Expression profile of protein kinase C isozymes in preimplantation mouse development

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Abstract

In the preimplantation mouse embryo, the protein kinase C (PKC) family has been implicated in regulation of egg activation, progression of meiotic and mitotic cell cycles, embryo compaction, and blastulation, but the involvement of the individual isozymes is largely unknown. Here, using semiquantitative immunocytochemistry and confocal microscopy we analyze the relative amount and subcellular distribution of ten isozymes of PKC (α , β I, β II, γ , δ , ϵ , η , θ , ζ , ι/λ) and a PKC-anchoring protein, receptor for activated C-kinase 1 (RACK1). Our results show that all of these isoforms of PKC are present between the two-cell and blastocyst stages of mouse preimplantation development, and that each has a distinct, dynamic pattern and level of expression. The data suggest that different complements of the isozymes are involved in various steps of preimplantation development, and will serve as a framework for further functional studies of the individual isozymes. In particular, there was a transient increase in the nuclear concentration of several isozymes at the early four-cell stage, suggesting that some of the PKC isozymes might be involved in regulation of nuclear organization and function in the early mouse embryo.

Reproduction (2005) **130** 441–451

Introduction

The protein kinase C (PKC) family is a family of serine/threonine protein kinases that plays an important role in cell signal transduction. Based on structural differences that produce differential sensitivity to activators and co-factors, the isozymes have been categorized into three subfamilies. The conventional PKCs (cPKCs) are α , β I, β II, and γ and are activated by phosphatidylserine, diacylglycerol, Ca^{2+} , and 4β -phorbol 12-myristate 13-acetate (4β -PMA). The novel PKCs (nPKCs) are δ , ϵ , η , and θ , and are not Ca^{2+} -dependent, but otherwise activated as the cPKCs. PKC μ is sometimes included in the nPKCs (Geiges *et al.* 1997), but it has homologies to both the PKC and calmodulin kinases, and is often placed into a PKC-like family (Hausser *et al.* 2002). The atypical PKCs (aPKCs) are ζ and ι/λ and are lipid-dependent, but cannot be activated by either Ca^{2+} or 4β -PMA (Newton 2001). Production of monoclonal antibodies and polyclonal antisera against peptides that distinguish the various isozymes has led to a number of experiments that indicate that individual PKC isozymes are localized differentially within cells before and after stimulation. Their restricted localization exposes them to different substrates and is believed to largely specify function (Schechtman & Mochly-Rosen

2001). It has been shown that phosphorylation of a group of conserved amino acids determines the activation state of PKC and affects the subcellular localization of each isozyme (Keranen *et al.* 1995). Also, binding of substrate and non-substrate proteins affects the ability of PKC isozymes to relocate and hence associate with particular substrates (Jaken & Parker 2000, Mochly-Rosen & Kauvar 2000, Schechtman & Mochly-Rosen 2001).

Several studies have established involvement of PKC in egg activation, fertilization, and progression through the meiotic cycles in mice and rats (Gallicano *et al.* 1995, 1997, Raz *et al.* 1998, Downs *et al.* 2001, Viveiros *et al.* 2001, 2003, Eliyahu & Shalgi 2002, Quan *et al.* 2003). However, there is less known of the role of the PKC during early cleavage. Activation with phorbol ester of the cPKC and nPKC blocks progression through the mitotic cycles (Quan *et al.* 2003). The synthesis of proteins that are required for embryonic gene activation is inhibited by H8 and H7, non-specific inhibitors of protein kinases A and C that act by competitive inhibition of their ATP-binding sites (Schultz 1993). During mouse preimplantation development, phorbol ester causes premature compaction of two-, four-, and early eight-cell-stage embryos (Ohsugi *et al.* 1993a, 1993b,

Ohsugi & Yamamura 1993), and the non-specific PKC inhibitor D-sphingosine blocks induced and natural compaction (Winkel *et al.* 1990). PKC α may be involved in compaction, since it redistributes to contact sites as compaction initiates and can phosphorylate β -catenin (Pauken & Capco 1999). It has also been demonstrated that blastocoele formation is regulated through phospholipase C (an enzyme that is known to activate PKC-dependent pathways; Stachecki & Armant 1996), and that targeted disruption of mouse phospholipase C β 3 (the most widely expressed member of the phospholipase C β family) results in early preimplantation death (Wang *et al.* 1998). Although all of these studies suggest a role for the PKC family in preimplantation development, the relative importance of each isozyme is unknown.

