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Specific PKC isoforms regulate blastocoel formation during mouse preimplantation development

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Abstract

During early mammalian development, blastocyst morphogenesis is achieved by epithelial differentiation of trophectoderm (TE) and its segregation from the inner cell mass (ICM). Two major interrelated features of TE differentiation required for blastocoel formation include intercellular junction biogenesis and a directed ion transport system, mediated by Na^+/K^+ ATPase. We have examined the relative contribution of intercellular signalling mediated by protein kinase C (PKC) and gap junctional communication in TE differentiation and blastocyst cavitation. The distribution pattern of four (δ, θ, ι/λ, ζ) PKC isoforms and PKCμ/PKD1 showed partial colocalisation with the tight junction marker ZO-1α+ in TE and all four PKCs (δ, θ, ι/λ, ζ) showed distinct TE/ICM staining patterns (predominantly at the cell membrane within the TE and cytoplasmic within the ICM), indicating their potential contribution to TE differentiation and blastocyst morphogenesis. Specific inhibition of PKCδ and ζ activity significantly delayed blastocyst formation. Although modulation of these PKC isoforms failed to influence the already established programme of epithelial junctional differentiation within the TE, Na^+/K^+ ATPase α1 subunit was internalised from membrane to cytoplasm. Inhibition of gap junctional communication, in contrast, had no influence on any of these processes. Our results demonstrate for the first time that distinct PKC isotypes contribute to the regulation of cavitation in preimplantation embryos via target proteins including Na^+/K^+ ATPase.

Keywords: Protein kinase C; Mouse embryo; Blastocyst; Trophectoderm; Inner cell mass; Tight junction; ZO-1; Na^+/K^+ ATPase; Cavitation

Introduction

Early mammalian development comprises a complex and well-orchestrated programme of gene expression, which, coupled with cell interactions and the spatial reorganisation of blastomeres, guides cell fate, lineage formation and blastocyst morphogenesis (reviewed in Fleming et al., 2001; Rossant et al., 2003). The maturation of cell membranes during cleavage and blastocyst formation reflects these developmental mechanisms (Johnson 1979; Fleming and Johnson, 1988; Fleming et al., 2001). At compaction in the mouse (8-cell stage), E-cadherin mediated adhesion and adherens junction formation occur between adjacent blastomeres and remain present in subsequent cell cycles within both inner cell mass (ICM) and trophectoderm (TE) lineages of the blastocyst (Ohsugi et al., 1997). In the compacted morula, gradual membrane assembly of tight junction (TJ) proteins is restricted to the outer cells as part of epithelial differentiation occurring within the TE lineage (reviewed in Fleming et al., 2001). The TJ paracellular seal between adjacent TE cells is required for epithelial integrity and the generation of the blastocoelic cavity by transepithelial ion transport processes mediated by the TE. Vectorial transport

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of mainly Na^+ influx through the apical Na^+/H^+ exchanger and efflux via the basolateral Na^+/K^+ ATPase is thought to drive osmotic transport of water across the epithelium (reviewed in Watson and Barcroft, 2001). The Na^+/K^+ ATPase enzyme consists of α and β subunits, each the product of multiple genes, that form dimers in different combinations with varying functions (see Therien and Blostein, 2000). The catalytic α subunit drives ion transport whilst the β subunit facilitates membrane delivery. A third γ subunit may also contribute to the enzyme structure. It determines active K^+ transport predominantly at the apical membrane and antisense inhibition delays cavitation without affecting Na^+/K^+ ATPase activity (Jones et al., 1997). During mouse blastocyst formation, the α1β1 isozyme appears to be the major driver of vectorial transport (MacPhee et al., 2000; Watson and Barcroft, 2001), although subunit redundancies have recently been suggested (Barcroft et al., 2004).

Little is known about the relative importance of these two interrelated membrane protein systems (adhesive junctions and Na^+/K^+ ATPase) and which signalling pathways may coordinate their activity during cavitation and TE differentiation. Pathways that have been suggested to contribute include many signalling molecules such as calcium, calcium-dependent enzymes, growth factor receptors and the large number of calcium-modulating agents (Dardik and Schultz, 1991; Kabir et al., 1996; Stachecki and Armant, 1996a,b; Stachecki et al., 1994), all of which could interact with protein kinase C (PKC)-mediated pathways. Signalling via PKCs remains controversial yet implicated in various reproductive and developmental events including oocyte maturation (Downs et al., 2001; Gangeswaran and Jones, 1997; Raz et al., 1998; Viveiros et al., 2003), fertilisation (Gallicano et al., 1995, 1997) and compaction (Ohsugi et al., 1993a,b; Pauken and Capco, 1999; Winkel et al., 1990). This may be due to the large number of PKC isoforms identified so far and classified according to structure and different activation requirements (Dempsey et al., 2000; Jaken and Parker, 2000; Mellor and Parker, 1998) into conventional PKCs (cPKCs; α, βI, βII, and γ), novel PKCs (nPKCs; δ, ε, η and θ) and atypical PKCs (aPKCs; ι/λ and ζ). PKCμ, formerly classified as novel-related PKC, is now called PKD1 and belongs to the PKD family that has been recently classified as novel subgroup of the CAMK family (Manning et al., 2002; Rykx et al., 2003). Biological function of PKCs is partly dependent upon the availability of various PKC isoforms and co-factors such as calcium, phospholipids or phorbol esters within the same cell. Their function is also regulated by PKC localisation to specific intracellular compartments mediated by association with specific anchoring proteins and limiting the proximity to PKC target proteins (Csukai and Mochly-Rosen, 1999; Mochly-Rosen and Gordon, 1998). Therefore, altered PKC localisation is indicative of a change in activation status and function, reflecting altered accessibility of the kinase to protein substrates (Dorn and Mochly-Rosen, 2002). During mouse preimplantation development, various PKC isoforms are available, regulated in a stage-specific manner and dependent upon the differentiation status of the blastomeres (Eckert et al., 2004; Pauken and Capco, 2000).

Another signalling mechanism whose role remains controversial is gap junctional intercellular communication (GJIC). Although communication via gap junctions may participate in recognition of different cell populations, the relative importance of this device within preimplantation mouse embryo development remains doubtful, because, to date, single or double knockouts of gap junction connexin proteins do not show an early embryo phenotype whilst antibody-mediated inhibition has perturbed compaction and blastocyst development (see Houghton et al., 1999, 2002; Kidder and Winterhager, 2001; Kidder et al., 1987; Vance and Wiley, 1999; White and Paul, 1999). On the other hand, in the mouse embryo, initiation of gap junction assembly coincides with compaction (Goodall and Johnson, 1982; Kidder et al., 1987; Lo and Gilula, 1979). GJIC becomes essential just after implantation when embryonic and extraembryonic regions of the conceptus are firmly established (see Kidder and Winterhager, 2001). These processes are initiated during TE differentiation and a reduced availability of connexin subunits in in vitro generated bovine embryos may contribute to their reduced developmental capacity (Boni et al., 1999; Wrenzycki et al., 1996). In addition, the localisation pattern of at least one connexin, Cx43, differed between ICM and TE cells in the nascent mouse blastocyst (De Sousa et al., 1993), suggesting that different mechanisms of communication occur early during emergence of these two cell phenotypes.

The relative contribution and interrelationship of both signalling mechanisms, GJIC and PKCs, during blastocyst morphogenesis to the establishment of a junctional seal and the process of ion transport driving cavitation is still poorly understood. For example, GJIC can be regulated by broad PKC activators in a manner dependent upon the system examined (Cruciani et al., 2001; Giepmans, 2004; Lampe and Lau, 2004). In connexin-knockout hepatocytes, newly expressed GJIC induced TJ formation and function, a process that could be inhibited by a chemical GJIC blocker (Kojima et al., 2002). Gap junction components have been reported to interact with TJ proteins in several cell types (Defamie et al., 2001; Giepmans, 2004; Giepmans and Moolenaar, 1998; Kausalya et al., 2001; Toyofuku et al., 1998). Certain connexins and TJ proteins may also be direct targets of phosphorylation via PKC (occludin: Andreeva et al., 2001; ZO-2: Avila-Flores et al., 2001) and these posttranslational modifications may contribute to the regulation of membrane assembly in the embryo (Fleming et al., 2001; Ogawa et al., 2000; Sheth et al., 1997, 2000a). Moreover, in epithelial Madin–Darby canine kidney (MDCK) cells, TJ membrane assembly has been shown to involve PKC-mediated mechanisms (Dodane and Kachar, 1996; Hurd et al., 2003; Matter and Balda, 1999; Stuart and Nigam, 1995; Suzuki et al., 2001). More recently, it has

been reported that structural organisation and vectorial ion transport via adherens junctions and Na^+/K^+ ATPase together are required to establish cell polarisation and TJ and desmosome formation in epithelial MDCK cells (Rajasekaran et al., 2001). Biological activity and function of Na^+/K^+ ATPase may also be regulated by PKC in various cellular systems, with the α1 subunit as the potential target (reviewed in Therien and Blostein, 2000). However, analysis of the roles of PKC signalling mechanisms is limited by the diversity, interaction and (potential) functional redundancy of PKC isoforms. Perhaps not surprisingly, and similar to the connexin knockouts, none of the knockouts of individual PKC isoforms reveal effects on early embryo development (β: Leitges et al., 1996; γ: Abeliovich et al., 1993; θ: Sun et al., 2000; ε: Khasar et al., 1999).

