

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

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**Running Title: PKC regulation in modeled microgravity**

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**ABSTRACT**

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 both in microgravity and modeled microgravity (MMG) as reflected in 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 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 1g and MMG-culture. Human lymphocytes were cultured and harvested at 24, 48, 72 and 96 hours and serial samples assessed for locomotion using type I collagen and expression of PKC isoforms. Expression of PKC- $\alpha$ , - $\delta$  and - $\epsilon$  was assessed by RT-PCR, flow cytometry and immunoblotting. Results indicated that PKC isoforms  $\delta$  and  $\epsilon$  were down-regulated by more than 50% at the transcriptional and translational levels in MMG-cultured lymphocytes compared with 1g controls. Events upstream of PKC such as phosphorylation of Phospholipase C $\gamma$  (PLC- $\gamma$ ) in MMG, revealed accumulation of inactive enzyme. Depressed Ca<sup>++</sup>-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, but after ligand-receptor interaction.

**Keywords:** Signal transduction, locomotion, immunity



## INTRODUCTION

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 prior to culture in MMG restores no locomotion (2). Activation redistributes cell surface molecules, alters cytokine production, the cytoskeleton reorganizes and changes gene transcription (13). PKC is central in T cell activation-triggered pathways, and in turn leads to other transcriptional and transnational 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 isozymes divided into three groups. The calcium dependent  $\alpha$ ,  $\beta_1$ ,  $\beta_2$ , and  $\gamma$  isozymes are dependent upon calcium, DAG and phosphatidyl serine (PS) for activation. Furthermore, they are sensitive to phorbol esters. The second group consists of novel isoforms, such as  $\epsilon$ ,  $\delta$ ,  $\theta$ ,  $\eta$ , and  $\mu$ , and are not dependent upon calcium, but require DAG and PS for activation and respond to phorbol esters. The third group is atypical and consists of the  $\zeta$  and  $\lambda/\iota$  isoforms that are calcium and DAG independent and they are insensitive to phorbol esters (7). In human T cells,  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\zeta$ ,  $\epsilon$ ,  $\delta$ , and  $\theta$  are expressed at both the mRNA and protein levels (1, 4).

Since the T cell receptor signaling pathway appears to be a pathway in locomotion, any molecules up-stream of PKC, such as PLC- $\gamma$ , may be the target of changes induced by microgravity. 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

### *Modeled Microgravity (MMG) culture*

*Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for the detection and quantitation of Protein Kinase C (PKC) isoforms from human Peripheral Blood Mononuclear Cells (PBMC) cultured in 1g and in MMG (RWV culture).* Human PBMCs were obtained as a buffy coat from the Gulf Coast Regional Blood Bank (Houston, TX). Cells were resuspended in Hanks' Balanced Salt Solution (HBSS) and layered onto a Ficoll-paque gradient then centrifuged at 2100 rpm at room temperature for 20 minutes. The mononuclear cell layer was isolated from each tube, washed with HBSS three times, pelleted and resuspended in RPMI-1640 supplemented with 10% Fetal Bovine Serum (FBS). Lymphocytes were counted and adjusted to  $1 \times 10^6$ /ml. Then they were cultured in a T75 flask and in an RWV (modeled microgravity) for up to 96 hours. Cells were sampled from the T flask and the RWV at 24, 48, 72 and 96 hours. The cells were harvested and total RNA extracted using tri-reagent. After isopropanol precipitation and ethanol wash, the RNA was quantitated based on  $A_{260/280}$ . A 300 ng sample was used for each quantitative RT-PCR reaction. RNA preparations with 260/280 ratios higher



than 1.8 were used for experiments. The RNA PCR kit (Perkin Elmer, NJ, USA) was modified based on primer choice. The reverse transcription phase of the reaction was performed first on all samples, followed by polymerase phase for 35 cycles. When primers were available as a combination, as for GAPDH, then 2  $\mu$ l of the total primer mix was used. The GAPDH primer set (Stratagene La Jolla, CA) amplified a 358 base pair fragment. The PKC isoform ( $\alpha$ ,  $\delta$ ,  $\epsilon$ ) primers (Oxford Biomedical Research, Inc, Oxford, MI) were manufactured as sets of 3' and 5' primers. One microliter of each was used for each reaction totaling 2  $\mu$ l per reaction. The predicted sizes of the PKC isoform products were as follows: PKC- $\alpha$ : 324 base pairs, PKC- $\delta$ : 351 base pairs, and PKC- $\epsilon$ : 731 base pairs. After thermal cycling, 20  $\mu$ l of each sample was electrophoresed on a 2.5% agarose gel with ethidium bromide at a final concentration of 0.5  $\mu$ g/mL. Isolates were analyzed using the Alpha Innotech semi quantitative imaging system.

