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Microgravity Modifies Protein Kinase C Isoform Translocation in the Human Monocytic Cell Line U937 and Human Peripheral Blood T-Cells

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Abstract Individual protein kinase C (PKC) isoforms fulfil distinct roles in the regulation of the commitment to differentiation, cell cycle arrest, and apoptosis in both monocytes and T-cells. The human monocyte like cell line U937 and T-cells were exposed to microgravity, during spaceflight and the translocation (a critical step in PKC signaling) of individual isoforms to cell particulate fraction examined. PKC activating phorbol esters induced a rapid translocation of several PKC isoforms to the particulate fraction of U937 monocytes under terrestrial gravity (1*g*) conditions in the laboratory. In microgravity, the translocation of PKC βII, δ, and ε in response to phorbol esters was reduced in microgravity compared to 1*g*, but was enhanced in weak hypergravity (1.4*g*). All isoforms showed a net increase in particulate PKC following phorbol ester stimulation, except PKC δ which showed a net decrease in microgravity. In T-cells, phorbol ester induced translocation of PKC δ was reduced in microgravity, compared to 1*g*, while PKC βII translocation was not significantly different at the two g-levels. These data show that microgravity differentially alters the translocation of individual PKC isoforms in monocytes and T-cells, thus providing a partial explanation for the modifications previously observed in the activation of these cell types under microgravity. J. Cell. Biochem. 87: 39–50, 2002.
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Key words: monocyte; microgravity; signal transduction; protein kinase C

Experiments performed in orbiting spacecraft have shown that the growth, differentiation, and cell cycle of mammalian cells are altered under low gravity or "microgravity" conditions. [Hughes-Fulford and Lewis, 1996; Cogoli and Cogoli-Greuter, 1997; Hashemi et al., 1999; Lewis et al., 2001]. Signal transduction processes appear sensitive to microgravity, particularly signaling by protein kinase C (PKC) isoforms [de Groot et al., 1991; Schmitt et al., 1996; Hatton et al., 1999]. Since gene expression is modulated by signal transduction processes and cytoskeleton reorganization, microgravity induced modifications in these processes could have important consequences for cell fate.

PKC isoforms [Mochly-Rosen and Kauvar, 2000], a family of serine threonine kinases, play a critical role in the regulation of monocyte differentiation, as well as the initiation of apoptosis (Fig. 1). Phorbol esters, which activate most PKC isoforms, are potent inducers of differentiation in the monocytic cell line U937, and sustained PKC activation is necessary for differentiation since this induces *c-jun* upregulation and *c-myc* downregulation proceeding

Abbreviations used: APC, antigen presenting cell; FBS, fetal bovine serum; GCAK, general cell activation kit; IL-1β, interleukin-1β; IL-2, interleukin-2; MTOC, microtubule organizing center; PBS, phosphate buffered saline; PDBu, phorbol-12,13-dibutyrate; PKC, protein kinase C; RACK, receptor for activated C kinase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, tris buffered saline; TNF-α, tumour necrosis factor alpha.

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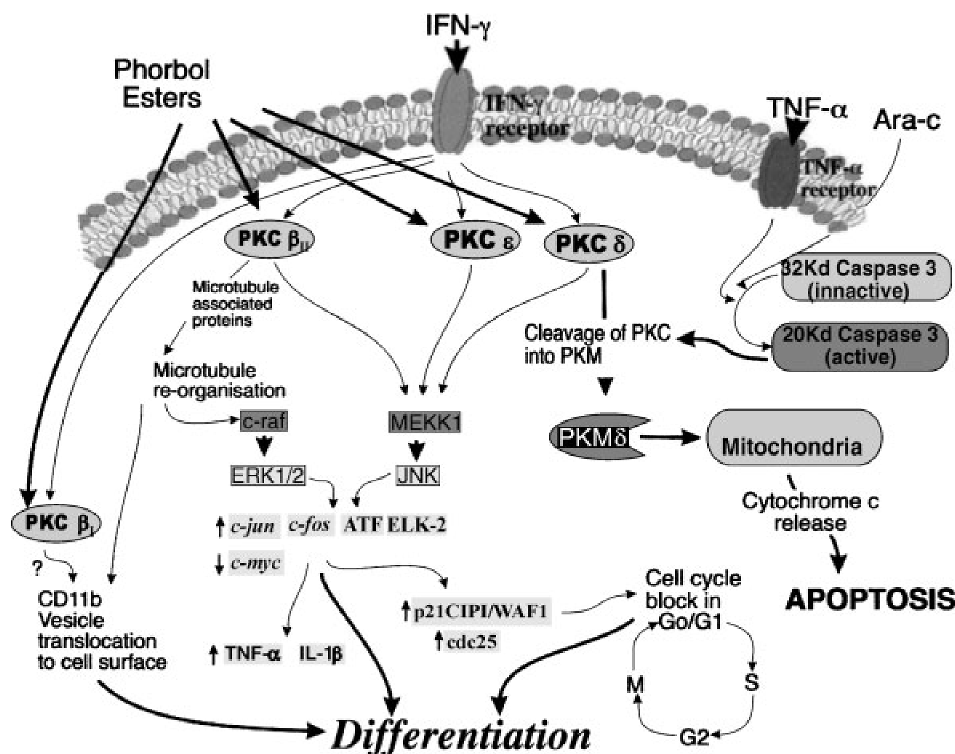