In rat oocytes, Raz *et al.* (1998) detected PKCs α , β I, β II, γ , δ , ϵ , λ , and ζ by Western blot and immunocytochemistry with isozyme-specific antibodies, but not PKCs θ or ι , and Eliyahu & Shalgi (2002) detected PKCs α , β I, and β II by immunocytochemistry. PKCs α , β I, and γ have been detected in pig oocytes (Fan *et al.* 2002). The studies of mouse oocytes have given inconsistent results. Gangewaran & Jones (1997) detected only PKCs δ and λ among nine isozymes using Western blot. Pauken & Capco (2000) detected PKCs α , γ , δ , λ , μ , and ζ by Western blot and immunocytochemistry, but not PKC β . While Luria *et al.* (2000) found that PKCs α , β I and β II were present by immunocytochemistry, and Downs *et al.* (2001) found that PKCs α , β I, δ , and ζ , but not ϵ were present by Western blot. Luria *et al.* (2000), Eliyahu & Shalgi (2002) and Baluch *et al.* (2004) have supported some of the results with PKC activator/inhibitor studies. In preimplantation embryos, Pauken & Capco (2000) detected PKCs α , γ , δ , λ , and ζ but not isoform β by Western blot and immunocytochemistry, but Eckert *et al.* (2004) detected PKCs β I and β II in freshly isolated inner cell masses of mouse blastocysts. Clearly neither Western blot nor immunocytochemistry has provided consistent results with PKC β .

In this study, we used immunocytochemistry to examine the expression of 10 members of the PKC family (α , β I, β II, γ , δ , ϵ , η , θ , ζ , ι/λ) between the two-cell and blastocyst stages of mouse development. We chose to use immunocytochemistry because it is very sensitive and provides the ability to localize individual PKC isozymes within cells. We included antibodies from two commercial sources that have been widely used in immunocytochemical detection of PKCs, and support the immunocytochemistry data with results from treatments with specific PKC activators. This study differs from previous ones in that we quantified the fluorescence resulting from binding of the antibody to each PKC isozyme. This was done in order to estimate concentrations of each isozyme in various subcellular compartments at various stages of cleavage. This is significant since it has been suggested that small changes in subcellular distribution can lead to functional changes in PKC action. It is difficult to obtain the numbers of preimplantation embryos

that would be required for cell fractionation and biochemistry. Quantifying immunofluorescence is one way to circumvent this problem and has been used by others for live cell imaging of PKC translocations (Almholt *et al.* 1999, Schechtman *et al.* 2004).

Materials and Methods

Embryo collection and treatment

Mice were maintained and handled in accordance with the guidelines of the Animal Care Committee, University of Guelph, Guelph, Ontario, Canada. Mice (CD1; Charles River, Saint-Constant, QC, Canada) were super-ovulated by intraperitoneal injection of 5 IU pregnant mare's serum gonadotropin followed 46 h later by 5 IU human chorionic gonadotropin (hCG). Each female mouse was placed with a male and was checked the following morning for the presence of a copulation plug. Embryos (two-cell, four-cell, 8–16-cell, morula, and early blastocyst) were flushed from oviducts or uterine horns with PBS (8.0 g/l NaCl, 0.2 g/l KCl, 0.15 g/l Na₂HPO₄, and 0.2 g/l KH₂PO₄) at 45, 52, 69, 76, and 93 h after hCG injection, respectively, as described by Hogan *et al.* (1994). At these time points, two-cell embryos are in the G₂ phase, and four-cell embryos should be in the G₁ or S phase of the cell cycle (Pratt 1987). For most immunofluorescence analysis, the embryos were fixed immediately after harvest and processed as described in the next section.

