PKC signalling regulates tight junction membrane assembly in the pre-implantation mouse embryo

Judith J Eckert1,2, Amanda McCallum1, Andrew Mears1, Martin G Rumsby3, Iain T Cameron2 and Tom P Fleming1

1University of Southampton, Division of Cell Sciences, School of Biological Sciences, Bassett Crescent East, Southampton SO16 7PX, UK, 2University of Southampton, Fetal Origins of Adult Disease Division, School of Medicine, Coford Road, Southampton SO 16 5YA, UK and 3University of York, Department of Biology, York YO1 5DD, UK

Correspondence should be addressed to J Eckert; Email: jje@soton.ac.uk

Abstract

Epithelial differentiation including tight junction (TJ) formation occurs exclusively within the trophectoderm (TE) lineage of the mouse blastocyst. Here we examine mechanisms by which TJ protein membrane assembly might be regulated by protein kinase C (PKC) in the embryo. To overcome the inherent staging asynchrony of individual blastomeres within intact embryos, we have used isolated inner cell masses (ICMs) from early blastocysts to induce epithelial differentiation in their outer cells responding to their new cell contact pattern. Two TJ proteins examined retain their order of membrane assembly in isolated ICMs in culture as during normal development (early-assembling ZO-2 and late-assembling ZO-1α1), but this process is highly accelerated. Using six chemical modulators of PKC activity, we show here that PKC signalling is involved in the regulation of TJ membrane assembly. While indolactam-mediated PKC activation stimulates membrane assembly of both TJ proteins, TPA-mediated PKC activation stimulates only that of ZO-1α1. The PKC inhibitors Ro-31-8220, Ro-31-8425 and Gö6983 suppress the stimulatory effect of both PKC activators on membrane assembly to varying extents according to inhibitor and TJ protein examined. Gö6983 similarly inhibits ZO-2 and ZO-1α+ membrane assembly. PKC inhibition by Gö6976 appeared to stimulate TJ membrane assembly. Despite the broad PKC isotype specificity of the inhibitors used, these data suggest that the two TJ proteins are differently regulated by PKC isotypes or subfamilies. As Gö6983 uniquely affects αPKC (particularly PKCζ) and we find that both PKCδ and ζ relocate upon activator treatment to co-localise partially with the TJ proteins in isolated ICMs, we suggest that at least PKCδ and ζ may play a central role in regulating TJ membrane assembly.


Introduction

Mammalian preimplantation development is driven by a spatially and temporally controlled gene expression programme regulating cell multiplication and differentiation. In the mouse, the newly activated embryonic genome coordinates the processes that upregulate epithelial differentiation in the outer trophectoderm (TE) layer of the blastocyst and suppress such differentiation in the pluripotent inner cell mass (ICM) (Fleming et al. 2001, Watson & Barcroft 2001). To generate a functional TE, these cells must engage in transepithelial transport driven by basolateral Na+/K+-ATPase to create the blastocoel cavity while at the same time acquiring intercellular junctional adhesion to maintain epithelial integrity and provide a permeability seal against blastocyst collapse.

TE differentiation is established gradually over three cell cycles and begins at compaction (eight-cell stage) when blastomeres become adhesive and polarised following activation of E-cadherin (reviewed in Fleming et al. 2001). Subsequently, differentiative divisions generate outer polarised and inner non-polarised progenitors of TE and ICM lineages respectively at the 16-cell morula stage before further division to the 32-cell stage, when differentiation is completed and the blastocyst forms (Johnson & Ziomek 1981, Fleming 1987, Fleming et al. 2001). During this period, apicolateral zonula adherens (ZA), tight junctions (TJ) and lateral membrane desmosomes are gradually assembled exclusively within outer polarised cells. At the TJ, the intracellular plaque proteins ZO-1α− and rab13 assemble first at the eight-cell stage and cingulin plaque protein 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 (Fleming et al. 1989, 1993, Sheth et al., 1997, 2000a,b). The ZO-2 plaque protein
also assembles at the 16-cell stage (B. Sheth, unpublished). After initiation of cavitation, desmosomes form during the 32-cell stage, comprising transmembrane desmocollin (DSC2) and the cytoplasmic protein desmoplakin, and stabilise epithelial cohesion (Fleming et al. 1991, Collins et al. 1995). During cleavage, adherens junctions (AJs) form in both ICM and TE lineages of the blastocyst (Ohsugi et al. 1997).

The underlying molecular mechanisms that coordinate the establishment of cell phenotype divergence in the blastocyst remain poorly understood. One major regulator of blastocoele formation is the release of intracellular calcium, partly mediated via G-protein-coupled receptors (Stachecki et al. 1994, Stachecki & Armant 1996a, b, Wang et al., 1998). During TE differentiation, membrane assembly of the majority of TJ components appears to be regulated by translation (Javed et al. 1993) and/or post-translational modifications (such as phosphorylation) rather than de novo transcription (Fleming & Hay 1991), since transcripts for TJ proteins are readily detectable within the ICM of the early blastocyst (Sheth et al., 1997, 2000a, b). For example, occludin appears to require post-translational changes in order to become competent for membrane insertion at the TJ in embryos (Sheth et al., 2000a). ZO-1α, however, may be an exception, since it is transcribed just prior to cavitation and may regulate completion of TJ membrane assembly and cavitation (Sheth et al., 1997, 2000a). Within models that already represent a differentiated epithelial cell phenotype, such as Madin-Darby canine kidney (MDCK) cells, transcription is generally not required for the assembly, maintenance and function of TJs, while activation of protein kinase C (PKC) is required, suggesting the occurrence of post-translational modifications (Ellis et al. 1992, Denisenko et al. 1994, Stuart & Nigam 1995; see also Denker & Nigam 1998, Mullin et al. 1998; Matter & Balda 1999, Suzuki et al., 2001, Tsukita et al. 2001, Brag 2002, Hurd et al. 2003). Moreover, occludin and ZO-2 are reported to be direct targets for PKC phosphorylation in vitro (Andreeva et al. 2001, Avila-Flores et al. 2001).