*Western Blotting.* SDS gel electrophoresis was performed by standard methods. Antibodies to different PKC isoforms were purchased from, BD Transduction labs, CA. PBMCs were adjusted to  $1-2 \times 10^6$  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 El Blotto (5% non fat milk, 50 mM Tris, 0.9% NaCl, 0.05% Tween 20, 0.02% NaN<sub>3</sub>, pH 7.8). The blots were incubated with the primary antibody at the appropriate dilution (1-2  $\mu$ g/ml) in El Blotto solution at 37° C for 3 hours. Blots were washed 3X five minutes each in El Blotto without non-fat milk. Then incubation was performed with the secondary antibody in el blotto at 37 ° C for 1 hour and unbound antibody 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  $1.5 \times 10^6$  cells/ml in both 1g and MMG conditions using an RWV bioreactor as described above. A total of  $50 \times 10^6$  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 was added to each sample and the resulting lysate was boiled for an additional 5 minutes. The lysate was then passed through a 26-gauge needle several times and centrifuged at 16000g for 5 minutes at 4° C to obtain the soluble fraction. To each sample the following were added: 400  $\mu$ l water, 100  $\mu$ l cell lysate (prepared as described above), 1 to 5  $\mu$ g of primary antibody (PLC- $\gamma$ 1, Transduction Labs, Lexington, KY), 500  $\mu$ l 2X immunoprecipitation buffer (100 mM Phenyl methyl sulfonyl fluoride [PMSF], 100mM Sodium orthovanadate [SOV], 100 mM EDTA, 100 mM EGTA, 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  $\mu$ g of rabbit anti mouse antibody was incubated with the sample after the first incubation for 1-2 hrs at 4° C. Ten microliters of Protein G: agarose (Sigma, USA) was added to the mixture and incubation continued for 30 minutes at 4° C. The sample was centrifuged at 16000g for 5 minutes at 4° C. The supernatant from this centrifugation was harvested; the pellet was washed three times with 1X immunoprecipitation buffer and then resuspended in 30  $\mu$ l of 2X concentrated electrophoresis sample buffer (250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2%  $\beta$ -mercaptoethanol). The sample was boiled for 5 minutes and centrifuged at 16000g at 4° C for 5 minutes. The supernatants from the above were loaded onto an SDS-PAGE gel and electrophoresed. The proteins were then transferred to nitrocellulose or PVDF membranes and probed with appropriate



antibodies. PLC- $\gamma$ 1 was immunoprecipitated, and immunoblotted, while 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<sup>TM</sup> Permeabilization Reagent (Immunotech, a Coulter Co., Miami, FL) according the manufacturer's protocol. Next, the cells were stained using appropriate specific and control mAbs. Indirect staining was performed using unconjugated mouse anti-human mAb followed by an additional 30-minute incubation with FITC-conjugated goat anti-mouse second 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, Miami, FL). Percent of positive cells and mean channel fluorescence were calculated by subtracting the appropriate control histograms from the test histograms using a cumulative subtraction routine (Oversub) within the Elite Immuno-4 software (Coulter Corp., Hialeah, FL).

*Scanning laser confocal microscopy.* Cells were covered with Elvanol (an anti fade), and the samples are scanned with an Applied Precision DeltaVision Scanning Microscope (Issaquah, WA), fitted with an Olympus IX70 microscope (Olympus America, Melville, NY) and a wide field-sectioning microscope that employs fluorescent light and equipped with a point spread function for better image quality (deconvolution). A 100-watt mercury arc lamp was used for illumination and excitation/emission wavelengths were produced by filters specific for each probe (Chroma Technology Corp., 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  $\mu\text{m}$  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<sup>TM</sup> software from Applied Precision using an algorithm based on the convolution of a point spread function (PSF) 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 using SoftWoRx<sup>TM</sup> 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  $1.5 \times 10^6$  cells/ml in two culture conditions, static (1g) and MMG (RWV). Lymphocyte locomotion was significantly inhibited in MMG and at 96 hours, the locomotion was negligible compared with static 1g 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 Figure 1 series shows calcium-independent (PKC- $\delta$ , - $\epsilon$ ) isoform protein expression levels by western blotting with



monoclonal antibodies directed against each isoform. PKC- $\delta$  protein expression in modeled microgravity was negligible through 96 hrs in MMG lymphocyte cultures (Figure 1a). In contrast, PKC- $\epsilon$  displayed evanescent expression in MMG when compared with static 1g culture (Figure 1b). At 24 hours, there was a significant decrease (57%) with near normal expression by 48 hours. The expression levels decreased again at 72 hours revealing negligible quantities by 96 hours. Results displayed are the average levels of three independent experiments. Protein concentrations used were 100  $\mu\text{g}/\text{lane}$ , and equal loading was documented by imaging prior to blotting. This was used as a parameter of equal loading, since common housekeeping genes, such as  $\beta$ -actin, were altered in RWV culture.

*Transcriptional down-modulation of  $\text{Ca}^{2+}$ -independent PKC isoforms.* RT-PCR was performed on 1g and MMG cultured lymphocyte total RNA. Protein kinase C- $\delta$  message was barely discernible at 24 and 48 hours in MMG and by 96 hours, it was still down-regulated by 56% in MMG (Figure 2a). PKC- $\epsilon$  message showed a similar trend in MMG, with an 83% decrease at 24 and 48 hours (Figure 2b). Oscillatory expression patterns were observed in of both these isoforms. The reduced transcription in modeled microgravity was reflected in the quantitative estimates by western blotting (Figures 1a and 1b). While there was reduced transcript expression of PKC- $\delta$  in modeled microgravity, protein expression was barely evident. Even though PKC- $\epsilon$  expression was comparable to static cultures at 48 hours, mRNA was decreased significantly, indicating that PKC- $\epsilon$  translated accumulation may initially occur prior to down-regulation of message. GAPDH transcriptional controls are displayed in Figure 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- $\epsilon$ , less obvious in PKC- $\delta$  and insignificant in PKC- $\alpha$ . There was an oscillatory expression of PKC- $\epsilon$  and - $\delta$  with a little recovery in expression of protein at 48 hours (Figure 3a). Furthermore, these isoforms translocate and localize with cytoskeletal components. Confocal microscopic analysis revealed more uniform distribution of PKC- $\epsilon$  in lymphocytes cultured in static conditions in comparison with cells cultured in MMG, where it was less uniform and patchy (Figure 3b).