To study the effects of activating PKC isozymes, two-cell embryos were flushed from oviducts at 45 h post-hCG with flushing-holding medium (Lawitts & Biggers 1993), and cultured in KSOM/AA medium at 37 °C under silicone oil in an atmosphere of 5% CO₂/5% O₂/90% N₂ (Lawitts & Biggers 1993, Ho *et al.* 1995) with or without PKC activator. Activation was with 100 nM 4 β -PMA for 15 min (LC Laboratories, Woburn, MA, USA) or with 100 nM of the cPKC-activating peptide, pseudo- β RACK (where RACK is receptor for activated C kinase) for 60 min (kindly donated by D Mochly-Rosen, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA, USA). The 4 β -PMA was dissolved in ethanol at 30 μ M (Watanabe *et al.* 2002), and used at 100 nM in medium as described for myocytes (Disatnik *et al.* 1994, Dorn *et al.* 1999) and similar to what has been used with oocytes (80, 100, and 160 nM; Gallicano *et al.* 1995, 1997, Raz *et al.* 1998, Luria *et al.* 2000). Control embryos were cultured in medium with 0.33% ethanol. We did not include inactive analogues of 4 β -PMA because others have shown that the analogues have non-specific activities (for example, see Doerner *et al.* 1990, Watanabe *et al.* 2002). The peptide activator pseudo- β RACK is cross-linked to a dimer of Antennapedia carrier peptide (CRQIKIWFQNRRMKWKK). This conjugate has been used extensively by Mochly-Rosen's group, and shown to enter cells and affect PKC distribution and cell function without compromising cell viability (Dorn *et al.* 1999). This group has also shown

that pseudo- β RACK does not affect distribution of other PKC isoforms at concentrations between 100 nM and 1 μ M, and that scrambled peptide and peptides derived from other parts of the PKC molecule do not affect distribution of the PKC. Negative-control embryos were cultured in medium containing 100 nM carrier-carrier dimer (also kindly donated by D Mochly-Rosen). After culture in treatment or control media, the embryos were immediately fixed and processed for immunocytochemistry.

Immunocytochemistry

Monoclonal antibodies against seven isozymes of PKC (α , γ , δ , ϵ , θ , ι , and λ) and RACK1 were purchased from Transduction Laboratories (Lexington, KY, USA). Polyclonal antibodies raised against specific peptides of nine isozymes of PKC (α , β I, β II, γ , δ , ϵ , ζ , η , and θ) were purchased from Research & Diagnostics Antibodies (Berkeley, CA, USA). These antibodies have been previously characterized and used in a number of studies on PKC in embryonic or adult cells (Disatnik *et al.* 1994, Johnson *et al.* 1996, Csukai *et al.* 1997, Miyamae *et al.* 1998, Dorn *et al.* 1999, Pauken & Capco 2000). Embryos were fixed in 2% paraformaldehyde in PBS for 30 min, and then permeabilized in 0.5% Triton-X 100 in PBS for 10 min. They were washed three times (20 min each) with 5 mM glycine in PBS, treated with 1% Tween-20 (10 min), and equilibrated in intracellular buffer (ICB; 100 mM KCl, 5 mM MgCl_2 , 3 mM EGTA, 1% BSA, and 20 mM Hepes, pH 6.8) for 5 min, as described by Pauken & Capco (1999, 2000). Groups of five embryos were transferred into 50 μ l drops of ICB containing 10% serum matching the secondary antibody, 0.1% Tween-20, and antibody against the PKC isozyme (at a final concentration of 10 μ g/ml for monoclonal antibodies and a final dilution of 1:100 for polyclonal antibodies), and incubated overnight at 4°C. Embryos were washed four times (30 min each) with ICB and transferred into 50 μ l drops of diluted secondary antibody at 20 μ g/ml for 2 h at room temperature in the dark. Fluorescein-conjugated, affinity-purified F(ab')₂ fragment of sheep anti-mouse IgG (Boehringer Mannheim, Mannheim, Germany) was used to detect the monoclonal antibodies, and affinity-purified F(ab')₂ fragment of goat anti-rabbit IgG labeled with Rhodamine Red-X (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was used to detect the polyclonal antibodies. The embryos were washed four times (30 min each at room temperature in the dark) with ICB and then mounted on 0.1% poly-lysine coated slides using Vectashield containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA). Negative controls for immunocytochemistry were embryos exposed to medium rather than primary antibody. For each isozyme at each stage, there were at least nine embryos analyzed from three independent experiments.

