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Relative contribution of cell contact pattern, specific PKC isoforms and gap junctional communication in tight junction assembly in the mouse early embryo

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Abstract

In mouse early development, cell contact patterns regulate the spatial organization and segregation of inner cell mass (ICM) and trophectoderm epithelium (TE) during blastocyst morphogenesis. Progressive membrane assembly of tight junctional (TJ) proteins in the differentiating TE during cleavage is upregulated by cell contact asymmetry (outside position) and suppressed within the ICM by cell contact symmetry (inside position). This is reversible, and immunosurgical isolation of the ICM induces upregulation of TJ assembly in a sequence that broadly mimics that occurring during blastocyst formation. The mechanism relating cell contact pattern and TJ assembly was investigated in the ICM model with respect to PKC-mediated signaling and gap junctional communication. Our results indicate that complete cell contact asymmetry is required for TJ biogenesis and acts upstream of PKC-mediated signaling. Specific inhibition of two PKC isoforms, PKCδ and ζ, revealed that both PKC activities are required for membrane assembly of ZO-2 TJ protein, while only PKCζ activity is involved in regulating ZO-1α+ membrane assembly, suggesting different mechanisms for individual TJ proteins. Gap junctional communication had no apparent influence on either TJ formation or PKC signaling but was itself affected by changes of cell contact patterns. Our data suggest that the dynamics of cell contact patterns coordinate the spatial organization of TJ formation via specific PKC signaling pathways during blastocyst biogenesis.

Keywords: Protein kinase C; Mouse embryo; Trophectoderm; Tight junction; ZO-1; Cell contact pattern; Inner cell mass; ZO-2

Introduction

Epithelial differentiation during development is a dynamic process and is spatially restricted to appropriate cell lineages by cell interactions and signaling mechanisms. The epithelial trophectoderm (TE) forms as progenitor of placental tissues and surrounds the inner cell mass (ICM), the progenitor of the fetus. Transepithelial transport processes driven by Na+/K+-ATPase on basolateral membranes of the TE generate the fluid-filled blastocoel cavity that further segregates the two cell phenotypes (Watson and Barcroft, 2001; Fleming et al., 2004).

In the mouse, cell phenotype divergence and TE differentiation are established gradually over three cell cycles between the 8-cell stage (compaction) and the 32-cell stage (cavitation). At compaction, blastomere polarization and differentiative division into outer polarized and inner non-polarized cells segregate the epithelial TE and pluripotent ICM within the blastocyst (Fleming et al., 2004). The outer TE cells assemble typical epithelial intercellular junctions (Tsukita et al., 2001; Braga, 2002; D'Atri and Citi, 2002) comprising apicolateral zonula adherens (ZA) and tight junctions (TJ) and lateral desmosomes which collectively mediate TE epithelial integrity, polarization and selective paracellular transport required for blastocoel formation. While ZA formation initiates at

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compaction with E-cadherin/catenin adhesion and persists throughout the entire embryo, the more apical TJ assembles gradually exclusively within the TE, with constituents incorporating into the complex at different times during cleavage. For example, the intracellular plaque protein ZO-1α− and the transmembrane protein JAM-1 first assemble at the membrane at the 8-cell stage, cingulin and ZO-2 plaque proteins at the 16-cell stage, followed by the transmembrane protein occludin and the second isoform of ZO-1, ZO-1α+, at the 32-cell stage (Sheth et al., in preparation; reviewed in Fleming et al., 2004). To stabilize epithelial cohesion after initiation of cavitation, desmosomes form during the 32-cell stage, comprising of transmembrane desmocollin (DSC2) and cytoplasmic desmoplakin (Fleming et al., 1991; Collins et al., 1995).

The mechanisms by which junctional maturation within the TE lineage is temporally and spatially regulated are complex and involve both exogenous cell-contact-mediated and endogenous biosynthetic processes. Exogenous processes mediated by cell contact patterns influence spatial restriction of epithelial differentiation and junctional maturation within the embryo. The asymmetric cell contacts within the outer TE lineage induce epithelial differentiation, while the symmetric contacts of the enclosed ICM lineage suppress differentiation (Fleming et al., 1984, 2004; Eckert et al., 2004a). This spatial control mechanism is reversible since provision of contact asymmetry to the ICM by immunosurgical isolation from the early blastocyst permits upregulation of TJ membrane assembly and reformation of a blastocoel (Spindle, 1978; Handyside, 1978; Rossant and Lis, 1979; Chisholm et al., 1985; Louvet-Vallee et al., 2001; Eckert et al., 2004a). Further developmental potential of isolated ICMs has been demonstrated previously (Azim et al., 1978; Rossant and Lis, 1979), underpinning the authenticity of the ICM model to investigate developmental mechanisms.

Among the endogenous biosynthetic processes, transcriptional regulation does not appear to be a limiting mechanism since transcripts for TJ constituents are often detectable well in advance of membrane assembly and within both TE and ICM lineages (Fleming et al., 2004). Thus, membrane assembly of the majority of TJ components during embryo development seems to be regulated by translation (Javed et al., 1993) and/or post-translational modifications such as phosphorylation (Fleming and Hay, 1991; Sheth et al., 2000a; Eckert et al., 2004a). Only ZO-1α+ is an exception, being transcribed just prior to blastocoel formation in both lineages and regulating the completion of TJ membrane assembly in the TE and immediate cavitation (Sheth et al., 1997, 2000a). (Post)Translational mechanisms are also found within non-developmental epithelial models comprising of already differentiated cultured cells, such as Madin–Darby canine kidney (MDCK) cells, where signaling systems, such as heterotrimeric G-proteins, intracellular calcium levels and protein kinase Cs (PKCs), regulate assembly, maintenance and function of TJs (Denker and Nigam, 1998; Matter and Balda, 1999; Braga, 2002; Hurd et al., 2003). Since certain TJ proteins are direct targets for PKC phosphorylation (occludin: Andreeva et al., 2001; ZO-2: Avila-Flores et al., 2001), the post-translational changes in occludin may be important in gaining competence to assemble on TE membranes during embryo development (Sheth et al., 2000a) and could be PKC-mediated.

To date, 10 different PKC isoforms (calcium-dependent conventional or cPKCs α, βI, βII, γ; calcium-independent novel or nPKCs δ, ε, η, θ; phospholipid-dependent atypical or aPKCs ι/λ, ζ) have been characterized that require different co-factors and exert a large number of different effects dependent upon the cellular context (Mochly-Rosen and Gordon, 1998). Altered PKC localization is indicative of a change in activation status and function, reflecting altered accessibility of the kinase to protein substrates (Csukai and Mochly-Rosen, 1999; Jaken and Parker, 2000; Newton, 2003). Although PKC signaling is significantly involved in regulation of early embryo development and expression and localization of several PKC isoforms are developmentally regulated, detailed functions remain unclear as mostly chemicals have been used that modulate broad PKC activity but do not target specific PKC isoforms (Bloom, 1989; Winkel et al., 1990; Ohsugi and Yamamura, 1993; Gangeswaran and Jones, 1997; Raz et al., 1998; Pauken and Capco, 1999, 2000; Eckert et al., 2004a,b). With this approach, we have identified a role for PKCs in TJ assembly in the mouse embryo (Eckert et al., 2004a) and are now using recently developed peptides that modulate localization or substrate binding of specific PKC isotypes (Souroujon and Mochly-Rosen, 1998).

The relative importance of gap junctional intercellular communication (GJIC) in early development remains controversial since pharmacological inhibition and a series of knockouts of connexins (Cx), the subunits of gap junctions, show no early lethality, while antibody-mediated inhibition has perturbed compaction and blastocyst development (Vance and Wiley, 1999; Kidder and Winterhager, 2001). However, GJIC is thought to play a critical role in growth and differentiation within developmental models (White and Paul, 1999). Some evidence suggests that GJIC could be involved in regulating formation and maintenance of cell lineage divergence by participating in TJ assembly control. Initiation of GJIC coincides with compaction paralleling the initiation of TJ membrane assembly (Houghton, 2005), and gap junction components were found to interact with ZO-1 and other TJ proteins (Toyofuku et al., 1998; Kausalya et al., 2001; Giepmans, 2004). Most importantly, in connexin-knockout hepatocytes, newly expressed GJIC induced functional TJ formation, a process preventable by a chemical inhibitor of GJIC (Kojima et al., 2002). PKC signaling may be involved in these processes since membrane assembly of Cxs depends upon PKC- and PKA-mediated phosphorylation (Ogawa et al., 2000) and broad chemical PKC activators such as phorbol esters affect GJIC (Lampe and Lau, 2004).

