inhibitors on the state of desmosome adhesion in MDCK cells (wounded 6-d cultures)

	Treatment		
Time	Wounded control	Chelerythrine (10 μM)	Gö6976 (10 nm)
7 h after wounding	47 ± 1.6	97.2 ± 7	95.5 ± 9

MDCK cultures were treated with drugs for the times indicated and then switched to drug-free LCM for 1 h. Data shown are percentages of confluent control values (mean of five replicates). Cell islands were formed by wounding 6-day-confluent monolayers in a grid pattern. The test substance was then applied for 7 h before LCM treatment began. Control treatments were performed by treating both islands and confluent cultures with vehicle for 7 h. The mean percentage of Ca-Ind cells in confluent controls was 89.5 ± 9.1.

Translocation of PKCα Correlates with Changes in the Calcium Sensitivity of Desmosomal Adhesion

The above results suggest that PKC is involved in intracellular signaling pathways that modulate desmosomal adhesion. Therefore, immunostaining and Western blotting were used to determine which isozymes of PKC were expressed by the MDCK cells used in this study. Both methods were able to identify PKCα, -δ, and -ζ in MDCK cells, whereas PKCβ and -ϵ were not detected (of these, α is a conventional isoform, δ is a novel isoform, and ζ is an atypical isoform).

Activation of PKC isozymes is often accompanied by a change in the distribution of the protein, so their locations were studied by immunostaining in cells with both Ca-Ind and Ca-Dep desmosomes. The distribution of PKCα was found to change during confluent culture and after wounding or treatment with drugs that affect PKC activity (Figure 6). Thus, in newly confluent cells, PKCα was found to be localized to the cell periphery (Figure 6A), but in cells with Ca-Ind desmosomes after confluent culture for 6 d, PKCα was no longer present at the membrane (Figure 6B). By contrast, PKCδ was always found in the cytoplasm, possibly associated with the cytoskeleton (our unpublished data), whereas PKCζ was always found at the cell periphery (Figure 6, C and D). Treatment of cells with Ca-Ind desmosomes with TPA caused PKCα to relocate to the cell periphery (Figure 6, E and F), and PKCα was present at the edges of lamellipodia. Conversely, treatment of cells with Ca-Dep desmosomes with PKC inhibitor caused PKCα to move from the cell periphery to the cytoplasm (our unpublished data). Wounding of 6-d-confluent cells caused an immediate (within 5 min) redistribution of PKCα to the cell periphery (Figure 6G). In addition, PKCα was found to be enriched at the leading edges of lamellipodia both at the edge of the sheet and submarginally (Figure 6H). Therefore, PKCα localization correlated with the calcium sensitivity of the desmosomes, being predominantly peripherally located and Triton X-100 insoluble when cells had Ca-Dep desmosomes but not when they had Ca-Ind desmosomes.

Western blotting was used to confirm this change in PKCα distribution. A cytosolic fraction released on cell homogenization, and Triton X-100–soluble and Triton X-100–insoluble protein fractions from the homogenization pellet, were prepared from newly confluent cells (Ca-Dep), 6-d-confluent cells (Ca-Ind), and 6-d-confluent cells that had been treated with solvent vehicle (DMSO) (Ca-Ind), treated with TPA (Ca-Dep), or wounded (Ca-Dep). Equal amounts of protein were then separated on 7% polyacrylamide gels, blotted for PKCα, and quantified (Figure 7). In 1-d-confluent cells with Ca-Dep desmosomes, substantial amounts of PKCα were associated with both the Triton X-100–soluble and Triton X-100–insoluble fractions (Figure 7, lane 1), but in 6-d-confluent cells with Ca-Ind desmosomes, the amount of PKCα in these fractions was greatly diminished (Figure 7, lane 2). Activation of PKC in 6-d-confluent cells by TPA treatment (Figure 7, lane 4) and multiple wounding of the monolayer (Figure 7, lane 5) caused increases in the amounts of PKCα in both Triton X-100 fractions, whereas treatment of cells with the TPA solvent vehicle, DMSO, caused no change in PKCα distribution (Figure 7, lane 3). These results are consistent with the results obtained by immunofluorescence of Triton X-100–extracted cells.

Relocalization of PKCα upon Wounding Precedes the Increase in PKC Activity and the Change of Desmosomes to Ca-Dep

From the experiments described above it appears that the relocalization of PKCα to the cell periphery after wounding occurs rapidly. To determine whether this relocalization coincides with or precedes the change of desmosomes to Ca-Dep, PKCα distribution in wounded monolayers was studied in more detail. It was found that PKCα became rapidly redistributed not only at the wound edge but also in cells deeper within the monolayer. The distribution of PKCα at 15 min after wounding is shown in Figure 8, A–C. The progression of the change to Ca-Dep from the wound edge occurred more slowly than this (Figures 5 and 8C).

