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E-cadherin mediates adherens junction organization through protein kinase C

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SUMMARY

Cultured human keratinocytes maintained in 30 μM Ca^{2+} do not form adherens junctions; however, when the extracellular Ca^{2+} concentration is raised to 1 mM, adherens junctions form very rapidly. The formation of a junction involves the coordinate organization of intracellular and extracellular components. Cadherins have been shown to mediate this coordinate organization. In this report we show that E-cadherin organizes the various junctional components by signalling through protein kinase C.

Key words: cadherin, keratinocyte, protein kinase C

INTRODUCTION

The adherens junction is an intercellular adhesion complex that is composed of a transmembrane protein (a cadherin) and numerous cytoplasmic proteins (α, β and γ-catenins, vinculin and α-actinin; see Takeichi, 1990, and Geiger and Ayalon, 1992, for reviews). The cadherins are directly responsible for adhesive interactions via a Ca^{2+}-dependent, homotypic mechanism; i.e. in the presence of sufficient Ca^{2+}, cadherin on one cell binds to an identical molecule on an adjacent cell. Cytoplasmic components of the adherens junction link the cadherin to actin filaments, thereby linking the cytoskeleton to the plasma membrane.

In cultures of human keratinocytes maintained in medium with 30 μM Ca^{2+}, no adherens junctions are present. However, elevation of the Ca^{2+} concentration to 1 mM induces the rapid organization of adherens junctions from preformed molecular components (O'Keefe et al., 1987; Green et al., 1987). This organization of adherens junctions can be demonstrated by the rapid redistribution of junctional components from a diffuse arrangement to a distinctive localization at cell-cell borders as well as by reorganization of the actin cytoskeleton (O'Keefe et al., 1987; Green et al., 1987; Zamansky et al., 1991; Wheelock and Jensen, 1992).

Little is known about how Ca^{2+} elevation and/or cadherin engagement lead to the redistribution of the cytoplasmic constituents of the adherens junction and the reorganization of the actin cytoskeleton; however, previous experiments from our laboratories and others strongly suggest that E-cadherin plays a distinct regulatory role (Wheelock and Jensen, 1992; Gumbiner et al., 1988). Antibodies that block E-cadherin homotypic interaction delay by several hours the Ca^{2+}-induced redistribution of adherens junction components, as well as the reorganization of the actin cytoskeleton (Wheelock and Jensen, 1992). In contrast, blocking antibodies against P-cadherin, the other cadherin present in squamous epithelial cells, have no demonstrable effect on the redistribution of any adherens junction components (Lewis et al., 1994).

The potential role of cadherin-generated intracellular signals that may direct the organization of the multicomponent adherens junction into a functional structure has not been explored; however, cadherins and other adhesion molecules have been implicated in signal transduction in a number of systems. One study (Winkel et al., 1990) suggested that protein kinase C (PKC) activation induces premature compaction in the mouse embryo by affecting E-cadherin. In addition, PKC has been implicated not only in the assembly but also in the disassembly of tight junctions (Balda et al., 1993; Citi, 1992; Citi et al., 1994). N-cadherin has been shown to influence cellular morphology directly through transmembrane signalling in PC12 cells (Doherty et al., 1991). These authors suggested that N-cadherin and NCAM signal neurite outgrowth through G-protein-dependent activation of Ca^{2+} channels. There is a wealth of evidence to show that integrins transmit signals across the plasma membrane, resulting in local regulation of cell adhesion and reorganization of the actin cytoskeleton (see Gumbiner, 1993, and Damsky and Werb, 1992, for reviews). Integrin signal transduction is mediated by tyrosine phosphorylation, presumably due to the association of the integrin molecule with cellular kinases. In all, several very different signalling pathways have been implicated in cell-cell and cell-substratum adhesive events. In the present study we tested the hypothesis that the regulatory role of E-cadherin in Ca^{2+}-induced adherens junction organization in cultured keratinocytes may be mediated through activation of PKC.

MATERIALS AND METHODS

Cell culture

Human keratinocytes from neonatal foreskins were propagated in

MCDB 153 medium (Sigma Chemical Co., St Louis, MO) with 30 μM Ca^{2+}, and the following additives: bovine pituitary extract (pituitaries from Pelfreez Biological, Rogers, AK), insulin, hydrocortisone (Sigma Chemical Co.), epidermal growth factor (Collaborative Research, Bedford, MA), and high amino acids, as previously described (Shipley and Prittlekow, 1987; McNeill and Jensen, 1990; Wheelock and Jensen, 1992). Cultures were used between the first and third passages. The medium was replaced every other day and cells were passaged when almost confluent. For antibody blocking studies, cells were grown on glass coverslips in Corning 24-well dishes. Cells were plated at a density of 2.5×10^4 cells in 1 ml complete medium per well. When the cells were almost confluent, the medium was removed and 300 μl of the appropriate antibody in complete medium was added.