Although activation of PKC has been shown to be involved in the regulation of oocyte maturation, fertilisation and compaction, whether it has a role during TE differentiation remains unclear (Winkel et al. 1990, Pauken & Capco 1999, Capco 2000). There are at least 11 different PKC isoforms (conventional or cPKCs, βI, βII and γ; novel or nPKCs, ε, η, θ and μ; and atypical or aPKCs/α and ζ) with different structure and activation requirements (Mochly-Rosen & Gordon 1998; Csukai & Mochly-Rosen 1999), and a variety of these have been detected throughout preimplantation embryo development (Gangeswaran & Jones 1997, Kaz et al. 1998, Pauken & Capco 2000, Downs et al. 2001, Eckert et al. unpublished). Moreover, expression and localisation of several isoforms appears to be developmentally regulated (Pauken & Capco 2000, Eckert et al. unpublished).

In the present study, we examine mechanisms by which TE differentiation and TJ protein membrane assembly might be governed by PKCs. However, due to the inherent asynchrony in developmental rates between individual blastomers within intact embryos, targeting treatment to modulate PKC activity to specific differentiative events becomes problematic. To overcome this concern, we have used the isolated ICM model in which immunosurgery acts as an experimentally controllable ‘switch’ to induce TE-like differentiation of outer blastomerers released from the inhibitory condition of cell contact symmetry experienced in the embryo interior (Fleming et al. 2001). Thus, for example, ICMs show ZO-1 membrane assembly within hours of isolation and form blastocyst-like vesicles with TE-like differentiation of the outer cells when isolated from early blastocysts, while ICMs isolated from later, more expanded blastocysts preferentially form endoderm-like structures (Handyside 1978, Spindle 1978, Rossant & Lis 1979, Fleming et al., 1984, Nichols & Gardner 1984, Chisholm et al., 1985, Fleming & Hay 1991). In addition, the ICM model concentrates on the effect of (post-) translational events rather than transcriptional influences, since the TJ transcripts are already present within the ICM. For example, isolated ICMs were used to show that initiation and stabilisation of cell polarisation may involve post-translational modification of ezrin, a cell-surface protein that mediates membrane–cytoskeletal interaction (Louvet-Vallee et al. 2001). Moreover, the justification for investigating developmental mechanisms using isolated ICMs has been indicated by their capacity to implant into the uterus (Rossant & Lis 1979) and develop into offspring when injected into host blastocysts (Azim et al. 1978).

In examining the isolated ICM model, our data, using a pharmacological approach with broad PKC specificity, show that PKC signalling is involved in regulation of membrane assembly of both TJ components ZO-2 and ZO-1α+ and also suggest the involvement of different PKC isotype networks for each TJ component. While modulation of PKC activity with broad chemical inhibitors and activators does not allow conclusive unravelling of the identity of the PKC isoforms involved, our results indicate a role for PKCα and ζ in TJ assembly. They also clearly demonstrate the complexity of the signalling cascades used by the early embryo to coordinate properly the assembly of a multimolecular complex such as a TJ during TE differentiation.

**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 mare’s serum (PMS; Folligon, Intervet UK Ltd, Milton Keynes, UK) followed by 5 i.u. human gonadotrophin (hCG; Chorulon, Intervet) and mating 48 h later. Eight-cell embryos were flushed from dissected oviducts with H6 medium.
supplemented with 4 mg/ml BSA (H6-BSA; Sigma) and cultured under our standard conditions up to the early blastocyst stage in T6 medium containing 4 mg/ml BSA (T6-BSA), in 5% CO₂ in air atmosphere at 37 °C, in micro-drops under oil (J.M. Loveridge, Southampton, UK), as described previously (Sheth et al., 1997, 2000a,b, Louvet-Vallee et al. 2001).

**Isolation and culture of ICMs**

ICMs were isolated in batches of 100–150 from early blastocysts (92–96 h after hCG; maximum of 2 h after the formation of a visible cavity) to ensure staining efficiency. The embryos/ICMs were stained 1997, 2000 with 1% formaldehyde (Analar, Merck, Poole, UK) in four-well plates (Nunc, Roskilde, Denmark) and incubated at 5% CO₂ in air and 37 °C in a humidified atmosphere for up to 29 h before fixation.

**Immunocytochemistry and confocal microscopy**

Zona-free embryos or ICMs were fixed in PBS supplemented with 1% formaldehyde (Alar, Merck, Poole, Dorset, UK or Sigma) for 7–15 min, attached onto cover slips coated with 1.5 mg/ml poly-L-lysine hydrobromide (Sigma) and processed for immunocytochemistry as described previously (Fleming et al. 1984, Chisholm et al. 1985) by immunosurgery, as previously described (Sheth et al. 1997) with modifications (Van Soom et al. 1996). After removal of the zona pellucida by acid Tyrode’s, blastocysts were incubated in 10 nM triunitrobenzene-sulphonic acid (TNBS; Sigma) in H6 medium containing 3 mg/ml PVP (Sigma, H6-PVP) (Sheth et al. 1997) for 10 min at room temperature to mark the outer TE cells. TNBS combines with N-terminal amines and thus labels proteins and some phospholipids. After extensive washing in H6-PVP, blastocysts were incubated for 10 min at room temperature in 0.3% goat anti-dinitrophenyl-antibody (anti-DNP-BSA; ICN Biochemicals (Basingstoke, Hampshire, UK); cross-reacts with trinitrophenol-groups) in H6-PVP, before three thorough washes in H6-PVP, and 10-min incubation in human (Sigma) or guinea pig (Cedarlane Laboratories Ltd, Hornby, ON, Canada) complement, reconstituted according to the manufacturer and diluted 1:10 in H6-BSA at 37 °C. Finally, after a 20-min recovery of the treated embryos in H6-BSA, the ICMs were shelled out mechanically with a thinly pulled and polished micropipette. The freshly isolated ICMs were placed in 500 μl DMEM (Gibco) plus 10% FCS (heat-inactivated; Labtech International, Rigmer, Sussex, UK) in four-well plates (Nunc, Roskilde, Denmark) and incubated at 5% CO₂ in air and 37 °C in a humidified atmosphere for up to 29 h before fixation.