In the present study, using the ICM immunosurgery model, we evaluate the relative contribution and interrelationship of exogenous regulators such as cell contact patterns combined with intrinsic PKC signaling and GJIC that are associated with TJ biogenesis in the embryo. We show that complete cell

contact asymmetry is mandatory to initiate TJ biogenesis and acts upstream of PKC-mediated stimulation of TJ biogenesis, provoking functional changes in specific PKC isoforms. We find no apparent role for gap junctions, but their function can also be influenced by cell contact pattern. We show that membrane assembly of individual TJ components is regulated by specific and different PKC isotypes. Membrane assembly of both ZO-2 and ZO-1α+ was found to be dependent upon PKCζ, while ZO-2 membrane assembly also required PKCδ activity.

Materials and methods

Embryo collection and culture

Embryos were collected from MF1 female mice (University of Southampton Biomedical Facility) after superovulation by intraperitoneal injection of 5 i.u. pregnant mares serum gonadotrophin (PMS; Folligon, Intervet) followed by 5 i.u. human chorionic gonadotrophin (hCG; Chorulon, Intervet) and mating 48 h later. Eight-cell embryos were flushed from dissected oviducts using H6 medium supplemented with 4 mg/ml BSA (H6-BSA; Sigma) and cultured up to the early blastocyst stage in T6 medium containing 4 mg/ml BSA (T6-BSA) in 5% CO_2 in air at 37°C in microdrops under oil (J.M. Loveridge, Southampton) as described previously (Sheth et al., 1997, 2000a,b; Thomas et al., 2004).

Isolation and culture of ICMs

ICMs were isolated from early blastocysts (92–96 h after hCG) by immunosurgery as described (Eckert et al., 2004a). ICMs were checked upon isolation to ensure all TE cells had been removed, initially on cohorts by incubation in propidium iodide (see below) to visualize lysed TE cells and subsequently using light microscopy. The freshly isolated ICMs were placed in 500 μl DMEM (Gibco) + 10% FCS (heat-inactivated; Labtech International) in 4-well plates (Nunc, Roskilde, Denmark) and incubated at 5% CO_2 in air and 37°C in a humidified atmosphere for up to 29 h before fixation. Initially, culture times adequate to allow for sufficient tight junction membrane assembly to occur were determined to provide the baseline for experimental endpoints. Treatment times were also deducted from these experiments and were related to previous results (Eckert et al., 2004a). In some cases, the lysed TE cells were left undisturbed, still surrounding the ICM (partial immunosurgery, 1st partIS) before culture. Some 1st partIS were subjected to a second round of immunosurgery (see above) with only 5-min exposure to complement without shelling out of the ICM (2nd partIS) before culture in parallel with intact blastocysts and 1st partIS. TE cell lysis was demonstrated by incubation in propidium iodide (Sigma; 0.05 μg/ml H6-BSA) and, in some experiments, with co-incubation with FITC-labeled dextran (4 kDa; Sigma; 1 mg/ml H6-BSA; Sheth et al., 2000b) for 20 min before confocal microscopy.

Antibodies

Antibodies to the junctional proteins occludin (rabbit polyclonal against human occludin, diluted 1:100; Van Itallie and Anderson, 1997), ZO-1 (R40.76, rat monoclonal against mouse ZO-1, neat culture supernatant; Anderson et al., 1988), ZO-1α− (rabbit polyclonal, diluted 1:250; Sheth et al., 1997), ZO-1α+ (rabbit polyclonal, diluted 1:250, or guinea pig polyclonal, diluted 1:250, both against the murine α-motif; Sheth et al., 1997), E-cadherin (rat polyclonal, Sigma; 1:1000) and desmoplakin (mouse monoclonal 11-5F against bovine desmoplakin 1 and 2, neat culture supernatant; Parrish et al., 1987; Fleming et al., 1991) were diluted in PBS and used as previously described. The antibody to ZO-2 (rabbit polyclonal, diluted 1:1000) was purchased from Zymed. The staining for PKC isoforms was performed with polyclonal antibodies against the rat sequences as detailed previously (Eckert et al., 2004a,b) at dilutions between 1:200 and 1:500. To verify the specificity of these antibodies, a set of commercial antibodies generated in mouse (Transduction Labs) or rabbit (PKCζ, Sigma) against the various PKC isoforms was used in parallel at the appropriate dilutions (1:100–1:300, and 1:1000 for PKCζ, respectively; see also Eckert et al., 2004a,b).

Immunofluorescence and confocal microscopy

Zona-free embryos or ICMs were fixed in PBS supplemented with 1% formaldehyde (Analar or Sigma) for 7–15 min, attached onto coverslips coated with 1.5 mg/ml poly-L-lysine hydrobromide (Sigma) and processed for immunofluorescence as described previously (Fleming et al., 1991; Sheth et al., 1997, 2000a,b; Eckert et al., 2004a,b; Thomas et al., 2004). For all experiments, each coverslip contained isolated ICMs and 2–3 intact control blastocysts to ensure staining efficiency. The embryos/ICMs were stained overnight at 4°C with the different primary antibodies as described above at the appropriate dilutions. In some experiments, embryos were co-stained for F-actin with Phalloidin (0.5 ng/ml PBS; Sigma). A set of cross-purified ALEXA-488-, ALEXA-546- or ALEXA-568-labeled anti-mouse, anti-rabbit or anti-rat secondary antibodies (Molecular Probes) was used at dilutions of 1:500 either alone or in combinations for double labeling experiments for 1 h at room temperature. After three final washes and mounting with an antifading agent (Citifluor in PBS; Citifluor Ltd), specimens were visualized with a ×63 oil-immersion Nikon inverted microscope linked to a Bio-Rad MRC-600 series confocal imaging system, equipped with a krypton–argon laser. Images were analyzed and processed using the Bio-Rad software system (Confocal assistant version 4.01). While linear staining within the membrane region was observed for TJs, desmosomes (desmoplakin) are punctate junctions by nature. The staining pattern for all junctional proteins in isolated ICMs was judged as *negative* if no staining was detected within cell membranes, *incomplete* if less than half of the ICM showed membrane staining or *continuous* if more than 50% of the ICM showed membrane staining (see Fig. 1). The incomplete pattern was seen as an intermediate state of membrane assembly as observed during junction biogenesis in intact embryos (Fleming et al., 1989, 1993; Sheth et al., 1997, 2000a,b).

Chemical PKC activation and inhibition

To evaluate the hierarchy between cell contact patterns and PKC signaling, intact control blastocysts or 1st partIS and 2nd partIS were cultured in the presence of up to 10 μM of the cell-permeable broad PKC activators TPA (phorbol ester; Calbiochem) or indolactam (alkaloid-type activator; Calbiochem) or 10 μM 4αPDD (inactive analogue; Calbiochem) or 0.1% DMSO (vehicle; Sigma) as controls. After 4 h or overnight culture (approximately 14 h to expanded blastocysts), the embryos were fixed and stained for PKCδ and ζ isotypes with ZO-1α+ double labeling in some experiments or ZO-2 or ZO-1α+ alone in others.