Antibodies

Production of rabbit polyclonal antiserum against human E-cadherin (Wheelock et al., 1987) and mouse monoclonal antibody against P-cadherin have been previously described (Lewis et al., 1994). Anti-human vinculin was purchased from Sigma Chemical Co.

Antibody blocking

Rabbit anti-E-cadherin IgG was purified on Protein A-Sepharose (Pharmacia, Piscataway, NJ) and diluted to 20 μg/ml in complete MCDB 153 containing 30 μM Ca^{2+}. After an overnight incubation with IgG, the Ca^{2+} concentration was elevated to 1 mM and incubation was continued for the indicated length of time.

Immunofluorescence

Keratinocytes were plated in complete medium on glass coverslips and grown until almost confluent. Ca^{2+} was elevated to 1 mM for the indicated length of time. Cells were fixed in 1% paraformaldehyde buffered with Hanks' balanced salt solution (Gibco Laboratories, Grand Island, NY) and 10 mM HEPES (Sigma Chemical Co.) and then permeabilized and blocked by incubating in phosphate buffered saline containing 10% goat serum, 0.1 M glycine and 0.05% saponin. Coverslips were then stained with the primary antibodies for 1 hour followed by fluorescein-conjugated anti-IgG (Jackson Immunoresearch Laboratories, West Grove, PA). Actin was visualized with rhodamine-conjugated phalloidin, diluted as recommended by the manufacturer (Molecular Probe, Eugene, OR). Fluorescence was detected with a Zeiss Axiophot microscope equipped with epifluorescence and filters appropriate for visualizing rhodamine or fluorescein and photographed using T-Max 3200 film. Pictures were taken using a ×40 (Figs 1, 2, 4) or ×100 (Fig. 3) objective.

Protein kinase activators and inhibitors

Phorbol-12,13-dibutyrate (PDBu, Gibco BRL, Gaithersburg, MD) was diluted to 1 mg/ml in dimethylsulfoxide (DMSO) and stored at −70°C. The working concentration was 18 nM. 12-*O*-tetradecanoylphorbol-13-acetate (PMA, Calbiochem, San Diego, CA) was diluted to 20 μM in ethanol and stored at −20°C. The working concentration was 1 nM. 4α-Phorbol-12,13-dibutyrate and 4α-12-*O*-tetradecanoylphorbol-13-acetate (Gibco BRL) were diluted to 1 mg/ml in DMSO, stored at −70°C and used at the same concentrations as the active phorbol esters. 1,2-Dioctanoyl-*sn*-glycerol (DiC8, Molecular Probes, Inc., Eugene, Oregon) was diluted to 50 mg/ml in DMSO, stored at −70°C and used at a concentration of 0.5 mM. 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7, Calbiochem) was diluted to 25 mM in distilled water, stored at 4°C and used at 400 μM. For PKC down-regulation, cells were treated with PDBu at 360 nM overnight (Ando et al., 1993).

RESULTS

Effect of PKC activation at low Ca^{2+}

In the first series of experiments the effect of PKC activation on distribution of adherens junction components at low (i.e. 30 μM) Ca^{2+} was examined. In control keratinocytes maintained at low Ca^{2+}, adherens junction proteins including E-cadherin, P-cadherin, α, β and γ-catenins, vinculin and α-actinin were diffusely localized throughout the cell (Wheelock and Jensen, 1992; see Fig. 1A and B for E-cadherin and P-cadherin distribution). Upon activation of PKC with phorbol esters (either 12-*O*-tetradecanoylphorbol-13-acetate (PMA) or phorbol-12,13-dibutyrate (PDBu)) or with 1,2-dioctanoylglycerol (diC8, a diacylglycerol analog), E-cadherin (Figs 1E, 2A), the catenins (not shown), vinculin (Fig. 3F) and α-actinin (not shown) were all transiently redistributed to regions of cell-cell contact. This redistribution resembled the pattern observed upon elevation of the Ca^{2+} concentration to 1 mM (Fig. 1C,D). Redistribution induced either by Ca^{2+} elevation or by PKC activation in low Ca^{2+} was rapid, being apparently complete within 30-60 minutes. However, while Ca^{2+}-induced redistribution was sustained indefinitely, redistribution induced by PKC activation was transient; two hours after PKC activation, no cell-cell border staining for any of the markers was observed. Importantly, H7, an inhibitor of PKC, blocked the redistribution of E-cadherin in response to PMA (Fig. 2B), consistent with the hypothesis that the effect of PMA is mediated through PKC activation. In further support of this hypothesis, the inactive isomers of PMA (αPMA) or PDBu (αPDBu) had no effect on the cells. Interestingly, in contrast to the other adherens junction components, P-cadherin distribution did not change when PKC was activated (Fig. 1F). Furthermore, desmosome components were not redistributed when PKC was activated in medium with 30 μM Ca^{2+} (data not shown).