In the present study, mechanisms contributing to the coordination and regulation of the complex developmental process of cavitation were investigated. Although initiated during cleavage and compaction, the process of cavitation occurs over a relatively defined time period at the 32-cell stage in the mouse and was therefore the focus of this study. The role and possible interaction of signalling systems such as GJIC and PKCs during cavitation and cell phenotype diversification into ICM and TE have been investigated for the first time. Whilst we found that GJIC had no influence on these processes, the distribution pattern of four (δ, θ, ι/λ, ζ) PKC isoforms suggests their contribution to TE differentiation. Modulation of the activity of two of these PKC isoforms, PKCδ and ζ, which represent nPKCs and aPKCs and for which isotype-specific peptides are available, confirmed their function in regulation of cavitation. Although the already established programme of junctional membrane assembly within the TE appeared unaffected, PKCδ and ζ regulated the process of cavitation at least in part by affecting Na^+/K^+ ATPase subunit distribution.

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) and cultured up to the early blastocyst stage in T6 medium containing 4 mg/ml BSA (T6-BSA; Sheth et al., 2000a,b). Culture was performed in 5% CO_2 at 37°C in microdrops under paraffin oil (J.M. Loveridge, Southampton) as previously described (Sheth et al. 1997, 2000a,b). Compacting 8-cell embryos, compact 16-cell embryos, and early (cavity <20% volume) and late (cavity >20% volume) blastocysts were fixed and stained for confocal microscopy as described (Sheth et al., 1997). ICMs were isolated from early blastocysts (92–96 h after hCG) by immunosurgery as described previously (Eckert et al., 2004).

PKC isotype-specific peptides

For PKC-isoform-specific inhibition or activation, PKC isozyme-specific activator and inhibitor peptides coupled to the antennapedia carrier (*Drosophila* antennapedia, positions 43–58, [RQIKIWFQNRRMKWKK]; Chen et al., 1999; Derossi et al., 1994) via a cysteine–cysteine bond for cell membrane permeability were kindly provided by Dr. Daria Mochly-Rosen (Stanford University, USA). The mechanisms by which the antennapedia carrier introduces other peptides into cells is yet unclear (Christiaens et al., 2004; Drin et al., 2003; Letoha et al., 2003), but it is estimated that 5% of the outside concentration applied are achieved intracellularly (Souroujon and Mochly-Rosen, 1998). 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]; Laudanna et al., 1998) and PKCδ (translocation inhibitor; δV1–1, positions 8–17 [SFNSYELGSL]; Chen and Mochly-Rosen, 2001; Chen et al., 2001; Inagaki et al., 2000) and an activator peptide for PKCδ (translocation activator; ψδRACK, positions 74–81 [MRAAEDPM]; Chen and Mochly-Rosen, 2001; Chen et al., 2001) were supplemented to DMEM culture medium containing 10% FCS in four-well dishes (Nunc) at 0.1, 0.5 or 1 μM. No peptide, antennapedia carrier monomer (1 μM) and dimer (0.5 μM) were used as control.

The zona pellucida of developmentally timed late morulae (20 h after compaction) was removed by acid Tyrode's. The embryos were then cultured for up to 4 h without oil in control medium or (i) in the presence of peptide with peptide-renewal after 2 h, (ii) in the presence of peptide for 2 h and without peptide for another 2 h (60–73 per treatment collected from six replicate experiments scoring at least 10 embryos per treatment group and replicate experiment). Alternatively, embryos were cultured overnight (approximately 12 h) in the presence of peptides to determine developmental capacity (25–30 embryos per treatment). Embryos were monitored hourly for cavitation. Embryos treated with peptides for 2 h were also fixed and stained for the α1 subunit of the Na^+/K^+ ATPase and counterstained with α-catenin to visualise localisation of the cell membrane (17–23 embryos per treatment collected from six replicates). Alternatively, embryos were fixed and stained for junctional proteins such as ZO-2, ZO-1α+, occludin or E-cadherin after 0, 2, 4 h or after overnight culture in the presence of peptides (5–37 embryos per treatment, antibody and time point, see Results for details).

Antibodies

Antibodies to the mouse junctional proteins ZO-1α+ isoform (guinea pig polyclonal, diluted 1:250; Sheth et al., 1997), ZO-2 (rabbit polyclonal, Zymed; 1:1000), occludin (rabbit polyclonal, 1:1000; Sheth at al., 2000b) and E-cadherin (rat polyclonal, Sigma; 1:1000) were used as previously described. Staining for PKC-isoforms was performed with rabbit polyclonal antibodies against 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. Antibodies for Na^+/K^+ α1 subunit (mouse monoclonal 6H, Upstate Biotechnology, Buckingham, UK; 1:100; MacPhee et al., 2000) and α-catenin (rabbit polyclonal VB1, 1:300; Braga et al., 1995; Santa Cruz Biotechnology) were used as previously described.

Immunocytochemistry and confocal microscopy

Zona-free embryos or isolated ICMs were fixed in PBS supplemented with 1% formaldehyde (Analar or Sigma) or methanol at −20°C (for labelling with Na^+/K^+ α1 and α-catenin) for 7–15 min, attached onto coverslips coated with 1.5 mg/ml poly-L-lysine hydrobromide (Sigma) and processed for immunocytochemistry as previously described (Fleming et al., 1991; Sheth et al., 1997, 2000a,b). The embryos/ICMs were stained overnight at 4°C with the different primary antibodies as described above. A set of cross-purified ALEXA-488, ALEXA-546 or ALEXA-568 labelled anti-mouse, anti-guinea pig, anti-rat or anti-rabbit secondary antibodies (Molecular Probes) was used at dilutions of 1:500 either alone or in combinations for double-labelling experiments for 1 h at room temperature. Specimens were visualised with a ×50 or ×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 analysed and processed using the Bio-Rad software system (Confocal assistant version 4.01). A representative number of embryos was double labelled with one of the PKC-isoforms and the TE TJ marker, ZO-1α+ or counterstained with the RNA/DNA dye propidium iodide (0.05 μg/ml) for 5 min to visualise the nuclei after the last wash for confocal microscopy.

Verification of antibody specificity and staining pattern

When we analysed the in-house antibodies (see above) by Western blotting, they 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; not shown). Preincubation with the respective peptide against which they were raised blocked either partially or completely immunofluorescent staining. To further verify the specificity of these antibodies, a set of commercially available antibodies generated in mouse (Transduction Labs) or rabbit (PKCζ, Sigma) against PKC isoforms was used in parallel at appropriate dilutions (1:100–1:300, and 1:1000 for PKCζ, respectively). The commercial antibodies have been previously used in different species or mouse embryos (e.g., Minichiello et al., 1999; Pauken and Capco, 2000). Negative controls (secondary antibody only; rabbit preimmune 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 1 primary and 2 secondary or 2 primary and 1 secondary antibody) ensured that there was no cross-reactivity between different primary and secondary antibodies. The staining pattern was identical using both sets of PKC isotype-specific antibodies when 19–20 embryos per PKC isotype were examined with the in-house antibodies and 15–20 embryos with the commercial antibodies in four replicate experiments.

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 (65 μM; AGA; Sigma) or 0.1% DMSO (vehicle control; Sigma). AGA is a relatively specific, stable, and reversible saponin that intercalates into the plasma membrane and binds to connexins (Davidson and Baumgarten, 1988). Cavitation was observed every 2 h (403–408 embryos per treatment collected from 8 replicates). Embryos (blastocysts or late morulae) were fixed at different time points after onset of AGA culture (18, 26 or 36 h of culture with or without AGA, respectively) and stained for junctional proteins or PKC isotypes (10–20 embryos per protein and time point). To ensure inhibition of gap junction communication, blastocysts were microinjected into a TE or ICM cell with 5% lucifer yellow (LY) in 70 mM KCl, 7 mM NaCl, 10 mM EGTA, 10 mM HEPES, pH 7.4, 280 mOsm (ICS). Propidium iodide (p.i., Sigma; 0.05 μg/ml in ICS) was co-injected to monitor the injection procedure because this dye does not travel easily through gap junctions. A pulled micropipette with inner filament (Clark Electromedical Instruments) was backfilled with the fluorescent dyes. Microinjection was performed at a constant injection pressure of 20–40 psi depending on the opening of the needle (Narishige). Embryos were maintained in an oil-covered (mineral oil, Sigma) microdrop of H6-BSA in the presence of AGA or DMSO. Dye distribution was monitored after 30 s and at 1, 3, 10 and 30 min under a fluorescence microscope (Nikon) at ×40 magnification (16–21 embryos per treatment collected in four replicates). Embryos were categorised as coupling when more than two cells were filled with LY whilst p.i. was visible in maximal two cells.

Statistical analysis

In the experiments involving PKC peptides, significant differences between treatments in cavitation rates over time and Na^+/K^+ ATPase localisation were analysed by a one-way ANOVA followed by a Tukey test (SigmaStat software package version 2.0, Jandel Scientific). TJ localisation was analysed by ChiSquare. In the experiments involving the GJIC inhibitor AGA, cavitation rates were compared by one-way ANOVA followed by a Tukey test and coupling rates were analysed by ChiSquare. Differences were considered as significant if $P < 0.05$.