Isotype-specific PKC activation and inhibition

PKC isozyme-specific activator and inhibitor peptides coupled to the antennapedia carrier (*Drosophila* antennapedia, positions 43–58, [RQIKIWFQNRRMKWKK]; Derossi et al., 1994; Chen et al., 1999, 2001) for cell membrane permeability were kindly provided by Dr. Daria Mochly-Rosen (Stanford University, USA) and used as described previously (Eckert et al., 2004b). Briefly, peptides were dissolved in PBS and diluted to their final concentration in DMEM with 10% FCS. Inhibitory peptides against PKCζ (from the pseudosubstrate region of the human PKC isozyme, positions 113–129 [SIYRRGARRWRKLYRAN], and PKCδ (translocation inhibitor; δV1-1), positions 8–17 [SFNSYELGSL], and an activator peptide for PKCδ (translocation activator; ψδRACK), positions 74–81 [MRAAEDPM]) were supplemented to DMEM culture medium containing 10% FCS in 4-well dishes (Nunc) at 0.1, 0.5 or 1 μM to generate a dose–response curve. These concentrations have previously been shown to be effective and non-toxic in other cell systems (e.g. Chen et al., 2001; Braun and Mochly-Rosen, 2003) and mouse embryos (Eckert et al., 2004b). Evidence for peptide specificity and cell entry mechanism has been discussed in detail previously (Eckert et

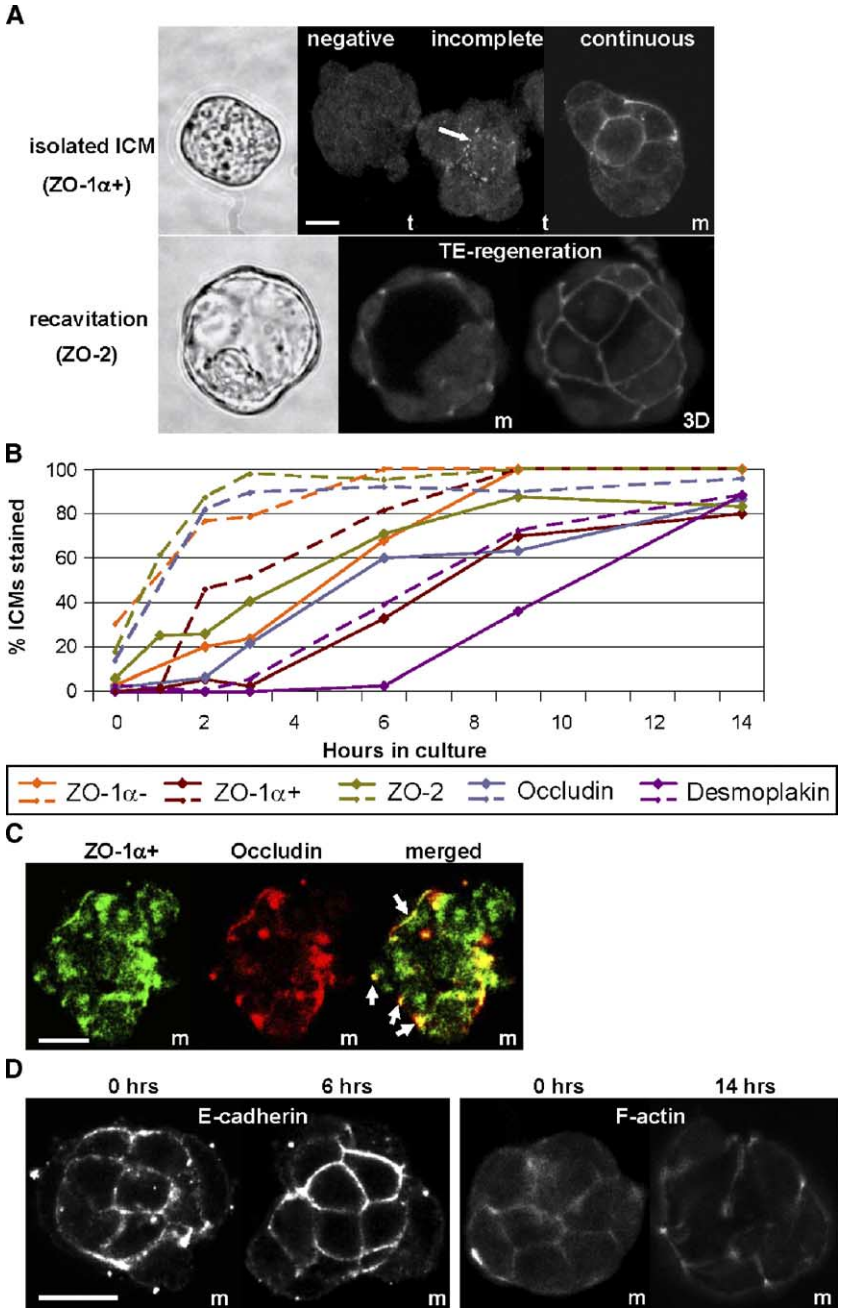

Fig. 1. Sequential membrane assembly of various TJ proteins in cultured isolated ICMs. (A) Bright field and single section confocal images of fixed and stained isolated ICMs showing negative, incomplete (arrow) or continuous membrane assembly of ZO-1α+ (upper panel) as well as complete TE regeneration and belt-like membrane assembly of ZO-2 (lower panel) in tangential (t) or midplane (m) sections or a 3D reconstruction of 1 μm sections (3D). Scale bar, 10 μm. (B) Each data point represents at least 5 replicates and a total number of 35–50 isolated ICMs. Shown are overall percentages of ICMs stained either continuous (solid line) or incomplete (dotted line, shown including continuos staining) for the different junctional proteins over time in culture. See Materials and methods for definition of incomplete and continuous. (C) Confocal midplane sections showing a representative isolated ICM after 6 h of culture double labeled for ZO-1α+ and occludin. Co-localization at the cell membrane and within the cytoplasm is indicated in the merged image (arrows). Scale bar, 10 μm. (D) Midplane confocal images showing E-cadherin (left) and F-actin (right) in isolated ICMs cultured for 0, 6 or 14 h. Scale bar, 10 μm.

al., 2004b; Chen et al., 2001; Braun and Mochly-Rosen, 2003). No peptide, antennapedia carrier monomer (0.05–1 μM) and dimer (0.025–0.5 μM) were used as controls. Isolated ICMs were fixed and stained for ZO-2 or ZO-1α+ for TJ membrane assembly assessment or PKCδ, θ or ζ to determine translocation modulator peptide specificity after 1 h of culture in our system. PKCθ was used as a control for specificity of PKCδ peptides as PKCθ and δ show a 52% amino acid identity in the V1 (first variable) domain (Chen et al., 2001). PKCζ was used as an unrelated control to demonstrate peptide specificity in our system. In some cases, isolated ICMs were cultured overnight (18–29 h) in the presence of peptides or carriers to determine recavitation capacity.

Analysis of gap junctional communication

Embryos were flushed at the 8-cell stage and cultured to blastocysts in the presence of the gap junction channel inhibitor 18 α-glycyrrhetinic acid (AGA, 65 μM; Sigma), 0.1% DMSO (vehicle control; Sigma) or no treatment. Freshly isolated or cultured ICMs (1, 3, 6 or 14 h of culture with or without AGA,

respectively) were stained for junctional proteins or PKC isotypes. To ensure inhibition of gap junction communication, isolated ICMs were microinjected with 5% lucifer yellow (LY) as described previously (Eckert et al., 2004b). To control for developmental capacity of isolated ICMs in the presence of AGA, a subset of ICMs was cultured for 14 h in its presence and monitored for recavitation.

Statistical analysis

Using the SigmaStat software package (Jandel Scientific), a general linear model was used to test for interactions between treatment and staining pattern. A 2-way ANOVA followed by a Tukey test or a 2-way ANOVA on Ranks followed by a Dunn's test where appropriate was used to identify significant differences. In the experiments involving the GJIC inhibitor AGA, coupling rates were compared by Chi-square. Differences were considered as significant if $P < 0.05$.

Results

Membrane assembly of junctional proteins in isolated ICMs is rapid and broadly mimics the sequence occurring during normal trophectoderm development

Isolated ICMs were either fixed immediately after immunosurgery or cultured for up to 14 h to determine the timing and order of membrane assembly of junctional proteins by immuno-confocal microscopy (Figs. 1A–C). Overall, 70% ICMs cultured for 14 h recavitated (n = 112). Isolated ICMs had between 12 and 16 cells irrespective of 0 or 14 h of culture (n = 40 at each time point). The ZA junction protein E-cadherin was detected at all cell contact sites in freshly isolated ICMs as well as after up to 14 h of culture (n = 14 at each time point; Fig. 1D). Similarly, the F-actin cytoskeleton remained undisturbed (n = 10 at each time point, Fig. 1D).