PKC activation at low Ca^{2+} also affected the organization of the actin cytoskeleton (Fig. 3C, D). When PDBu was added to cells in low Ca^{2+}, the actin filaments were reorganized to form a peripheral band, similar but not identical to the pattern observed at 1 mM Ca^{2+} (see below).

The data presented above indicate that PKC activation at low Ca^{2+} caused a change in the localization of adherens junction components, including E-cadherin, cytoplasmic components and actin filaments. Although the patterns observed were similar to those elicited by elevation of the extracellular Ca^{2+} concentration, examination at higher magnification revealed that they were not identical. Specifically, at high Ca^{2+} concentration, short actin filaments extending from the peripheral actin bands were lined up between adjacent cells and appeared to insert into the broad band of E-cadherin that was found at the cell periphery (Fig. 3C, arrow). In cells treated with PDBu at low Ca^{2+}, these short actin filaments between cells were not observed (Fig. 3D), and E-cadherin was present as a thinner line at the cell periphery (Fig. 3B). There was also a difference in the distribution of vinculin, a cytoplasmic component of the adherens junction. At high Ca^{2+} concentration, vinculin appeared at cell-cell borders as a broad band resembling the E-cadherin band (Fig. 3E, arrow), a pattern consistent with its being organized, along with E-cadherin and actin, into adherens junctions. Activation of PKC at low Ca^{2+} concentration resulted in a thin vinculin band that appeared to be just inside the plasma membrane; in adjacent cells, vinculin was detected as a double line resembling railroad tracks (Fig. 3F, arrow). These pattern differences suggest that although PKC activation at low Ca^{2+} concentration can induce translocation of adherens junction components to the cell periphery, it