Phosphorylation of Phospholipase C  $\gamma$ 1-1 (PLC- $\gamma$ 1) expression in MMG. Phospholipase C- $\gamma$ 1, the primary enzyme for generation of diacylglycerol (DAG) via the hydrolysis of IP<sub>3</sub> and PIP<sub>2</sub> in T cells, undergoes tyrosine phosphorylation to become the active form. Modeled microgravity-cultured lymphocytes, even early in culture showed a 56% decrease in phosphorylated PLC- $\gamma$ 1 compared with 1g cultured lymphocytes (Figure 4). Samples were immunoprecipitated with a PLC- $\gamma$ 1 antibody and detected using 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*.

Modeled microgravity-induced inhibition of locomotion was restored up to 87% by addition of phorbol myristate acetate (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- $\epsilon$  was markedly reduced at both the RNA and protein levels, suggesting inhibition at both levels in MMG. The change in PKC- $\delta$  was similar to, although not as pronounced as PKC- $\epsilon$  by flow cytometry. However, while PKC- $\delta$  was negligible at the protein level by western blotting at all assay times, at 72 and 96 hours, mRNA was detectable by RT-PCR and transcriptional inhibition was evident at 24 and 48 hours. 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- $\epsilon$  and - $\delta$  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- $\alpha$  levels did not change significantly in 1g and MMG-cultured lymphocytes (data not shown). However, locomotion was not augmented in MMG, even though PKC- $\alpha$  levels were optimal. This suggests that calcium-dependent PKC isoforms may not participate in the signal pathway for lymphocyte locomotion. Transcriptional changes in PKC- $\delta$  and - $\epsilon$  expression levels indicate that MMG has selective effects on transcription and transcript stability of PKC isoforms, either directly acting on the gene or via up-stream effects such as Phospholipase C- $\gamma$ 1 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- $\epsilon$  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- $\delta$  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 (FAK) was observed. Inhibition of Protein Tyrosine Kinases (PTK) using Genistein significantly decreased spontaneous locomotion, while 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 MTs with Colcemid recruited previously nonlocomotory cells and demonstrated an increase in the duration of locomotion (9). This pro-locomotory role of Colcemid is due to chemoattractant behavior (9). Activated T lymphocyte locomotion is triggered by LFA-1 and is PKC $\beta$ -1 dependent (9). Our studies reveal a down-regulation of calcium-independent PKC- $\delta$  and  $\epsilon$  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  $CA^{++}$ -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- $\gamma$ 1 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- $\epsilon$  isoform to the cytoskeleton may be the pathway to inhibition of locomotion in MMG.

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**FIGURE LEGENDS**

Fig. 1a. PBMCs were obtained from normal human donors and suspended in culture in static (1g) culture or modeled microgravity (MMG). Cells were sampled at 24, 48, 72 and 96 hours from both culture conditions. They were then lysed and run on 10% SDS-PAGE gels, transferred to nitrocellulose and probed with antibodies to PKC- $\delta$ . Protein Kinase C- $\delta$  was not detectable in MMG cultures. All western blots and RT-PCRs were performed on an experimental 'n' of three and the graph shown is an average of three experiments with a representative blot image. Paired sample *t*-tests were performed between groups and time points for all experiments.

Fig. 1b. PKC- $\epsilon$  protein expression levels in lymphocytes in modeled microgravity (MMG) revealed a significant decrease of this isoform (57%,  $p < 0.001$ ) even at 24 hours in MMG compared with 1g. This decrease was evident even at 72 hours, followed by a very negligible amount detected at 96 hours. All Western blots and RT-PCRs were performed on an experimental 'n' of three, and the graph shown is an average of three experiments with a representative blot image. Paired sample *t*-tests were performed between groups and time points for all experiments.



Fig. 2a. RT-PCR analysis of PKC- $\delta$ . Revealed that the transcription of PKC- $\delta$  was down-regulated to negligible levels at 24 and 48 hours and by 56% ( $p<0.001$ ) at 96 hours. Therefore the decrease of this isoform occurs transcriptionally in MMG. All western blots and RT-PCRs were performed on an experimental 'n' of three, and the graph shown is an average of three experiments with a representative blot image. Paired sample *t*-tests were performed between groups and time points for all experiments.

Fig. 2b. Transcriptional down-regulation of PKC- $\epsilon$  was evident at 24 and 48 hours in MMG (83%,  $p<0.001$ ) with a 53% decrease at 96 hours. All western blots and RT-PCRs were performed on an experimental 'n' of three, and the graph shown is an average of three experiments with a representative blot image. Paired sample *t*-tests were performed between groups and time points for all experiments.

Fig. 2c. GAPDH mRNA levels revealed consistent expression in both 1g and MMG-cultured lymphocytes. All western blots and RT-PCRs were performed on an experimental 'n' of three, and the graph shown is an average of three experiments with a representative blot image. Paired sample *t*-tests were performed between groups and time points for all experiments

Fig. 3a. FACS analysis revealed down-regulation of PKC isoforms  $\delta$  and  $\epsilon$ . In addition, this figure also shows that the levels of calcium-dependent isoform PKC- $\alpha$  was not significantly altered in MMG.

Fig. 3b. The distribution of PKC- $\epsilon$  was uniform in lymphocytes cultured in 1g and very patchy in those cultured in MMG. This could be attributed to down-regulation of the isoforms, as well as changes in translocation properties of PKC- $\epsilon$  within the cell in MMG. Human lymphocytes were incubated in static and RWV cultures for 24 hours then harvested, permeabilized, and stained with isoforms-specific antibody. Deconvolution microscopy was used, and stacked images were analyzed by three-dimensional modeling and image rotation to observe the deployment of PKC isoforms (green) and the counter-stained nuclei (blue).