To determine whether PKC activity in the insoluble fraction increased after wounding and whether the time course corresponded with PKCα relocalization, the activity of PKC was measured at intervals in multiply wounded monolayers. The results (Figure 9) show that PKC activity does increase in the insoluble membrane fraction after wounding, but more slowly than expected from the relocalization of PKCα. Thus, by 1 h after wounding changes in activity were hardly measurable, but thereafter a progressive increase was found in the insoluble membrane fraction and a progressive decrease was found in the cytosolic fraction.

Specific Depletion of PCKα Modulates Desmosomal Adhesion

The above evidence implicates PKCα as a regulator of desmosome adhesion because (1) its location in the cell correlates with the calcium sensitivity of the desmosomes, and (2) inhibition of conventional isoforms with the use of Gö6976 causes desmosomes to become Ca-Ind, and PKCα is the only detectable conventional isoform in MDCK cells. To provide direct evidence for the role of PKCα, antisense oligonucleotides were used to deplete PKCα specifically from the cells, and the consequences for the desmosomal adhesion state were investigated.

Canine PKCα had not been sequenced, but rabbit, human, and rat sequences were available. Therefore, the 5′ end of

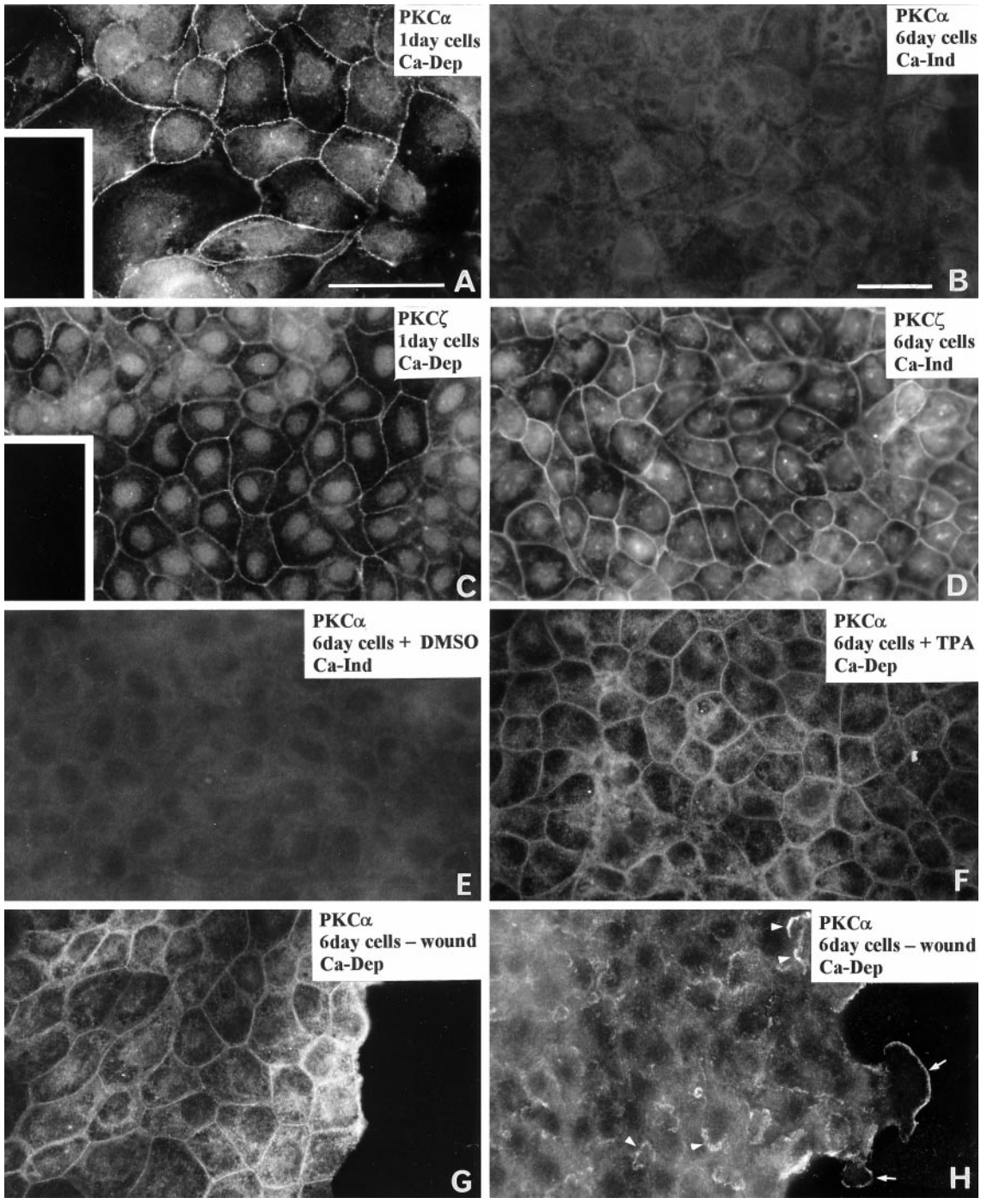

Figure 6. The cellular distribution of PKCα but not other PKC isoforms correlates with desmosomal adhesion state. MDCK cells with desmosomal adhesion in either the Ca-Dep or Ca-Ind state were extracted with 0.5% Triton X-100, fixed with methanol, and stained with specific antibodies to PKC isoforms α (A, B, E, F, G, and H) and ζ (C and D). (Ca-Dep and Ca-Ind of desmosomes was tested on parallel cultures.) (A and C) One-day-confluent cells. (B, D, E, and F) Six-day-confluent cells. (G and H) Edges of 1-h-old wounds in 6-d-confluent cell sheets; G is focused on the junctional region and H is focused on the substratum. (E) Cells treated with DMSO for 1 h. (F) Cells treated with 5 nM TPA for 1 h. Insets in A and C show that antibody staining was abolished by previous incubation with the isoform-specific peptide to which the antibody was raised. (A–D) The cell-peripheral distribution of PKCα is lost in cells that have acquired Ca-Ind desmosomes, but the distribution of PKCζ does not change. (E and F) Treatment with TPA, but not DMSO vehicle control, restores Ca-Dep desmosomes and the cell-peripheral distribution of PKCα. (G) Wounding causes relocation of PKCα to the cell periphery. (H) Wound edge, with the microscope focused on the basal cell surface; note PKCα staining at the leading edges of lamellipodia both at the edge of the sheet (arrows) and submarginally (arrowheads). Bar, 20 μm.