**Antibodies and verification of staining patterns**

Antibodies to the junctional protein ZO-1α⁺ (rabbit polyclonal, diluted 1:250, or guinea pig polyclonal, diluted 1:250, both against the murine α-motif; Sheth et al., 1997) were used 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 in-house polyclonal antibodies against the rat sequences, as detailed elsewhere (Drew et al. 1994, Gott et al. 1994, Littlebury et al. 1997), at dilutions between 1:200 and 1:500 (Eckert et al. unpublished). These in-house antibodies were analysed by Western blotting and showed similar protein sizes for PKCβI, βII, γ, δ, ε and ζ in mouse liver and/or brain lysates, as described previously (Drew et al. 1994, Gott et al. 1994, Littlebury et al. 1997) (data not shown). Preincubation with the respective peptide against which they were raised blocked immunofluorescent staining either partially or completely.

For further verification of the specificity of these antibodies, a set of commercial antibodies generated in mouse (Transduction Labs, BD Bioscience, Oxford, UK) 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). The commercial antibodies have been used previously in different species or mouse embryos (e.g. Minichiello et al. 1999, Pauken & Capco 2000). Negative controls (secondary antibody only; rabbit pre-immune serum, neat) did not show background staining. Respective negative controls for the double labelling (successive incubation in respective primary antibodies at room temperature for 1 h instead of simultaneous incubation in antibody-cocktail; cross-controls with one primary and two secondary or two primary and one secondary antibody) ensured that there was no cross-reactivity between different primary and secondary antibodies. The staining pattern was identical with both sets of PKC isotype-specific antibodies.

**Chemical PKC activation and inhibition**

Isolated ICMs were cultured in the presence of 0.1, 1, or 10 nM of the cell-permeable PKC activators TPA (phorbol
ester; Calbiochem-Novachem Ltd, Nottingham, UK) or indolactam (alkaloid-type activator; Calbiochem) or 10 nM 4aPDD (inactive analogue; Calbiochem) or 0.1% DMSO (vehicle; Sigma) as controls. Both activators directly affect cPKCs and nPKCs, but while TPA preferably binds the calcium-dependent cPKC isoforms (Kazanietz et al. 1993) indolactam binds to cPKC and nPKC subtypes without preference (Kazanietz et al. 1993, Lee et al. 1993, Geiges et al. 1997). After 1, 2 or 3 h (n = 34–57 per time point, treatment and antibody) or 6 h (n = 15–38 per treatment and antibody), the ICMs were fixed and stained in parallel for ZO-2 or ZO-1α+ to determine the capacity for membrane insertion of an ‘early’ and a ‘late’ assembling TJ protein. For each time point, data were collected from at least five replicate experiments (that is, different batches of embryos).

Isolated ICMs were also cultured in the presence of the cell-permeable protein kinase inhibitors Gö 6976 (indolocarbazole; 100 or 10 nM), Gö 6983, Ro-31-8220 or Ro-31-8425 (bisindolylmaleimides; 1 μM or 100 nM; all from Calbiochem) for 1 h before fixation and staining for ZO-2 or ZO-1α+. In parallel, ICMs were first incubated in inhibitor for 15 min, washed three times, and then incubated for the remaining 45 min in the presence of either PKC activator to examine the reversibility of inhibitor treatment (n = 38–60 per treatment and antibody in five replicate experiments). The inhibitor was washed out before activator treatment to avoid interaction between the two chemicals. These protein kinase inhibitors inhibit PKC at nanomolar concentrations (100–1000-fold more efficient for PKC than, for example, for PKA) and can be effective within minutes of treatment within other systems (Staendart et al. 1997, Lin & Chen 1998, Keller et al. 2000). The inhibitors have been shown to affect a broad range of PKC isoforms with slightly varying efficiency (summarised in Way et al. 2000). For example, Gö 6076 has been used to inhibit cPKCs over nPKCs, and it uniquely affects PKCζ at nanomolar concentrations, while Gö 6983 is the only inhibitor among the ones tested that affects PKCζ (see Table 1 and references therein).

Activator (10 nM) and inhibitor (10 nM for Gö 6976 and 100 nM for the other inhibitors) treatments for 1 h were also followed by staining for PKC isotypes (n = 19–25 per treatment and antibody in 3–5 replicate treatments).

Table 1 Effects of modulation of PKC activity in murine isolated ICMs on membrane assembly of the TJ proteins ZO-2 and ZO-1α+.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>TPA</th>
<th>Indolactam</th>
<th>TPA</th>
<th>Indolactam</th>
<th>PKC isoforms affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>c/nPKCs (α, βI, μ) (Martiny-Baron et al. 1993, Gschwendt et al. 1996)</td>
</tr>
<tr>
<td>Gö 6976</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>c/nPKCs (α, βI, γ, δ, ε, ζ) (Gschwendt et al. 1996)</td>
</tr>
<tr>
<td>Gö 6983</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>c/nPKCs (α, βI, γ, δ, ε, ζ) (Staendart et al. 1997)</td>
</tr>
<tr>
<td>Ro-31-8220</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>c/nPKCs (α, βI, γ, δ, ε) (Wilkinson et al. 1993)</td>
</tr>
</tbody>
</table>

*: no effect; †: stimulation of membrane assembly; ‡: inhibition of membrane assembly. *Inhibitor effective only at very high dosages.
PKC regulation of tight junction formation

657

Fixation and staining for the two TJ proteins ZO-2 and ZO-1α+ established that, like the sequence within the intact embryo, ZO-2 assembles earlier than ZO-1α+ in isolated ICMs, with a clear distinction between the two up to 6 h after onset of in vitro culture. For example, at 1, 2 and 6 h after ICM isolation, ZO-2 continuous membrane assembly was observed in 25%, 26%, and 71% (n = 35–45) respectively, while ZO-1α+ membrane assembly was observed in 2%, 5% and 33% (n = 37–50) of ICMs stained. After overnight culture, some 80% of ICMs showed membrane assembly of these proteins, indicative of their viability (Fig. 1). Moreover, recavitation was first observed after 9 h of culture in a small percentage of isolated ICMs (1–3%; n = 112), but, by 14 h, 70% had recavitated.