Fig. 1. The roles of PKC isoforms in the regulation of differentiation and apoptosis in U937 cells: Individual PKC isoforms play distinct roles in the regulation of differentiation and apoptosis of monocytic cells. PKC βII regulates CD11b integrin translocation to the cell surface (necessary for cell adhesion) and early gene expression controlling the commitment to differentiate to a macrophage like phenotype and cell cycle arrest. PKC ε appears to regulate the expression of anti-apoptotic genes. PKC δ is also involved in the commitment to differentiation following phorbol ester stimulation. However, apoptosis inducing agents such as TNF-α activate caspase 3, which in turn cleaves PKC δ into a constitutively active catalytic fragment (PKM). The PKM translocates to the mitochondria where it mediates cytochrome c release, a key step in the induction of apoptosis.

growth arrest which signals commitment to differentiation [Ways et al., 1994]. Additionally, cell cycle arrest can be induced by phorbol esters which promote induction of the cyclin inhibitor p21CIP/WAF1 and downregulation of cdc25 [Vrana et al., 1998]. Individual PKC isoforms appear to play distinct roles in regulation of monocyte differentiation. In U937 cells, PKC βI phosphorylation appears to correlate with critical cell density [Hansra et al., 1999], while PKC βII regulates microtubule reorganization, CD11b transport to the cell surface and early gene expression necessary for differentiation expression [Kiley and Parker, 1997; Whelan et al., 1999]. PKC ε appears to have an anti-apoptotic role [Mayne and Murray, 1998], while PKC δ is implicated in the initiation of apoptosis [Majumder et al., 2000]. Similarly, PKC isoforms in T-cells appear to fulfill specific roles, with PKC θ playing a critical role in signaling from the T-cell receptor [Coudronniere et al., 2000]. Other isoforms including PKC α, β, δ, and ε have been implicated in the regulation of the expression of Interleukin-2, Interleukin 2 receptor [Szamel et al., 1998] and induction of apoptosis by FAS ligand [Scheel-Toellner et al., 1999].

Activation of PKC isoforms is generally accompanied by relocalization of the isoform from one cellular compartment to another, a process known as translocation (Fig. 2). PKC isoforms are targeted to specific intracellular locations during translocation by receptors, known as RACK's, for the activated form of the isoform [Mochly-Rosen and Gordon, 1998]. Blocking PKC ε translocation with peptides which compete with the isoform for the RACK binding site inhibited the anti-apoptotic effect of phorbol ester on U937 cells [Mayne and Murray, 1998], indicating the critical role translocation plays in PKC isoform signaling.

Previously, we reported that during spaceflight the proportion of PKC in the nuclear fraction of both U937 and Jurkat cells (a T-cell

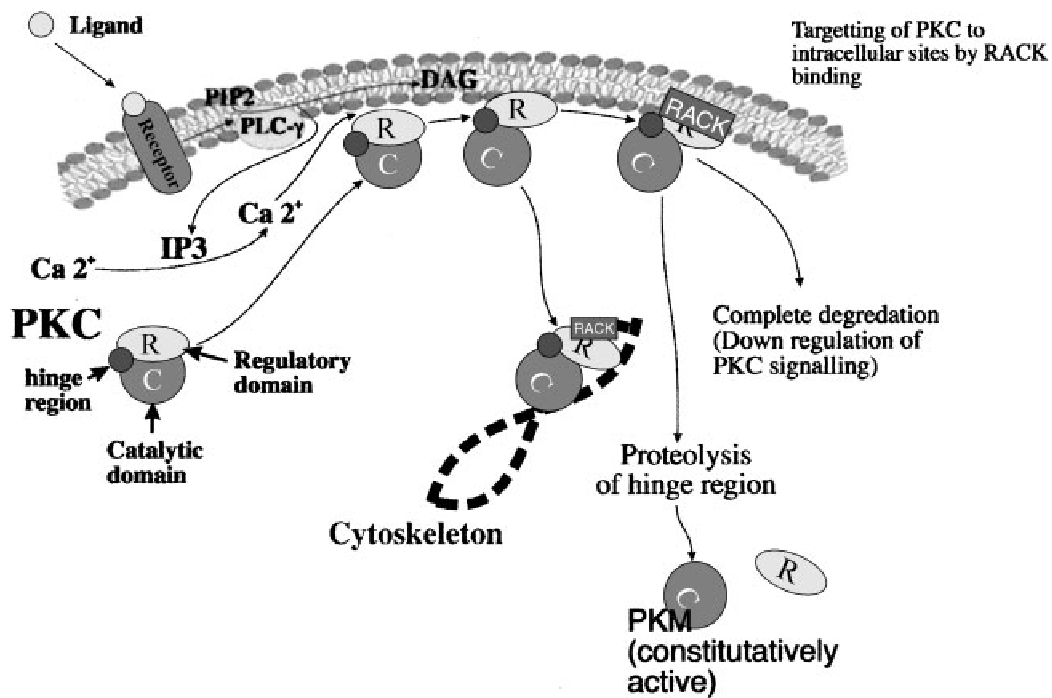

Fig. 2. Mechanism of PKC activation and targeting: PKC consists of a regulatory subunit which binds to diacylglycerol and a catalytic subunit separated by a proteolysis sensitive hinge. Activated phospholipase C γ (PLCγ) generates inositol triphosphate (IP3), which stimulates Ca^{2+} release, and diacylglycerol which activates PKC. Classical PKC isoforms have a Ca^{2+} binding site; binding of Ca^{2+} to PKC promotes association with the membrane, making it easier for PKC to bind DAG in the membrane. Following activation PKC isoforms are targeted in an isoform specific manner to intracellular sites by binding to receptors for activated PKC. PKC signaling is often down-regulated by proteolysis of PKC. However, cleavage of PKC at the hinge region can generate a constitutively active catalytic fragment (PKM).