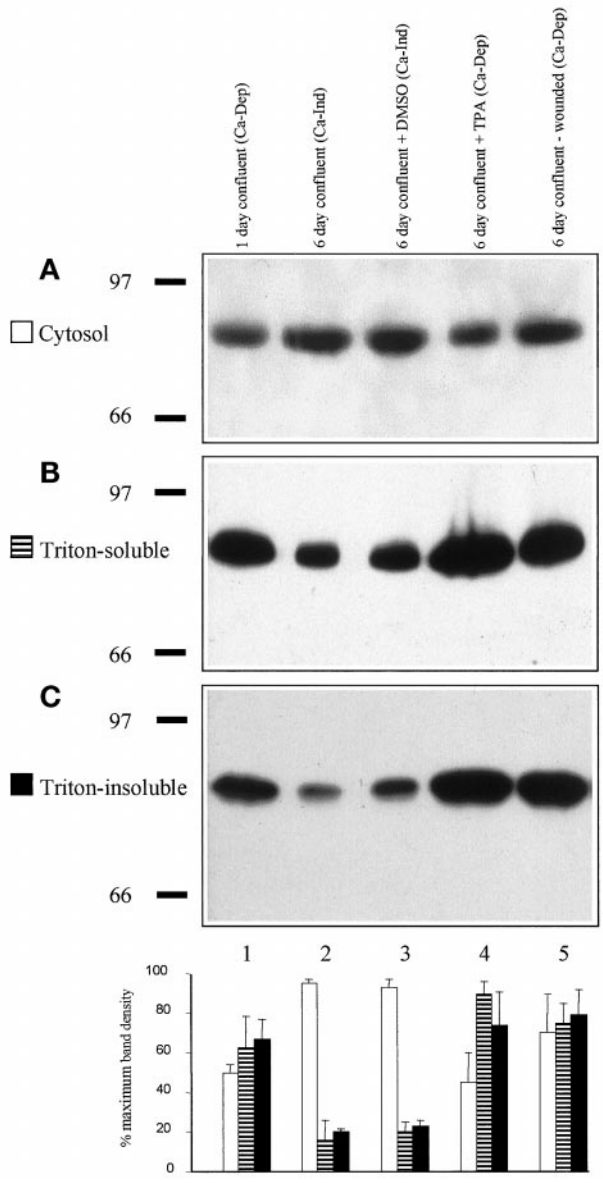

Figure 7. Increase of PKCα in the Triton X-100–soluble and Triton X-100–insoluble fractions correlates with Ca-Dep of desmosomes. Immunoblotting for PKCα of cytosolic (A), Triton X-100–soluble (B), and Triton X-100–insoluble (C) fractions prepared from cells that had been cultured and treated as follows: lane 1, 1-d-confluent cells (Ca-Dep desmosomes); lane 2, 6-d-confluent cells (Ca-Ind desmosomes); lane 3, 6-d-confluent cells treated with DMSO for 1 h (Ca-Ind desmosomes); lane 4, 6-d-confluent cells treated with 5 nM TPA for 1 h (Ca-Dep desmosomes); lane 5, 6-d-confluent cells from cultures extensively wounded 1 h previously (mostly Ca-Dep desmosomes). In each case, the five lanes were equally loaded. Molecular weight markers (numbers) $\times 10^3$. Chart shows quantification of band density (pixel intensity) from two immunoblots. Each triplet of bars lies below the appropriate gel lane. (White bars) Cytosolic fraction. (Striped bars) Triton X-100–soluble fraction. (Black bars) Triton X-100–insoluble fraction. Note that the presence of greater amounts of PKCα in the Triton X-100–insoluble fraction correlate with the presence of Ca-Dep desmosomes (lanes 1, 4, and 5) and smaller amounts correlate with the presence of Ca-Ind desmosomes (lanes 2 and 3).

canine PKCα was cloned with the use of reverse transcription-PCR with primers based on homology to these sequences. Sequence analysis of the 147-bp insert revealed that it was 94.6% identical to human, 96.6% identical to rat, and 74.2% identical to rabbit PKCα in this region (Figure 10). An antisense oligonucleotide was designed against the first 18 bp from and including the AUG start codon of PKCα cDNA. Sense and nonsense (same bases but scrambled so that they recognized no known sequences) oligonucleotides were also designed.

Transfection of the antisense oligonucleotide into freshly confluent MDCK cells resulted in depletion of PKCα to 29.8% of the level in untreated and nonsense or sense oligonucleotide-treated cells after 48 h (Figure 11A). No changes in the levels of PKCδ or PKCζ were seen (Figure 11, B and C). This resulted in a 20% increase in the proportion of cells with Ca-Ind desmosomes (Figure 11). This was the maximum increase possible because >70% of control cells already had Ca-Ind