Results

Distinct distribution of PKC isoforms in blastocysts

We first examined the distribution of PKC isoforms using isotype-specific antibodies and confocal microscopy in

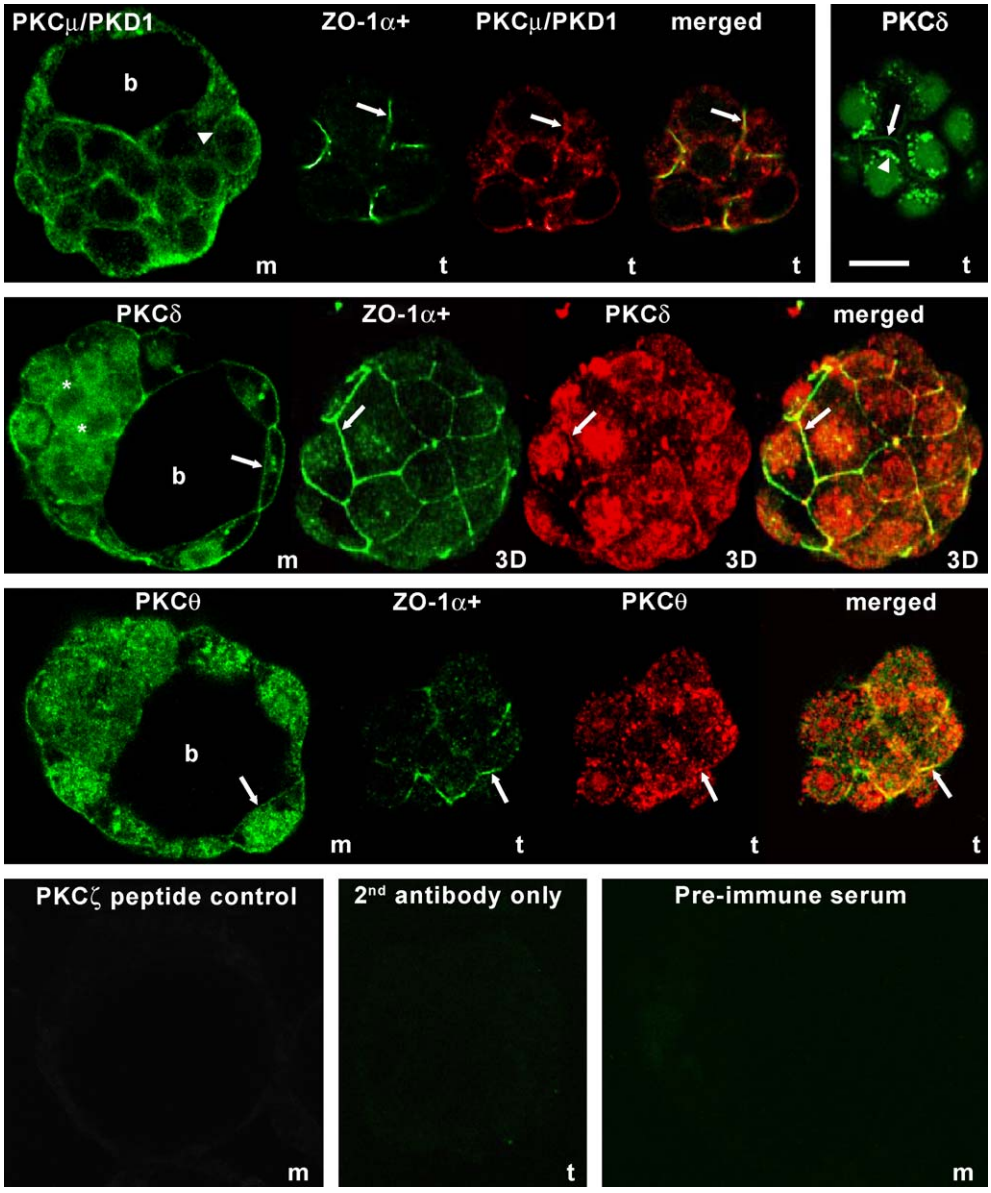

Fig. 1. Distribution pattern of nPKC isoforms in murine blastocysts after formaldehyde fixation, immunofluorescence and confocal microscopy is shown in either tangential (t) or midplane (m) sections or reconstructed z-series of 1-μm sections (3D). For each PKC isotype, 19–20 embryos were examined with the in-house antibodies and 15–20 embryos with the commercial antibodies in four replicate experiments. Staining pattern of PKCμ, PKCδ and PKCθ is shown using the in-house isoform-specific rabbit primary antibodies together with ALEXA-488-labelled secondary antibodies. Some embryos were double labelled with ZO-1α+ (green) and respective PKC isoforms (red). Control blastocysts incubated with secondary antibody only or rabbit preimmune serum showed background staining when images were brightened. Similarly, background staining was observed in blastocysts when the in-house PKCζ antibody was preincubated with the peptide it was raised against. Perinuclear and nuclear envelope (arrowheads) or membrane (arrow) staining are indicated; asterisks = absence of PKCδ from the nucleus when viewed in the midplane; b = blastocoel. Scale bar, 30 μm.

blastocysts to evaluate which, based upon distribution, might be involved in TE membrane differentiation. Of the complete range examined (cPKCs α, βI, βII, γ, nPKCs δ, ε, η, θ, aPKCs ι/λ, ζ), four (nPKCs δ, θ, aPKCs ι/λ, ζ) and PKCμ/PKD1 gave staining patterns showing membrane presence. These were examined in relation to the TE junction marker protein ZO-1α+ and are shown in Fig. 1 (PKCμ/PKD1 and nPKCs) and Fig. 2 (aPKCs). The staining pattern was independent of blastocyst age or expansion (not shown). PKCμ/PKD1 was present at membranes (cell membrane and nuclear envelope) similarly within ICM and TE cells and therefore overlapped with the TJ marker ZO-1α+ within the TE (Fig. 1). The distribution pattern of nPKCδ and θ was distinct between ICM and TE. These isoforms were concentrated at the cell membranes of the TE and were distributed within the cytoplasm within the ICM (Fig. 1). Within the TE, PKCδ and θ partially overlapped with ZO-1α+ (Fig. 1) but were also found within the