To establish whether PKC signalling is involved in regulating junction membrane assembly in our developmental model, we investigated the effect of two different PKC-specific activators, TPA and indolactam, on the membrane assembly of ZO-2 and ZO-1α+ in cultured isolated ICMs (Fig. 2). Overall, continuous membrane assembly of both TJ proteins was accelerated (see Materials and Methods and Fig. 1 for definition; Fig. 2A and B), while discontinuous staining was minimally affected (not shown; deduced from Fig. 2). This stimulation was observed after a short culture period only and most potently with indolactam (1-2 h for ZO-2 and 2–3 h for ZO-1α+). Due to increasing spontaneous continuous membrane assembly of both TJ proteins (significant shift towards the continuous pool in all treatment groups), the stimulatory effects of PKC activators became undetectable after 6 h. Specificity of the effects was confirmed, as the inactive TPA analogue 4αPDD did not affect TJ assembly rate in any case.

The two activators had different effects that depended upon dosage, time point, and TJ protein examined (Fig. 2A and B). Continuous membrane assembly of ZO-2 was not significantly elevated compared with the controls at any time point by TPA treatment. However, a rapid (1 h) and significant (P < 0.05) shift of ZO-2 from the negative towards the continuous pool was observed in all concentrations of TPA. Indolactam appeared more potent than TPA, as increasing doses rapidly (1 h) and significantly (P < 0.05) elevated membrane assembly of ZO-2 in continuous pools by decreasing the discontinuous and negative pools. For the later assembling protein, ZO-1α+, all concentrations of TPA significantly (P < 0.05) reduced the negative pool by shifting ZO-1α+ towards the discontinuous and/or continuous pools. While the highest 10 nM dose was most potent in this respect early on (2 h), lower doses became more effective later (3–6 h), suggesting dose-dependent downregulation when higher doses of TPA were used. In contrast, the lowest dose of indolactam (0.1 nM indolactam) was most potent to reduce significantly (P < 0.05) the negative pool of ZO-1α+ in favour of continuous membrane assembly early on (2 h), and the middle dose was the most potent after 3 h.

**Results**

**Activation of PKC stimulates TJ protein membrane assembly in isolated ICMs**

To confirm the specificity of PKC action and to elucidate which PKC isotypes might be involved, four PKC-specific inhibitors with slightly different isotype-specific inhibitory efficiency (see Methods and Methods; Table 1) were used at two different concentrations. These treatment groups were compared with vehicle control (DMSO), minimal effective dose of activator alone (TPA: 10 nM for ZO-1α+, indolactam: 10 nM for ZO-2 and 0.1 nM for ZO-1α+; derived from Fig. 2), and inhibitor followed by activator treatment (Fig. 3A and B) to examine the reversibility of inhibitor treatment. To minimise variable and (potentially) non-specific effects of drug treatment, culture time was restricted to 1 h when activator effects had been obvious previously (see above). Non-specific toxicity of drug treatment was excluded, as 60–80% of ICMs derived from each treatment group (n = 20–30 recavitated after culture in drug-free medium for an additional 12 h.

Overall, again the discontinuous pool of TJ membrane assembly remained similar, and mainly the distribution between the continuous and negative staining pools was changed. Moreover, the lower inhibitor concentrations showed more severe inhibition than the higher concentrations, as the majority of ICMs remained within the negative staining pool. As with the activators, the effects of different inhibitors varied between the two TJ proteins examined. In the case of ZO-2, due to a high and rapid spontaneous membrane assembly in this experimental series, the effect of indolactam alone remained slightly below significance level compared to vehicle control (P = 0.058). The opposite was observed in the case of ZO-1α+. The low spontaneous membrane assembly occurring within the time frame of 1 h used in this experimental series was not further reduced by any inhibitor type or concentration but was significantly (P < 0.05) increased by both activator treatments. Ro-31-8220 prevented (P < 0.05) activator-induced membrane assembly
Figure 2 Membrane assembly of (A) ZO-2 or (B) ZO-1α+ over time in mouse isolated ICMs cultured in vitro in the presence or absence of either PKC activator TPA (left panel) or indolactam (right panel). Each column contains 34–57 (1 or 2 h respectively) and 15–38 (6 h) isolated ICMs in at least five replicates. Significant differences (P < 0.05) are indicated by different letters within either the continuous (filled bars) or negative (open bars) pools and separately for each time point examined. Asterisks indicate significant differences between the continuous and negative pool within one treatment group and time point. The discontinuous pool is not shown (no significant effect of treatment) but can be deduced from the continuous and negative pools.
Figure 3 Membrane assembly of (A) ZO-2 or (B) ZO-1α+ in mouse isolated ICMs cultured in vitro either with different PKC-specific inhibitors alone for 1 h or for 15 min followed by 45-min culture in the presence of the activators indolactam (10 nM for ZO-2 or 0.1 nM for ZO-1α+) or TPA (10 nM for ZO-1α+). In the left panel, 10 nM (Go¨ 6976) or 100 nM (Go¨ 6983, Ro-31-8220 or Ro-31-8425) inhibitor were used, and in the right panel 100 nM (Go¨ 6976) or 1 μM (Go¨ 6983, Ro-31-8220 or Ro-31-8425) inhibitor were used. Data were collected from at least five replicates and a total number of 38–60 isolated ICMs per treatment. Significant differences (P < 0.05) are indicated by different letters within either the continuous (filled bars) or negative (open bars) pools respectively. Asterisks indicate significant differences between the continuous and negative pools within one treatment group. The discontinuous pool is not shown (no significant effect of treatment) but can be deduced from the continuous and negative pools.
of both ZO-2 and ZO-1α+ in a dose- and activator-type-dependent manner, while Ro-31-8425 inhibited only TPA-induced ZO-1α+ membrane assembly. TPA-induced continuous membrane assembly of ZO-1α+ was more effectively prevented by both inhibitors compared to indolactam-induced membrane assembly. Furthermore, most ICMs remained within the discontinuous pool of ZO-1α+ staining rather than the negative one (deduced from Fig. 3), suggesting that these inhibitors could not prevent the initiation of membrane assembly (discontinuous staining), but only its completion (continuous staining), during this time frame of 1 h. However, the most potent inhibitor of membrane assembly of both TJ proteins induced by both activator types was Gö 6983, which appeared to reduce both initiation and completion of membrane assembly of both TJ proteins, with the majority of ICMs remaining within the negative pool. Notably, a speckled intracellular staining pattern for ZO-2 was observed in 80% of ICMs examined in all five replicates (Fig. 4). This pattern could also be seen occasionally (2/5 replicates) in the presence of Ro-31-8220 in approximately 30% of the ICMs investigated, and could only partially be overcome by indolactam-induced activation. The inhibitor Gö 6976 did not have any significant effect (P > 0.05) on either the rapid spontaneous or indolactam-induced ZO-2 membrane assembly. Surprisingly, similar continuous ZO-2 membrane assembly levels were observed as compared to indolactam activation, as also evidenced by a significant shift towards the continuous pool. In the case of ZO-1α+, Gö 6976 stimulated the low spontaneous membrane assembly, although this was slightly less evident when followed by the respective activator treatment. Results are summarised in Table 1.