desmosomes after 48 h of confluent culture. In four similar experiments, a mean increase of 18.7% (SE = 0.56%) was found. This was highly significantly different from nonsense controls by paired t test ($p < 0.001$). For reasons that are not clear, attempts to carry out similar experiments on subconfluent cells resulted in extremely poor transfection efficiencies and, consequently, minimal depletion of PKCα. The results obtained with confluent cells show that the proportion of cells with Ca-Ind desmosomes increased after the specific depletion of PKCα, thus confirming a role for PKCα in regulating desmosomal adhesion.

DISCUSSION

These results demonstrate several important novel aspects of epithelial cell adhesion. (1) They provide the first, and possibly unique, example of an adhesion system that is reversibly modulated between Ca-Dep and Ca-Ind. (2) They show that desmosomal adhesion may be rapidly modulated in response to wounding and that a modulating signal generated at the free edge can be propagated through the cell sheet. (3) They demonstrate that the α isoform of PKC is involved in a signaling pathway that results in modulation of desmosomal adhesions (summarized in Figure 12).

Our observations on mouse tissues indicate that desmosomal adhesion in intact cell sheets is normally Ca-Ind. Borysenko and Revel (1973) showed that desmosomes in some tissues were Ca-Ind and others were disrupted by prolonged treatment with EDTA. Similar observations have been reported for colonic mucosa (Collins *et al.*, 1990). In our experiments, the Ca-Dep state of desmosomes was associated with areas where cell motility would be expected to be enhanced (subconfluent monolayers and wound edges), whereas Ca-Ind was associated with less motile cells in confluent sheets. We suggest that Ca-Dep desmosomes may be more readily broken and reformed than Ca-Ind desmosomes, which stabilize adhesion in cell sheets. Rapid reversion to Ca-Dep at the wound edge and the propagation of this effect are thus seen as important components of a response that may facilitate reepithelialization.

Involvement in a signaling pathway that modulates desmosomal adhesion in response to wounding is a novel role for the α isoform of PKC. The evidence in support of this involvement is: (1) phorbol esters that activate conventional