Imaging was performed using a Bio-Rad MRC 600 confocal laser-scanning microscope attached to an Optiphot-II Nikon fluorescence microscope (with a 60 $\times$, 1.4

numerical aperture plan-apochromat oil-immersion objective). Excitation was with 488 and 568 nm wavelengths from a krypton-argon laser.

Quantification of fluorescence intensity

We used the same confocal microscope settings for images from all embryos regardless of antibody, and for all trials. The settings were based on the fluorescence intensity of embryos immunostained for PKC β II, because this antibody gave us the strongest signal (see Fig. 2C, below). The settings were selected to ensure that the PKC β II images were always less than 256 on the gray scale in the highest part of the linear range.

A minimum of three embryos were imaged in three trials on separate days. For each blastomere of each embryo up to the blastocyst stage, an image (optical slice) was selected from the stack that bisected the nucleus and included apical and basal membranes. In our experience, this mid-blastomere slice provides the best single representation of blastomere asymmetry, since the polarized distribution of components in blastomeres is radially disposed. At the blastocyst stage, 10 cells were randomly chosen from the trophectoderm or inner cell mass. The images were converted to TIFF files using Bio-Rad's Confocal Assistant software (version 4.02), and the fluorescence analyzed using Scion Image (Scion Corporation, Frederick, MD, USA). For quantification of fluorescence within the nucleus and cytoplasm, the whole area for nucleus and cytoplasm was selected manually. The cytoplasm was further subdivided by selecting 10 sub-regions (four pixels each) that were immediately sub-adjacent to plasma membrane that was not in contact with another cell (apical cytoplasm), immediately sub-adjacent to plasma membrane that was in contact with another blastomere (basal cytoplasm), or that was midway between these two areas and the nucleus (central cytoplasm).

The mean fluorescence intensity for each subcellular area of each blastomere was calculated by dividing the sum of all gray values in the sub-regions by the total number of pixels enclosed. The background gray value in each image (a region outside of the embryo) was subtracted from the mean fluorescence intensity. Analysis of variance did not show a significant difference between the three trials, and thus we were able to treat the readings from each subcellular area of each blastomere at a given time point as a single population. The error bars in the graphs are the standard deviations of these readings, and represent the variations among blastomeres, embryos, and trials.

Difference in fluorescence intensity between the nucleus and cytoplasm was analyzed using a Student's *t*-test ($P < 0.05$), and is indicated in Figs 2–5 (see below) by an asterisk. Differences in fluorescence intensity among the apical, central, and basal regions of the cytoplasm were compared using ANOVA ($P < 0.05$), and the statistically different groups were identified by Tukey's test

($P < 0.05$). Statistical difference is indicated by lower-case letters on Figs 2 and 5.