line) decreased with increasing g-level between microgravity and 1.4*g*, while PKC in the cytosolic fraction increased with g-level, suggesting a gravity dependant redistribution of PKC between these cellular fractions [Schmitt et al., 1996]. Additionally, IL-1β synthesis by U937 cells following phorbol ester stimulation was significantly decreased in microgravity compared to 1*g* samples. Furthermore, the kinetics of PKC translocation in U937 cells were modified during spaceflight compared to 1*g* ground conditions, while total PKC content of spaceflown cells was elevated relative to cells cultured on the ground [Hatton et al., 1999]. Given the apparent sensitivity of PKC translocation to microgravity and the diverse roles of individual PKC isoforms in both U937 and T-cells, we investigated the effect of microgravity culture on the translocation of selected PKC isoforms during the STS-81 space shuttle mission. Here, we show that the phorbol ester translocation of PKC βII, δ, and ε in U937 cells was inhibited in microgravity and enhanced in 1.4*g* hypergravity, compared to 1*g* conditions, resulting in different relative levels of each isoform at each g-level. In peripheral blood T-cells, PKC βII and δ showed differential sensitivities to the microgravity environment depending upon the method used to activate PKC. Given the importance of PKC isoforms in monocyte and T-cell signal transduction, these data provide a partial explanation for the alterations in gene expression and cell function which have been reported to occur in these cell types when exposed to microgravity or hypergravity.

MATERIALS AND METHODS

Materials

Reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Lymphoprep 1.077 medium (ficoll solution), penicillin, streptomycin sulphate, L-glutamine, and penicillin-streptomycin-neomycin antibiotic mix were from Gibco-BRL (Paisley, UK). Rabbit polyclonal antibodies against PKC βII, PKC δ, PKC ε were from Santa Cruz biotechnology (Santa Cruz, CA). Tween 20 was from Biorad

France (Ivry-sur-seine, France). The Bichrotinic Acid protein assay kit and Supersignal and Supersignal ultra enhanced chemiluminescent substrate were obtained from Pierce (Rockford, IL). Nitrocellulose membranes (0.45 μm pore size) were from Schleicher and Schuler (Dassel, Germany). Anti-CD14, -CD19, and IgG2 antibody coated magnetic beads were from Dynal France (Compiegne, France). Anti-CD11b-PE, -CD11c-PE, -CD13-FITC, -CD16-FITC, -CD64-FITC, -CD71-FITC, -CD56 antibody was obtained from Immunotech (Marseille, France). Genosys blocking buffer was from Genosys (Pampisford, UK). Peroxidase couple goat anti-mouse and goat anti-rabbit polyclonal antibodies were from Jackson (West Grove, PA).

Cell Culture

The human leukemic myelomonocytic cell line U937 [Sundström and Nilsson, 1976] and peripheral human blood T-cells were used for this investigation. U937 cells were obtained from the American Cell Type Collection and maintained in a 37°C/5% CO_2 incubator at the Life Science Support Facility (LSSF), Kennedy Space Center prior to the Space Shuttle mission. Cultures were maintained in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% (v/v) penicillin-streptomycin-neomycin antibiotic mix. Medium was changed every 2 days, with cell concentration adjusted to 5×10^5 cells/ml at each medium change.

Human T-cells were purified from fresh peripheral blood of healthy volunteers. Approximately 150 ml of whole peripheral blood was subject to a 20 min centrifugation at 400*g* on a ficoll gradient to separate leukocytes from erythrocytes. After separation, the leukocyte fraction was washed twice in RPMI-1640 medium with supplements before being incubated with Dynal magnetic beads coated with anti-CD14, -CD19, and -CD56 antibodies for 2×30 min at 4°C to eliminate monocytes, B-cells, and natural killer cells. This treatment yielded better than 98% pure human T-cells, as determined by cytofluorimetry. After purification the T-cells were resuspended in medium at 2×10^6 cells/ml.

For the space shuttle experiments, U937 and T-cells were loaded into special GCAK-1 and -2 culture modules (described in detail in [Hatton et al., 1998]) which permit cell culture manipulations in microgravity. Prior to loading into GCAK-1 cassettes, cell cultures were transferred to RPMI-1640 medium supplemented to permit culture in the absence of CO_2: RPMI-1640 at pH 7.4, 25 mM HEPES, 12 mM sodium bicarbonate, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% (v/v) heat inactivated FBS, 1% (v/v) penicillin-streptomycin-neomycin antibiotic mix. For experiments evaluating PKC isoform translocation, cells were cultured in the GCAK-2 modules and stimulated with agonists as indicated in the figures. At the end of the incubation period, samples were fixed with a digitonin-based inhibitor mix (0.5 mg/ml digitonin, 50 mM MOPS, pH 7.2, 5 mM NaF, 5 mM EDTA, 10 mM EGTA, 2 mM sodium ortho-vanadate, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin final concentration with cells). This mixture permeabilizes the cell to release the cytosol and preserve the translocation state of PKC to the particulate fraction of the cell [Hatton et al., 1999]. Samples were maintained at −20°C until post flight analysis. In experiments evaluating surface marker expression, cells were cultured in GCAK-1 cassettes. Samples were fixed inflight with 3.7% paraformaldehyde, washed once with PBS, then maintained at +4°C until postflight analysis.