In general, the TJ and desmosomal proteins assembled in the sequence ZO-2, ZO-1α− isoform, occludin, ZO-1α+ isoform and desmoplakin (Fig. 1B). Freshly isolated ICMs already showed 15–30% and 3–8% of incomplete or continuous staining respectively (see Materials and methods for definition) for early assembling proteins (ZO-2, ZO-1α−) but were negative for late assembling proteins (ZO-1α+, desmoplakin). This presumably reflects nascent assembly during the immunosurgery period (20–30 min) since ICM cells within intact blastocysts at this stage do not display TJ protein membrane assembly (see Introduction). By 2 h of culture, rapid upregulation of TJ protein membrane assembly was observed with 20–25% ICMs showing continuous and

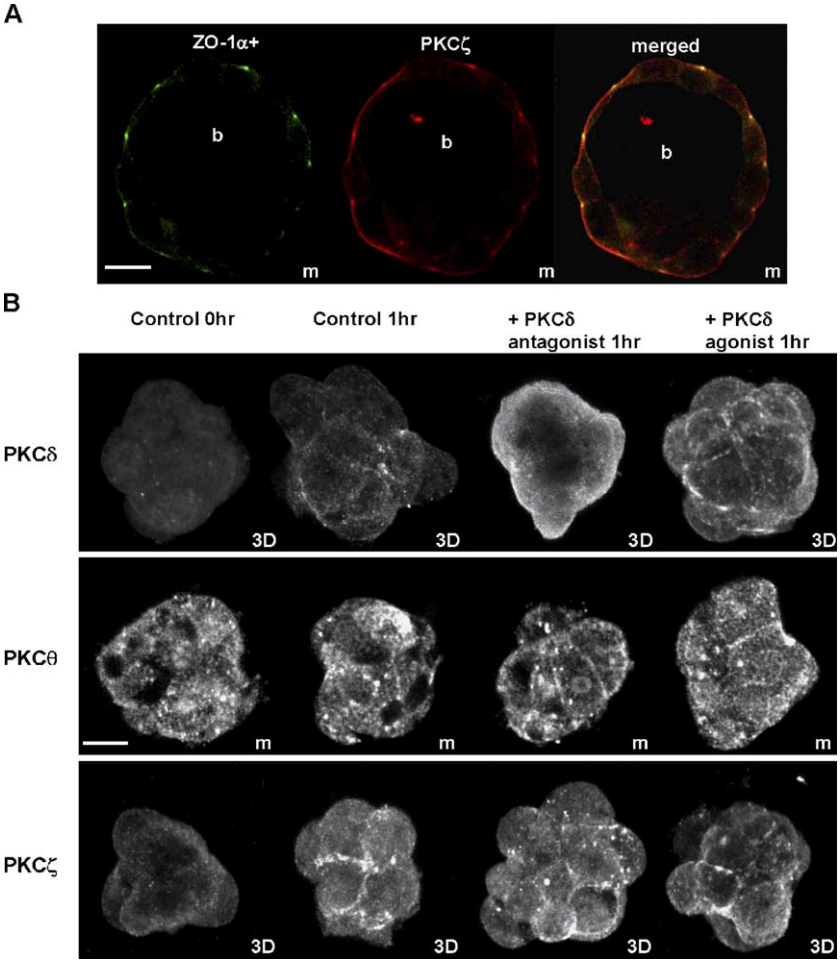

Fig. 2. (A) Representative distribution of PKCζ in relation to ZO-1α+ within intact blastocysts. b = blastocoel. Scale bar, 30 μm. (B) Representative confocal images (n = 8–11 ICMs per treatment, time point and antibody from 2 to 3 replicates) showing isolated ICMs immediately after isolation (control 0 h) or cultured for 1 h in the presence or absence of PKCδ antagonist peptide or PKCδ agonist peptide and stained for either PKCδ (upper panel, 3D z-series reconstructions of 1 μm sections), PKCθ (middle panel, midplane sections) or PKCζ (lower panel, 3D z-series reconstructions of 1 μm sections). Scale bar, 10 μm.

60–72% ICMs showing incomplete membrane assembly of ZO-2 and ZO-1α−. By 9 h of culture, continuous staining for all TJ proteins was observed in most (>65%) ICMs. Finally, continuous membrane staining for the desmosome junction protein desmoplakin was evident between 9 and 14 h of culture in most ICMs. Double labeling of isolated ICMs for occludin and ZO-1α+ showed co-localization within the majority of ICMs (n = 14; Fig. 1C). This order and pattern of membrane assembly broadly resemble the pattern identified for intact embryos during compaction and cavitation (see Introduction), however, it is extremely accelerated and takes place over approximately one cell cycle. These experiments established a model suitable to examine mechanisms regulating membrane insertion for early (ZO-2) and late (ZO-1α+) TJ assembly events. These two proteins also differ in their binding capacity to adherens junction proteins. While ZO-1α+ is specific for TJs, ZO-2 is also transiently associated with adherens junctions during early stages of epithelial differentiation (Itoh et al., 1999; Sheth et al., 1997; 2000a,b; Sheth et al., in preparation).

Specific PKC isotypes influence membrane assembly of different TJ proteins

In a separate study, we have found that certain PKC isotypes, the nPKCδ and θ and the aPKCι/λ and ζ, show lineage-specific distribution in intact blastocysts (predominantly membrane-associated in TE and cytoplasmic in ICM) and in the TE co-localize to varying extents with TJ proteins (Eckert et al., 2004b). Co-localization is most evident with PKCζ and ZO-1α+ in TE, and a representative sample is shown in Fig. 2A. We have also found that, upon isolation of ICMs, PKCδ and ζ rapidly relocate (within minutes; see e.g. Nowicki et al., 2000) from cytoplasmic to membrane sites in ICM cells both spontaneously and in an increased manner in response to chemical PKC activation (TPA and indolactam) and subse-

