Modeled microgravity-induced protein kinase C isoform expression in human lymphocytes

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Sundaresan, A., D. Risin, and N. R. Pellis. Modeled microgravity-induced protein kinase C isoform expression in human lymphocytes. J Appl Physiol 96: 2028–2033, 2004. First published February 13, 2004; 10.1152/japplphysiol.01248.2003.—In long-term space travel, the crew is exposed to microgravity and radiation that invoke potential hazards to the immune system. T cell activation is a critical step in the immune response. Receptor-mediated signaling is inhibited in both microgravity and modeled microgravity (MMG) as reflected by diminished DNA synthesis in peripheral blood lymphocytes and their locomotion through gelled type I collagen. Direct activation of protein kinase C (PKC) bypassing cell surface events using the phorbol ester PMA rescues MMG-inhibited lymphocyte activation and locomotion, whereas the calcium ionophore ionomycin had no rescue effect. Thus calcium-independent PKC isoforms may be affected in MMG-induced locomotion inhibition and rescue. Both calcium-dependent isoforms and calcium-independent PKC isoforms were investigated to assess their expression in lymphocytes in 1 g and MMG culture. Human lymphocytes were cultured and harvested at 24, 48, 72, and 96 h, and serial samples were assessed for locomotion by using type I collagen and expression of PKC isoforms. Expression of PKC-α, -δ, and -ε was assessed by RT-PCR, flow cytometry, and immunoblotting. Results indicated that PKC isoforms δ and ε were downregulated by >50% at the transcriptional and translational levels in MMG-cultured lymphocytes compared with 1-g controls. Events upstream of PKC, such as phosphorylation of phospholipase Cγ in MMG, revealed accumulation of inactive enzyme. Depressed calcium-independent PKC isoforms may be a consequence of an upstream lesion in the signal transduction pathway. The differential response among calcium-dependent and calcium-independent isoforms may actually result from MMG intrusion events earlier than PKC, but after ligand-receptor interaction.

Lymphocyte locomotion is integral to the immune response and is adversely affected in microgravity and modeled microgravity (MMG) (8). Wound healing, migration to antigenic sites, and phagocytosis require cellular locomotion. Activation of lymphocytes before culture in MMG restores locomotion (2). Activation redistributes cell surface molecules and alters cytokine production; the cytoskeleton reorganizes, followed by changes in gene expression (13). PKC is central in T cell activation-triggered pathways and in turn leads to other transcriptional and translational changes occurring in the cell. It was hypothesized from previous experiments that MMG induces a lesion either at or upstream of PKC. To test this hypothesis, lymphocytes were treated with phorbol myristate acetate (PMA), which directly activates PKC, bypassing the second messenger diacylglycerol (DAG). The cells were then assessed for locomotion in type I collagen gels. The locomotion inhibition observed under microgravity culture conditions was reversed by up to 87% by prior activation with PMA. However, the incorporation of ionomycin was not synergistic with PMA (10). Thus it is possible that the calcium-independent, and not the calcium-dependent, isoforms of PKC primarily may be necessary for locomotion.

PKC is a family of 12 isoforms divided into three groups. The calcium-dependent α, β1, β2, and γ isozymes are dependent on calcium, DAG, and phosphatidylycerine for activation. Furthermore, they are sensitive to phorbol esters. The second group consists of novel isoforms, such as ε, δ, η, and μ, and are not dependent on calcium but require DAG and phosphatidylycerine for activation and respond to phorbol esters. The third group is atypical and consists of the ζ and λ/θ isozymes, which are calcium and DAG independent and they are insensitive to phorbol esters (7). In human T cells, α, β, γ, ε, δ, and θ are expressed at both the mRNA and protein levels (1, 4).

The objective of this investigation is to assess the expression of key calcium-dependent and -independent isoforms in MMG and to determine whether the isoform deployment is a consequence of an upstream lesion in the signal pathway. It is less likely that MMG affects ligand-receptor interactions (2), but any stage in the activation sequence through PKC may be affected and observed as decreased proliferation and locomotion in MMG. The investigation presented herein documents the PKC isoform profiles in human lymphocytes and suggests that the peculiar isoform profiles are likely due to upstream changes induced by MMG.