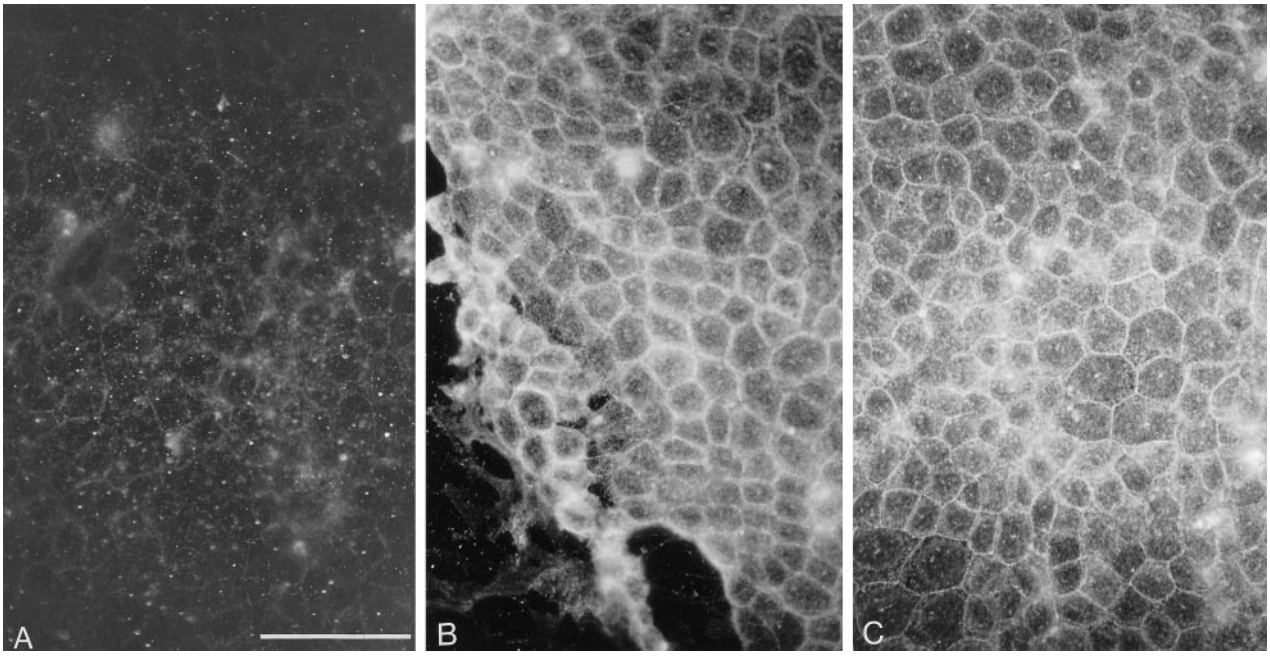

Figure 8. Relocalization of PKCα to the cell periphery after wounding precedes the change of desmosomes to Ca-Dep. A–C show monolayers of MDCK cells extracted with Triton X-100, fixed with methanol, and stained for PKCα. (A) Confluent 6-d monolayer (parallel culture showed 98% Ca-Ind cells) showing the absence of PKCα from the cell periphery. (B and C) Confluent 6-d monolayer 15 min after wounding showing the localization of PKCα to the cell periphery both at the edge of the wound (B) and one microscope field within the monolayer (C). At this stage, only one or two rows of cells at the edges of comparable wounds show reversion to Ca-Dep, which shows cells at the edge of a wound in a parallel culture after LCM treatment stained for desmoplakin. Note that at most only two rows of cells at the edge show reversion to Ca-Dep. Bar, 50 μm.

isoforms of PKC, including the α isoform, change desmosomes to Ca-Dep (Stabel and Parker, 1991); (2) Gö6976, which inhibits conventional PKC isoforms (Martiny-Baron *et al.*, 1993), changes desmosomes to Ca-Ind; (3) localization of PKCα to a cell-peripheral, Triton X-100–insoluble fraction correlates with desmosome Ca-Dep; and (4) specific depletion of PKCα by transfection with antisense oligonucleotide promotes desmosome Ca-Ind. Consistent with our results, Godson *et al.* (1993) have found translocation of PKCα to the membrane of MDCK cells after TPA treatment.

Targeting within the cell is a crucial aspect of the regulation of PKC and other kinases (Mochly-Rosen, 1995; Colledge and Scott, 1999). Clearly, a specific effect on cell junctions requires that the kinase concerned should be localized at the cell periphery. The translocation of PKCα that we have found is clearly consistent with the necessity to regulate cell junctions in response to wounding. It was surprising to find that the relocalization of PKCα after wounding precedes both the increase in PKC activity in the membrane fraction and the reversion of desmosomes to Ca-Dep. This suggests that different signals are involved in targeting and activation of PKCα.

Although strongly supporting the involvement of PKCα in these events, our results do not rule out the involvement of other PKC isoforms. For example, the cell-peripheral location of PKCζ suggests that it is suitably placed to regulate junctional adhesion. However, the latter isoform is neither activated by phorbol esters nor inhibited by Gö6976, and it did not show altered localization in association with desmosomal adhesive changes.