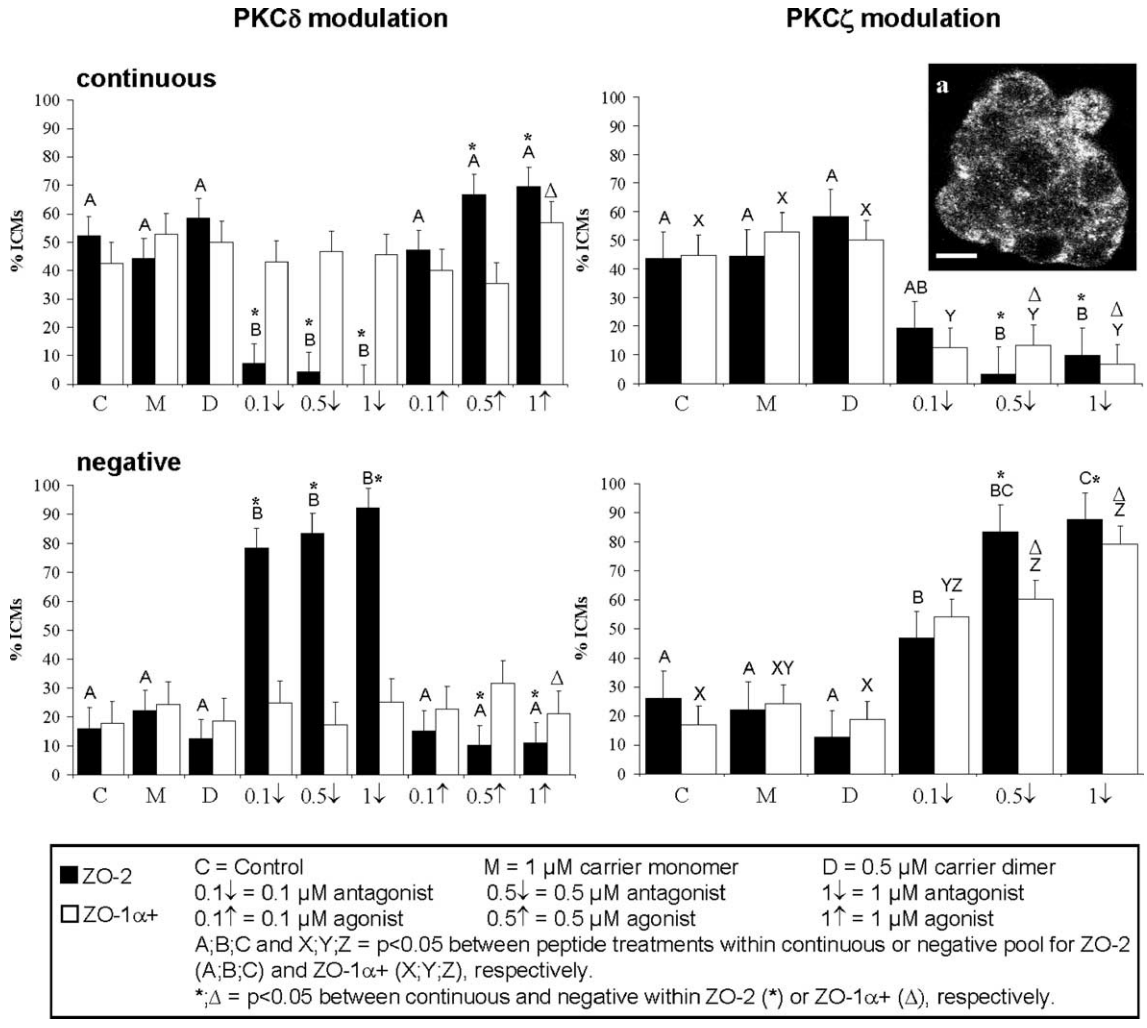

Fig. 3. Membrane assembly of ZO-2 or ZO-1α+ in isolated ICMs cultured for 1 h with different PKC isoform-specific inhibitory or activator peptides coupled to the antennapedia carrier for cell membrane permeability. Data were collected from at least 5 replicates, and a total number of 17–30 isolated ICMs per treatment. Significant differences ($P < 0.05$) are indicated by different superscripts within either the continuous or negative pool, respectively. Asterisks (ZO-1α+) or triangles (ZO-2) indicate significant differences between the continuous (upper panel) and negative pools (lower panel) within one treatment group. The incomplete pool is not shown (no significant effect of treatment) but can be deducted from the continuous and negative pools. (A) A representative midplane confocal section of an isolated ICM cultured for 1 h in the presence of 0.5 μM PKCζ antagonist is shown after fixation and staining for ZO-2. Scale bar, 10 μm.

quently co-localize with TJ proteins (Eckert et al., 2004a). Representative examples of spontaneous PKCδ and ζ relocation at 0 h and 1 h after ICM isolation are shown in Fig. 2B.

To elucidate more precisely the role of PKCδ or ζ, isolated ICMs were cultured for 2 h in the presence of different concentrations of PKCδ translocation activating or inhibiting peptide or PKCζ pseudosubstrate inhibitor. Specificity and potency of inhibition or activation of PKCδ translocation were confirmed in isolated ICMs cultured for 1 h in the presence of PKCδ agonist or antagonist or control antennapedia carrier dimer (Fig. 2B; Eckert et al., 2004b). Due to high amino acid similarity between PKCδ and θ within the V1 domain, the area of peptide design, PKCθ was used as a control for isoform specificity against PKCδ (Fig. 2B, middle panel), while PKCζ served as unrelated control. PKCδ antagonist or agonist inhibited or stimulated membrane accumulation of PKCδ, while PKCζ and θ distribution remained unaffected. Long-term culture of isolated ICMs in the presence of carrier peptide controls (1 μM monomer or 0.5 μM dimer, 25–28 isolated ICMs per treatment group) did not affect recavitation rate ($P > 0.05$; 12–25% and 40–63% recavitation after 18 and 29 h of culture in treatment groups, respectively) excluding toxicity of the carrier peptides as suggested for other cell types (Drin et al., 2003; Christiaens et al., 2004).

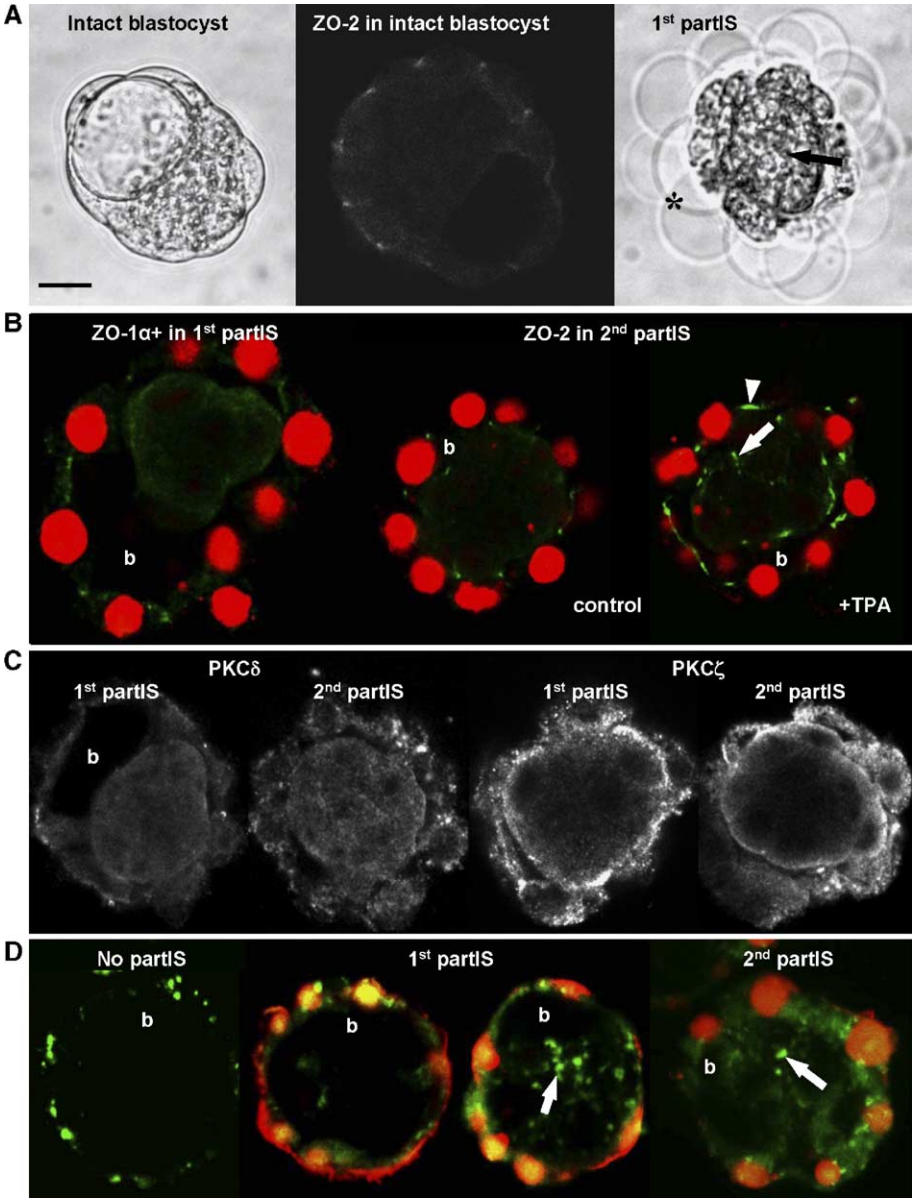

Fig. 4. (A–D) Bright field or representative single confocal sections of intact blastocysts or blastocysts after one or two rounds of partial immunosurgery (1st and 2nd partIS, respectively; $n = 13–25$ from 3 to 4 replicates). (A) After partIS, the lysed TE cells (asterisks) surround the ICM (arrow) in the bright field picture and (B, D) their nuclei stained with propidium iodide (red) in the confocal images. Shown are representative single confocal images of 1st or 2nd partIS blastocysts stained for (B) the TJ components ZO-1α+ or ZO-2 (ZO-2: cultured with or without the PKC activator TPA; see Table 1 for distribution and treatment groups; arrowhead: remnants of TJ seal stained for ZO-2 in the lysed TE, arrow: initiation of ZO-2 membrane assembly in areas within the ICM) or for (C) PKCδ or ζ. (D) Representative distribution of FITC-Dextran (green) and propidium iodide (red) in relation to no immunosurgery (no partIS), 1st or 2nd partIS is shown; arrow: FITC-Dextran distribution within the ICM. b = blastocoel. Scale bar, 25 μm.