MATERIALS AND METHODS

MMG culture. To model some aspects of microgravity, a specialized rotating-wall vessel culture system (RWV) developed at the National Aeronautics and Space Administration-Johnson Space Center and commercially available from Synthecon (Friendswood, TX) was utilized. The RWV bioreactor (Synthecon, Houston, TX) consists of a cylindrical culture vessel (with zero headspace and a silicon membrane for direct gas exchange with the culture medium) that is rotated along a horizontal axis on a base that also pumps air over the silicon-O2 exchange membrane.

This very-low-shear culture system provides two conditions that model microgravity: 1) randomization of cell’s orientation to the gravity vector and 2) sustaining of cells in continuous free fall at terminal velocity through the culture medium. This culture system

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was used previously to profile the potential effects of microgravity on peripheral blood mononuclear cell (PBMC) locomotion performed during shuttle missions STS-54 and STS-56. Controls were cultured in stationary conditions in plastic tissue culture flasks. All cultures were maintained at 37°C in atmosphere of 95% air-5% CO₂.

RT-PCR for the detection and quantitation of PKC isoforms from human PBMC cultured in 1 g and in MMG (RWV culture). Human PBMCs were obtained as auffy coat from the Gulf Coast Regional Blood Bank (Houston, TX). Cells were resuspended in HBSS, layered onto a Ficoll-paque gradient, and then centrifuged at 2,100 rpm at room temperature for 20 min. The mononuclear cell layer was isolated from each tube, washed with HBSS three times, pelleted, and resuspended in RPMI-1640 supplemented with 10% FBS. Lymphocytes were counted and adjusted to 1 x 10⁶/ml. Then they were cultured in a T75 flask and in an RWV (MMG) for up to 96 h. Cells were sampled from the T flask and the RWV at 24, 48, 72, and 96 h. The cells were harvested, and total RNA was extracted by use of TRI Reagent. After isopropanol precipitation and ethanol wash, the RNA was quantitated on the basis of A₂₆₀/₂₈₀. A 300-ng sample was used for each quantitative RT-PCR reaction. RNA preparations with 260-to-280 nm ratios higher than 1.8 were used for experiments. The RNA PCR kit (Perkin-Elmer, Wellesley, MA) was modified on the basis of primer choice. The reverse transcription phase of the reaction was performed first, followed by polymerase chain reaction (PCR) cycles. When primers were available as a combination, as for GAPDH, then first on all samples, followed by polymerase phase for 35 cycles.

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Western blotting. SDS gel electrophoresis was performed by standard methods. Antibodies to different PKC isoforms were purchased from BD Transduction Laboratories. PBMCs were adjusted to 1–2 x 10⁶ cells per sample and solubilized in SDS sample buffer. The soluble protein was electrophoresed in a 10% SDS-PAGE gel. Then the proteins were transferred to Immobilon membrane and blocked in 5% nonfat milk, 50 mM Tris, 0.9% NaCl, 0.05% Tween 20, 0.02% NaN₃, pH 7.8. The blots were incubated with the primary antibody at the appropriate dilution (1–2 μg/ml) in Blotto solution at 37°C for 3 h. Blots were washed 3 times 5 min each in Blotto without nonfat milk. Then incubation was performed with the secondary antibody in Blotto at 37°C for 1 h, and unbound antibody was removed. Luminescence solution (Amersham Scientific, Arlington Heights, IL) was added, and blots were photographically developed.

Immunoprecipitation. PBMCs were prepared according to previously described methods (10). Cells were cultured at 1.5 x 10⁶ cells/ml in both 1-g and MMG conditions with an RWV bioreactor as described above. A total of 50 x 10⁶ cells were obtained at each time point and were lysed with boiling denaturing lysis buffer (1% SDS, 10 mM Tris, pH 7.4). Five hundred microliters of lysis buffer were added to each sample, and the resulting lysate was boiled for an additional 5 min. The lysate was then passed through a 26-gauge needle several times and centrifuged at 16,000 g for 5 min at 4°C to obtain the soluble fraction. To each sample the following were added: 400 μl water, 100 μl cell lysate (prepared as described above), 1–5 μg of primary antibody (PLC-γ1, Transduction Laboratories, Lexington, KY), 500 μl 2% immunoprecipitation buffer (100 mM PMSF, 100 mM NaCl, 20 mM Tris pH 7.4, 1% Triton X-100, 0.5% NP 40). Samples were vortexed and mixed in a rocking platform overnight at 4°C. When the primary antibody was a mouse monoclonal, then 5 μg of rabbit anti-mouse antibody was incubated with the sample after the first incubation for 1–2 h at 4°C. Ten microliters of protein G-agarose (Sigma Chemical) were added to the mixture, and incubation continued for 30 min at 4°C. The sample was centrifuged at 16,000 g for 5 min at 4°C. The supernatant from this centrifugation was harvested; the pellet was washed three times with 1 x immunoprecipitation buffer and then resuspended in 30 μl of 2x concentrated electrophoresis sample buffer (250 mM Tris, pH 6.8, 4% SDS, 10% glycerol, 0.006% bromphenol blue, 2% β-mercaptoethanol). The sample was boiled for 5 min and centrifuged at 16,000 g at 4°C for 5 min. The supernatants from the above were loaded onto an SDS-PAGE gel and electrophoresed. The proteins were then transferred to nitrocellulose or polyvinylidene difluoride membranes and probed with appropriate antibodies. PLC-γ1 was immunoprecipitated and immunoblotted, whereas the PKC isoforms were detected by immunoblotting alone.