Fig. 4. Immunoprecipitation and detection of activated PLC- $\gamma$ 1, revealed a 56% decrease ( $p < 0.001$ ) in the activated form in MMG at 48 and 72 hours. This molecule is important in lymphocyte activation upstream of PKC, since it brings about the generation of second messenger DAG, which is a PKC activator in T cells. All western blots and RT-PCRs were performed on an experimental 'n' of three, and the graph shown is an average of three experiments with a representative blot image. Paired sample *t*-tests were performed between groups and time points for all experiments



# Expression of PKC delta protein in 1g and MMG

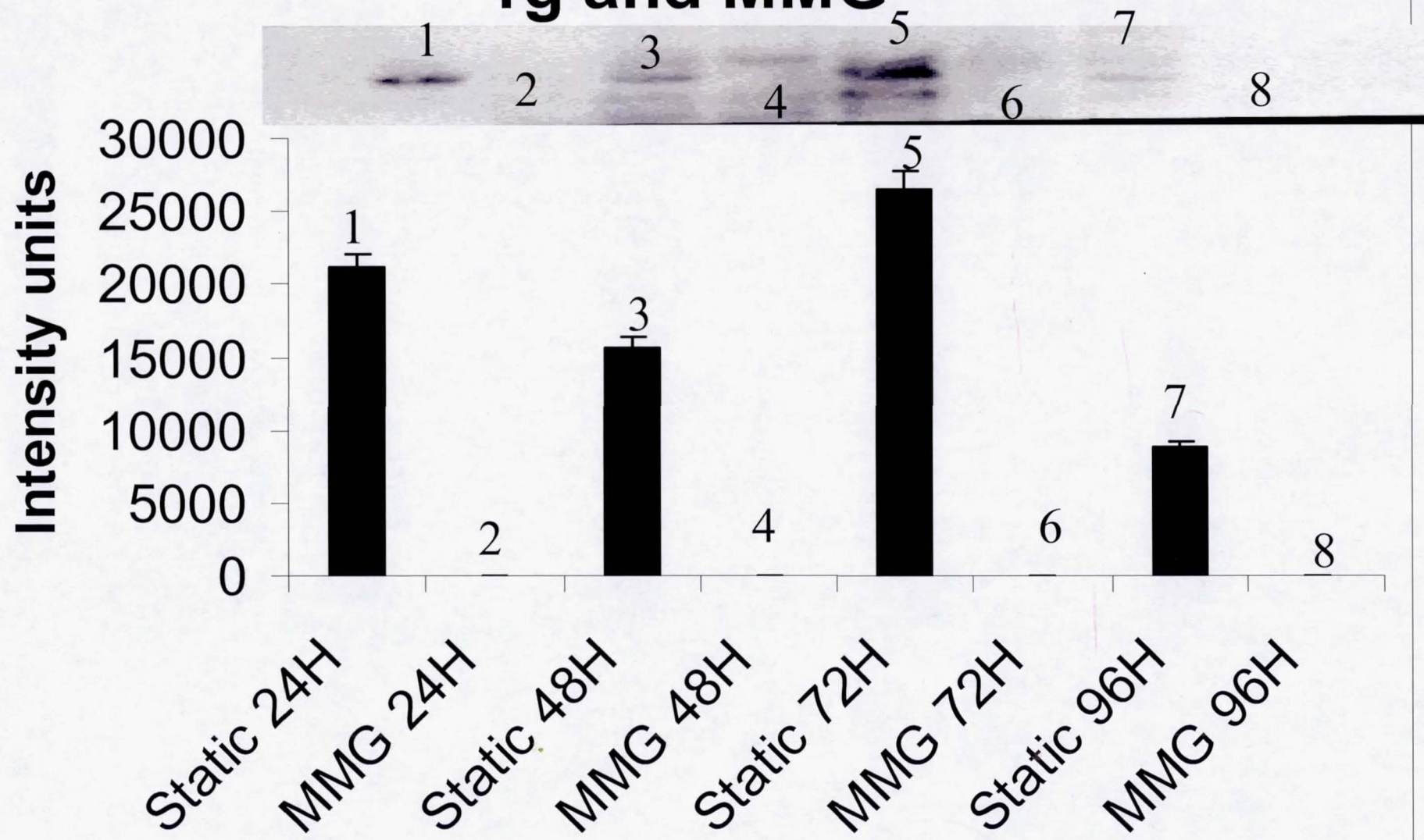


Fig. 1a

# Expression of PKC-epsilon protein in 1g and MMG

Fig. 1a

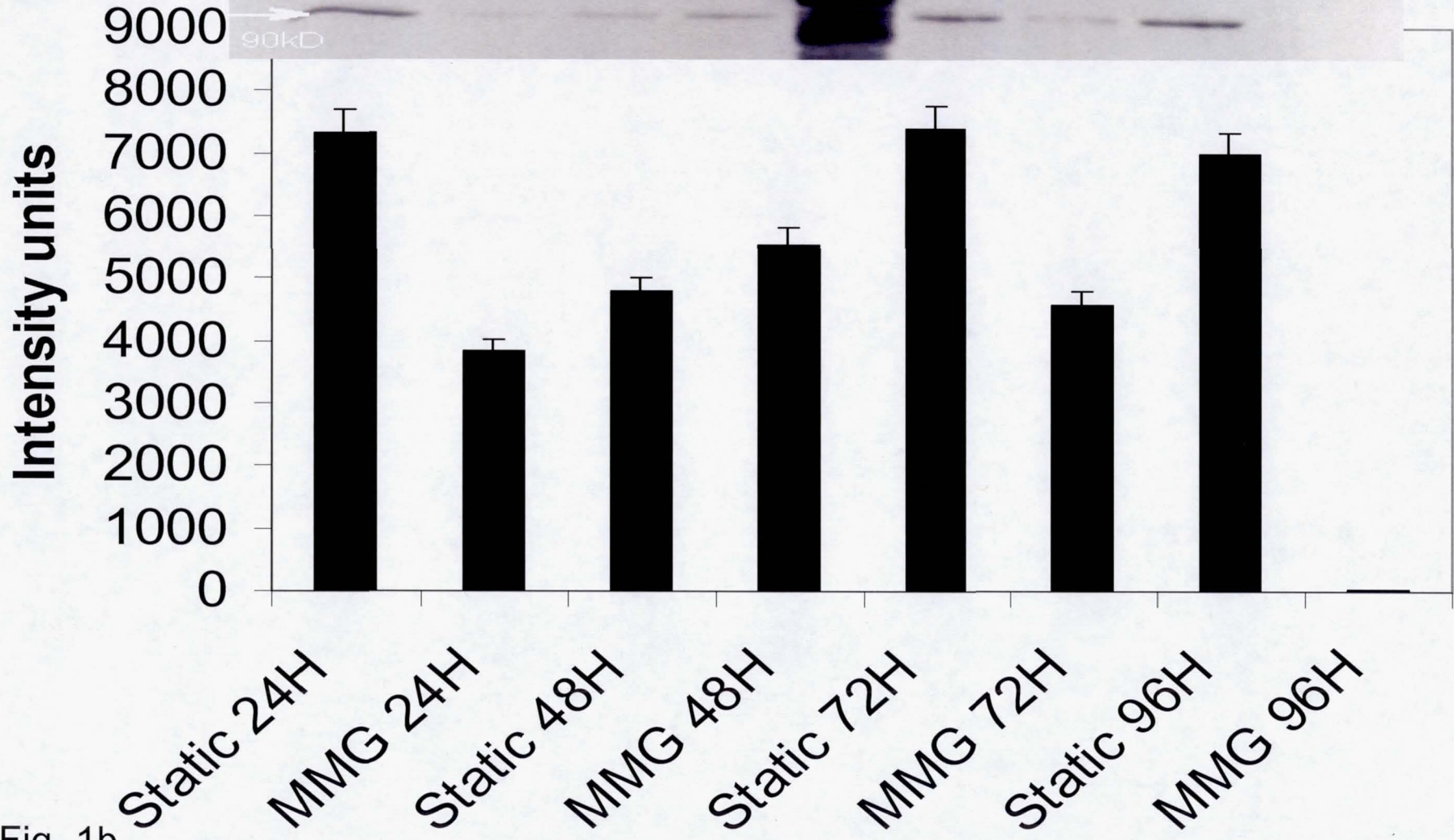
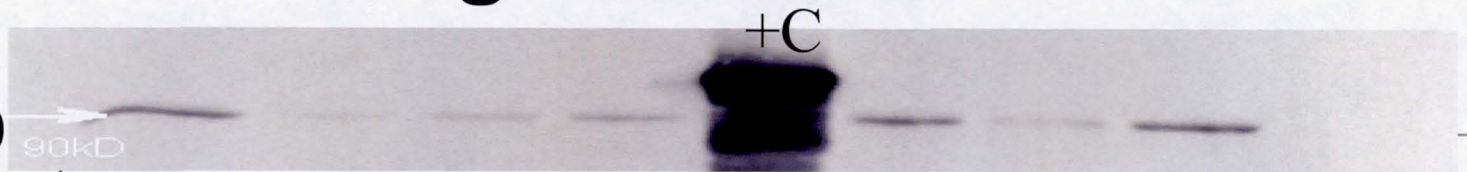