A number of previous studies have demonstrated a role for PKC, in some cases specifically PKCα, in relation to cell adhesion and wounding. Thus, PKC has been shown to be involved in the regulation of adhesion of focal contacts (Woods and Couchman, 1992; Vuori and Ruoslahti, 1993) and regulation of the affinity of binding of LFA-1 to I-CAM1 (Dustin and Springer, 1989). PKC activation has also been implicated in the initial assembly of desmosomes and other epithelial junctions (Kitajima *et al.*, 1988; Sheu *et al.*, 1989; Balda *et al.*, 1991; Pasdar *et al.*, 1992; Sneyd *et al.*, 1994; van Hengel *et al.*, 1997). Translocation of PKCα from focal contacts to lamellipodia after wounding was seen in REF52 rat embryo fibroblasts (Liao and Jaken, 1993). Association of PKCα with membrane ruffles has been induced by phorbol ester treatment in rat renal proximal tubule epithelial cells, in which it was reported to be Triton X-100 soluble (Dong *et al.*, 1993), and in E10 mouse lung epithelial cells (Dwyer *et al.*, 1996) and VACO 10MS human colon carcinoma cells, in which it was reported to be Triton X-100 insoluble (Cowell and Garrod, 1999). Interestingly, Osada *et al.* (1997) have shown translocation of several PKC isoforms, including α, to the particulate fractions of human keratinocytes upon treatment with pemphigus autoantisera, which weaken intercellular adhesion. Lin and Brazen (1995) have demonstrated specific activation of PKCα in wounded corneal epithelium. The latter results, together with our results, lead us to pro-

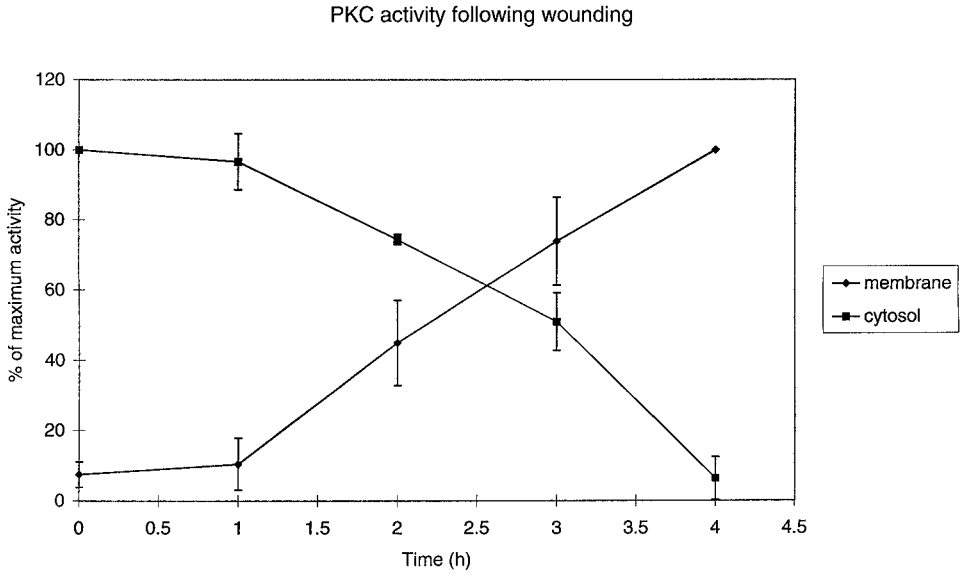

Figure 9. Change in PKC activity after wounding. Multiple wounds were made in a grid pattern in dishes of 6-d-confluent MDCK cells, and PKC activity was assayed at intervals after wounding in the soluble cytosolic fraction and the particulate membrane fraction. The activity progressively decreases in the cytosolic fraction and increases in the membrane fraction. The data shown represent the combined results from three identical experiments, in each of which four 9-cm dishes of cells were used for each point. The bars represent SEs. The results are shown as percentage of maximum activity because the specific activity of ^{32}P varied between experiments. The mean cpm in the cytosol fraction was 2878 at time 0 and 663 at 4 h, and the mean cpm in the membrane fraction was 282 at time 0 and 3534 at 4 h.

pose that PKCα is an important component of the signal transduction process that leads to cell migration into epithelial wounds. It appears to be associated with both the activation of a motile organelle, the lamellipodium, and the modulation of stable cell–cell adhesions.

It has recently been shown that PKCζ is involved in integrin-dependent adhesion and chemotaxis of polymorphonuclear neutrophils (Laudanna *et al.*, 1998). In these studies, the conventional PKC isoform inhibitor Gö6976 did not inhibit adhesion or chemotaxis but the broad-spectrum inhibitor chelerythrine chloride was effective, suggesting that an atypical PKC isoform was involved. This was confirmed by treatment of cells with synthetic myristoylated peptide with sequence based on the endogenous PKCζ pseudosubstrate region. Chemoattractants induced rapid translocation of PKCζ to the plasma membrane of the neutrophils. In this case, as in our study, the mechanism by which PKCζ regulates these functions is not clear.