Membrane assembly of ZO-2 or ZO-1α+ remained unaffected by carrier monomer or dimer used in the highest concentration corresponding to isoform-specific peptides (Fig. 3, incomplete staining pool not shown but deducted from continuous and negative pools, respectively). This therefore suggests that, in our hands and with the concentrations used here, the carrier does not cause broad disturbance of membrane protein distribution in early embryos as discussed controversially in other cell lines (Drin et al., 2003; Christiaens et al., 2004). ZO-2 membrane assembly was significantly reduced in a dose-dependent manner ($P < 0.05$) by PKCδ and ζ inhibitory peptides, respectively (Fig. 3, black bars), mainly due to a significant ($P < 0.05$) shift towards the negative staining pool (all three doses of PKCδ inhibitor and 0.5 and 1 μM PKCζ inhibitor, respectively). ZO-2 was found within the cytoplasm in a speckled pattern (Fig. 3A, insert) when ICMs were cultured with PKCζ inhibitory peptide. Although PKCδ agonist did not further increase continuous membrane staining for ZO-2 compared to controls, a significant shift towards the continuous pool away from the incomplete (not shown; deducted from Fig. 3) or negative one was observed in the presence of 0.5 or 1 μM PKCδ agonist. In contrast to ZO-2, membrane assembly of ZO-1α+ was only significantly ($P < 0.05$) decreased by PKCζ pseudosubstrate, also reflected in a significant shift towards the negative pool compared to the continuous and incomplete pools (0.5 or 1 μM PKCζ inhibitor) but remained largely unaffected by PKCδ peptides (Fig. 3, open bars). Only in the presence of 1 μM PKCδ agonist was there a significant shift towards the continuous membrane staining pool compared to the incomplete (not shown; deducted from Fig. 3) or negative pools. Overall, these data show that PKCζ plays a role in membrane assembly of both ZO-2 and ZO-1α+, while PKCδ only regulates membrane assembly of ZO-2 in our ICM model. We next examined what mechanisms might regulate PKC signaling to drive TJ membrane assembly.

Cell contact asymmetry is obligatory for PKC-mediated upregulation of TJ protein membrane assembly

To elucidate the role of cell contact pattern in relation to PKC-mediated regulation of TJ membrane assembly in ICMs, we either cultured intact early blastocysts with chemical PKC activators (TPA, indolactam) or used a model in which the generation of a contact-free cell surface in ICMs remained incomplete (one or two rounds of partial immunosurgery; 1st or 2nd partIS). For 1st or 2nd partIS, ICMs were left surrounded by the lysed TE cells after one or two treatments of complement-mediated lysis as performed during immunosurgery and shown by propidium iodide counterstaining of lysed TE cells, while ICM cells remain unstained, demonstrating their membrane integrity (Fig. 4). To ensure viability of the ICMs after 1st or 2nd partIS, a small percentage was shelled out after up to 6 h of initial TE lysis and cultured for an additional 9–14 h. Of these, 80% recavitated ($n = 34$), indicating no loss in viability of the ICM by this treatment.

Membrane assembly of both TJ proteins (ZO-2 and ZO-1α+) remained absent from the ICM of intact embryos when cultured overnight to expanded blastocysts in the presence of 10 μM broad PKC activators or their control reagents 0.1% DMSO or 10 μM 4αPDD ($n = 32–47$ embryos per treatment). Similarly, membrane distribution of both TJ proteins and both PKCδ and ζ was not seen in ICMs after 1st partIS treatment and cultured for 4 h either in the presence or absence of either PKC activator (1 μM; Table 1; Fig. 4). The same was observed after 2nd partIS treatment and 4 h culture for ZO-1α+ and both PKC isotypes, while ZO-2 was occasionally detectable at ICM cell membranes but only after additional PKC activator treatment (Table 1; Fig. 4). To evaluate the extent to which partIS indeed did destroy the seal function of the TE after cell lysis, propidium iodide and the larger FITC-Dextran were used. While propidium iodide clearly demonstrated membrane penetrability of the lysed TE after the 1st partIS and intact membranes of the remaining ICM cells, FITC-Dextran was taken up by some ICMs after the 1st and by all ICMs after the 2nd partIS. This clearly demonstrated the remainder of some barrier function even after TE lysis after the 1st partIS. This was also indicated by the remainder of a visible blastocoelic cavity in the majority of 1st partIS embryos. On the other hand, the PKC and TJ protein distributions after partIS treatment appeared to be independent of the penetration capacity of FITC-Dextran into TE or ICM cells: while dextran was undetectable within the ICMs of intact controls after 20 min of culture, 40% of 1st partIS ICMs and 100% of 2nd partIS ICMs contained dextran despite the absence of membrane distribution of TJ and PKC proteins (Table 1, Fig. 4). Taken together, this may suggest that two rounds of IS are indeed required to effectively destroy the barrier function of the TE entirely but that this is not sufficient to allow upregulation of signaling networks leading to epithelial differentiation.

Our data demonstrate that the maintenance of a symmetric cell contact pattern, even with lysed cells, overrules PKC-mediated upregulation of TJ membrane assembly in ICM cells. The data suggest that a specific membrane interaction is responsible for maintaining the suppression of phenotype change within ICMs. Moreover, relocation of PKC isoforms in ICM cells is also suppressed, even when chemical activators are employed.

Table 1
Effects of incomplete cell contact modulation and activation of PKC on TJ membrane assembly within ICMs after 4 h

	Drug treatment	No IS	1st partIS	2nd partIS
		ICM localization *x*/*n* (%)	ICM localization *x*/*n* (%)	ICM localization *x*/*n* (%)
Dextran	None	0/5 (0)	8/20 (40)	19/19 (100)
p.i.	None	0/5 (0)	0/20 (0)	0/19 (0)
ZO-1α+	0.1% DMSO	0/19 (0)	0/19 (0)	0/22 (0)
	1 μM TPA	0/21 (0)	0/20 (0)	0/15 (0)
	1 μM Ind.	0/23 (0)	0/17 (0)	0/13 (0)
ZO-2	DMSO	0/23 (0)	0/21 (0)	0/24 (0)
	1 μM TPA	0/25 (0)	0/21 (0)	2/17 (12)
	1 μM Ind.	0/22 (0)	0/20 (0)	1/15 (7)

p.i.: propidium iodide; Dextran: FITC-Dextran.

Intercellular communication via gap junctions is not required for cavitation or tight junction formation in isolated ICMs

To examine whether communication via gap junctions is involved in organizing TJ membrane assembly or PKC relocation in response to cell contact modulation, we investigated GJIC in isolated ICMs by lucifer yellow (LY) injection. While all intact control blastocysts showed dye coupling over the entire embryo within 3 min irrespective of injection into an ICM or TE cell, 56% of freshly isolated ICMs did not show any dye transfer between blastomeres within 30 min (Figs. 5A, B). To elucidate whether this closure of gap junctions has an effect on TJ membrane assembly or PKC signaling, we chemically inhibited gap junctional coupling by incubation in AGA. Embryos were cultured in the presence of AGA or DMSO from the 8-cell stage onwards until blastocyst formation and isolated ICMs were cultured in the presence of AGA for up to another 14 h. When fixed after 0–14 h of culture, membrane assembly of ZA junctions (E-cadherin), TJs (ZO-2; ZO-1α+; occludin) and desmosomal (desmoplakin) proteins and the distribution of PKCδ or ζ remained unaffected by AGA incubation of isolated ICMs (Fig. 5C; see Fig. 5D for representative junctional and PKC images). Neither blastocyst formation (Eckert et al., 2004b) nor recavitation of isolated ICMs was affected ($P > 0.05$) after overnight culture in the presence of AGA, DMSO or no drug, respectively (46, 52 and 70% ICMs recavitated, $n = 24–30$ per treatment). LY injection confirmed that AGA treatment abolished gap junctional coupling between blastomeres in isolated ICMs (only 8% showed dye coupling within 30 min; Figs. 5A, B).