Space Shuttle Flight

The experiments on PKC translocation and surface receptor expression in U937 and T-cells during microgravity were performed aboard the Space Shuttle *Atlantis* during the STS-81 mission, launched from the Kennedy Space Center on 12 January 1997 for a 10 day mission, which included several days docked to the Russian *Mir* space station. The g-level aboard during the mission was generally better than $10^{-3}g$. Cell cultures in GCAK modules were loaded into the Space Shuttle ~17 h prior to launch and were maintained at ambient temperature (21.4–24.9°C) until 24 h after launch when they were transferred to the ESA Biorack facility which provides a 37°C incubator containing on-board 1*g* centrifuge as well as a glovebox. For each experimental condition, one cassette was placed in the static (microgravity) racks of the incubator while an identical cassette was placed on a centrifuge within the incubator exposing samples to 1*g* (1*g* in flight control). The cassettes containing U937 or T-cell cultures were incubated for 17 h at 37°C until the start of experimental manipulations. Experiment cassettes were transferred briefly to the

Biorack glovebox where a crew member injected activators into the cell culture as described in the results section. Cultures were incubated for 0', 10', or 60' at 37°C in the Biorack incubator. At the end of the incubation period, cassettes were transferred back to the glovebox where the digitonin-based inhibitor mix (described above) was added to the cell cultures, before the samples were transferred to −20°C. A near identical experiment was performed in parallel on the ground at the NASA LSSF, delayed 2 h with respect to the flight experiment, with static rack (1*g* ground) and centrifuge (1.4*g*) samples.

Cells for cytofluorimetric analysis were incubated for 17 h after launch at ambient temperature, then fixed with paraformaldehyde as described above. An identical experiment was performed on the ground at the NASA LSSF, delayed 2 h with respect to the flight experiment.

Western Blot

Samples stored at −20°C were unfrozen and vortexed followed by centrifugation at 14,000*g* for 30 min at 4°C. The supernatant which contained the cytosolic fraction of the cells as well as culture medium was transferred to a separate tube. The pellet, containing the particulate fraction of the cell, was washed once in MOPS buffer with inhibitors (50 mM MOPS, pH 7.2, 5 mM NaF, 5 mM EDTA, 10 mM EGTA, 2 mM sodium ortho-vanadate, 10 mM sodium pyrophosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin final concentration with cells), then resuspended in 250 μl of buffer. The particulate fraction was sonicated twice for 5 s on ice, followed by addition of 20% SDS to give a final concentration of 1% w/v. A small aliquot of particulate fraction was taken for determination of protein concentration using a commercial bichrotinic acid assay kit (Pierce, Rockford, IL). Reducing buffer (5 × concentration; 10% SDS, 25% β-mercaptoethanol, 25% glycerol) was added to particulate fraction samples, which were then heated at 70°C for 5 min.

Equal quantities of protein from each sample were loaded on a SDS–PAGE minigel, migrated, then transferred onto nitrocellulose membranes (Miniprotein electrophoresis kit, Biorad, Ivry-sur-seine, France). Reference samples of constant PKC concentration were run on the same gel to act as a standard for band intensity quantification. Membranes were blocked for 1 h in 10% Genosys blocking buffer, 0.05% Tween 20 in Tris-buffered saline (TBS; 16 mM Tris-HCl, 3 mM Tris, 150 mM NaCl, pH 7.5) followed by 2 × 5 min washes in 0.5% Tween 20/TBS before incubation with the primary antibody. Rabbit polyclonal antibodies directed against PKC βII, PKC δ, PKC ε were used at between 0.1 and 0.25 μg/ml concentrations. Membranes were incubated with primary antibodies for 1.5 h in 4% blocking buffer, 0.05% Tween 20 in TBS, washed three times in 0.5% Tween 20/TBS, then incubated for 1.5 h with peroxidase coupled goat anti-rabbit antibodies, in the same buffer used for the primary antibodies. The membranes were then washed in 0.5% Tween 20 before ECL detection by Supersignal or Supersignal ultra reagents (Pierce, Rockford, IL) according to the manufacturer's instructions. The intensity of the bands recorded on film was quantified by a computer image analysis program (Biocom image analysis system, Les Ulis, France).

Cytofluorimetry

Samples previously fixed and washed as described above were centrifuged at 400*g* for 5 min then resuspended in 500 μl of PBS. Each sample was split into five separate aliquots for labeling. Five μl of FITC or PE directly labeled antibodies (CD11b, CD11c, CD16, CD25, CD64, CD71) were incubated with 100 μl aliquots of sample for 30 min, followed by 400*g* centrifugation and resuspension of the sample in 250 μl of PBS. Five μl of unlabelled antibodies (CD13, CD71) were incubated for 30 min with sample, followed by 400*g* centrifugation. The sample was then resuspended in 100 μl of buffer to which 1 μl of FITC labeled secondary antibody was added. The sample was incubated for 30 min then centrifuged at 400*g*, followed by resuspension in 250 μl of PBS. Labeled samples were analyzed using a Becton Dickinson FACScan.

RESULTS

We have previously reported that the intracellular distribution and translocation of phorbol ester binding PKC is sensitive to microgravity. In standard 1*g* laboratory conditions phorbol ester treatment of U937 cells induced a rapid translocation of PKC βI, βII, and ε from the cytosol to particulate fraction of the cell (Fig. 3). Little or no PKC βI, βII, or ε was associated with the particulate fraction of unstimulated cells, but within 5 min of stimulation with 100 ng/ml

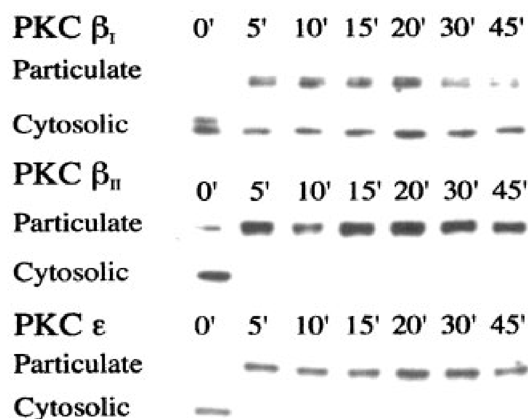

Fig. 3. PKC βI, βII, and ε translocation to the particulate fraction of U937 cells following stimulation with the phorbol ester PDBu: U937 cells were stimulated with 100 ng/ml PDBu and a digitonin-based inhibitor mix (0.5 mg/ml digitonin, 50 mM MOPS, pH 7.2, 5 mM NaF, 5 mM EDTA, 10 mM EGTA, 2 mM sodium ortho-vanadate, 10 mM sodium pyrophosphate, 10 µg/ml leupeptin, 10 µg/ml aprotinin) added to samples at the times indicated to fix the translocation state of PKC isoforms. Samples were fractionated into particulate and cytosolic fractions, followed by Western blotting of PKC βI, βII, and ε as indicated in the text.