**Specific PKC isotypes relocate in ICMs after isolation and after activation or inhibition of PKC**

To help identify the candidate PKC isotypes potentially involved in membrane assembly of TJ proteins in isolated and cultured ICMs, we next examined the localisation of PKC isotypes either immediately following isolation, or after culture for 1 h in the presence of either vehicle control, the two activators (10 nM), or the lower concentration of one of the inhibitors. Isolated ICMs were both fixed immediately after isolation or after 1 h in culture and stained for different isotypes (Fig. 5A and B). Two factors determine biologically relevant activity of PKCs: the accessibility of their substrate binding site and their subcellular distribution. Both are in turn regulated by second messengers, specific anchoring proteins and the phosphorylation status of PKCs (Mochly-Rosen & Gordon 1998, Jaken & Parker 2000, Newton 2003). When many PKC isoforms are present within the same cell, a change in localisation reflecting altered accessibility of the kinase to protein substrates is indicative of a change in activation status and function in response to a certain event – in this case, ICM isolation and culture.

The cPKCs (Fig. 5B) and PKCη (not shown) remained diffusely located within the cytoplasm in freshly isolated or cultured ICMs in all treatment groups. The novel-related isoform PKCλ also did not change location over time in any treatment group (Fig. 5B). In contrast, nPKCε, δ and θ, and aPKCζ and λ changed their distribution pattern in ICMs after isolation, but a high variation of treatment effects was observed according to isotype (Fig. 5A and B). While PKCε was found distributed diffusely within the cytoplasm of freshly isolated ICMs, it appeared to be concentrated at the membrane or as a combination of membrane and focal cytoplasmic staining (Fig. 5B) within outer blastomeres in ICMs cultured for 1 h (P < 0.05; Fig. 5A). As expected, PKCε remained significantly (P < 0.05) more within the cytoplasm when cultured in the presence of Ro-31-8220 and Ro-31-8425, the two inhibitors reportedly affecting this isoform. PKCθ, ζ and λ were already located within the membrane of approximately 25–83% (Fig. 5A and B) of freshly isolated ICMs, while ICMs within intact blastocysts showed no membrane staining for any of these isoforms (not shown; Eckert et al. unpublished). This suggested a very rapid (within minutes of handling time) relocation towards the ICM membrane region upon provision of a contact-free cell surface for these isoforms. However, PKCθ and ζ showed a tendency (P < 0.1; Fig. 5A) to relocate increasingly towards the membrane region in isolated ICMs during the 1-h incubation period. As expected, PKCθ was shifted to the membrane by both activators (P < 0.01 and P < 0.05 respectively) while PKCζ was significantly (P < 0.01) increased within the...
Figure 5 Distribution of specific PKC isotypes in mouse ICMs freshly isolated or cultured in the presence of PKC activators or inhibitors for 1 h. In 3–5 replicates, a total number of 19–25 ICMs were examined per treatment and PKC isoform. (A) Significant differences (* P < 0.05 or ** P < 0.001) between cytoplasmic or linear staining in the membrane region are indicated by asterisks for the specific PKC isoform and treatment. (B) Representative localisation of different PKC isoforms in ICMs either freshly after isolation (0 h) or after 1 h of in vitro culture (1 h) are shown in midplane sections (m) or 3-D reconstructions (3D). Arrows indicate membrane distribution; arrowhead indicates cytoplasmic foci. Bar = 20 μm.
membrane pool upon TPA, but not indolactam, activation. However, none of the inhibitors had any significant effect on the localisation of these two isotypes. On the other hand, PKCα/γ was significantly (P < 0.01) increased within the cytoplasmic pool by Gö 6976, an inhibitor not reported to affect this PKC isotype. Finally, PKCθ rapidly relocated towards the membrane in the controls, but this was even more pronounced in the presence of both activators and all four inhibitors (P < 0.01–0.05).

To examine a possible interaction between PKC isoforms and TJ proteins during membrane assembly, isolated ICMs cultured with activators or inhibitors were double-labelled with ZO-1α⁺ and the different PKCs. A close proximity was found between the TJ protein ZO-1α⁺ and PKCζ, ψ, θ or δ in the membrane region of blastomeres after 1-h culture in vitro in all treatments, such that in some domains colocalisation was evident but in other areas the two proteins were separately located (Fig. 6 shows double-labelling for ZO-1α⁺ and PKCθ).