Fig. 1b



# Expression of PKC delta mRNA in 1g and modeled microgravity

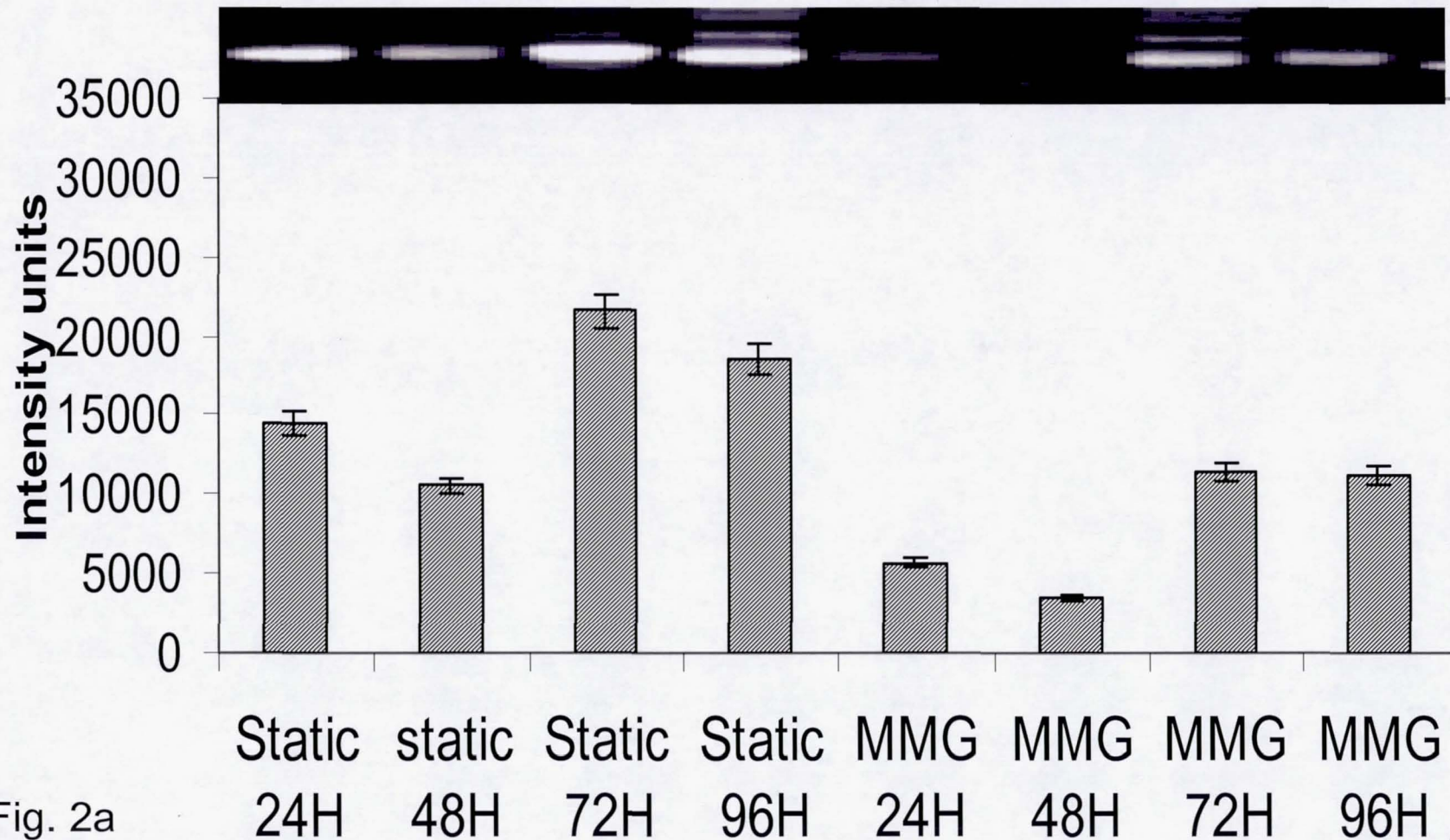


Fig. 2a

# Expression of PKC-epsilon mRNA in 1g and mmg

+C -C

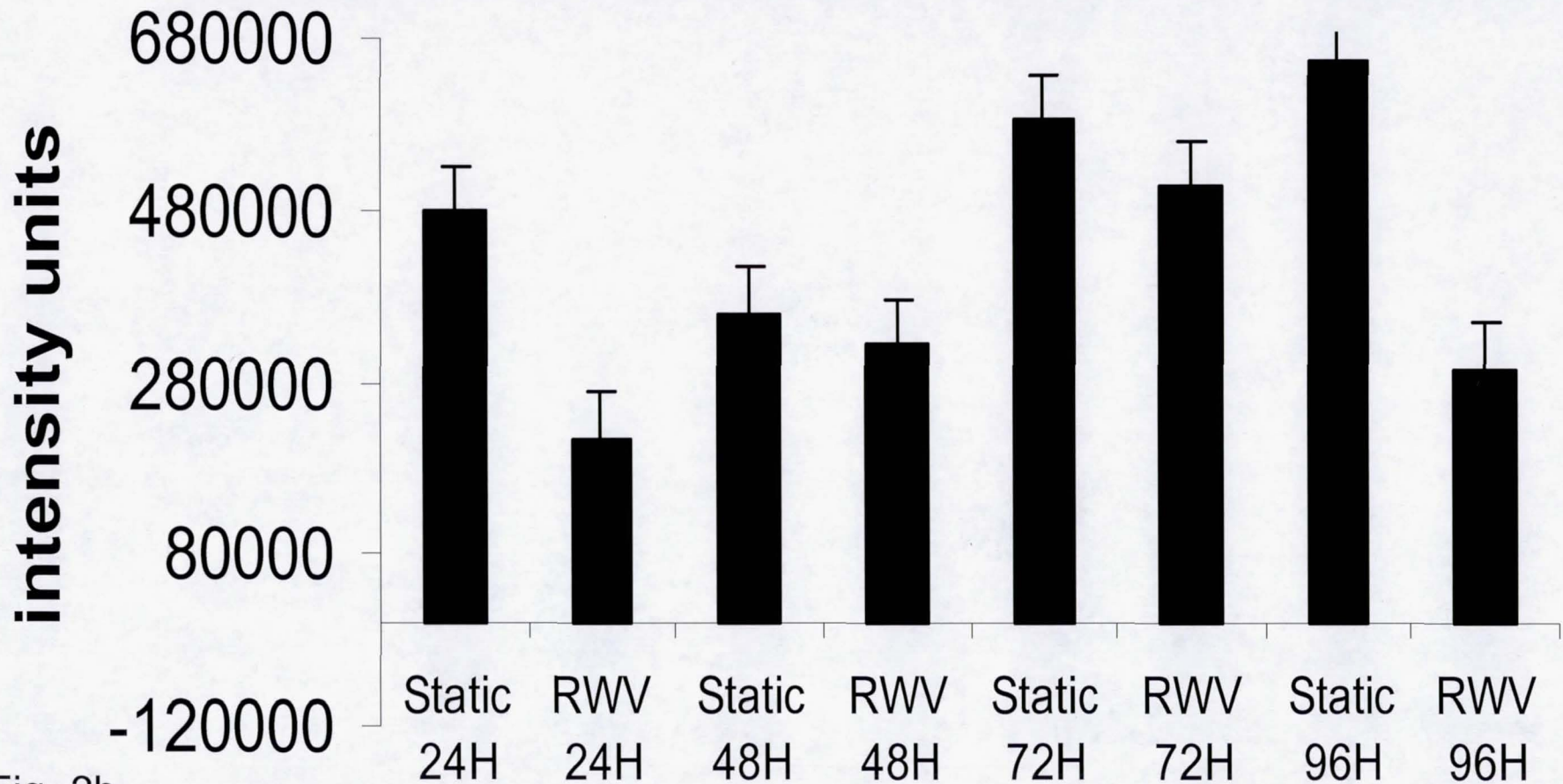


Fig. 2b



## Expression of GAPDH mRNA in 1g and modeled microgravity

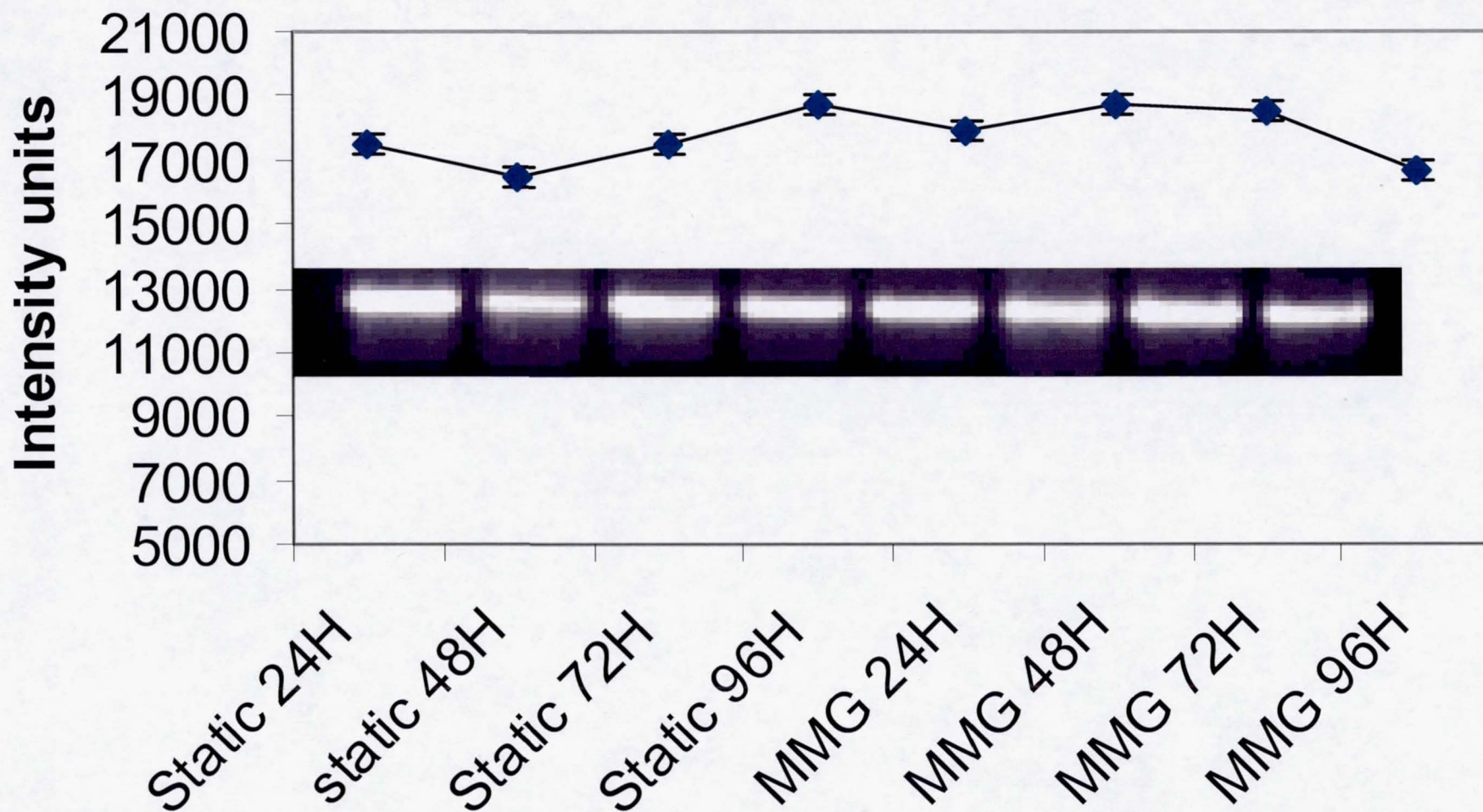