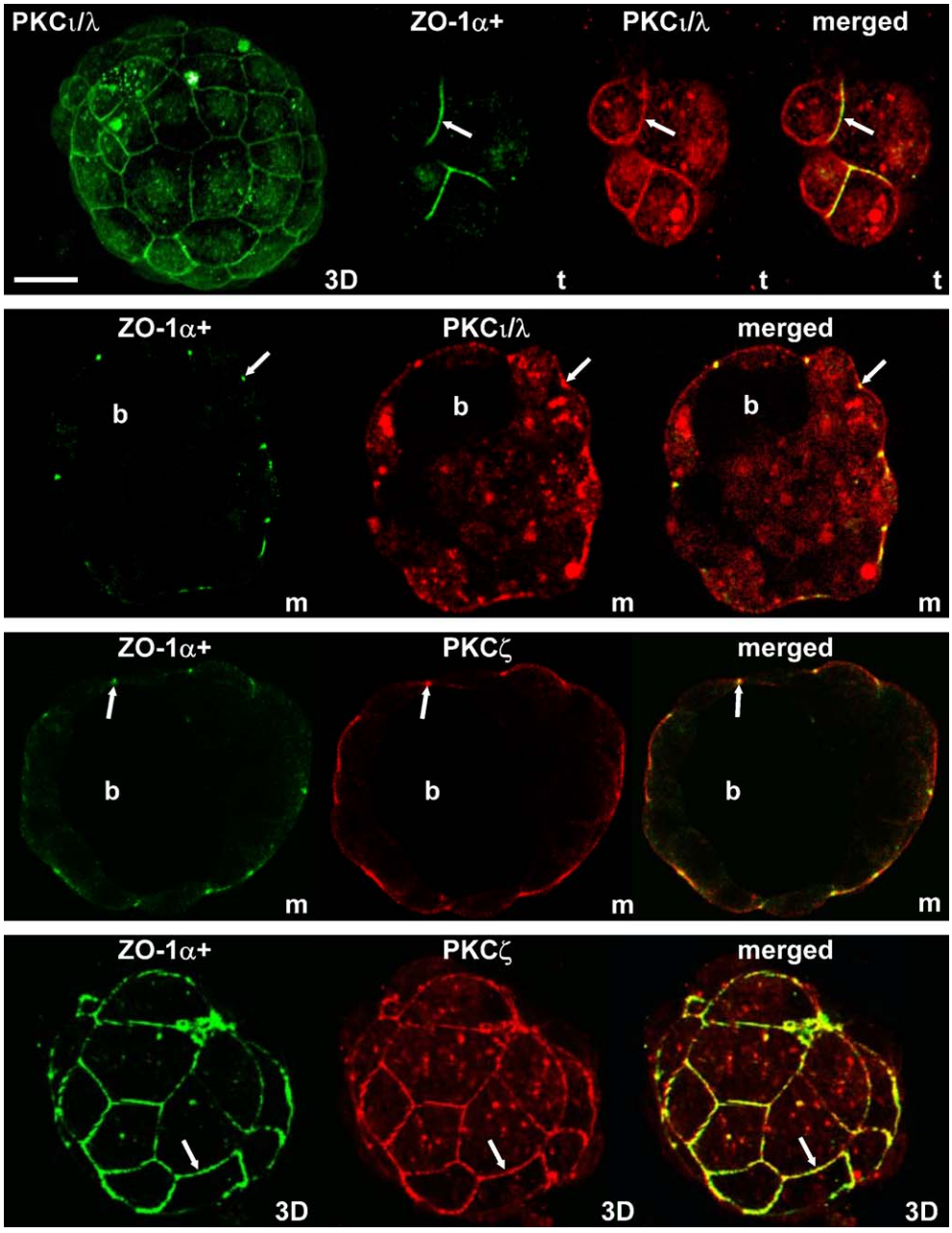

Fig. 2. Distribution pattern of aPKC isoforms in murine blastocysts after formaldehyde fixation, immunofluorescence and confocal microscopy is shown in either tangential (t) or midplane (m) sections or reconstructed z-series of 1-μm sections (3D). For each PKC isotype, 18–20 embryos were examined with the in-house antibodies and 15–20 embryos with the commercial antibodies in four replicate experiments. Staining pattern of PKCι/λ and PKCζ is shown using the in-house isoform-specific rabbit primary antibodies (except top left image of ι/λ was stained with the commercial antibody) together with ALEXA-488-labelled secondary antibodies. Some embryos were double labelled with ZO-1α+ (green) and respective PKC isoforms (red). Membrane (arrow) staining is indicated; b = blastocoel. Scale bar, 30 μm.

cytoplasm of both cell lineages. The aPKCs ι/λ and ζ also showed lineage-distinct staining patterns in blastocysts. Whilst PKC ι/λ, like the nPKCs, was distributed at the TE membrane and cytoplasm (Fig. 2), PKCζ demonstrated the most distinct difference in distribution within TE and ICM in all embryos examined, being present predominantly at the apical membrane of the TE and just detectable above background level within the ICM. PKCζ, therefore, showed the clearest colocalisation with ZO-1α+ (Fig. 2).

To exclude the possibility of inefficient antibody penetration for isoforms with weak or absent staining within the ICM of intact blastocysts, ICMs were isolated immunosurgically, fixed immediately and stained for different isoforms. Isolated ICMs and intact control blastocysts were treated in parallel on the same slides during the staining procedure. The staining pattern in isolated ICMs did not differ from that seen in intact blastocysts for all (PKCμ/PKD1) or for some (PKCδ, θ, ι/λ, ζ) ICMs examined, indicating the reliability of lineage-distinct staining patterns in blastocysts (Eckert et al., 2004). In remaining ICMs, PKC relocalisation to membranes was observed (Eckert et al., 2004) reflecting the change in phenotype of outer ICM cells to TE-like cells in response to altered cell contact patterns (Fleming et al., 1984; Handyside, 1978).

PKCδ and ζ are involved in regulating blastocoel formation

Amongst the PKC isoforms that showed a different distribution pattern within TE and ICM (see above), isoform-specific peptides were only available designed for PKCδ (activating and inhibitory) and ζ (inhibitory only). More importantly, PKCδ represents an nPKC and PKCζ represents an aPKC, the two PKC subfamilies we have previously identified as being involved in de novo TJ membrane assembly within the ICM model (Eckert et al., 2004; see Discussion). For these reasons, further studies focused on these two PKC isotypes. Developmentally timed late morulae (from 20 h post compaction) were cultured for up to 4 h in the presence of PKCδ and/or ζ-specific inhibitory or PKCδ activating peptides and compared to controls for timing of cavitation. Culture of intact embryos in the presence of PKCδ activator peptide caused a significant increase in the rate of cavitation in a dose-dependent manner (Fig. 3). Cavitation was found to be delayed in response to PKCδ and ζ inhibitory peptides partially in a dose-dependent manner. When used in combination, low concentrations of both inhibitory peptides significantly and effectively delayed cavitation (Fig. 3). The inhibitory effects of PKCδ and ζ inhibitors on cavitation were reversed when embryos where released from peptide-

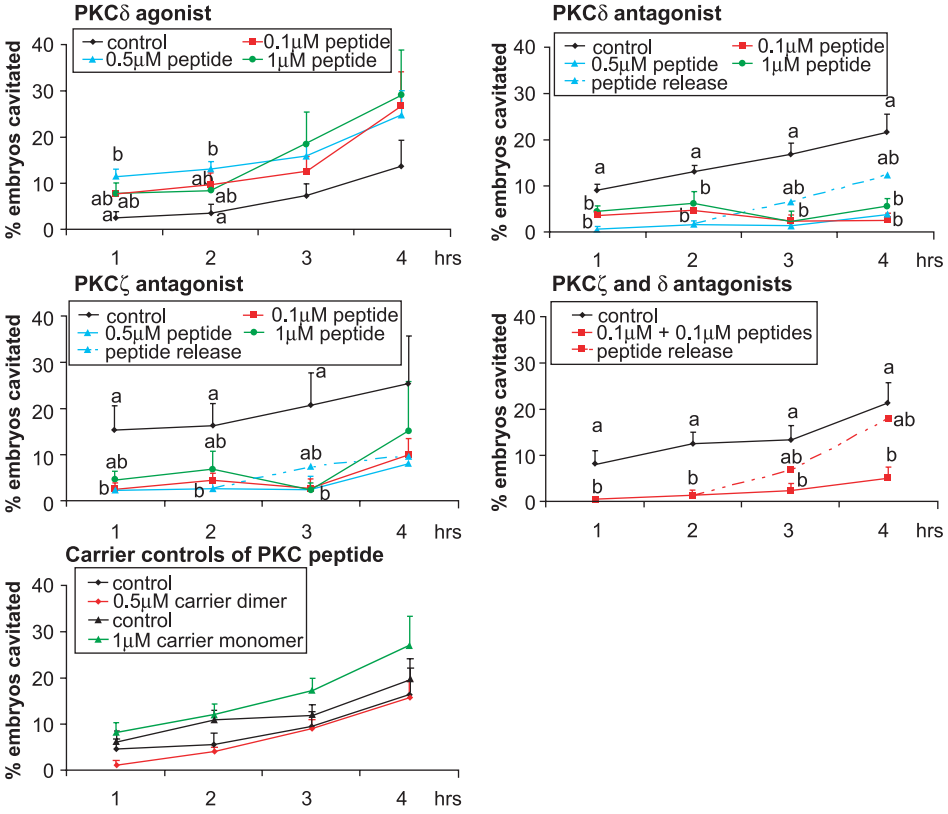

Fig. 3. Cavitation of developmentally timed late morulae during culture in the presence or absence of PKC isoform specific inhibitory or activating peptides is shown as means ± SEM (n = a total of 60–73 per treatment collected from six replicate experiments scoring at least 10 embryos per treatment group and replicate experiment). Late morulae were incubated in the presence of 0, 0.1, 0.5 or 1 μM PKCδ agonist or antagonist or PKCζ antagonist or a combination of 0.1 μM PKCδ and ζ antagonist or up to 1 μM control carrier peptide for up to 4 h. After the initial 2 h, peptides were either renewed or embryos were washed and cultured in medium alone (release). Significant differences ($P < 0.05$) are shown by different letters at each time point.

Table 1
Verification of PKC peptide specificity and potency

PKC isoform	ICMs showing PKC membrane presence in *x*/*n* (%) after 0 h	1 h		
		Control carrier	PKCδ↓	PKCδ ↑
δ	4/11 (36)	7/11 (64)	1/10 (10)	7/8 (88)
θ	6/8 (75)	7/7 (100)	7/9 (78)	8/9 (89)
ζ	3/11 (27)	7/12 (58)	5/9 (56)	5/10 (50)

Control carrier: antennapedia monomer; PKCδ↓: PKCδ inhibitory peptide (0.1 μM); PKCδ ↑: PKCδ activating peptide (0.1 μM).

culture after the first 2 h (Fig. 3). The antennapedia carrier alone did not influence the timing of cavitation or caused morphological changes to the embryos (Fig. 3). This therefore suggests that, in our hands and with the concentrations used here, the carrier does not cause unspecific toxic effects on early embryos as discussed controversially in other cell lines (Christiaens et al., 2004; Drin et al., 2003). For reasons of clarity of the staining pattern, the potency and specificity of these PKC peptides was confirmed in isolated ICMs after immunosurgery (Table 1; Fig. 4). Thus, PKCδ antagonist inhibited PKCδ membrane relocation evident in controls but had no effect on PKCθ or ζ relocation to cell membranes; moreover, PKCδ activator peptide induced enhanced membrane distribution of PKCδ in ICMs.