**Discussion**

In the present study, we have used the isolated ICM model for reformation of TE epithelial differentiation to examine the role of PKC isoforms in TJ biogenesis. Collectively, our results from investigating the effects of broad PKC activators and inhibitors on TJ protein membrane assembly and PKC isotype localisation demonstrate for the first time that PKCs regulate this process under our culture conditions in vitro. The switch in cell contact patterns experienced by ICM cells following immunosurgery thus appears to activate PKCs to mediate this step in differentiation.

In vitro cultured ICMs isolated from early blastocysts within the first few hours after formation of a visible cavity develop a TE-like outer cell layer with polarised cytoplasm and TJs resembling a miniature blastocyst (Fleming et al. 1984, Chisholm et al. 1985, Fleming & Hay 1991). This miniature blastocyst can induce a decidual reaction upon embryo transfer, further confirming TE-like identity of the outer cells (Rossant & Lis 1979). The capacity to recavitate and reform TE-like outer cells is progressively lost when ICMs are isolated from older, more expanded blastocysts, which then form predominantly endoderm-like structures (Fleming et al. 1984, Chisholm et al. 1985). In the present study using cultured ICMs isolated from very early blastocysts (within 2 h of cavitation), we further demonstrate the suitability of this model to examine underlying mechanisms of TJ protein membrane assembly. Our confocal microscopy time course shows that both TJ proteins examined kept their characteristic membrane assembly timing as during normal development (ZO-2 early and ZO-1α⁺ late). However, the acceleration of assembly from three cell cycles to one cell cycle helped overcome the problem of exact timing of developmental stage and asynchrony. Cell adhesion (E-cadherin) and cytoskeleton (F-actin) remained unchanged in cultured ICMs (not shown; Louvet-Vallee et al. 2001), and it has been shown previously that in this model polarisation of outer cells, including microvilli-formation, takes place as in normal development (Fleming et al. 1984, Louvet-Vallee et al. 2001). Our results and those of others confirm the notion that reversal of the cell contact symmetry within the ICM in intact blastocysts releases the suppression of TJ protein membrane assembly, and illustrate the capacity of the ICM to compensate rapidly and repair potential cellular loss in the outer TE wall (see Introduction; reviewed in Fleming et al. 2001). Moreover, a number of PKC isoforms redistribute rapidly within ICMs during isolation from the blastocyst (discussed below). During compaction and cavitation of intact embryos, changes of PKC distribution have similarly been shown, and certain PKC isoforms are distributed in a lineage-specific manner within the nascent blastocyst (Pauken & Capco 2000, Eckert et al. unpublished). However, we cannot exclude the possibility that in vitro culture methods may influence any of these processes.

In the present study, stimulation of PKC activity by either the phorbol ester, TPA (which activates only the cPKCs and nPKCs and preferably binds the calcium-dependent cPKC isoforms; Kazanietz et al. 1993) or the alkaloid-type activator, (−)-indolactam V (which binds to cPKC and nPKC subtypes without preference; Kazanietz et al. 1993, Lee et al. 1993, Geiges et al. 1997), showed a dose- and time-dependent acceleration of membrane assembly dependent upon the TJ protein examined. PKC activator treatment was most effective during shorter incubation times and was no longer evident after 6-h culture. This may suggest that, by 6 h, those ICMs that will recavitate within the next few hours (indicating reformation of predominantly TE) have accomplished TJ membrane assembly (approximately 55–70% show assembly, corresponding to an approximately 70% cavitation rate a few hours later respectively), and PKC activation cannot further increase this percentage. Alternatively, the activator may have caused downregulation of the activity of certain PKC isoforms. This has been widely reported previously as a consequence of longer-term exposure and/or high concentrations of TPA (e.g. Ellis et al. 1992). To avoid this effect, the concentrations of phorbol ester used in our study were well below those that induced downregulation or inhibited compaction as reported previously by others (Bloom 1989, Winkel et al. 1990). In addition, only a short-term exposure to the drugs was employed to avoid other side effects on embryo development (Ohsumi & Yamamura 1993).

Modulation of PKC activity had different effects for the two TJ protein markers examined. While assembly of ZO-1α⁺ was readily stimulated by both activators, although with different potency, ZO-2 assembly was stimulated by indolactam and at a higher concentration than that required for ZO-1α⁺ while TPA had only marginal effects. This distinction may in part reflect the rapidity of ZO-2 assembly in controls compared with ZO-1α⁺, making further stimulation more difficult to induce. Alternatively,
different PKC isotypes may be involved for each TJ protein. The latter idea is supported further by the variable capacity of different inhibitors to prevent spontaneous and/or activator-induced membrane assembly within a restricted time frame. For example, the broad cPKC and nPKC inhibitor Ro-31-8425 reduced TPA-induced membrane assembly of ZO-1α+ but not that of ZO-2. On the other hand, Ro-31-8220, which is another broad inhibitor reaching cPKCs and nPKCs with similar efficiency as Ro-31-8425, prevented activator-induced and/or spontaneous upregulation of membrane assembly of both TJ proteins. This surprising discrepancy may be explained by the fact that only Ro-31-8220 has been shown to inhibit also aPKCs (PKCζ; Staendart et al. 1997), although at a much higher concentration than the one used in the present study. Involvement of aPKCs in TJ membrane assembly is indicated further by very efficient inhibition of membrane assembly of both proteins in the presence of Gö 6983, an inhibitor reaching

![Representative midplane (m) or tangential (t) sections of confocal images showing the relative distribution of the TJ marker ZO-1α+ and PKCδ in mouse ICMs freshly after isolation (control 0 h) or after 1 h of culture in vitro in the absence (control 1 h) or presence of chemical PKC activators TPA or indolactam. Twelve to 15 ICMs were analysed per treatment and time point respectively. Bar = 30 μm.](image-url)
cPKCs and nPKCs, like Ro-31-8425 and Ro-31-8220, but also inhibiting αPKCζ more potently than Ro-31-8220. However, only ZO-2 protein, not ZO-1αγ, was distributed in a speckled pattern within the cytoplasm of ICMs in the presence of Gö 6976, suggesting different underlying mechanisms regulating membrane assembly of the two TJ proteins, as suggested previously (Dodane & Kachar 1996). The ZO-2 staining pattern may, for example, represent protein storage within specific cytoplasmic regions and could suggest that degradation or transport, but not expression, of ZO-2 protein is regulated by αPKCs. ZO-1αγ, however, could be more diffusely distributed within the blastomeres by different mechanisms of protein storage or degradation, it could be less stable than ZO-2, or, indeed, its protein expression could be downregulated. Further experiments are needed to clarify this difference.