Immunodetection of the intracellular proteins by flow cytometry. PBMCs were washed with PBS, permeabilized, and fixed by using IntraPrep permeabilization reagent (Immunotech, Coulter, Miami, FL) according to the manufacturer’s protocol. Next, the cells were stained with appropriate specific and control MAb. Indirect staining was performed using unconjugated mouse anti-human MAb followed by an additional 30-min incubation with FITC-conjugated goat antimouse secondary antibody. Control for indirect staining included incubation with FITC-conjugated secondary antibody alone. Stained cells were washed twice in cold HBSS with 2% FBS. All incubations were at 4°C. Antibodies were used as suggested by the manufacturer. Stained cells were analyzed by flow cytometry (EPICS XL; Coulter). Percentage of positive cells and mean channel fluorescence were calculated by subtracting the appropriate control histograms from the test histograms via a cumulative subtraction routine (Oversub) within the Elite Immuno-4 software (Coulter, Hialeah, FL).

Scanning laser confocal microscopy. The protocol in the previous section was used to stain cells for scanning laser confocal microscopy. Cells were covered with Elvanol (an anti-fade), and the samples were scanned with an Applied Precision DeltaVision scanning fluorescence microscope (Issaquah, WA), fitted with an Olympus IX70 microscope (Olympus America, Melville, NY) with deconvolution capabilities. The confocal instrumentation exists as one unit. A 100-W mercury arc lamp was used for illumination, and excitation and emission wavelengths were produced by filters specific for each probe (Chroma Technology, Brattleboro, VT). The filter set combination for the low-emission probe was a 490-nm excitation filter with a band-pass width of 20 nm and an emission filter of 518 nm with a band pass of 38 nm. Filter combination for the high-emission probe was a 555-nm excitation filter with a band pass of 20 nm and emission filter of 617 nm with a 73-nm band-pass width. Image scans for each emission probe were acquired in series at a step-size thickness of 0.2 μm by a Photometrics (Tucson, AZ) PXL CCD camera. At least 30 sections were scanned per sample for each probe (i.e., yielding 60 total images when 2 probes were used).

Image analysis was performed by transferring each data set to a Silicon Graphics workstation for deconvolution using SoftWoRx software from Applied Precision using an algorithm based on the convolution of a point spread function to differentiate and reduce extraneous or scattered light captured by the camera. All data sets were subjected to five deconvolution iterations and then used for image reconstructions and modeling. Baseline subtraction of background fluorescence and change of intensity gain were optimally set for each emission and consistent for each analysis. An image projection is rendered by SoftWoRx software, by stacking each of the individual sections, which produces a three-dimensional image on a two-dimensional screen.

RESULTS

Human PBMCs were isolated from normal human donors and cultured at 1.5 x 10⁶ cells/ml in two culture conditions,
static (1 g) and MMG (RWV). Lymphocyte locomotion was significantly inhibited in MMG, and at 96 h the locomotion was negligible compared with static 1-g cultures (10). Cells from the same cultures were examined for their expression of PKC isoforms. Locomotion inhibition in microgravity analog culture is accompanied by a parallel decrease in the expression of specific calcium-independent PKC isoforms. The Fig. 1 series shows calcium-independent (PKC-δ, ε) isoform protein expression levels by Western blotting with monoclonal antibodies directed against each isoform. PKC-δ protein expression in MMG was negligible through 96 h in MMG lymphocyte cultures (Fig. 1A). At 24 h, there was a significant decrease in PKC-δ expression in MMG lymphocyte cultures. In contrast, PKC-ε displayed evanescent expression in MMG when compared with static 1-g culture (Fig. 1B). All Western blots and RT-PCRs were performed on an experimental n of 3, and the graph shown is an average of 3 experiments with a representative blot image. Paired sample t-tests were performed between groups and time points for all experiments. Values are means ± SE.