Fig. 2c

# PKC Isoform expression in lymphocytes by flow cytometry in static and modeled microgravity culture

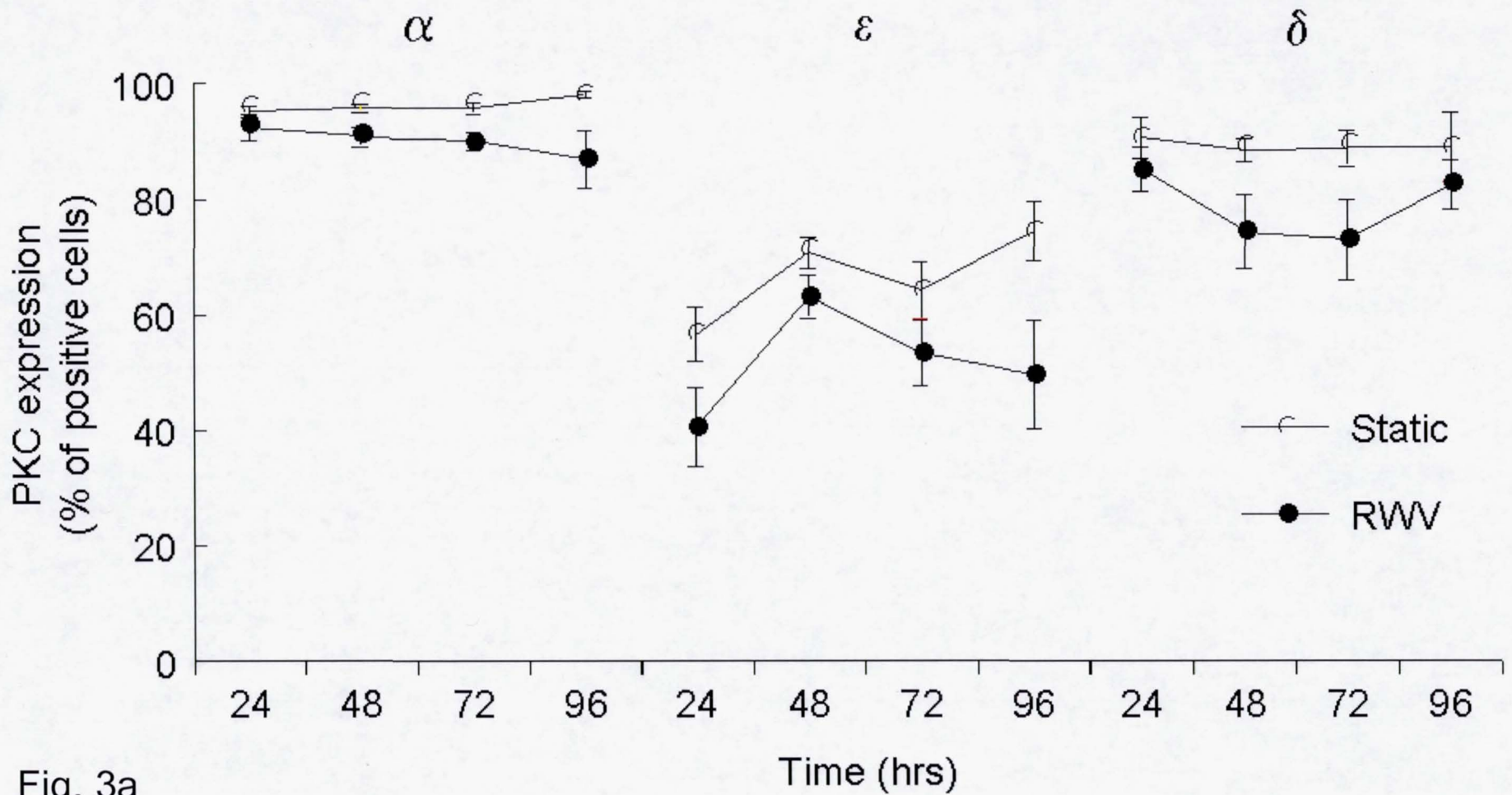


Fig. 3a



## Expression of Protein Kinase C Isoforms in Human Lymphocytes

Human lymphocytes were incubated in static and RWV cultures for 24 hours then harvested, permeabilized, and