A surprising observation was the stimulation of TJ membrane assembly not only upon PKC activator treatment but also upon PKC inhibition with Gö 6976. Gö 6976 reaches cPKCs just like the other three PKC inhibitors discussed above but also efficiently inhibits nPKCζ. However, in our hands, PKCζ localization in isolated ICMs remained unaffected by Gö 6976. Since PKCζ was predominantly found at the membrane, access of the inhibitor could have been limited or membrane anchorage could have remained unchanged (Way et al. 2000, Slater et al. 2002). On the other hand, relocation of αPKCζ, an isoform not reportedly affected by this inhibitor, was prevented. Similarly, clear relocation upon PKC activator treatment indicative for activity change could be demonstrated only for PKCβ and ζ in isolated ICMs. Although this may support the idea that nPKCs and αPKCs are involved in TJ membrane assembly, as evident particularly for αPKCs in other systems (see D’Atri & Citi 2002, Gonzalez-Mariscal et al. 2003), it also indicates the presence of a complex network of different mechanisms and second messengers that regulate this process in a hierarchical manner (Dodane & Kachar 1996). The latter idea is supported by the fact that the unexpected relocation of αPKCζ towards the membrane upon TPA treatment (αPKCs would not be reached directly by TPA) observed in the present study has been reported previously in neutrophils where more complex signalling through Rho GTPases and DAG-sensitive PKCs was involved in causing this relocation (Laudanna et al. 1998). The presence of a very complex hierarchical signaling network may also be one explanation for the lack of clear effects of inhibitor and activator treatment on PKC isoform localization in isolated ICMs. The ‘switch’ to induce TE-like differentiation upon provision of a contact-free cell surface by immunosurgery could not be overridden, although the ICM isolation procedure was performed in the presence of the respective inhibitor to ensure immediate effectiveness of the chemical. It must be noted, though, that although a steady-state effectiveness of various PKC inhibitors was reached within 15 min in other cell-culture systems (e.g. Staendart et al. 1997, Lin & Chen 1998, Keller et al. 2000), the kinetics within the early embryo or isolated ICM remain unknown.

It is, therefore, possible that the time frames of (spontaneous) relocation of specific isotypes upon provision of a contact-free cell surface and (possibly) shutting between cell compartments might be very short. In other systems, PKC isoforms can change their distribution within minutes as a result of cellular manipulations (e.g. Nowicki et al. 2000). Therefore, it cannot be excluded that the inhibitors might show different effects at different time points (such as minutes after ICM isolation), when intact blastocysts are pre-incubated with inhibitors for some time or when the inhibitors are used at higher dosages. However, since such treatments could provoke unwanted side effects and a loss in specificity for PKC (Beltman et al. 1996, Morreale et al. 1997, Lingameneni et al. 2000), this was avoided. It must be noted that although broad chemical PKC activity modulators are useful tools to establish participation of PKC signalling, interpretation of the data obtained is difficult, in particular when a large number of PKC isoforms are available, as in the early embryo (Pauken & Capco 2000, Eckert et al. unpublished). Insufficient or absent PKC isoform selectivity of the chemicals, variable binding efficiency that depends upon PKC localization and accessibility, and the presence of and interference with intracellular co-factors contribute to the limitations of interpretation. More PKC isoform-specific approaches are, therefore, required to identify clearly the nature of participating PKC-signalling networks or specific isoforms that regulate certain developmental events (see Way et al. 2000, Slater et al. 2002).

One alternative and more selective approach to isolate individual participating PKC isoforms is to determine their localization relative to the protein of interest. This has been used in various cell types. For example, co-localisation between PKC isoforms and TJ proteins suggests the involvement of certain PKC isoatypes, particularly αPKCs, in TJ protein membrane assembly in epithelial cell models (Stuart & Nigam 1995, Dodane & Kachar 1996, Suzuki et al. 2001). In our ICM model, the lack of complete co-localisation between PKC isoatypes and ZO-1αγ during the membrane assembly process suggests that a permanent direct interaction between the two protein systems is not required for TJ assembly. This was suspected previously in MDCK cells (Dodane & Kachar 1996). However, it is possible that PKCs and TJ proteins do interact during tightly regulated time periods and in certain subcellular compartments, as a newly emerging model for temporal and spatial regulation of oscillating PKC phosphorylation may suggest (Violin et al. 2003). Our confocal data showing partial PKC and TJ co-localisation support this notion, but more rigorous biochemical analyses would be required for confirmation.

Taken together, our data showing that PKC inhibitors can, to varying extents, suppress the stimulatory effect of PKC activators on TJ protein membrane assembly, confirm a role for PKCs in this differentiation event. The data also indicate that (i) the two TJ proteins are differently regulated by PKC isoatypes or subfamilies, (ii) different steps in the assembly process (reflected in different
membrane and cytoplasmic staining patterns of the TJ proteins) may be separately regulated by different PKC iso-
types, and (iii) from contrary effects of the different
inhibitors, a role for aPKC (particularly PKC\(\zeta\)) is impli-
cated from the data using G\(\delta\) \(6983\). Given the broad PKC
isotype specificity of the inhibitors used, there is evidence
also for a potential role for both cPKCs and nPKCs. How-
ever, since PKC\(\delta\) and \(\zeta\) undergo clear relocation upon
activator treatment in isolated ICMs and are differentially
distributed within the TE and ICM of the nascent blasto-
cyst (Eckert et al. unpublished), those two PKC isotypes
could play a central role in regulating TJ membrane
assembly.