Results

Specificity of PKC antibodies

To ascertain that the expression pattern and intracellular location of PKC isozymes are correctly identified, we applied the following measures. First, we compared expression of PKC isozymes using monoclonal and polyclonal antibodies from two sources. Figure 1A shows the staining of both an isozyme-specific monoclonal antibody and polyclonal antiserum from two manufacturers for PKCs γ , δ , ϵ , and θ . In each case the monoclonal antibodies and polyclonal antisera were raised against two distinct regions of the PKC isozyme, and both antibodies produced the same distribution patterns, although intensity of staining was usually less with the monoclonal antibodies (Fig. 1A). Hence, all further experiments used polyclonal antisera for PKCs α , β I, β II, γ , δ , ϵ , η , θ , and ζ , and monoclonal antibodies for PKCs ι and λ , and RACK1. The monoclonal antibodies that recognize mouse PKCs ι and λ cross-react, and the proteins are 99% homologous (Transduction Laboratories). They are thought to be equivalent genes of human (ι) and mouse (λ ; Kazanietz 2000), so it was reassuring that they displayed almost identical patterns of binding on mouse embryos (see Fig. 5 below). Secondly, the evidence that the antisera against PKCs β I and β II detected these PKCs is that 4 β -PMA (a general activator of cPKCs and nPKCs) and pseudo- β RACK (a specific peptide activator of cPKCs) affected expression of the molecules detected by these antisera at the two-cell stage. The changes were isozyme-specific, and the same with both types of activation (Fig. 1B); the distributions of

PKCs β I, β II, and γ changed, but PKC α staining remained more concentrated in nuclei, although the nuclear/cytoplasmic ratio decreased. The peptide pseudo- β RACK was designed using PKC β sequence, but from the constant (C2) region shared by all cPKCs. Thirdly, whereas all isozymes were detected during preimplantation development, not all isozymes were expressed at every stage. There were negative embryos for some isoforms with each of the two secondary antibodies, one that recognized the polyclonal antisera, and one that recognized the monoclonal antibodies. The stages that were negative for PKCs α and γ (Figs 2A and 3A) were stained with the polyclonal secondary, and the stages that were negative for PKCs ι and λ and RACK1 (see Fig. 5 below) were stained with the monoclonal secondary. Finally, fluorescence intensity was above that of negative control embryos (no primary antibody) that were part of each trial.

Intracellular distribution of PKC isozymes

Summary

The intracellular location of each isozyme was distinctive and dynamic (Figs 2–5). Whenever detected, PKCs β II, ι , and λ and RACK 1 were more concentrated in the cytoplasm than in the nucleus (Figs 2 and 5). The opposite was true of PKC η ; its nuclear concentration was consistently higher than the cytoplasmic concentration except at the late two-cell stage (Fig. 4). For the other PKCs, there were changes in the nuclear/cytoplasmic distribution between stages of preimplantation development. It is interesting that in early four-cell embryos there were more PKCs localized to the nuclei than at any other stage; the concentrations of seven out of 10 isozymes were higher in nuclei than in the cytoplasm.

Figure 1 Analysis of antibody specificity. (A) Representative confocal images of two-cell (for PKC δ), four-cell (for PKC γ), blastocyst (for PKC ϵ), and eight-cell (for PKC θ) mouse embryos which were immunostained with antibodies from two different sources. TD, monoclonal antibody, Transduction Laboratories; R&D, polyclonal antiserum, Research & Diagnostics Antibodies. (B) Treatment of two-cell embryos with two different activators of cPKC: 15 min in 100 nM 4 β -PMA or 60 min in 100 nM pseudo- β RACK. Embryos subsequently were immunostained for each of the cPKC isozymes and examined with a confocal microscope.

Figure 2 Subcellular localization of PKCs α , βI , and βII in preimplantation mouse embryos. A representative confocal image for each preimplantation stage is shown above the histogram. Mean fluorescence intensity (average gray value of pixels enclosed minus background gray value) was calculated for the nucleus or whole of cytoplasm, and apical, central, and basal cytoplasmic regions of all blastomeres in the embryos of a given stage over three trials. Each bar of the histogram represents the mean fluorescence intensity of all blastomeres in that group, except for blastocysts where 10 blastomeres were selected. Error bars show s.d. values. Significant differences in mean fluorescence intensity between cytoplasmic and nuclear regions are shown by *, $P < 0.05$. The letters a and b represent the significant differences among apical, central, and basal regions of the cytoplasm (analyzed by ANOVA and Tukey's test; $P < 0.05$). Two bars with the same letter are statistically similar, and any given bar with unique letter is significantly different from the others. Scale bars, 20 μm . Note that the optical slice chosen to represent the whole embryo does not bisect all blastomeres in the mid-plane used for analysis. Therefore the image suggests more interblastomeric heterogeneity than exists.