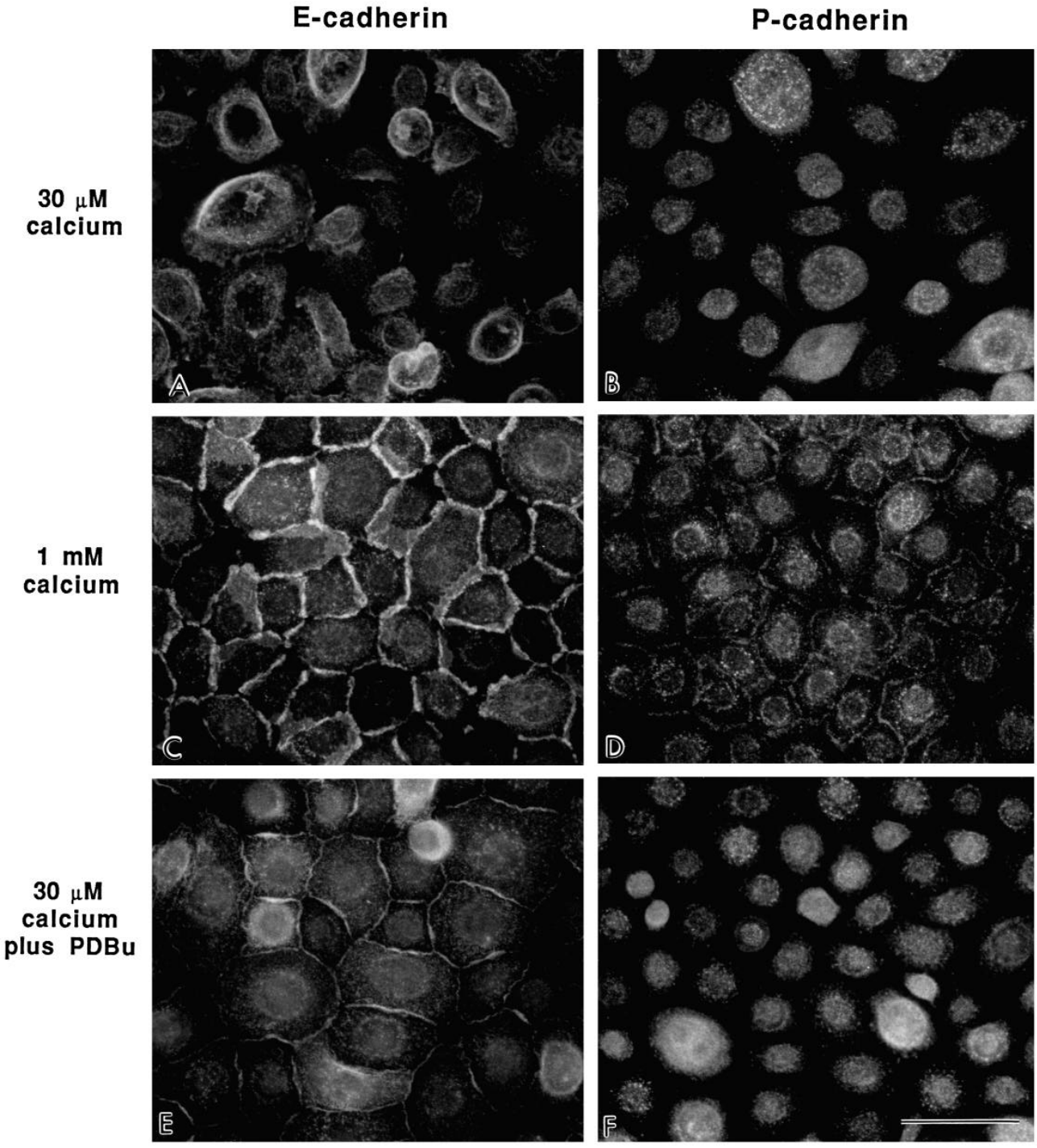

Fig. 1. Activation of PKC results in redistribution of E-cadherin but not P-cadherin. E-cadherin (A, C, E) or P-cadherin (B, D, F) was localized with immunofluorescence before treatment (A, B), after a 1 hour incubation in 1 mM Ca^{2+} (C, D), or after a 1 hour incubation in 30 μM Ca^{2+} containing 18 nM PDBu (E, F). Cell-cell border distribution for E-cadherin was observed when cells were treated with either Ca^{2+} or PDBu; a similar pattern was found when cells were incubated with PMA. In contrast, P-cadherin redistribution was observed only after Ca^{2+} elevation. Bar, 100 μm.

cannot allow proper organization of these components into a functional adherens junction.

Involvement of PKC in Ca^{2+}-induced adherens junction organization

The results presented thus far suggest that PKC activation can mediate the translocation of adherens junction components to the cell periphery, thereby generating staining patterns that are similar, but not identical, to those observed upon elevation of the extracellular Ca^{2+} concentration to 1 mM. These findings raise the possibility that PKC might mediate Ca^{2+}-induced adhesion molecule redistribution. To test this hypothesis, PKC was down-regulated by an overnight incubation in PDBu under low Ca^{2+} conditions (Ando et al., 1993); Ca^{2+} was then