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References
Andreeva AY, Krause E, Muller EC, Blasig IE & Utepebergenov DI
2001 Protein kinase C regulates phosphorylation and cellular
localization of occcluding. Journal of Biological Chemistry 276
38480–38486.
Avila-Flores A, Rendon-Huerta E, Moreno J, Islas S, Betanzos A,
Robles-Flores M & Gonzalez-Mariscal L 2001 Tight-junction pro-
tein zona occuludens 2 is a target of phosphorylation by protein
kinase C. Biochemical Journal 360 295–304.
Azim M, Surani H, Torchiana D & Barton SC 1978 Isolation and
development of the inner cell mass after exposure of mouse
embryos to calcium ionophore A23187. Journal of Embryology and
Bellman J, McCormick F & Cook SJ 1996 The selective protein kinase
C inhibitor, Ro-31-8220, inhibits mitogen-activated protein kinase
phosphatase-1 (MKP-1) expression, induces c-Jun expression, and
activates Jun N-terminal kinase. Journal of Biological Chemistry
271 27018–27024.
Bloom TL 1989 The effects of phorbol ester on mouse blastomers: a
role for protein kinase C in compaction Development 106
159–171.
Braga VM 2002 Cell–cell adhesion and signaling Current Opinion in
Cell Biology 14 546–556.
Capco DG 2001 Molecular and biochemical regulation of early
mammalian development. International Review of Cytology 207
195–235.
Chisholm JC, Johnson MH, Warren PD, Fleming TP & Pickering SJ
1985 Developmental variability within and between mouse
expanding blastocysts and their ICMs. Journal of Embryology and
Experimental Morphology 86 311–336.
Collins JE, Lorrimer JE, Garrod DR, Pidley SC, Buxton RS & Fleming
TP 1995 Regulation of desmocollin transcription in mouse preim-
plantation embryos. Development 121 743–753.
Csukai M & Moehly-Rosen D 1999 Pharmacologic modulation of
protein kinase C isozymes: the role of RACKS and subcellular
D’Atri F & Citi S 2002 Molecular complexity of vertebrate tight junc-
tions. Molecular Membrane Biology 19 103–112.
Denisenko N, Burighel P & Citi S 1994 Different effects of protein
kinase inhibitors on the localization of tight junctional proteins at
Denker BA & Nigam SK 1998 Molecular structure and assembly of
the tight junction. American Journal of Physiology 274 (Renal
Physiology 43) F1–F9.

Dodane V & Kachar B 1996 Identification of isoforms of G proteins
and PKC that colocalize with tight junctions. Journal of Membrane
Biology 149 199–209.
Downs SM, Cotom J & Hunzicker-Dunn M 2001 Protein kinase C
and meiotic regulation in isolated mouse oocytes. Molecular
Drew L, Groome N, Hallam TJ, Warr JR & Rumby MG 1994 Changes
in protein kinase C subtypes protein expression and activity in a series of multidrug-resistant human KB carcinoma
Ellis B, Schneeberger EE & Rabito CA 1992 Cellular variability in
the development of tight junctions after activation of protein kinase C. American Journal of Physiology 263 (Renal Fluid Electrolyte Physi-
ology 32) E293–E300.
Fleming TP 1987 Quantitative analysis of cell allocation to tropho-
derm and inner cell mass in the mouse blastocyst. Developmental
Biology 119 520–531.
Fleming TP & Hay MJ 1991 Tissue-specific control of expression of the
tight junction polypeptide ZO-1 in the mouse early embryo. Development 113 295–304.
Fleming TP, Warren PD, Chisholm JC & Johnson MH 1984 Tropho-
teronal processes regulate the expression of totipotency within
the inner cell mass of the mouse expanding blastocyst. Journal of
Embryology and Experimental Morphology 84 63–90.
Fleming TP, McConnell J, Johnson MH & Stevenson BR 1989 Devel-
opment of tight junctions de novo in the mouse early embryo: con-
trol of assembly of the tight junction-specific protein, ZO-1.
Fleming TP, Garrod DR & Elsmore AJ 1991 Desmosome biogenesis in
the mouse preimplantation embryo. Development 112
527–539.
Fleming TP, Hay M, Javed Q & Citi S 1993 Localisation of tight junc-
tion protein cingulin is temporally and spatially regulated during
Fleming TP, Sheth B & Fesenko I 2001 Cell adhesion in the preim-
plantation mammalian embryo and its role in trophoblast differen-
tiation and blastocyst morphogenesis. Frontiers in Bioscience 6
D1000–D1007.
Gangswarwar R & Jones KT 1997 Unique protein kinase C profile in
mouse oocytes: lack of calcium-dependent conventional isoforms
suggested by RT-PCR and Western blotting. FEBS Letters 412
309–312.
Geiges D, Meyer T, Marte B, Vanek M, Weissgerber G, Stabel S,
Pfeilischlifer J, Fabbro D & Huwiler A 1997 Activation of protein
kinase C subtypes alpha, gamma, delta, epsilon, zeta, and eta by
tumor-promoting and non-tumor-promoting agents. Biochemical
Pharmacology 53 865–875.
Gonzales-Mariscal L, Betanzos A, Nava P & Jaramillo BE 2003 Tight
junction proteins. Progress in Biophysics and Molecular Biology
81 1–44.
Gott AL, Mallon BS, Paton A, Groome N & Rumby MG 1994 Rat
brain glial cells in primary culture and subculture contain the \(\delta, \epsilon\) and \(\zeta\) subtypes of protein kinase C as well as the conventional
Gschwendt M, Dieterich S, Rennecke J, Kittstein W, Mueller HJ &
Johannes FJ 1996 Inhibition of protein kinase mu by various
inhibitors. Differentiation from protein kinase C isozymes. FEBS
Handside AH 1978 Time of commitment of inside cells isolated
from preimplantation mouse embryos. Journal of Embryology and
Experimental Morphology 45 37–53.
Hurd TW, Gao L, Roh MH, Macara IG & Margolis B 2003 Direct
interaction of two polarity complexes implicated in epithelial
Javed Q, Fleming TP, Hay M & Citi S 1993 Tight junction protein cin-
gulin is expressed by maternal and embryonic genomes during


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