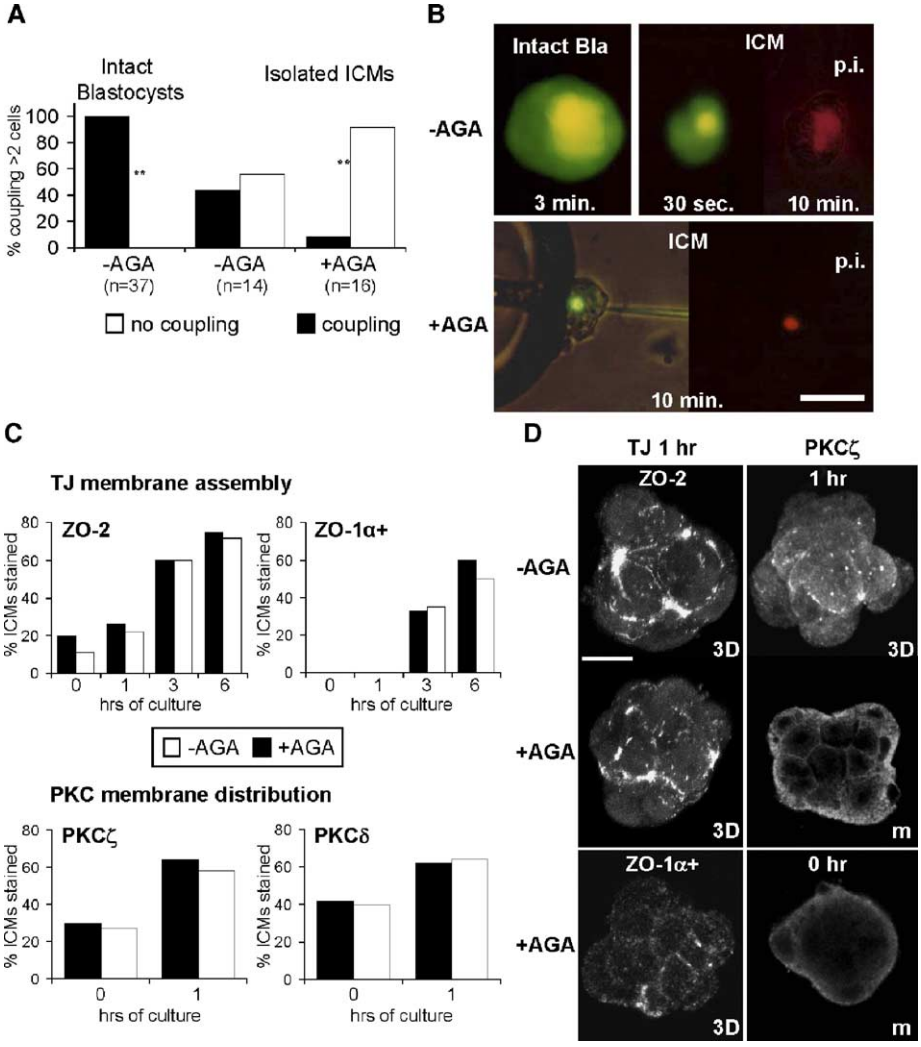

Fig. 5. (A) Gap junctional coupling between blastomeres as monitored by lucifer yellow injection into intact blastocysts or isolated ICMs cultured with or without 65 μM AGA. Significant differences ($P < 0.05$) between coupling and no coupling are indicated by asterisks within intact and isolated ICMs, respectively. Shown are overall percentages collected during 5 replicates ($n = 14–37$). (B) Representative fluorescent photographs of lucifer yellow (green) distribution in relation to propidium iodide (p.i.; red) when injected into intact blastocyst (intact bla) or isolated ICMs cultured with or without AGA. Scale bar, 50 μm. (C) Representative data from 3 to 4 replicates showing continuous TJ membrane assembly (upper panel; data shown for ZO-2 and ZO-1α+; $n = 10–18$ ICMs per antibody and time point) or membrane distribution of PKCδ or ζ (lower panel; $n = 10–14$ per antibody and time point) in isolated ICMs cultured with or without 65 μM AGA for up to 6 h before fixation. (D) Representative confocal images of isolated ICMs cultured in the presence or absence of AGA for 0 or 1 h showing the distribution of the TJ components ZO-2 or ZO-1α+ (left panel) after 1 h of culture or PKCζ (right panel) after 0 or 1 h of culture. Shown are reconstructed z-series projections of 1 μm sections (3D) or individual midplane sections (m). Scale bar, 10 μm.

These data suggest that, although GJIC appears to decrease upon ICM isolation and provision of a contact-free cell surface, it is not the signaling system involved in regulating PKC relocation or junction membrane assembly in response to changes in cell contact patterns.

Discussion

In the present study, we have used the isolated ICM model for reformation of TE epithelial differentiation and have established for the first time that cell contact patterns act through PKC signaling but not via GJIC to induce TJ protein membrane assembly. While we find that cell contact patterns act as primary or obligatory regulators of TJ membrane assembly, distinct PKC isoforms, namely, at least PKCδ and ζ, are involved in regulating membrane assembly of different TJ components. Finally, contact patterns appeared to modulate all cellular processes examined (TJ membrane assembly, GJIC, PKC signaling).

Isolated ICMs as an accelerated developmental model for TJ formation

Our confocal microscopy time course of TJ membrane assembly in isolated and cultured ICMs characterized this system as a suitable and synchronized model to investigate underlying mechanisms. ZA junction formation (E-cadherin) which is required for TJ formation in the embryo (Ohsugi et al., 1997; Fleming et al., 2004) and the cytoskeleton (F-actin) were unaffected by ICM immunosurgery or culture. Moreover, membrane assembly of junctional proteins, upon provision of a contact-free cell surface in isolated ICMs, followed a similar sequence (see Introduction) but was accelerated to less than one cell cycle (9–14 h in this study; similar cell numbers were present within each ICM freshly isolated or cultured for up to 24 h) compared to normal embryo development (three cell cycles). Duration of the cell cycle within ICM cells can vary substantially between 12 and 24 h depending upon blastocyst stage, whether they remain within the intact blastocyst, in vivo vs. in vitro development, and cell cycle number (Spindle, 1978; Rossant and Lis, 1979; Handyside, 1978; Nichols and Gardner, 1984; Chisholm et al., 1985). Our results confirm that cell contact symmetry suppresses TJ protein membrane assembly within the ICM (Fleming et al., 2004) and illustrate the capacity of the ICM to compensate rapidly and repair cellular loss in the outer TE wall.