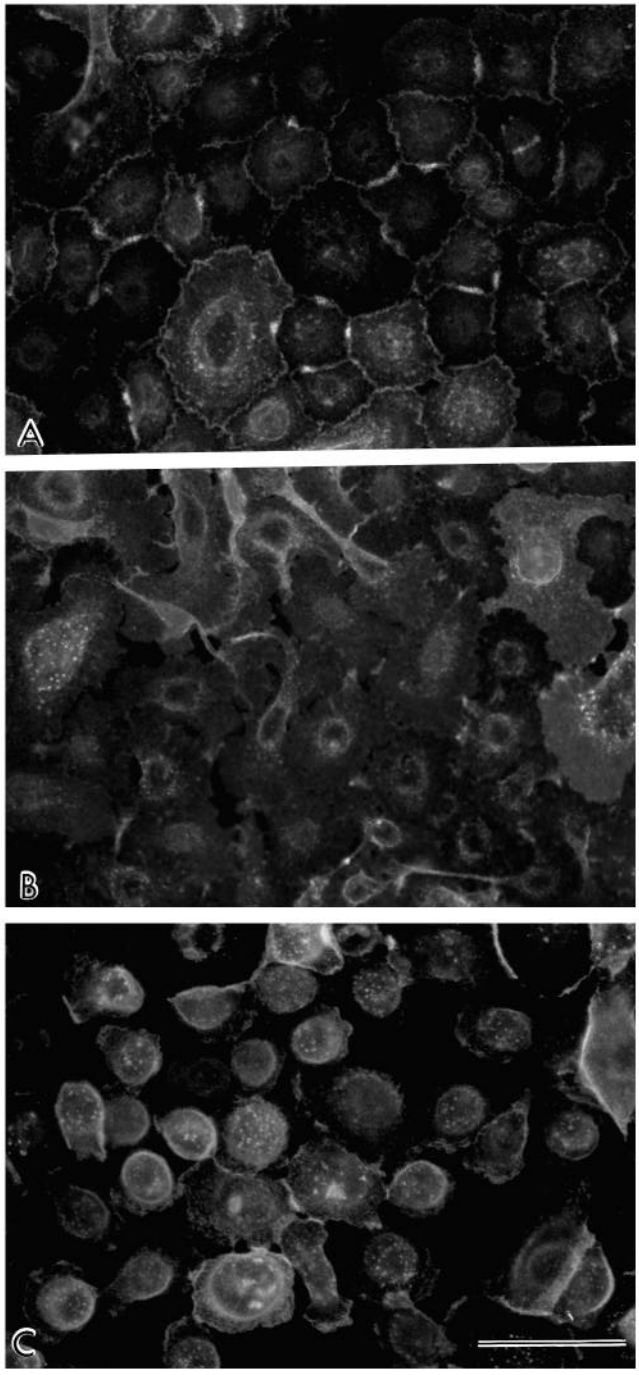

Fig. 2. The redistribution of E-cadherin is specific to PKC activation. Nearly confluent cultures of keratinocytes in 30 μM Ca^{2+} were treated for 1 hour with 0.5 mM DiC8 (A), or with 1 nM PMA in the presence of 400 μM H7 (B); or for 12 hours with 360 nM PDBu, followed by elevation of Ca^{2+} to 1 mM for 1 hour (without changing the medium). Immunofluorescence localization of E-cadherin showed cell-cell border staining in cells treated with DiC8 (A) and a diffuse pattern, analogous to that seen at 30 μM Ca^{2+} in cells in which PKC was inhibited with H7 (B) or downregulated with an overnight treatment with PDBu (C). Bar, 100 μm.

elevated to 1 mM and the distributions of E-cadherin and P-cadherin were examined. Neither molecule was redistributed in response to Ca^{2+} elevation in the cells that had been treated overnight with PDBu (Fig. 2C). The preincubation in PDBu was not toxic, as the cells recovered the ability to redistribute both cadherins normally in response to Ca^{2+} elevation if PDBu was removed from the medium. These data indicate that PKC is required for Ca^{2+}-induced adhesion marker redistribution.

Evidence that E-cadherin regulates junctions through PKC

E-cadherin has been shown to be a regulator of the organization of adherens junctions in keratinocytes and other epithelial cells. To consider the possible involvement of PKC in the regulation of adherens junction organization by E-cadherin, the following experiment was performed. Cells were preincubated with inhibitory anti-E-cadherin antibody; the Ca^{2+} concentration was then elevated in the presence or absence of PDBu. The immunocytochemical localization of P-cadherin was determined one hour later. As previously described (Wheelock and Jensen, 1992), blocking antibodies to E-cadherin delayed the Ca^{2+}-induced redistribution of P-cadherin; i.e. in cells preincubated in anti-E-cadherin IgG (Fig. 4D), P-cadherin distribution after 1 hour in high Ca^{2+} resembled that seen in the control with 30 μM Ca^{2+} (Fig. 4B). In contrast, when PDBu was added concurrently with Ca^{2+} elevation, blocking antibodies to E-cadherin had no effect on the redistribution of P-cadherin (Fig. 4F); i.e. P-cadherin was redistributed normally. Furthermore, this P-cadherin redistribution was stable with time, presumably because Ca^{2+} elevation stabilized homotypic P-cadherin interactions. This experiment indicates that the inhibitory effects of anti-E-cadherin antibody can be overcome if PKC is activated at the time Ca^{2+} is elevated. Hence these findings argue that E-cadherin modulates P-cadherin distribution through activation of PKC.

DISCUSSION

Cultured human keratinocytes grow as individual cells with minimal cell-cell contact in low extracellular Ca^{2+}. When the Ca^{2+} concentration is elevated to 1 mM the cells very rapidly make contact and begin to organize adherens junctions. Adherens junction formation is reflected by a redistribution of the junctional components, including the cadherins, the catenins and actin, from a diffuse localization to a very distinct cell-cell border localization. The experiments described in the present study show that the activation of PKC at 30 μM Ca^{2+} induces redistribution of junctional components similar to that observed upon Ca^{2+} elevation. However, stable junctions do not form when PKC is activated at 30 μM Ca^{2+}, as evidenced by the transient nature of the molecular redistributions and the lack of short actin filaments extending between cells. The lack of stable junctions is not surprising, as the extracellular domain of cadherin, which mediates homotypic cell-cell adhesion, is not functional at this low Ca^{2+} concentration. The redistribution of vinculin and actin together with E-cadherin to the cell-cell borders when PKC is activated in low Ca^{2+} suggests that the cytoplasmic components of adherens junctions are induced to organize upon PKC activation. A logical extension of this observation is that PKC may be a mediator of Ca^{2+}-induced junctional organization. Previous data have demonstrated that E-cadherin can regulate Ca^{2+}-induced junctional organization in the keratinocyte (Wheelock and Jensen, 1992); the present