Cytoplasmic versus nuclear distributions

At the two-cell stage (45 h post-hCG, G_2), PKCs βI , δ , ϵ , η , θ , and ζ were distributed equally between the cytoplasmic and nuclear compartments (Figs 2–4). Only PKC α was more concentrated in the nucleus ($P < 0.05$), whereas PKCs βII , γ , ι , and λ were more concentrated in the cytoplasm ($P < 0.05$).

At the four-cell stage (52 h post-hCG, G_1/S), the distributions of PKCs α , βI , γ , δ , ϵ , η , and θ changed, with six out of seven (all but PKC α) becoming more concentrated in nuclei ($P < 0.05$). PKC α became equally distributed

after being more concentrated in two-cell nuclei. PKC ι was below the detection limits of the assay.

At the eight-cell stage (69 h post-hCG), three out of six PKC isoforms with high nuclear concentrations at the four-cell stage became equally distributed between the cytoplasm and nucleus (βI , δ , and θ) or became more concentrated in the cytoplasm (ϵ ; $P < 0.05$). Only PKCs γ and η retained higher nuclear fluorescence intensity at this stage ($P < 0.05$). The concentration of PKC ζ became higher in the cytoplasm from the equal distribution of the four-cell stage ($P < 0.05$).

Figure 3 Subcellular localization of PKCs γ , δ , and ϵ in preimplantation mouse embryos. All details are as for Fig. 2.

At the morula stage (76 h post-hCG), the concentrations of PKC α , β I, and ζ once again increased in nuclei ($P < 0.05$), whereas PKCs δ and ϵ became evenly distributed between the nucleus and cytoplasm. Immunostaining for PKCs γ , ι , and λ and RACK1 decreased to negative-control levels.

Of the eight PKC isozyms detectable at the blastocyst stage (93 h post-hCG), PKC η was the only isozyne that was more concentrated in the nucleus ($P < 0.05$).

Cytoplasmic distributions

In the first photomicrographs, there seemed to be uneven distribution within the cytoplasm of some isozyms. Therefore, we separately measured fluorescence in the apical, basal, and central regions of the cytoplasm. At the two-cell stage, PKCs β II, θ , ι , and λ were localized preferentially to the apical cytoplasm ($P < 0.05$; Figs 2, 4, and 5), and at the four-cell stage RACK1 was localized to the basal cytoplasm ($P < 0.05$; Fig. 5).

Figure 4 Subcellular localization of PKCs η , θ , and ζ in preimplantation mouse embryos. All details are as for Fig. 2.

Discussion

Subcellular localization of PKC isozymes

In this study, we have quantified the relative subcellular concentrations of 10 PKC isozymes and the anchoring protein RACK1 during mouse preimplantation development. To do this we measured the mean fluorescence intensity from subcellular regions (nucleus, cytoplasm, apical cytoplasm, central cytoplasm, and basal cytoplasm) of all blastomeres of embryos immunostained for a PKC

isozyme up to the blastocyst stage. Since the fluorescence intensity of the embryo depends on the quantity of primary antibody–secondary antibody associations, it is an indicator of the amount of each isozyme. Because the number of antibody-binding sites and affinities are uncertain, the assay does not measure the absolute amount of isozyme, or allow quantitative comparison between isozymes. Therefore, the assay is semi-quantitative. However, by maintaining antibody reaction conditions, by using the same confocal microscope settings, and by averaging three independent trials, the assay does produce