PDBu a large fraction of PKC βI and all of PKC βII and ε were translocated to the particulate fraction.

U937 and T-cells were exposed to microgravity during a space shuttle flight and stimulated with phorbol esters. Post flight the intracellular distribution of PKC isoforms was assessed by Western blot. In unstimulated U937 cells, neither PKC βII nor ε were detectable in the particulate fraction at any g-level. In contrast, PKC δ was present in the particulate fraction at all g-levels in unstimulated U937 cells (Fig. 4a). Stimulation of the cells with phorbol ester for 10 min induced the translocation of PKC βII and ε to the particulate fraction at all g-levels (Fig. 4b). For both isoforms, the level of translocation was lowest in the microgravity and 1*g* flight samples and highest in the 1.4*g* ground sample. Likewise, quantity of particulate PKC δ increased with g-level, being lowest in the microgravity sample and highest in the 1.4*g* sample. However, in contrast to the other isoforms, PKC δ showed a net decrease in the particulate fraction of the microgravity sample following phorbol ester stimulation (Fig. 5). After 10 min of PDBu stimulation, the quantity of PKC δ in the 1*g* flight samples did not change significantly from that in unstimulated cells, while the ground 1 and 1.4*g* samples showed a net increase. By 60 min after PDBu stimulation, no

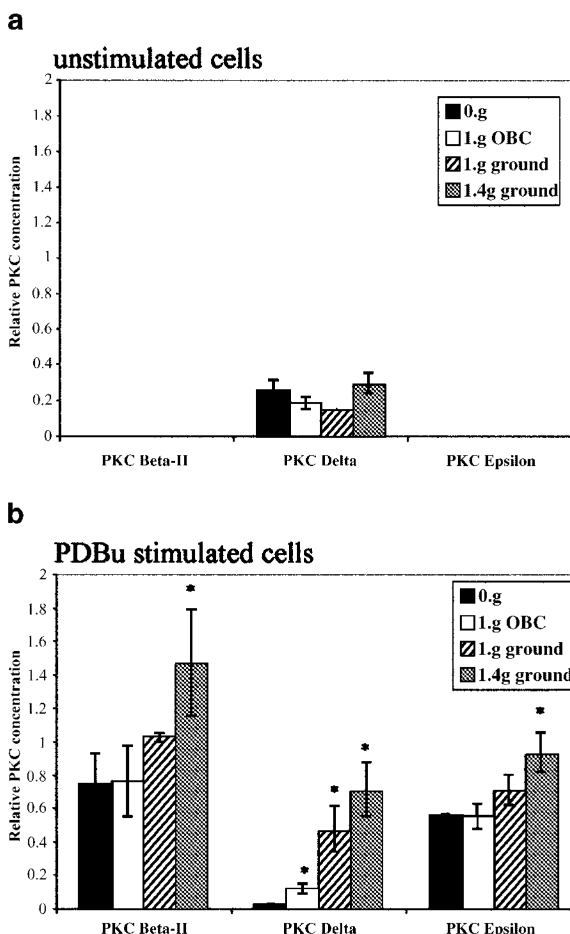

Fig. 4. PKC isoform translocation in U937 cells during the STS-81 Space Shuttle mission: U937 cells were cultured in the Biorack facility for 17 h at 37°C in either microgravity (0*g*) or on a 1*g* centrifuge (1*g* OBC), while identical samples were maintained at 1*g* on the ground (1*g* ground) or 1.4*g* in a centrifuge (1.4*g* ground) in the Biorack ground model. U937 cells were stimulated with 100 ng/ml PDBu and samples fixed at 0', 10', and 60' after the start of stimulation. The relative quantity of each PKC isoform in the particulate fraction of the cell was quantified postflight by densitometry of Western blot images. Neither PKC βII nor ε was detectable in the particulate fraction at any g-level prior to phorbol ester stimulation (**a**), but within 10' of stimulation a significant translocation to the particulate fraction was detectable at all g-levels (**b**). By 60' after stimulation, when PKC signaling is being downregulated, no significant differences were detectable between g-levels (data not shown). For each sample, n = 3 ± standard deviation of the mean. Samples which were statistically different at the $P < 0.05$ level from the microgravity sample are indicated on the graph by an asterix (*Students *t*-test).

significant differences in particulate PKC βII, δ, and ε were detectable between g-levels.