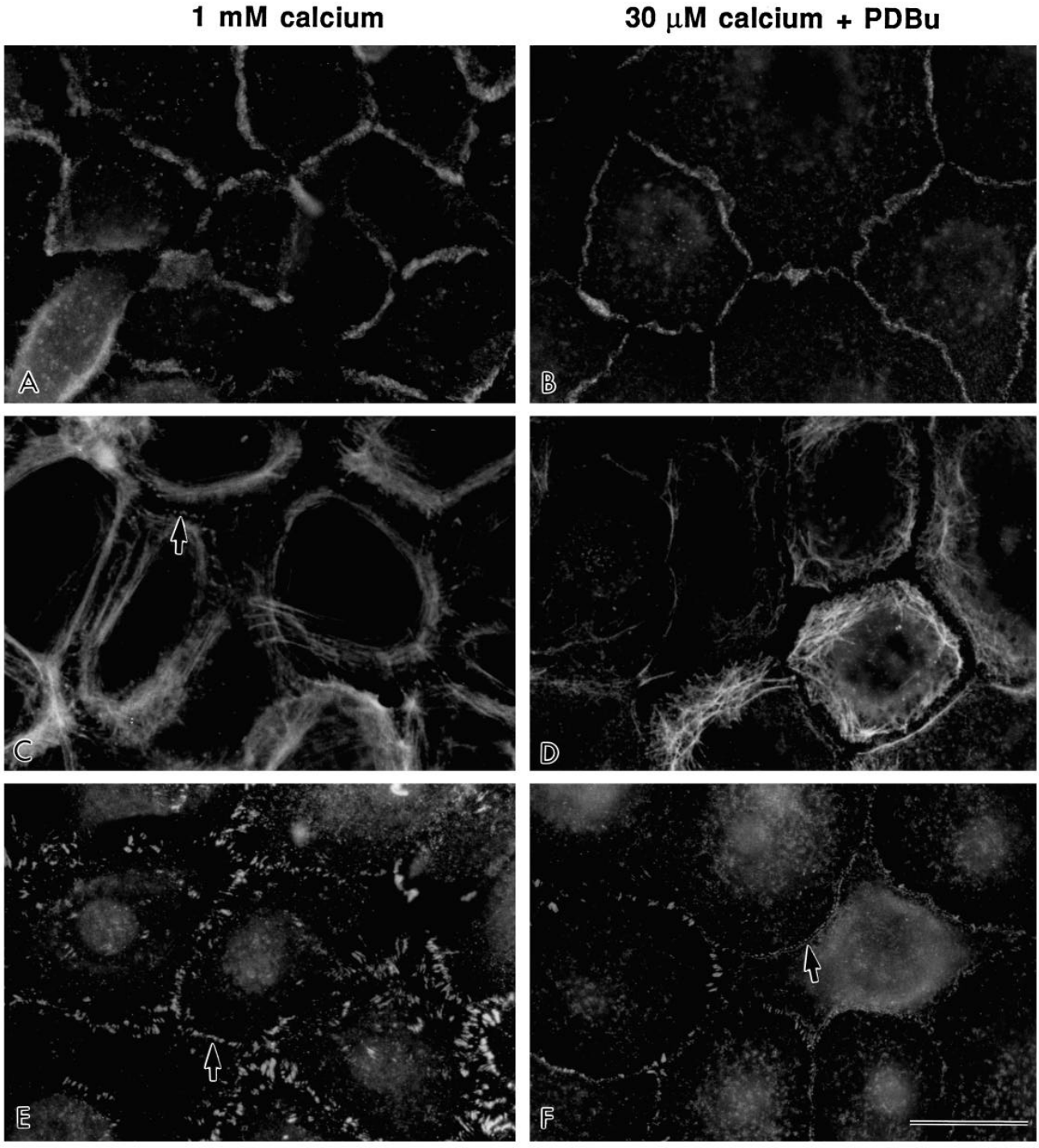

Fig. 3. Activation of PKC mimics elevation of Ca^{2+}. Cells were treated for 1 hour either with 1 mM Ca^{2+} (A, C, E) or with 18 nM PDBu in 30 μM Ca^{2+} (B, D, F). Immunofluorescence localization of E-cadherin (A and B) and vinculin (E and F) revealed cell-cell border staining under both conditions. Actin filaments were reorganized to a peripheral band when cells were treated for 1 hour with 1 mM Ca^{2+} (C) or with 1.8 nM PDBu in 30 μM Ca^{2+} (D). An arrow in C points out typical short actin strands seen between adjacent cells only upon Ca^{2+} elevation. An arrow in C points out cell-cell border staining of vinculin in cells treated with high Ca^{2+}. An arrow in F points out double membrane staining with vinculin in cells treated with PDBu. Cell-substratum adhesion plaques are also evident in cells stained for vinculin whether they are in high Ca^{2+} (E) or low Ca^{2+} plus PDBu (F). Cell-substratum adhesion plaques are more evident in cells that have not been treated with PDBu. Bar, 40 μm.

finding that PKC activation can reverse the effect of compromised E-cadherin function strongly suggests that E-cadherin mediates junctional organization through activation of PKC.

Thus we suggest the following hypothesis for initiation of adherens junction organization: upon Ca^{2+} elevation, homotypic engagement of E-cadherin transmits an intracellular signal that activates PKC. PKC activation leads to the rapid redistribution of adherens junction components, both intracel-