Modulation of PKCδ and ζ activity does not affect the TJ protein membrane assembly programme

After 4 h of culture of developmentally timed late morulae (from 20 h post compaction) in the presence of PKCδ or ζ modulators, embryos (morulae and blastocysts) were fixed and stained for the TJ proteins ZO-2 and ZO-1α+ as markers of TE differentiation. The percentage of embryos showing membrane assembly of both proteins appeared unaffected by treatment or the presence or absence of a cavity (Table 2; Fig. 5). Whilst ZO-2 membrane assembly is clearly visible within embryos even at onset of peptide culture at 20 h after compaction, that of ZO-1α+ is found only in approximately 50% of late morulae and appears subsequently over the following 4 h when cavitation is initiated. This corresponds well with previous observations where even the late assembling ZO-1α+ was always found at the cell contact sites before cavitation (Novak et al., in preparation; Sheth et al., 1997). Because similar percentages of embryos cultured overnight (approximately 12 h) in the presence of the PKC peptides reached the late blastocyst stage by the next morning (n = 25–30 embryos per treatment; 70–90% cavitated in controls and peptide-treated groups, respectively), the peptides do not show embryo toxicity following extended treatment. Similarly, membrane localisation of all junctional proteins examined after extended overnight culture remained unaltered in the presence or absence of peptides in the culture medium (all cavitated embryos showed linear membrane staining for E-cadherin, ZO-2, occludin or ZO-1α+; n = 5–10 per treatment and antibody; data not shown; see Fig. 7D for localisation examples).

Membrane localisation of Na^+/K^+ ATPase is affected by PKC modulation

These same treatments were also followed by examination of the localisation of the α1 subunit of the Na^+/K^+ ATPase.

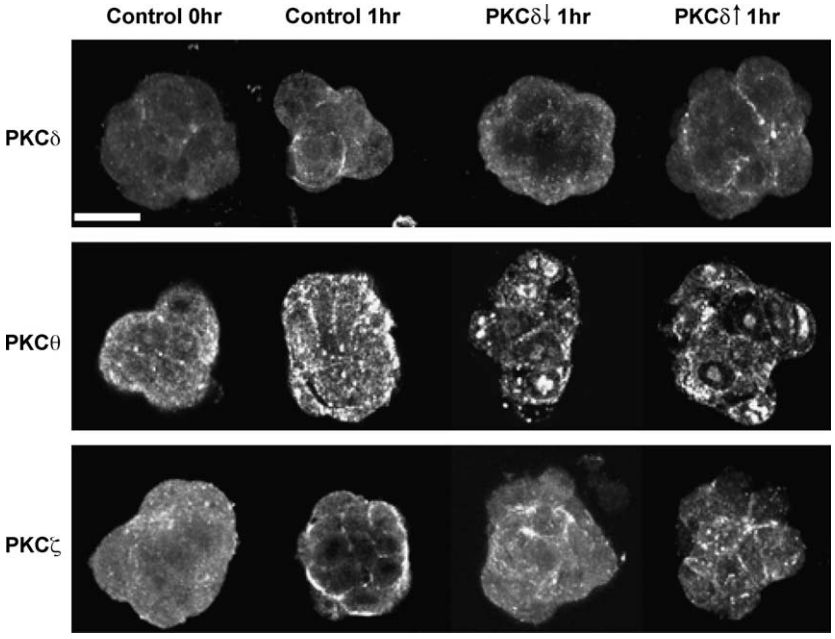

Fig. 4. Representative confocal images showing isolated ICMs immediately after isolation (control 0 h) or cultured for 1 h in the presence or absence of PKCδ inhibiting peptide (PKCδ↓) or PKCδ activating peptide (PKCδ ↑) and stained for either PKCδ (upper panel, 3D z-series reconstructions of 1-μm sections), PKCθ (middle panel, midplane sections) or PKCζ (lower panel, 3D z-series reconstructions of 1-μm sections). Scale bar, 10 μm.

Table 2
TJ localisation in intact embryos[a] after modulation of PKCδ and ζ activity for 4 h

Treatment	Protein detected at membrane within the TE [*x*/*n* (%)]	
	ZO-1α+	ZO-2
0.1% DMSO	9/12 (75)	28/37 (76)
0.5 μM monomer	15/33 (45)	26/31 (84)
0.25 μM dimer	12/19 (63)	30/34 (88)
0.5 μM ζ↓	15/21 (71)	24/31 (77)
0.5 μM δ↓	16/19 (84)	28/32 (88)
0.5 μM δ↑	17/27 (63)	28/37 (76)
0.1 μM ζ↓ + 0.1 μM δ↓	9/17 (53)	21/26 (81)

Monomer: antennapedia carrier monomer. Dimer: antennapedia carrier dimer. ↓: PKC isoform inhibitory peptide. ↑: PKC isoform activating peptide.
[a] Data from cavitated and noncavitated embryos were combined because TJ protein membrane assembly was not affected by the presence or absence of a cavity.

After 2-h culture in the presence of PKCζ antagonist or PKCδ agonist, the α1 subunit of the Na^+/K^+ ATPase was found concentrated in foci within the cytoplasm away from the membrane (as marked by α-catenin localisation) in a significantly higher percentage of embryos compared to controls (Figs. 6A,B). This internalisation occurred similarly within cavitated and noncavitated embryos. The PKCδ antagonist had no effect on Na^+/K^+ ATPase localisation whilst a combination of PKCδ and PKCζ inhibitory peptides had an intermediate effect. These data are shown quantitatively in Fig. 6B. In contrast, when further cultured overnight without peptide or in the presence of PKCδ agonist or antennapedia carriers in a limited set of experiments, predominant membrane localisation of the α1 subunit of the Na^+/K^+ ATPase was observed irrespective of peptide treatment in 67–100% of expanded blastocysts (n = 6–12 per treatment group collected in two replicate experiments; see supplementary data Fig. 6).

Intercellular communication via gap junctions does not affect cavitation, junction membrane assembly or PKC localisation

Gap junctional coupling was chemically inhibited by culturing 8-cell embryos in the presence of AGA overnight until cavitation. Speed and rate of cavitation were unaffected by this treatment (Fig. 7A). AGA treatment, however, effectively blocked coupling between blastomeres within both cell lineages as monitored by LY

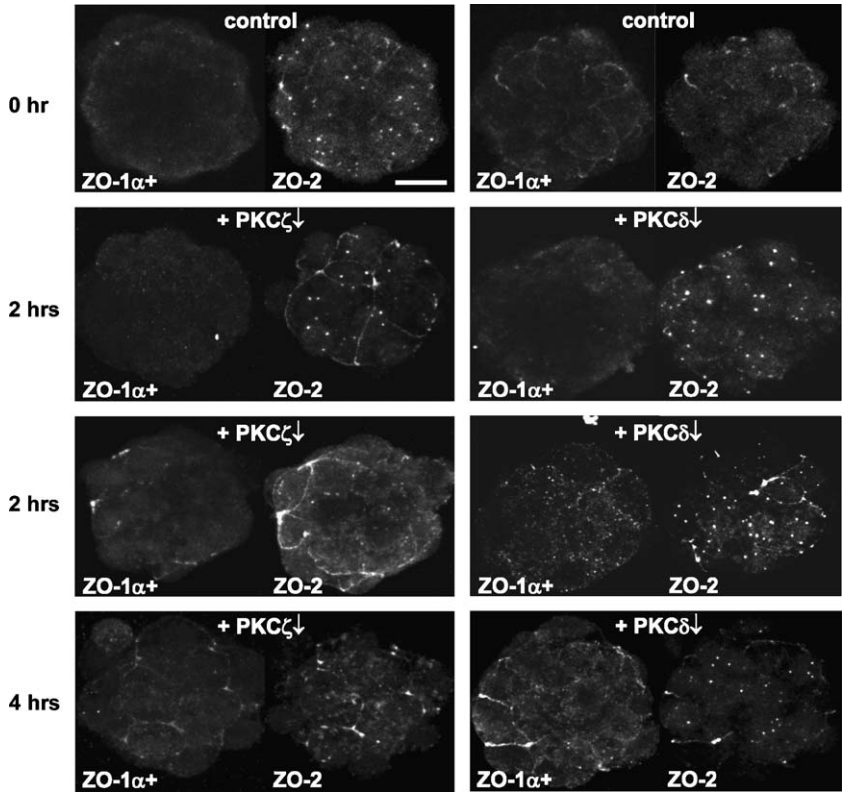

Fig. 5. Representative localisation of ZO-1α+ (left image) and ZO-2 (right image) in double-labelled late morulae (n = 14–18 or 10–14 per treatment and time point) cultured in the presence or absence of PKC peptides for 0, 2 or 4 h is shown as 3D z-series reconstructions of 1-μm sections (images of embryos cultured with 0.5 μM PKCζ antagonist or 0.5 μM PKCδ antagonist are shown as examples; for quantitative data see Table 2). Whilst ZO-2 membrane assembly is clearly visible within the embryos even at onset of peptide culture, that of ZO-1α+ appears subsequently over the following 4 h. PKCζ↓ = PKCζ antagonist; PKCδ↓ = PKCδ antagonist. Scale bar, 40 μm.