T-cells were treated with either PDBu/Ionomycin, anti-CD3 beads, or medium (unstimulated control) for 60 min. PDBu/Ionomycin is a potent PKC activator, while anti-CD3 beads

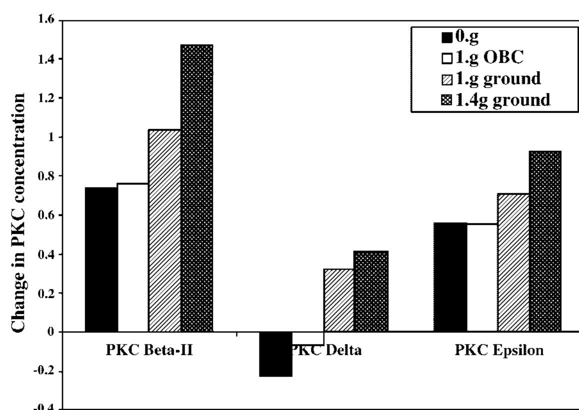

Fig. 5. Relative change in PKC isoforms in the particulate fraction of U937 cells following activation: The net increase or decrease in each PKC isoform going from the unstimulated cell to 10′ stimulated cell for each g-level is indicated. PKC δ shows a net decrease in quantity in the microgravity sample, going from unstimulated to 10′ stimulated, while all other isoforms show an increase. This difference in the pattern of PKC isoform translocation could result in important differences in gene expression at different g-levels.

simulate activation of the T-cell by an antigen presenting cell. Both PKC βII and δ were detectable in the particulate fraction of unstimulated T-cells at all g-levels (Fig. 6A,C). PDBu/I stimulated a significant increase in particulate PKC βII and δ compared to unstimulated cells at all g-levels (Fig. 6A). Anti-CD3 bead stimulated cells showed comparable or lower levels of particulate PKC to unstimulated cells (Fig. 6C,F). This is likely because the 60 min timepoint is after the peak in PKC translocation. No statistically significant differences in particulate PKC βII were detectable between any of the g-levels for each activation condition. However, the data suggests a trend of increasing particulate PKC βII with increasing g-level for PDBu/I stimulation, but decreasing particulate PKC βII with increasing g-level for unstimulated and anti-CD3 stimulated cells. In contrast, particulate PKC δ levels were significantly higher in the 1*g* samples than microgravity samples for unstimulated and PDBu/I stimulated samples. No significant difference in particulate PKC δ could be detected between g-levels for anti-CD3 bead stimulated cells, although the data suggests an increase in this isoform in the particulate fraction of the cell with increasing g-level. Taken together, these data indicate that microgravity has a differential effect on PKC βII and δ isoform activation in T-cells.

Expression of selected surface activation in markers in unstimulated U937 cells exposed to 17 h of microgravity was compared to markers in 1*g* ground cells by cytofluorimetry. The relative abundance of CD 11b (aL subunit of integrin LFA-1, inducible expression), CD11c (aM subunit of integrin CR3, inducible expression), CD13 (aminopeptidase N, myeloid cell marker), CD16 (FcγRIII), CD64 (FcγRI receptor, inducible expression), CD71 (Transferrin receptor) were assessed. A significant upregulation of CD11b, CD11c, CD13, and CD64 was observed in microgravity (Table I). No significant changes were observed for CD16 and CD71. These data suggest that microgravity may induce a weak activation of the unstimulated cell. However, the level of upregulation is small in comparison to that which occurs when U937 cells are treated with differentiation inducers such as γ-interferon (Fig. 7).

DISCUSSION

PKC isoforms play crucial roles in monocyte differentiation and T-cell activation. Translocation of PKC isoforms to the plasma membrane and other cellular compartments is a key step in signal transduction by this family of kinases, as experiments inhibiting the translocation of individual isoforms have shown [Mochly-Rosen and Kauvar, 2000]. Our data shows that PKC isoform translocation is generally reduced in microgravity, suggesting microgravity impairs PKC translocation and thus signal transduction through these kinases. Furthermore, the pattern of PKC isoform translocation varies with g-level. The isoforms we examined in the space shuttle experiment, play key roles in U937 cell differentiation, while those in T-cells are believed to play important roles in cell activation following stimulation of the T-cell receptor. PKC βII activation plays a critical role in U937 differentiation including CD11b vesicle translocation to the cell surface [Kiley and Parker, 1997], MAP kinase activation [Kaneki et al., 1999], and early gene expression [Hass et al., 1993]. In T-cells, PKC β appears to regulate the secretion of IL-2 [Long et al., 2001], probably through a regulation of the microtubule organizing center (MTOC), which PKC β associates and is also necessary for regulation of cell polarization during locomotion following stimulation of LFA-1. Therefore, it is possible that the changes, we observed in PKC translocation, could have important consequences for the responiveness of these cell types to mitogens.