Figure 5 Subcellular localization of PKCs ι and λ , and the anchoring protein RACK1 in preimplantation mouse embryos. All details are as for Fig. 2.

information on relative concentrations (fluorescence intensity) of a single isozyme between subcellular compartments and between stages. In the accompanying study (Dehghani *et al.* 2005), the ratio of nuclear to cytoplasmic fluorescence is calculated as a way to accommodate inter-trial variation in staining intensity. This calculation was not performed in this study because various parts of the cytoplasm as well as the whole of the cytoplasm are examined. Also, fluxes in concentration between stages are indicated by the data in this form that would not be seen with ratios. On the other hand, variability in staining

between trials may have reduced sensitivity of the analysis. Finally, the zona pellucida was not removed prior to immunostaining, following the protocol of Pauken & Capco (1999). This was to fix embryos as soon as possible after recovery. Trapping of antibody in the zona pellucida could have caused the absence of staining of blastocysts with some of the antibodies. However, it seemed that the amount of antibody trapped varied more with trial than stage or antibody.

In most studies with adult cell types, PKC in different subcellular fractions has been detected by Western blots

using 200 µg–1 mg protein, depending on isozyme abundance. Similar studies with preimplantation embryos certainly would be hampered by lack of material. A mouse oocyte has approximately 23 ng total protein (Hogan *et al.* 1994). However, live-cell imaging of translocation in individual cells, imaging for co-localization of intracellular signaling molecules, and photobleaching studies have quantified relative fluorescence intensity (Almholt *et al.* 1999, Ron *et al.* 1999, Echevarria *et al.* 2003, Schechtman *et al.* 2004). Thus whereas quantifying fluorescence intensity is not novel, it has not been used to localize signaling molecules in preimplantation mammalian embryos in quite this manner. The success of the approach is seen in the fact that differences were detected between the nucleus and cytoplasm and different areas of the cytoplasm. Thus, this approach should be effective for studying intracellular distribution and associations of molecules in preimplantation embryos where starting material is too limited for cell fractionation.

There are differences between this study and that of Pauken & Capco (2000) especially in the subcellular distribution of PKCs α , γ , and ζ . More recently, Quan *et al.* (2003) and Baluch *et al.* (2004) have shown PKC α distribution similar to ours. However, discrepancies in distribution will likely remain since distribution of PKC is dynamic, and precisely when and how embryos are collected could affect results. In this study, we controlled timing of gonadotrophin administration and embryo collection as much as possible. The major difference between this study and that of Pauken & Capco (2000) is the presence of PKCs β I and β II. Pauken & Capco found no PKC β in preimplantation mouse embryos. However, Eckert *et al.* (2004) detected both PKCs β I and β II in freshly isolated mouse inner cell masses. The expression of the PKC β isoforms in mouse oocytes has been similarly controversial using both Western blots and immunofluorescence (Gangeswaran & Jones 1997, Luria *et al.* 2000, Pauken & Capco 2000, Downs *et al.* 2001). Because of this, we decided to use activation as a means to verify antibody specificity. The fact that both general and specific peptide activators of the cPKC caused translocation of the molecules recognized by our antibodies against PKCs β I and β II suggests that what is recognized are PKC β , or at least PKC β -like, molecules.

Localization of PKC isozymes to subcellular compartments can be mediated by binding to isozyme-specific anchoring proteins or through the cytoskeleton (Mochly-Rosen & Gordon 1998, Jaken & Parker 2000, Newton 2001, Schechtman & Mochly-Rosen 2001). Known anchoring proteins include substrates that interact with C kinases (STICKs) and isozyme-specific RACKs. Most known STICKs are located at interfaces between membranes and cytoskeletal structures, and phosphorylation by PKC modifies their function. They may or may not be multi-enzyme scaffold proteins. An example of the latter is AKAP 79, which binds to PKCs α and β II (Klauck *et al.* 1996), as well as at least two other signal transducers,