Identification of specific PKC isoforms involved in the regulation of TJ membrane assembly

Recently improved design of PKC isotype-specific peptide modulators (Mochly-Rosen and Gordon, 1998; Souroujon and Mochly-Rosen, 1998) combined with new transmembrane delivery systems (Derossi et al., 1994) helps overcome specificity problems experienced with chemical PKC modulators. In our hands, the use of these isoform-specific peptide modulators indicated that more than one PKC isoform and different isotype combinations (dependent upon the TJ component) are involved in regulating TJ formation in the ICM model. In the case of ZO-2 membrane assembly, both PKCδ and ζ activity were required, while ZO-1α+ assembly only required PKCζ activity. These data therefore extend our previous finding that chemical PKC activators stimulate TJ assembly in isolated ICMs (Eckert et al., 2004a). It should be noted, however, that the specific requirements for TJ protein membrane assembly could differ dependent upon the cell type and the combinations of PKC isoforms and/or other second messengers present within the same cell. Nevertheless, involvement of certain PKC isotypes, particularly aPKCs, is evident for both ZO-2 and ZO-1 in different systems that represent an established epithelial phenotype (see D'Atri and Citi, 2002; Gonzales-Mariscal et al., 2003). In MDCK cells, for example, the phosphorylation state of different sites of ZO-2 determines its capacity to function at the junctional complex, although, in these cells, increased ZO-2 phosphorylation via aPKCs was suggested to cause TJ degradation, while cPKCs and nPKCs were thought to promote TJ reassembly. In addition, PKCβ, ε, ι/λ and ζ were the identified isotypes to directly phosphorylate ZO-2 (Avila-Flores et al., 2001). ZO-1 translocation regulated via cPKCs α/β was observed during toxin-induced TJ membrane disassembly in colon epithelium (Chen et al., 2002). ZO-1 may also be a direct target for PKCζ in MDCK cells (Stuart and Nigam, 1995), and overexpression of dominant-negative aPKCs could disrupt ZO-1 membrane assembly (Suzuki et al., 2001). In our ICM model, data reported here together with the co-localization evident between PKC isotypes and the ZO-1α+ isoform (Eckert et al., 2004b) may suggest some direct interaction possibly at the cell membrane between PKCs and this TJ component during membrane assembly. This was suspected previously in MDCK cells (Dodane and Kachar, 1996). Alternatively, PKCs and TJ proteins could interact within the cytoplasm to coordinate the assembly process. During membrane assembly of new TJs, it is more likely that various PKC isoforms and/or other second messengers are involved in a complex signaling cascade which regulates the different steps for membrane assembly of each individual TJ protein (Dodane and Kachar, 1996). These pathways, which might be very adaptable, specifically in the early embryo, remain to be explored further. Potential PKC isoform redundancy during early development might also explain why knockouts of specific PKC isotypes do not show early lethal effects (β: Leitges et al., 1996; γ: Abeliovich et al., 1993; θ: Sun et al., 2000; ε: Khasar et al., 1999).

Interrelationship between cell contact pattern, gap junctional communication and PKC signaling networks in the regulation of TJ membrane assembly

Cell contact symmetry plays an important role in regulating cell phenotype and fate during tissue differentiation including the preimplantation embryo (Johnson, 1979; Kimber et al., 1982; Schoeck and Perrimon, 2002; Fleming et al., 2004). It is widely acknowledged that cell contact patterns induce signal

transduction pathways, but these remain poorly understood (Fagotto and Gumbiner, 1996). Although it is emerging that signaling via extracellular matrix/integrins integrates cell morphology and signal transduction in three-dimensional tissues or cells grown on different substrates, it is unclear which intracellular signaling pathways are affected (Boudreau, 2003; Bourdeau and Jones, 1999). Moreover, it remains elusive which mechanisms are involved in the early embryo as the first epithelial TE develops independently of extracellular matrix/integrin components, although many are beginning to be present in the preimplantation embryo (Sueoka et al., 1997; Bowen and Hunt, 2000). Provision of a contact-free cell surface alone changes the gene expression program and cellular organization in the embryo (Johnson, 1979; Fleming and Hay, 1991; Fleming et al., 2004). We have shown in the present study that some of this reorganization is mediated by PKC signaling. Thus, membrane assembly of two TJ components and membrane distribution of PKCδ and ζ were abolished when cell contact asymmetry was absent or incomplete following partIS treatment when only remnants of porous TE cells remained surrounding the ICM as suggested by propidium iodide penetration and increased dextran-tracer uptake. This block of membrane distribution was maintained even when broad chemical PKC activators were used, a treatment that enhanced membrane relocation of at least PKCδ and ζ in the ICM model (Eckert et al., 2004a). These results suggest that cell contact pattern is mediated by a specific membrane interaction situated upstream of the PKC signaling cascade controlling PKC protein localization and membrane delivery of TJ components. The exact (membrane) protein(s) systems responsible for this suppressive effect of cell contact symmetry remain to be determined in future studies but could include growth factor receptors, integrins, adherens junction or extracellular matrix components (see above). Cell contact patterns may further sustain the change in cellular phenotype by downregulating the expression of the transcription factor Oct-4 involved in ICM and germline pluripotency (Pesce and Scholer, 2001), thereby further enhancing an epithelial phenotype possibly involving TJ function (Nichols et al., 1998; Niwa et al., 2000). A recent study may support this idea since it showed upregulation of Oct-4 expression and downregulation of H19 expression in response to a functional inhibitor of occludin during blastocyst formation (Kim et al., 2004).

Evidence from other cell types may suggest interrelationships (see Introduction) between GJIC, PKC signaling and TJ membrane assembly as candidate mechanisms by which cell contact pattern coordinates the PKC signaling cascade within adjacent ICM cells. For example, in bovine embryos, dye transfer was enhanced in isolated ICMs compared with TE (Boni et al., 1999). Furthermore, localization of Cx43 differed between ICM and TE cells in the nascent mouse blastocyst (De Sousa et al., 1993), suggesting different mechanisms of communication in these two cell phenotypes. Regulation of GJIC is very complex, depends upon channel composition and reacts rapidly to extra- and intracellular changes. Due to this complexity of influences on GJIC, its relative importance in developmental and organ functions may be obscured and is difficult to assess as reflected by controversial discussions in the literature (White and Paul, 1999; De Sousa et al., 1993; Vance and Wiley, 1999; Cruciani et al., 2001; Saez et al., 2003; Giepmans, 2004; Lampe and Lau, 2004). The objective of the present study was, therefore, to establish whether GJIC is or is not involved in regulating TJ membrane assembly or PKC signaling by chemical inhibition of GJIC by AGA. In the present study, 56% isolated ICMs did not show any dye coupling, suggesting a rapid spontaneous closure of GJIC upon isolation since, within the intact embryo, ICM cells show as extensive dye transfer as in the TE (Eckert et al., 2004b). This is in contrast with the bovine embryo (Boni et al., 1999) and suggests that regulation of GJIC may not be conserved over different mammalian species. In addition, in our hands, development to the blastocyst, recavitation of isolated ICMs and distribution of different PKC isoforms and TJ proteins were not altered after total chemical inhibition of GJIC, similar to a previous report in embryos (Vance and Wiley, 1999) and in lens epithelial cells (Le and Musil, 1998) but contrary to Cx-knockout hepatocytes (Kojima et al., 2002). Our findings indicate, therefore, that contact-mediated changes in PKC signaling involved in epithelial differentiation of ICM cells are independent of GJIC. We speculate that the spontaneous decrease in GJIC in isolated ICMs could be due to PKC-mediated phosphorylation of connexins as reported in liver cells or fibroblasts (Ren et al., 1998; Lampe et al., 2000), indicating that suppression of gap junctional activity, like TJ membrane assembly, may be influenced by cell contact disturbance and PKC signaling. Alternatively, we cannot exclude the possibility of short-term closure of gap junction channels as a result of cellular stress or injury (see De Maio et al., 2002, for review) experienced during immunosurgery (although cell death was not observed nor was damage to the membranes (exclusion of propidium iodide), cytoskeleton (F-actin), ZA (E-cadherin) or viability (rapid TJ membrane assembly, high recavitation rate)).

Conclusions

We have shown that isolated ICMs are a suitable time-controlled model to study mechanisms governing TJ membrane assembly during early mammalian development. The sequence of membrane delivery of TJ components in ICMs induced to differentiate by provision of cell contact asymmetry broadly mimics, but at a protracted time course, that occurring within intact embryos. Specific PKC isoforms (δ, ζ) have been identified which promote TJ membrane assembly. The pattern of isoforms involved differed for the two TJ proteins examined, indicating that each TJ component has its own mechanism of membrane assembly. Manipulation of contact patterns and PKC activation in the early embryo have further demonstrated that cell contact asymmetry acts upstream of PKC-mediated signaling mechanisms governing TJ membrane assembly. This pathway is not dependent upon GJIC, which, in turn, may be subject to regulation by disturbances in cell contact patterns. We propose that cell position and the dynamics of cell contact acting via PKC signaling provide a spatially and temporally controlled mechanism to coordinate TE differentiation and blastocyst morphogenesis.

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