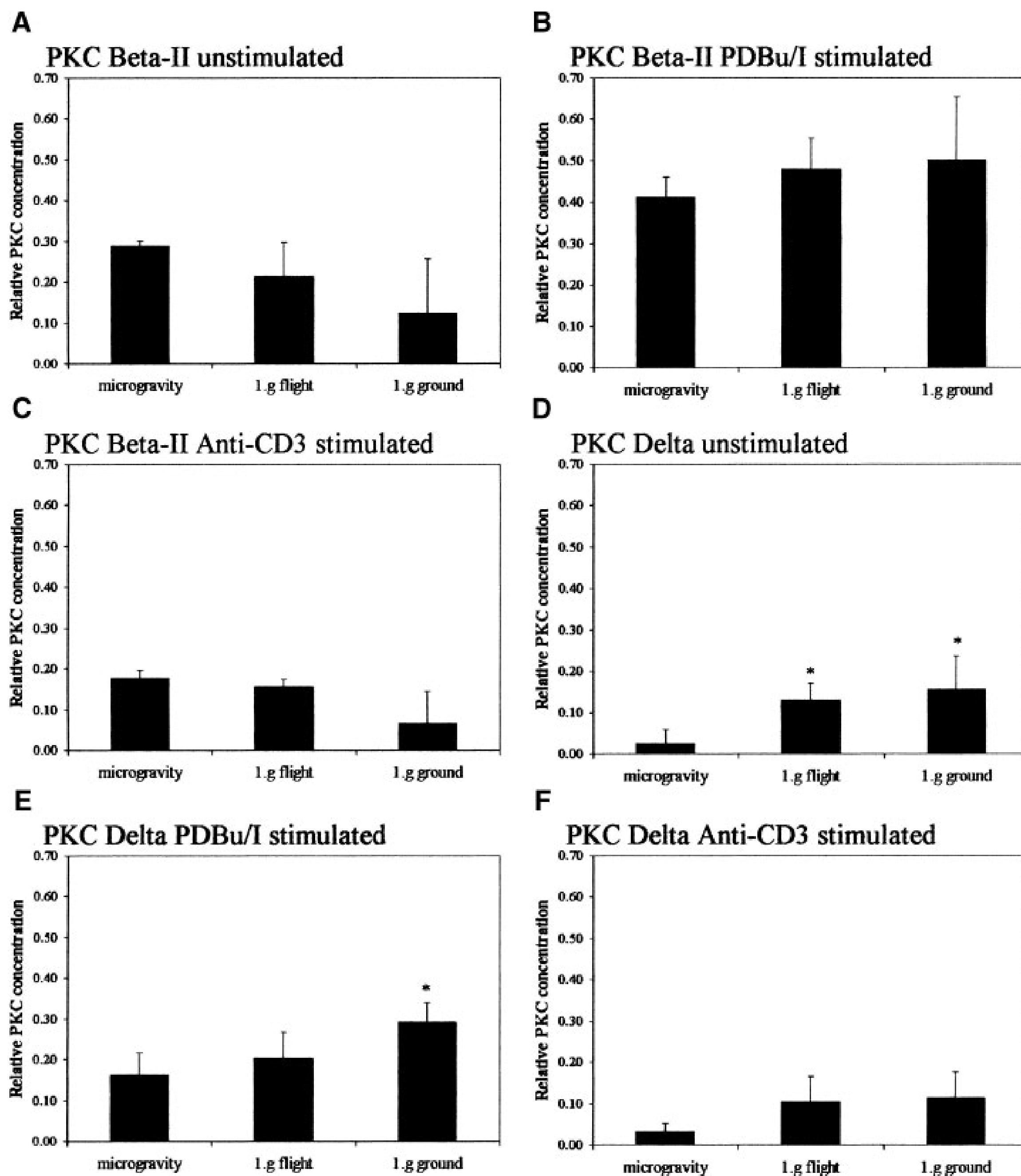

Fig. 6. PKC isoform translocation in human peripheral blood T-cells during the STS-81 Space Shuttle mission: T-cells were cultured in the Biorack facility for 17 h at 37°C in either microgravity (0*g*) or on a 1*g* centrifuge (1*g* OBC), while identical samples were maintained at 1*g* on the ground (1*g* ground) or 1.4*g* in a centrifuge (1.4*g* ground) in the Biorack ground model. T-cells were stimulated with either medium alone (unstimulated control), 100 ng/ml PDBu/0.4 μM Ionomycin or anti-CD3 coated beads (2.5×10^7 beads/ml) and samples fixed at 60′ after the start of stimulation. The relative quantity of each PKC isoform in the particulate fraction of the cell was quantified postflight by densitometry of Western blot images. For each sample n = 4 ± standard deviation of the mean. Samples which were statistically different at the $P < 0.05$ level from the microgravity sample are indicated on the graph by an asterix (*Students *t*-test).

In T-cells, other investigators have found functional changes in microgravity, which could be a result of a perturbation of PKC signaling. IL-2 synthesis and secretion by T-cells stimulated by mitogen is inhibited [Pippia et al., 1996] a processes which is believed to be regulated by PKC θ and PKC β [Altman et al., 2000, Long et al., 2001]. The polarization of the microtubule

TABLE I. Expression of Selected Surface Markers of U937 Cells in Spaceflight and Ground Cultures During the STS-81 Space Shuttle Mission Measured by Cytofluorimetry

Marker	CD11b*	CD11c*	CD13*	CD16	CD64*	CD71
R.F.U. flight	29.4 ± 1.4	29.5 ± 0.4	61.4 ± 7.1	37.9 ± 1.5	34.4 ± 1.1	116.0 ± 6.4
R.F.U. ground	25.6 ± 0.4	24.7 ± 0.2	47.2 ± 2.2	35.9 ± 0.4	29.4 ± 0.2	114.0 ± 1.5

For each condition n = 3 ± standard deviation of the mean. Differences which are significant at the $P < 0.05$ level as determined by *t*-test are indicated by an asterix (*).
R.F.U., mean of relative fluorescent units measured from a fluorescence histogram of cytofluorimetric fluorescence measurements.

organizing center (MTOC) in the T-cell towards the site of contact with antigen presenting cell (APC) is necessary for cytokine containing vesicles to be targeted to the APC [Kupfer and Dennert, 1984]. The localization of the MTOC in human peripheral blood T-cells stimulated with anti-CD3 bead, which simulate the APC, was examined in microgravity under very similar conditions to those used in our experiment (same experiment hardware and activation conditions). Polarization of the MTOC towards the anti-CD3 beads was found to be completely inhibited in microgravity, while in the 1*g* flight samples polarization was found to be normal [Hashemi et al., 1998]. Both PKC β and PKC θ are believed to play roles in the regulation of MTOC organization [Monks et al., 1997; Volkov et al., 2001].