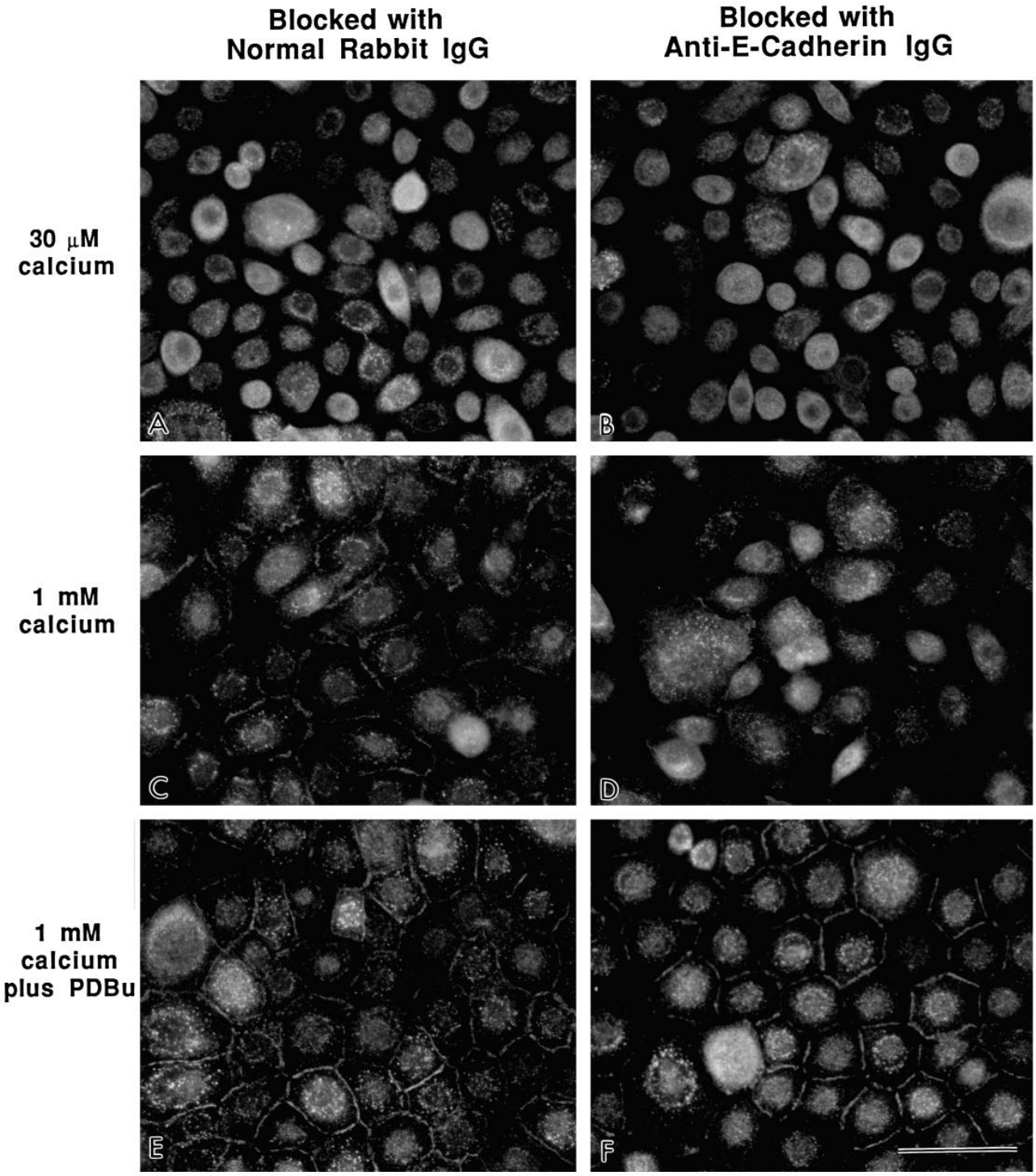

Fig. 4. Activation of PKC can overcome the effect of blocking E-cadherin function. Keratinocytes in 30 μM Ca^{2+} were treated overnight with normal rabbit IgG (A, C, E), with anti-E-cadherin IgG (B, D, F), or with IgG, followed by PDBu (E, F). The Ca^{2+} concentration was maintained at 30 μM (A, B) or raised to 1 mM for 1 hour (C, D, E, F). P-cadherin localization was determined by immunofluorescence. Control cells revealed a typical change from diffuse to cell-cell border stain upon Ca^{2+} elevation (compare A and C). Antibodies to E-cadherin delayed this redistribution (compare C and D). However, if PKC was activated with PDBu, cell-cell border localization of P-cadherin was observed at 1 mM Ca^{2+} even in the presence of anti-E-cadherin IgG (compare D and F). Bar, 100 μm.

lular and cytoskeletal, as well as the recruitment of additional E-cadherin molecules to the cell-cell borders. In this hypothesis, E-cadherin has two roles with regard to adherens junction organization: (i) to mediate Ca^{2+}-dependent homotypic adhesion between cells; and (ii) to transmit an intracellular signal that, through PKC activation, leads to recruitment and organization of junctional components.

Our data also demonstrate that E-cadherin itself can be redis-

tributed after PKC activation, even in low Ca^{2+}, indicating that E-cadherin can respond to PKC as well as activate PKC. In these respects, E-cadherin differs from P-cadherin even though these molecules are highly homologous. Specifically, our data suggest that redistribution of P-cadherin requires not only PKC activation, but also a second signal mediated by Ca^{2+} elevation. Our previous data have indicated that E-cadherin and P-cadherin can at least partially substitute for one another with regard to regulation of adherens junction organization (Wheelock and Jensen, 1992; Lewis et al, 1994). However, the present findings suggest that the two cadherins differ significantly with respect to regulatory involvement in adherens junction formation and that E-cadherin exerts its selective role through PKC activation.