Our results provide a new insight into the mechanism by which desmosomal adhesion may be regulated. How might PKCα signaling give rise to adhesive changes? Several accessory proteins can be recruited to desmosomes as monolayers mature. For example, the phosphoprotein pinin associates with the plaques of mature desmosomes (Ouyang and Sugrue, 1992, 1996). However, pinin recruitment occurs in subconfluent MDCK cultures (Ouyang and Sugrue, 1992) when desmosomes do not become Ca-Ind, and doubt has recently been cast on the desmosomal role of pinin (Brandner *et al.*, 1997). The microtubule-binding protein pp170 localizes to desmosomes in mature monolayers (Wacker *et al.*, 1992), and plectin associates with desmosomes in polarized MDCK cells (Eger *et al.*, 1997). However, we have found the PKC inhibitors cause desmosomes to become rapidly (within 15 min) Ca-Ind, even in immature, subconfluent monolayers. This is unlikely to be sufficient time for new protein synthesis, and the transition is not prevented by cycloheximide, although recruitment of existing proteins is conceivable. The rapid effects of PKC activators and inhibitors, and the phosphatase inhibitor okadaic acid, on the state of desmosomal adhesion suggest that direct signaling events involving protein phosphorylation/dephosphorylation are involved. The desmosomal proteins and glycoproteins are good candidates for phosphorylation targets. However, it is possible that the major desmosomal components are not the primary phosphorylation targets involved in these events. Thus, keratins 8 and 18, the predominant keratins of MDCK

Antisense oligo 1

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Dog      ATGGCTGACGTTTATGCGACCAACGACTCCACGGCGTCTCAGGACGTGGCCAACCGCTTCGCCCGCAAAGGGGCG
Human    ATGGCTGACGTTTTCCCGGGCAACGACTCCACGGCGTCTCAGGACGTGGCCAACCGCTTCGCCCGCAAAGGGGCG
Rat      ATGGCTGACGTTTACCCGGCCAACGACTCCACGGCGTCTCAGGACGTGGCCAACCGCTTCGCCCGCAAAGGGGCG
Rabbit   ATGGCTGACCCGGCTGCGGGGCAGCCGCCGAGCGAGGGCGAGGAGAGCACCGTGCGCTTCGCCCGCAAAGGCGCC

Dog      CTGAGGCAGAAAAACGTGCACGAGGTGAAGGACCACAAATTCATCGCCCGCATCTTCAAGCAGCCCCACCTTC
Human    CTGAGGCAGAAGAACGTGCATGAGGTGAAGGACCACAAATTCATCGCCCGCTTCTTCAAGCAGCCCCACCTTC
Rat      CTGAGGCAGAAGAACGTGCACGAGGTGAAAGACCACAAATTCATCGCCCGCTTCTTCAAGCAACCCCACCTTC
Rabbit   CTCCGGCAGAAGAACGTGCACGAGGTGAAGAACCACAAATTCACCGCCCGCTTCTTCAAGCAGCCCCACCTTC
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Figure 10. Alignment of the first 160 bp of dog PKCα cDNA sequence (GenBank accession number AF096837) with the human, rat, and rabbit sequences. Boldface letters indicate nucleotides that differ from those in the dog sequence. The antisense oligonucleotide used in the experiments depicted in Figure 11 is indicated.