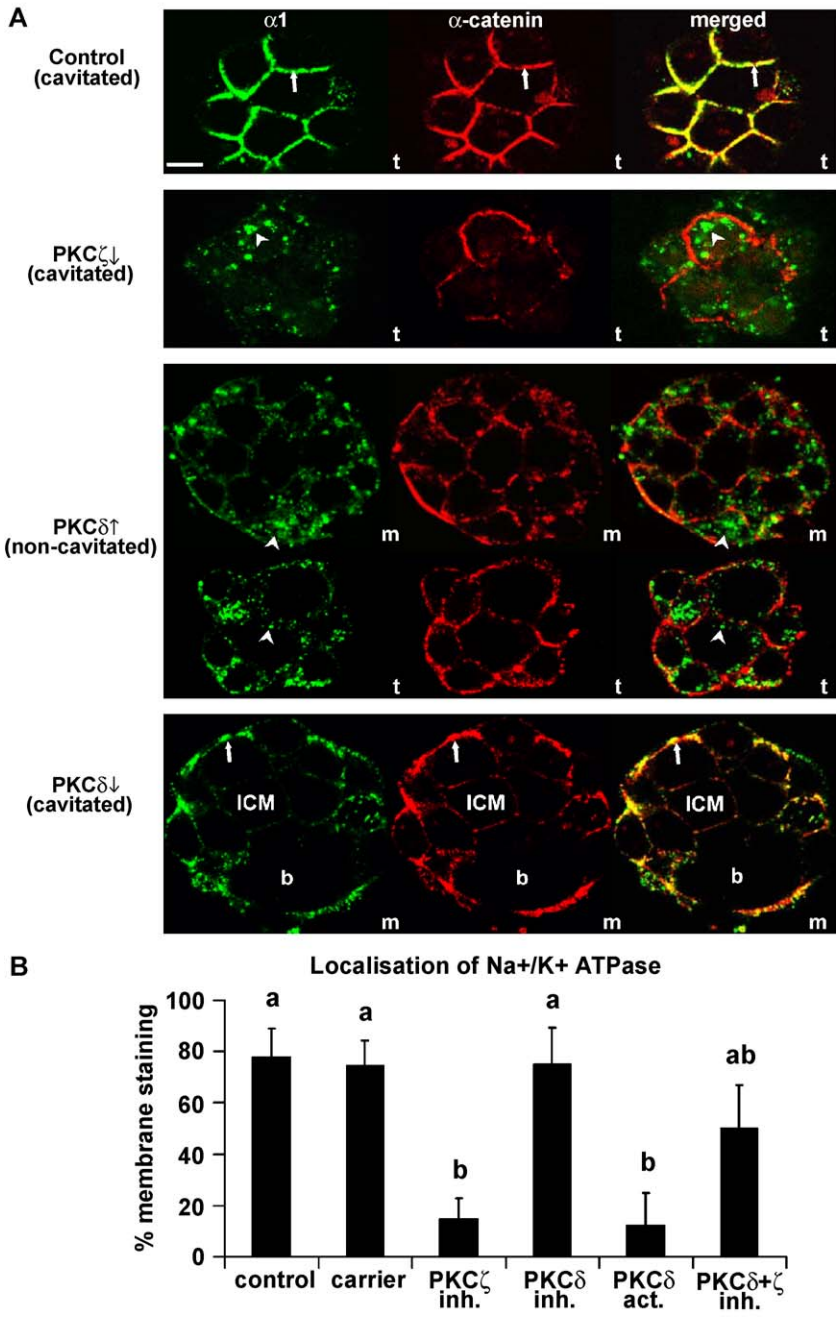

Fig. 6. (A) Representative confocal images of embryos cultured in the absence (c, control) or presence of PKC peptides (PKCδ↓ = PKCδ antagonist; PKCδ ↑ = PKCδ agonist; PKCζ↓ = PKCζ antagonist) after fixation and double-labelling to visualize the relative position of the α1 subunit of the Na^+/K^+ ATPase (green) to the cell membrane (α-catenin; red) in overlays (merged) of tangential (t) or midplane (m) sections. Cytoplasmic (arrowheads) or membrane (arrow) staining of α1 (green) are indicated in relation to α-catenin membrane staining (red). Shown are cavitated or noncavitated embryos. For quantitative results, see (B) and text. b = blastocoel; ICM = inner cell mass; Scale bar, 10 μm. (B) Relative localization of the α1 subunit at the membrane dependent upon peptide treatment during 2 h of culture is shown as means ± SEM (n = a total of 17–23 embryos per treatment collected from six replicates). Significant differences are indicated by different superscripts. Inh. = inhibiting peptide; act. = activating peptide.

injection into ICM or TE cells (Fig. 7B). In 100% control embryos, LY was distributed over the entire blastocyst within 30 s to 1 min (Fig. 7C) compared to 11–15% whole blastocysts that showed dye coupling after AGA treatment ($P < 0.05$). Membrane localisation of the junctional protein systems involved in compaction and TE differentiation and the distribution patterns of PKC isotypes were unaffected by AGA treatment. Thus, membrane localisation of adherens junctions (E-cadherin), TJ (ZO-2, ZO-1α+, occludin) and desmosomal (desmoplakin) proteins and the distribution of PKC isotypes remained unchanged after AGA incubation for up to 36 h

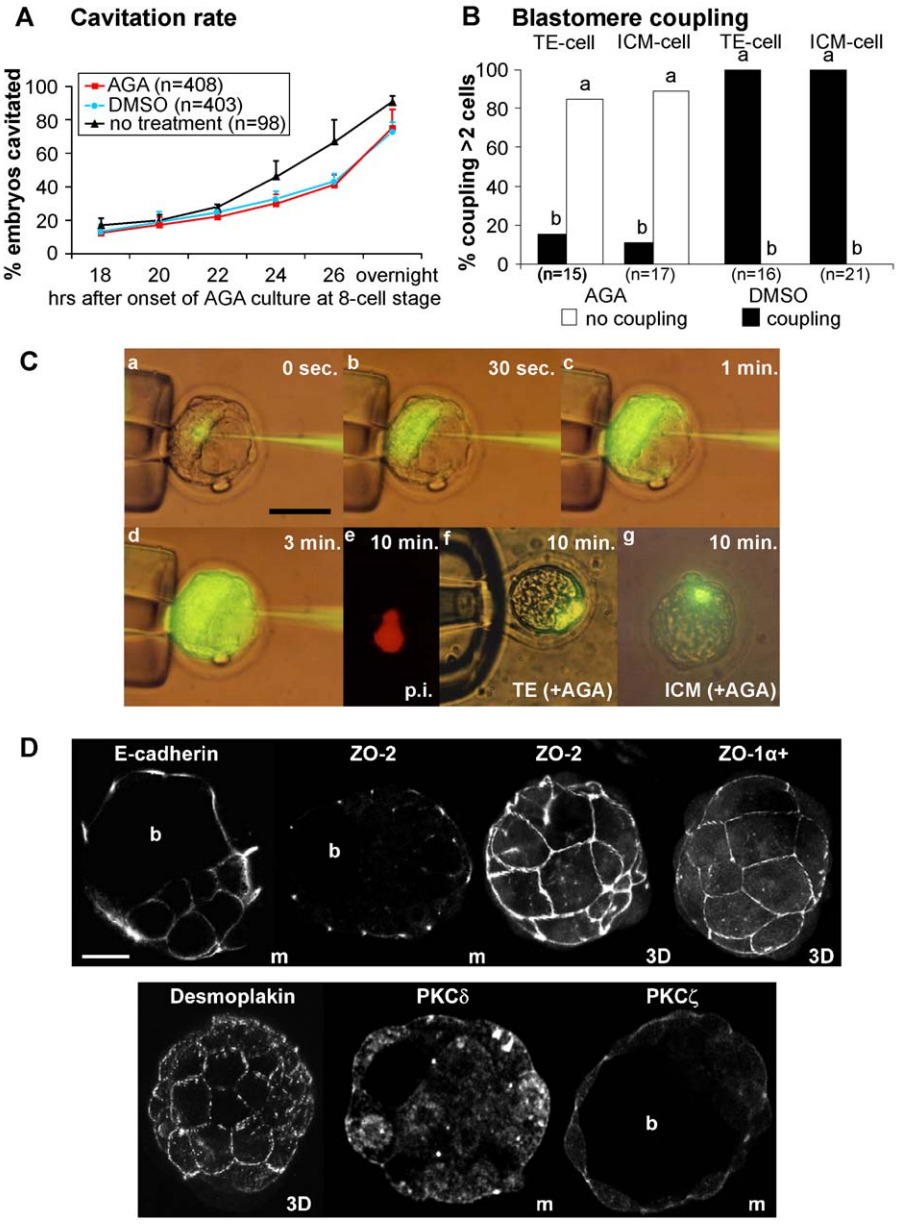

Fig. 7. (A) Blastocoel formation of embryos cultured with or without 65 μM AGA or vehicle control (DMSO) from the 8-cell stage onwards overnight is shown as means ± SEM from eight replicates. (B) Gap junctional coupling between blastomeres within 30 s after dye injection into either an ICM or TE cell in the presence of 65 μM AGA or DMSO is shown as overall percentage from four replicates. Significant differences ($P < 0.05$; ChiSquare) between coupling and no coupling are indicated by different letters in each group. (C) Representative fluorescent photographs of dye coupling after lucifer yellow injection into whole blastocysts. Lucifer yellow spread over the whole embryo within 3 min after injection (a–d) in control blastocysts. Coinjection of propidium iodide (p.i.; e) ensured injection of only one single cell and injection location (ICM shown). Dye coupling was inhibited by AGA treatment in both cell phenotypes (f, g). Scale bar = 50 μM. (D) Representative localisation (n = 15–20 embryos per protein) of the junctional proteins E-cadherin, ZO-2, ZO-1α+ and desmoplakin or the PKC isotypes δ and ζ in blastocysts after incubation in the presence of 65 μM AGA for 24 h is shown in midplane (m) or 3D z-series reconstructions of 1 μm sections (3D). b = blastocoel. Scale bar, 30 μm.