PKC δ is implicated in the transduction of pro-apoptotic signals in both U937 and T-cells. Generally, PKC activation appears to be anti-

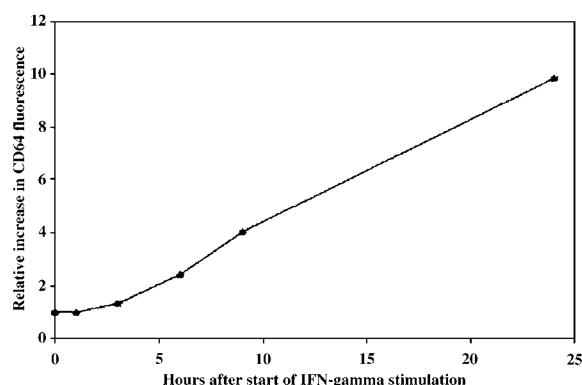

Fig. 7. Timecourse of Fc-gamma-RI (CD64) expression in U937 cells treated with γ-interferon: U937 cells were treated with 200 U/ml of γ-interferon to induce differentiation into a macrophage phenotype. Cells were incubated at 37°C/5% CO_2 and fixed at different time intervals. Fc-gamma-RI expression was assessed by use of a fluorescently labeled anti-CD64 antibody in cytofluorimetry. Each datapoint is the mean of a duplicate. Fluorescence is expressed relative to unstimulated cells (RFU = 1). Note how the CD64 receptor is significantly upregulated within 6 h of IFN-γ treatment.

apoptotic [Kaneko et al., 1999], while apoptosis promoting agents often induce cleavage of PKC isoforms at the hinge region, to yield a constitutively active PKM fragment [Mizuno et al., 1997]. In T-cells, PKC δ translocates to the nucleus during apoptosis, a translocation which can be reversed by anti-apoptic factors such as IFN-β [Scheel-Toellner et al., 1999]. In U937 cells, pro-apoptotic agents such as TNF-α induces PKC δ translocation to the mitochondria, which mediates cytochrome c release from mitochondria, an important step in apoptosis [Majumder et al., 2000]. During apoptosis, caspase 3 is activated which in turn cleaves PKC δ to a PKM fragment [Koriyama et al., 1999]. In contrast, PKC ε activation blocks TNF-α induced apoptosis in U937 cells [Mayne and Murray, 1998]. Therefore, the apparent reversal of PKC δ translocation, from particulate to cytosolic fraction, in U937 cells under microgravity (Fig. 5) as well as the significant differences in PKC δ and ε isoforms present in the cell particulate fraction could result in either the induction or suppression of apoptosis. Unfortunately, the analysis methods used for the spaceflight experiment do not allow us to differentiate between translocation to specific cellular compartments such as the mitochondria or nucleus, nor the presence of PKM fragments. However, evidence that apoptosis is increased in microgravity has been provided by Lewis et al. [Cubano and Lewis, 2000] who reported that Jurkat cells (a T-cell line) exposed to microgravity in three different spaceflights have higher levels of apoptosis and FAS-L expression than 1*g* cultured cells. This report is particularly interesting since PKC activation and FAS mediated apoptosis are antagonistic processes [Mizuno et al., 1997].

The translocation of some PKC isoforms in microgravity is similar to that of the 1*g* in-flight control, but significantly different to the 1*g* control (Fig. 4), while in other cases the response of the 1*g* flight sample is intermediate

between that of the microgravity and 1*g* controls (Fig. 6). We observed a similar phenomena in our previous spaceflight experiments [Schmitt et al., 1996, Hatton et al., 1999]. The most likely explanation for this discrepancy between flight and ground 1*g* controls is that all flight samples were exposed to microgravity after launch until loading into Biorack at least 24 h later, when the 1*g* flight samples were placed on the centrifuge. During this time, it is possible that the cells were sensitive to microgravity and that subsequent culture at 1*g* did not reverse changes which may have occurred in the cell. Additionally, it was necessary to remove the experiment cassettes from the 1*g* centrifuge for manipulations, hence the cell cultures were exposed to microgravity for a brief period immediately before and after addition of activator.

The mechanisms by which microgravity affects PKC isoform translocation is unknown. However, the fact that individual isoforms show distinct differences in sensitivity which may vary depending on the activation conditions provides some clue to the mechanism. The U937 and T-cells in our experiments were exposed to microgravity for an extended period prior to stimulation, hence modifications in cell architecture may have occurred. Although PKC isoforms translocation is diffusion limited [Schaefer et al., 2001], changes in expression of RACK's or other PKC binding sites could affect PKC translocation. Many PKC isoforms associate with cytoskeleton, both prior to and after activation [Keenan and Kelleher, 1998]. Since cytoskeleton organization has been reported to be modified in both immune and bone cells exposed to microgravity [Hughes-Fulford and Lewis, 1996; Lewis et al., 1998], it is possible that changes in cytoskeleton organizations could cause modifications to PKC translocation. In turn, many cytoskeletal proteins are PKC substrates, so inhibited PKC signaling could result in altered cytoskeleton reorganization following cell stimulation. The recent observation by Tabony's group [Papaseit et al., 2000] that self organization of microtubule polymerization in vitro is drastically reduced in microgravity provides a potential mechanism by which gravity, or absence of gravity, could be perceived directly by the cell. Altered microtubule architecture and microtubule disruption under microgravity conditions has been observed in several different cell types including Jurkat, HL-60 and human breast cancer cells [Piepmeier et al., 1997; Lewis et al., 1998; Vassy et al., 2001]. Microtubule disruption is believed to activate several different signaling pathways, including PKC, which induce apoptosis [Wang et al., 1999]. Microtubule disruption and mitochondrial abnormalities occurred in Jurkat cells exposed to microgravity with these events preceding apoptosis [Schatten et al., 2001]. Therefore, it is possible that modifications in the microtubule cytoskeleton induced by microgravity could be responsible for changes in PKC translocation that we obsesrved. However, further experiments will be necessary to determine how the changes in cytoskeleton, signal transduction and apoptosis that have been observed in microgravity are related. Ultimately, information from these experiments will help in identifying and understanding the underlying mechanisms by which mammalian cells perceive gravity.

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