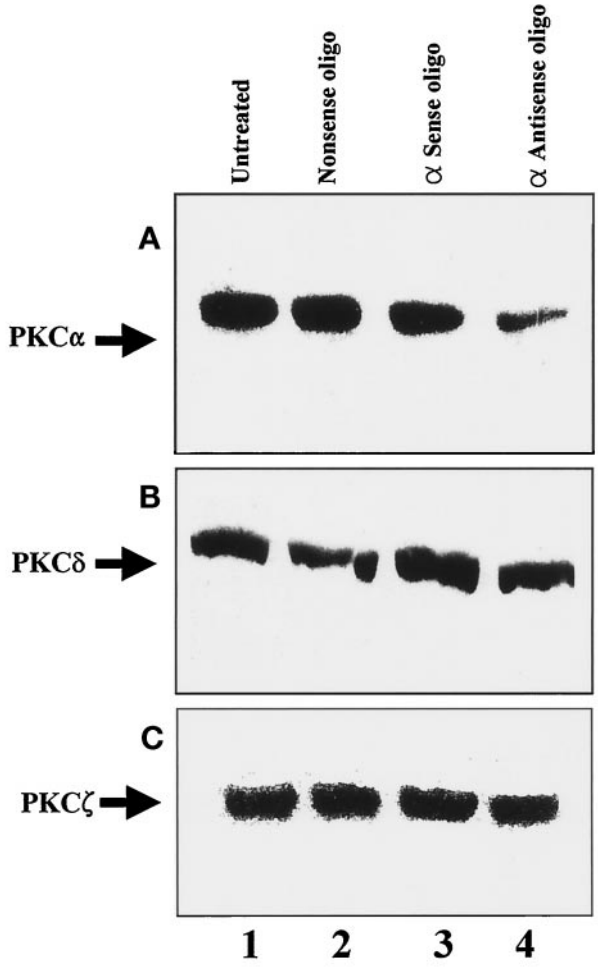

Figure 11. Depletion of PKCα increases the proportion of cells with Ca-Ind desmosomes. MDCK cells were seeded at confluent density and, when attached, were treated with 1 μM PKCα antisense oligonucleotide with the use of Superfect reagent. Control cells were untreated or treated with sense or nonsense oligonucleotide. After 48 h, cultures were lysed in sample buffer for blotting with antibodies to PKCα, -δ, and -ζ, whereas parallel cultures were used to quantify Ca-Ind of desmosomes. (Lane 1) Untreated cell; (lane 2) nonsense oligonucleotide–treated cell; (lane 3) sense oligonucleotide–treated cell; (lane 4) antisense oligonucleotide–treated cell. Blots show PKCα (A), PKCδ (B), and PKCζ (C). The table shows quantification of the immunoblots and determination of Ca-Ind in parallel cultures. The experiment was repeated on four separate occasions. There was a mean 18.7% increase of cells with Ca-Ind desmosomes in cells transfected with the antisense oligonucleotide compared with cells transfected with the nonsense oligonucleotide. The difference was highly significant ($p < 0.001$) by paired *t* test.

Oligo	Band density (% of maximum for each antibody)			Proportions of cells with Ca-Ind desmosomes (%), (± S.E)
	PKCα	PKCδ	PKCζ	
Untreated	100	95.2	100	72.4 ± 2.3
Nonsense	93.4	90.2	98.3	74.7 ± 2.2
α sense	81.9	100	99.0	75.7 ± 2.0
α antisense	29.8	94.0	98.1	94.0 ± 1.2

cells, can be hyperphosphorylated by PKC (Yano *et al.*, 1991; Ku and Omary, 1994), and EGF-induced serine phosphorylation of keratins 8 and 18 caused reorganization of the cytoskeleton in rat hepatocytes (Baribault *et al.*, 1989). Further investigations to identify phosphorylation targets associated with changes in desmosomal adhesion are currently in progress. Study of the phosphorylation of proteins that are incorporated into desmosomes is not straightforward because of the great insolubility of these junctions.

Wounding of mature monolayers of MDCK cells caused cells at the wound edge to acquire Ca-Dep desmosomes. This change in desmosomal adhesion is rapid, being detectable within 1 h of wounding, and thus can be regarded as part of the "kick-starting" response. This response appears to be triggered in some way by the sudden creation of a free edge within the confluent monolayer (i.e., sudden loss of confluence, the condition required for the development of Ca-Ind). The change was then transmitted to neighboring

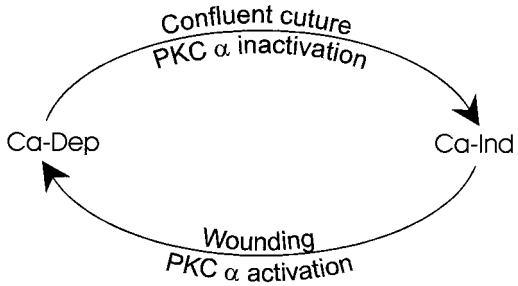

Figure 12. Diagram showing factors that change the adhesive state of desmosomes.

cells, thus being propagated across the monolayer. We considered that release of a diffusible factor by damaged cells could cause these changes but could find no evidence for such a factor in wound-conditioned medium. Furthermore, a number of growth factors were unable to affect the state of desmosomal adhesion. These growth factors may activate PKC, but we found that none of them caused translocation of PKCα to the cell periphery (our unpublished data). This may explain why they were unable to modulate desmosomal adhesion. Our data do not exclude the possibility that a diffusible factor passes within the extracellular space between cells, beneath the tight junctions. Another possibility is that signals pass from cell to cell through gap junctions. Indeed, it has been suggested that propagation of calcium waves across cell monolayers from a point of mechanical stimulation takes place via gap junctions (Charles *et al.*, 1992; Sneyd *et al.*, 1994). Our preliminary data show that calcium ionophores and the internal calcium chelator bis-(*o*-aminophenoxy)-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) have small and opposite effects on desmosomal adhesion [ionophores promote Ca-Dep; BAPTA promotes Ca-Ind], suggesting that this is a promising area for further investigation.

In conclusion, we have described a new phenomenon, the rapid modulation of the adhesive state of desmosomes in response to wounding of an epithelial cell sheet. Furthermore, we have discovered a type of PKCα-mediated "inside-out" signaling that modulates desmosomal adhesion. This may be functionally important in embryonic development, wound healing, cancer metastasis, and tissue dynamics, e.g., in relation to desmosome turnover in tissues such as epidermis (North *et al.*, 1996). Our results provide a new system to study how such a change can be signaled and propagated and an important illustration of how the properties of cells in a tissue-like organization, an epithelial cell sheet, differ from those in isolated cell groups, which are used for the majority of studies of epithelial cell behavior in culture.

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