(n = 10–15 per treatment, antibody and time point of fixation; see Fig. 7D).

Discussion

Our study is the first to examine the cellular distribution pattern of PKC isotypes in mouse blastocysts at the time of cell phenotype diversification and cavitation. We found four PKC isoforms (θ, δ, ι/λ and ζ) and PKCμ/PKD1 concentrated within cell membranes of blastocysts overlapping with the TJ marker ZO-1α+. All four PKCs showed a cell-lineage-distinct distribution pattern (θ, δ, ι/λ and ζ) accumulating at the apico- and apicolateral membranes of the TE whilst remaining cytoplasmic within the ICM. This particular distribution pattern may indicate that PKC

signalling activity participates in regulating the process of cavitation and TE differentiation. For example, colocalisation of PKCζ with ZO-1 reported previously in MDCK epithelial cells was used as indication of an interrelation between the two protein systems, and this was confirmed by modulating PKC with broad PKC-activators and/or inhibitors (Ellis et al., 1992; Denisenko et al., 1994; Dodane and Kachar, 1996; Mullin et al., 1998; Stuart and Nigam, 1995).

In the early embryo, cavitation depends upon two interrelated events, (i) fluid accumulation along an ion gradient within the TE due to organised Na^+/K^+ ATPase localisation and function and (ii) formation of a TJ seal between the TE cells to retain the fluid within the blastocoelic cavity (Fleming et al., 2001; Watson and Barcroft, 2001). Membrane localisation, organisation and function of both groups of proteins are reported targets for PKC regulation in various tissue types (see D'Atri and Citi, 2002; Gonzalez-Mariscal et al., 2003; Hurd et al., 2003; Ohno, 2001; Suzuki et al., 2001; Therien and Blostein, 2000). In our study, PKCζ showed the most distinct difference in distribution pattern within the two cell lineages and clearest colocalisation with the TJ marker ZO-1α+. Whilst it was barely detectable above background levels within the ICM, it concentrated at the apical and apicolateral region of the membrane in the TE. Other epithelial cell types have shown a similar apical distribution of aPKCs (e.g., Minichiello et al., 1999).

When we examined the role of two of the four PKC isoforms during cavitation, which represent nPKCs and aPKCs and for which isoform-specific cell-permeable peptides were available, we found that reduced activity of PKCδ and ζ delayed cavitation but did not completely inhibit blastocoel formation. One possible explanation for this transient inhibition of cavitation could be peptide decay. The half-life of the peptides in our system is not known. Whilst the overnight incubations were designed to exclude toxic side effects without medium replacement, peptide renewal after 2 h in the short-term experiments ensured continuous peptide availability. The relatively fast catch-up cavitation rate of embryos after peptide-release may be indicative of a rapid peptide clearance from the embryo. However, in other systems, peptides remained effective for several hours without renewal, even when injected into the blood stream of live animals (Braun and Mochly-Rosen, 2003; Chen et al., 2001; Souroujon and Mochly-Rosen, 1998). Alternatively, the transient effect of the peptides could suggest protein redundancies. In the case of the latter, PKC-targeted proteins could be replaced by nontargeted ones in the early embryo, an example of developmental plasticity. Recent experiments using a Na^+/K^+ ATPase null mutation may support this idea (discussed below; Barcroft et al., 2004). Also, cavitation of human blastocysts was not entirely prevented by poor membrane assembly of certain TJ components (Ghassemifar et al., 2003).

Different effects of peptide treatment on junctional proteins and Na^+/K^+ ATPase were revealed (see Fig. 8 for a working model of cavitation regulation). Although Na^+/K^+ ATPase localisation was altered (discussed below), TJ proteins or α-catenin were not differentially localised between experimental and control treatments, further indicating that nonspecific disturbance of membrane molecule distribution by the antennapedia carrier peptides as suggested previously (Christiaens et al., 2004; Drin et al., 2003) does not occur. One interpretation of apparent junctional insensitivity may relate to the timing of PKC modulation. At 20 h post compaction, the time point peptides were added to the culture medium in the present study, the process of TJ membrane assembly is already well advanced with all late morulae showing linear ZO-2 assembly and approximately 50% late morulae showing ZO-1α+ assembly (Novak et al., in preparation; Sheth et al., 1997; similar control data not shown in detail). Even the late assembling ZO-1α+ has always been found at the cell contact sites before cavitation (Sheth et al., 1997). Thus, PKC modulation in this study focused on attempting to block or reverse an already established TJ membrane assembly programme for ZO-2 and ZO-1α+, which it failed to do. Inherent asynchrony in developmental rates between blastomeres within intact embryos causes difficulty in targeting treatment to specific differentiative events. We have been able to overcome this concern in our related studies of ZO-2 and ZO-1α+ membrane assembly in isolated ICMs during culture where immunosurgery acts as a 'switch' to induce TE-like differentiation of outer ICM cells (Eckert et al., 2004). In this model, we have been able to demonstrate unambiguously that PKCs, particularly nPKCs and aPKCs, are involved in regulating de novo TJ membrane assembly (Eckert et al., 2004). Therefore, we interpret the current data to imply that pre-assembled TJ protein is relatively insensitive to PKC modulating agents, presumably reflecting their enhanced stabilisation by cytoskeletal anchorage. Also, it may be possible that TJs were affected at the functional level rather than morphologically in the current data set and that TJ permeability may have been increased. Further studies are needed to clarify this.

In the present study, inhibition of cavitation with PKCζ inhibiting peptide was found to coincide with rapid internalisation of the α1 subunit of the Na^+/K^+ ATPase. In renal cells, Na^+/K^+ ATPase inhibition was associated with rapid PKC-dependent endocytosis of the α- and β-subunits (Chibalin et al., 1997) and internalisation of the α-subunit was associated with its PKC-mediated phosphorylation (Chibalin et al., 1998). Whilst both events were found to result in decreased Na^+/K^+ ATPase activity, subunit internalisation was the key regulator of enzyme activity in these cells, a process possibly involving receptor-mediated PI3K signalling that could participate in regulating PKCζ activity (Chibalin et al., 1999; Efendiev et al., 1999). Our data may suggest that, indeed, during early embryo development, a decrease of PKCζ activity in the late morula could delay cavitation due to reduced Na^+/K^+ ATPase function by