![Graphs and images](Figures.png)

Fig. 1. A: peripheral blood mononuclear cells (PBMCs) were obtained from normal human donors and suspended in culture in static (1 g) culture or modeled microgravity (MMG). Cells were sampled at 24, 48, 72, and 96 h from both culture conditions. They were then lysed and run on 10% SDS-PAGE gels, transferred to nitrocellulose, and probed with antibodies to PKC-δ. PKC-δ was not detectable in MMG cultures. All Western blots and RT-PCRs were performed on an experimental n of 3, and the graph shown is an average of 3 experiments with a representative blot image. Paired sample t-tests were performed between groups and time points for all experiments. Values are means ± SE. B: PKC-ε protein expression levels in lymphocytes in MMG revealed a significant decrease of this isoform (57%, P < 0.001) even at 24 h in MMG compared with 1-g. This decrease was evident even at 72 h, followed by a very negligible amount detected at 96 h. All Western blots and RT-PCRs were performed on an experimental n of 3, and the graph shown is an average of 3 experiments with a representative blot image. Paired sample t-tests were performed between groups and time points for all experiments. Values are means ± SE. C: GAPDH mRNA levels revealed consistent expression in both 1-g and MMG-cultured lymphocytes. All Western blots and RT-PCRs were performed on an experimental n of 3, and the graph shown is an average of 3 experiments with a representative blot image. Paired sample t-tests were performed between groups and time points for all experiments. Values are means ± SE.
with near-normal expression by 48 h. The expression levels decreased again at 72 h, revealing negligible quantities by 96 h. Results displayed are the average levels of three independent experiments. Protein concentrations used were 100 μg/lane, and equal loading was documented by imaging before blotting. This was used as a parameter of equal loading, because common housekeeping genes, such as β-actin, were altered in RWV culture.

Transcriptional downmodulation of calcium-independent PKC isoforms. RT-PCR was performed on 1-g- and MMG-cultured lymphocyte total RNA. Protein kinase C-δ message was barely discernible at 24 and 48 h in MMG and by 96 h, it was still downregulated by 56% in MMG (Fig. 2A). PKC-ε message showed a similar trend in MMG, with an 83% decrease at 24 and 48 h (Fig. 2B). Oscillatory expression patterns were observed in both of these isoforms. The reduced transcription in MMG was reflected in the quantitative estimates by Western blotting (Fig. 1). Although there was reduced transcript expression of PKC-δ in MMG, protein expression was barely evident. Even though PKC-ε expression was comparable to static cultures at 48 h, mRNA was decreased significantly, indicating that PKC-ε-translated accumulation may initially occur before downregulation of message. GAPDH transcriptional controls are displayed in Fig. 2C. These figures also reflect averages of three independent experiments.

Distribution of PKC isoforms is altered in MMG. In situ PKC isoform expression determined by flow cytometry revealed a decrease in PKC isoform deployment in MMG. The decrease was significant in PKC-ε, less obvious in PKC-δ, and insignificant in PKC-α. There was an oscillatory expression of PKC-ε and -δ with a little recovery in expression of protein at 48 h (Fig. 3A). Furthermore, these isoforms translocate and localize with cytoskeletal components. Confocal microscopic analysis revealed more uniform distribution of PKC-ε in lymphocytes cultured in static conditions compared with cells cultured in MMG, where it was less uniform and patchy (Fig. 3B).


Phosphorylation of PLC-γ1 expression in MMG. PLC-γ1, the primary enzyme for generation of DAG via the hydrolysis of inositol 1,4,5-trisphosphate and phosphatidylinositol 4,5-bisphosphate in T cells, undergoes tyrosine phosphorylation to become the active form. MMG-cultured lymphocytes, even early in culture, showed a 56% decrease in phosphorylated PLC-γ1 compared with 1-g-cultured lymphocytes (Fig. 4). Samples were immunoprecipitated with a PLC-γ1 antibody and detected with a phosphotyrosine secondary antibody.