Increased extracellular Ca^{2+} is known to enhance cytoplasmic Ca^{2+} concentration in the keratinocyte. To begin to consider the possible role of increased cytoplasmic Ca^{2+} as a direct mediator of adherens junction protein redistribution, we incubated keratinocytes in 30 μM Ca^{2+} with the Ca^{2+} ionophore A23187 (10 μM) for times ranging from 5 to 60 minutes. No change could be detected in the distribution of E-cadherin in the presence of A23187 at any time point. These data strongly suggest that increased intracellular Ca^{2+} on its own does not induce reorganization of adherens junction components.

Although the mechanism is unknown, there is considerable evidence that increased extracellular Ca^{2+} leads to activation of PKC in keratinocyte culture. Specifically, in a human squamous cell carcinoma line, elevations in extracellular Ca^{2+} rapidly increase PKC in the membrane fraction (Nagao et al., 1989), which is a classical indication of its activation (Nishizuka, 1984). Furthermore, in mouse keratinocyte culture, Ca^{2+} elevation promotes an increase in inositol phospholipid metabolism, as well as an increase in diacylglycerol, one of the products of phospholipid hydrolysis and the classical endogenous activator of PKC (Jaken and Yupsa, 1988; Lee and Yupsa, 1991; Nishizuka, 1984, 1992). From our data, we suggest that the mechanism of this PKC activation may involve homotypic engagement of E-cadherin. While we have focused on adhesion marker redistribution consequent to E-cadherin-mediated activation of PKC, previous investigators have provided evidence that the Ca^{2+}-induced expression of several keratinocyte differentiation markers also depends on PKC activation (Dlugosz and Yupsa, 1993, 1994). Hence, increases in extracellular Ca^{2+} may influence a number of keratinocyte parameters by activation of one or more PKC enzymes. The particular PKC isozyme involved in E-cadherin signal transduction and its potential relationship to differentiation marker expression remain intriguing topics for further investigation.

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