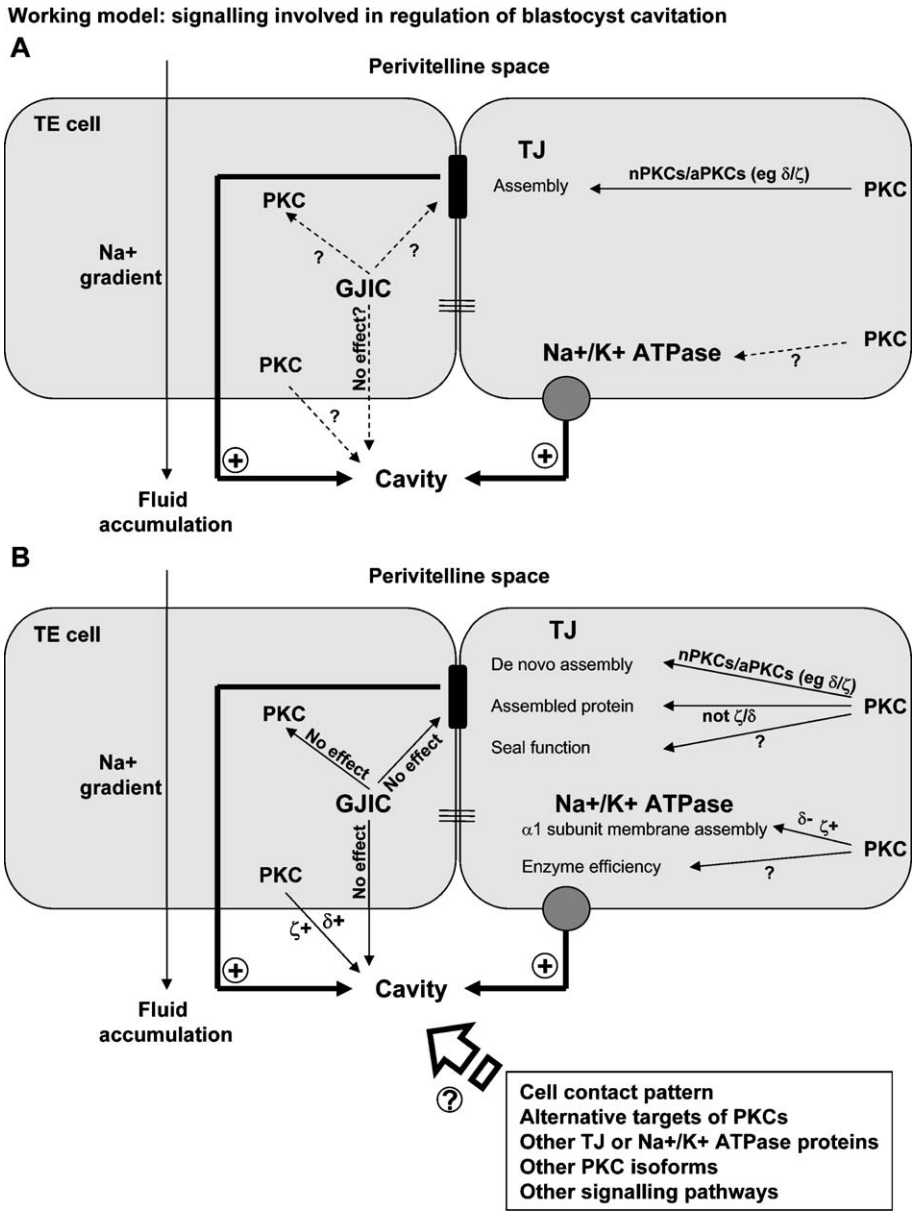

Fig. 8. Working model of signalling mechanisms involved in regulating blastocyst cavitation. Blastocoel formation and fluid accumulation are driven by transepithelial fluid transport along a sodium gradient generated by the Na^+/K^+ ATPase. A tight junctional seal (TJ) is required to provide TE integrity to generate the sodium gradient and seal off the fluid-filled blastocoelic cavity. The interrelationship between Na^+/K^+ ATPase, TJs and signalling via PKCs or gap junctional intercellular communication (GJIC) during the complex process of cavitation is presented. (A) We have shown previously that TJ membrane assembly can be influenced by PKC signalling (particularly nPKCs or aPKCS). The current study investigates whether PKC signalling is involved in regulating cavitation, how PKCs may contribute (Na^+/K^+ ATPase, TJs) and which PKCs are involved. In addition, the role of signalling via GJIC in cavitation is examined including whether GJIC affects PKC signalling or TJ membrane assembly. (B) From our current results, we have shown that whilst GJIC has no effect on cavitation, TJ assembly or PKC signalling, two specific PKCs, δ and ζ, promote cavitation. Whilst PKCζ is suggested to have an important role in Na^+/K^+ ATPase membrane insertion, thus promoting cavitation, the role of PKCδ remains unclear and we suggest that PKCδ, although inhibiting Na^+/K^+ ATPase membrane insertion, may promote cavitation via alternative targets (see text for suggestions). It is also suggested that the different levels of organization of cavitation (morphology or assembly of protein complexes, protein complex function, timing of events) are affected by different pathways and may be highly adaptable within the early embryo. Moreover, we suggest that more players may have key functions during cavitation and need to be added to the simplified model.

reversing or inhibiting membrane insertion or increasing turnover of at least one functional enzyme subunit. In MDCK cells, overexpression of dominant negative consensus ι/λ and ζ aPKCs similarly disrupted the distribution of Na^+/K^+ ATPase but not without disruption of TJ formation leaving some ambiguity about the order of events and interrelationships between TJ, Na^+/K^+ ATPase and PKC (Suzuki et al., 2001). The current data suggest that, in the early mouse embryo, PKCζ may solely affect the Na^+/K^+ ATPase but not TJ assembly indicating that different and

possibly non-interdependent signalling pathways may be involved in regulating Na^+/K^+ ATPase and TJs.

In the present study, activation of PKCδ, but not its inhibition, caused internalisation of α1, and α1 internalisation was slightly reduced when both PKCδ and ζ inhibitors were present together, confirming opposite effects of these PKC isotypes on α1 localisation during blastocyst formation. This may appear to contradict the synergistic effects of both PKC antagonists seen during cavitation timing but demonstrates the need for a simultaneous analysis of several molecules and contexts affected by PKC signalling as previously shown in other systems. For example, in a permanent lung epithelial cell line, the α1 subunit is endocytosed from the membrane upon PKCζ-mediated phosphorylation without involvement of PKCδ (Dada et al., 2003), whilst in primary lung epithelia, exocytosis of Na^+/K^+ ATPase molecules from late endosomes into the basal membrane is regulated by PKCδ and not ζ (Ridge et al., 2002). Yet, in kidney cells, α1 is a poor substrate for PKCδ-mediated phosphorylation compared to other PKCs (Kazanietz et al., 2001). Another complication is the fact that Na^+/K^+ ATPase in turn can stimulate PKC autophosphorylation hereby altering PKC localisation and activity (Feschenko et al., 1997).

Precisely how PKCδ activity both enhances cavitation over time in our system yet provokes membrane internalisation of Na^+/K^+ ATPase awaits further clarification. PKCδ may preferentially promote TJ function, for example impermeability, in a time-dependent manner. This could explain why PKCδ activator enhances cavitation possibly by tightening the TJ seal so that the simultaneous internalisation of α1 subunit of the Na^+/K^+ ATPase is insufficient to cause a delay in cavitation. It has recently been shown that homozygous null embryos for the α1 subunit are able to cavitate initially but then undergo cell dissociation and the cavity collapses (Barcroft et al., 2004). This agrees well with our current findings that cavitation did occur, although delayed, when the α1 subunit of the Na^+/K^+ ATPase was internalised. PKCζ may, in contrast, act synergistically promoting both TJ function and α1 subunit membrane insertion, therefore eliciting clearer effects on cavitation. In addition, the duration of the different peptide effects remains unknown and may differ, dependent upon the different target proteins and their turnover rates. The presence of the α1 subunit within the membrane after overnight culture irrespective of peptide treatment may indeed suggest a loss of peptide activity (discussed above) but could also indicate a high α1 subunit turnover rate, an increase of α1 protein production or a quick recycling of the α1 subunit from the cytosol to the membrane. The exact interrelationship between the timing and establishment of a TJ seal, Na^+/K^+-ATPase activity and blastocoel formation and the role of PKC isoforms awaits further refinement. In addition, more protein systems are likely employed during blastocoel formation, including the contribution of another potential target of PKC, the aquaporins (Barcroft et al., 2003; Han et al., 1998; Van Balkom et al., 2002; reviewed in Fleming et al., 2001; Watson and Barcroft, 2001). It is also well established that elevated cytoplasmic cAMP or calcium levels, two components that interact with PKCs, can accelerate the process of cavitation or affect embryonic gene expression (Dardik and Schultz, 1991; Rout et al., 1997; Stachecki and Armant, 1996a,b; Stachecki et al., 1994; Wang et al., 1998). We suggest a working model (Fig. 8) that shows the complexity of the cavitation process, some key players involved and how they may interrelate, and that demonstrates the importance of careful consideration of the different levels of potential effects (morphology, function, timing).

Because evidence from other cell types may suggest interrelationships (see Introduction) between GJIC, PKC signalling, TJ membrane assembly and, hence, the process of cavitation, we also examined the relative contribution of GJIC to blastocyst biogenesis. Regulation of GJIC is very complex, depends on channel composition and reacts rapidly to extra- and intracellular changes. Due to this complexity of factors influencing GJIC, its relative contribution to cellular, developmental and organ functions may be obscured and is difficult to assess as reflected by the controversial literature (Buehr et al., 1987; Cruciani et al., 2001; De Sousa et al., 1993; Giepmans, 2004; Lampe and Lau, 2004; Saez et al., 2003; Vance and Wiley, 1999; White and Paul, 1999; Wrenzycki et al., 1996). The objective of the present study was, therefore, to establish whether GJIC is or is not involved in regulating cavitation, TJ membrane assembly or PKC signalling by chemical inhibition of GJIC by AGA. Similar concentrations of AGA as used previously (Haghighat and Van Winkle, 1990; Houghton et al., 2002; Guo et al., 1999; Vance and Wiley, 1999) do not affect cavitation, junction membrane assembly and PKC mediated signalling within the blastocyst. This supports the idea that communication via this device may be dispensable during preimplantation differentiation events in the mouse (Houghton et al., 2002; Kidder and Winterhager, 2001).

Overall, our present data suggest that certain PKC isotypes (δ, θ, ι/λ and ζ) and PKCμ/PKD1 play a role in cavitation and blastocyst differentiation in the mouse independently of GJIC. All four membrane-concentrated PKC isoforms (δ, θ, ι/λ and ζ), but not PKCμ/PKD1, although present in TE and ICM, showed a distinct cell-type-dependent distribution pattern within the blastocyst and demonstrated colocalisation with a marker for epithelial TE differentiation, the TJ protein ZO-1α+. This may be indicative of a role for PKCs, particularly nPKCs and aPKCS, during TJ membrane assembly or function as shown from our parallel studies in isolated ICMs (Eckert et al., 2004). Amongst these four PKCs, we have identified two representing nPKCs and aPKCs for which isotype-specific activity modulators are available (PKCδ and ζ) that have a direct role in the cavitation process. This effect may be exerted at least in part via internalizing the α1 subunit of the Na^+/K^+ ATPase. Our results demonstrate for the first time that different PKC isotypes have distinct signalling functions and several

specific target proteins during the process of cavitation in preimplantation embryo development.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.07.027.

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