DISCUSSION

Spontaneous locomotion in human lymphocytes begins with homotypic aggregation. Shortly thereafter, the cells polarize, form podia, and then commence random locomotion. The pathway from cell-cell contact to locomotion involves intracellular signaling pathways and activation of contractile mechanisms within the cell. The experiments reported herein map the changes that occur in intracellular signaling pathways when the gravitational orientation of the cell is randomized by clinostatic rotation. The result in MMG is similar to microgravity when locomotion is assessed in type I collagen (10). Locomotion in both settings ceases and yet is restored by several activation paradigms (8, 10), suggesting a signal transduction lesion rather than an “uncoupling” of the locomotion apparatus per se.

We have shown previously that MMG-induced inhibition of locomotion was restored up to 87% by addition of PMA but not by calcium ionophore. Calcium signaling pathways were presumed intact in MMG. When the role of calcium-independent PKC isoforms predominant in T cells was investigated, PKC-ε was markedly reduced at both the RNA and protein levels, suggesting inhibition at both levels in MMG. The change in PKC-δ was similar to, although not as pronounced, as that in PKC-ε by flow cytometry. However, although PKC-δ was negligible at the protein level by Western blotting at all assay times, at 72 and 96 h mRNA was detectable by RT-PCR and transcriptional inhibition was evident at 24 and 48 h. The low levels of expression of these isoforms coincided with serial locomotory inhibition in lymphocytes (10). PKC activation is one of the earliest events in the signal transduction cascades that lead to several cellular functions, such as cell growth, differentiation, and gene expression. Decreased PKC-ε and -δ expression may lead to loss of appropriate interactions with nuclear and membrane-associated proteins and transcriptional factors, ultimately resulting in functional losses within the cell. PKC-α levels did not change significantly in 1-g- and MMG-cultured lymphocytes (data not shown). However, locomotion was not augmented in MMG, even though PKC-α levels were optimal. This suggests that calcium-dependent PKC isoforms may not participate in the signal pathway for lymphocyte locomotion. Pretranslational changes in PKC-δ and -ε expression levels indicate that MMG hasselective effects on transcription and transcript stability of PKC isoforms, either directly acting on the gene or via upstream effects such as PLC-γ1 activation, resulting in inadequate hydrolysis and DAG formation to activate PKC. Shape changes in cells in spaceflight and MMG may initiate the effect on signaling and transport of PKC. Altered cytoskeleton organizational changes in microgravity may lead to signaling and functional defects in the T cell. PKC-ε is known to bind to actin and to other cytoskeletal components, and its translocation to specific cellular compartments is recognized as critical for cardiomyocyte contraction and other cytoskeletal events. PKC-δ binds and phosphorylates stat 3, regulating important genes in the T cell (5). Entschladen et al. (3) found that in purified T cell populations (CD4+, CD8+), a spontaneously locomoting population of 25% developed once they were incorporated into a three-dimensional collagen matrix. A concurrent increase in tyrosine phosphorylation of focal adhesion kinase was observed. Inhibition of protein tyrosine kinases using genistein significantly decreased spontaneous locomotion, whereas direct activation of PKC using PMA rescued locomotion (3). Investigations with T lymphocytes positively correlate PKC activity with locomotion (11), yet others associate inhibition of PKC with augmented locomotion (12). Also, disruption of microtubules with Colcemid recruited previously nonlocomotory cells and demonstrated an increase in the duration of locomotion (9). This prolocomotory role of Colcemid is due to chemoattractant behavior (9). Activated T lymphocyte locomotion is triggered by leukocyte function-associated antigen-1 and is PKC-δ dependent (9). Our studies reveal a downregulation of calcium-independent PKC-δ and -ε isoforms in MMG. This is consistent with previous results with ionomycin, which did not restore locomotion, suggesting that calcium pathways in lymphocytes cultured in MMG are basically intact. PMA activation of PKC isoforms together with the ineffectiveness of ionomycin indicated involvement of calcium-independent PKC isoforms. However, whether calcium-independent isoforms play a direct role in locomotion inhibition in MMG or an indirect role was not determined by the experiments herein. Inhibition of PLC-γ1 activation indicates potential MMG-induced lesions upstream. Nevertheless, the results show that the downstream effects are partitioned to calcium-independent isoforms of PKC, and the relationship of the PKC-ε isoform to the cytoskeleton may be the pathway to inhibition of locomotion in MMG.

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REFERENCES