protein kinase A and calcineurin (a Ca^{2+} /calmodulin-dependent phosphatase). RACK1 is a non-substrate receptor for PKC β II, although it also binds other PKC isoforms and other molecules (Jaken & Parker 2000, Rigas *et al.* 2003). Since RACK1 is involved in the translocation and association of PKC β II with a normal substrate in cardiac myocytes (Ron & Mochly-Rosen 1995, Csukai *et al.* 1997), it is interesting that RACK1 and PKC β II distributions differ in preimplantation embryos. In particular, RACK1 is concentrated in the basal region of cytoplasm in four-cell stage embryos, whereas PKC β II is concentrated in the apical cytoplasm. Other RACKs, STICKs, or a receptor for inactivated C kinase (RICK) may govern PKC β II localization at this stage. Further studies to find molecular partners of PKC isozymes are needed to elucidate their localization and function.

Although translocation of PKC is associated with activation in other cell types (Csukai & Mochly-Rosen 1999, Teruel & Meyer 2000), it must be noted that the state of activation of each isozyme during preimplantation development still needs to be determined. Also, there are several sources of change in the relative concentration of an isozyme observed in this study. First, there could be translocation of existing molecules between the cytoplasm and nucleus or of newly synthesized protein from the cytoplasm into the nucleus. Second, new synthesis of an isozyme without translocation would increase the relative cytoplasmic concentration. Or thirdly there could be differential degradation of an isozyme among subcellular compartments, which would decrease the relative concentration in that compartment. Further studies with individual isozymes to separate these possibilities are in order.

Functional implications of subcellular localization of PKC isozymes

This study defines the intracellular distribution of each PKC isozyme at specific times during cleavage, because it is subcellular location of PKC isozymes that correlates with different functions in a cell (Newton 2001). In adult cells, the PKC isozymes have a large and varied number of regulatory roles. Regulation of nuclear architecture (Hocavar *et al.* 1993, Maraldi *et al.* 1999), cell cycle (Livneh & Fishman 1997), and cellular polarization (Plant *et al.* 2003) are just a few examples. The PKC isozymes translocate to different subcellular areas during preimplantation mouse development, and likely are involved in multiple steps in preimplantation development. We found that nuclear localization of PKC α (early two- and four-cell nuclei) overlaps with that of PKC γ (four- and eight-cell nuclei), whereas PKCs δ and ϵ are more concentrated in nuclei only at the early four-cell stage (Figs 2 and 3). Furthermore, we found that PKC η localizes to the nuclei of eight-cell- to blastocyst-stage embryos (Fig. 4). Nuclear localization may indicate involvement in regulation of transcription. Hamatani *et al.* (2004) have shown increases in the number of genes transcribed at each stage of mouse preimplantation development. There is also

evidence for chromatin remodeling in early mouse embryos (Thompson *et al.* 1998, Schultz *et al.* 1999). Recently, Santos *et al.* (2003) and Sarmiento *et al.* (2004) have described constant modulation of histones during this period. It is very likely that some of the PKCs are involved in changing the relative lengths of the cell-cycle components during early cleavage (Pratt 1987, Livneh & Fishman 1997).

In conclusion, this study suggests fertile areas for further research. Activation state, effects of specific inhibitors and activators, co-localization with potential substrates and regulators, and temporal and spatial correlation of the isozymes with specific processes of mouse preimplantation embryogenesis are studies that can be undertaken in the future using this semi-quantitative approach. In the accompanying paper (Dehghani *et al.* 2005), we study the relationship between translocation of PKCs δ and ϵ into four-cell nuclei and transcription and nuclear morphology.

Acknowledgements

We thank Dr Mochly-Rosen for providing the isozyme-specific translocation activator of cPKC. This work was supported by a NSERC Canada grant to A C H, and H D was supported by a scholarship from MCHE Iran. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 15 November 2004

First decision 6 January 2005

Revised manuscript received 22 March 2005

Accepted 21 June 2005