stained with isoform specific antibody. Deconvolution microscopy was used and stacked images were analyzed by 3D modeling and image rotation to observe the deployment of PKC isoforms (green) and the counterstained nuclei (blue).

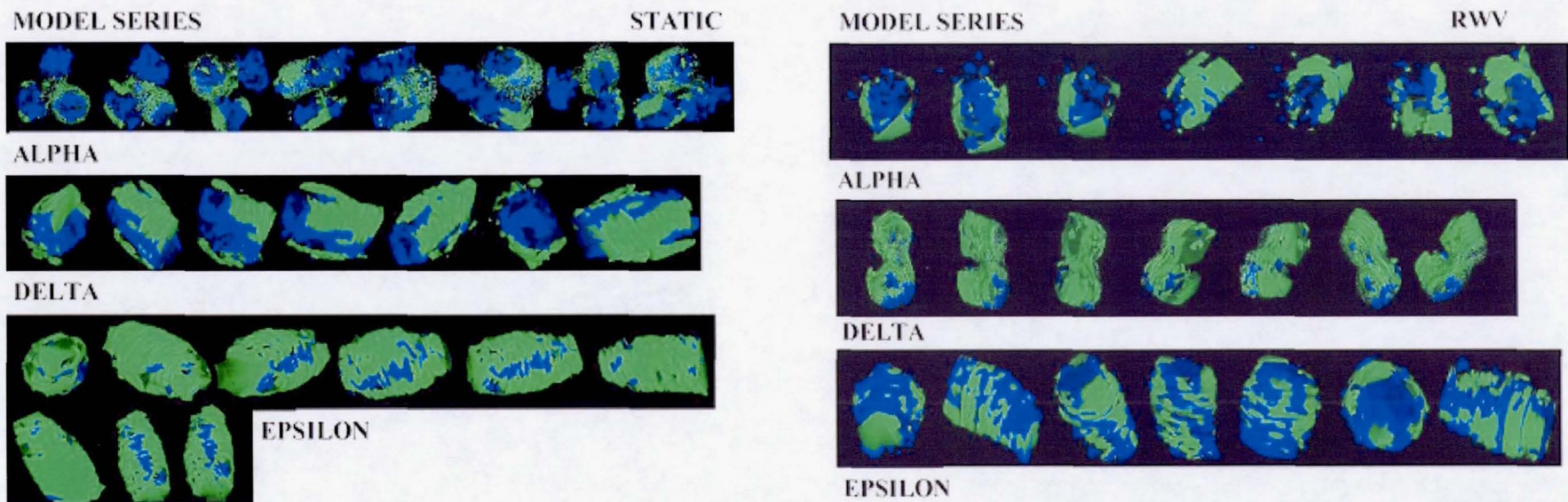


Fig. 3b



# Expression of activated PLC-gamma 1 in MMG

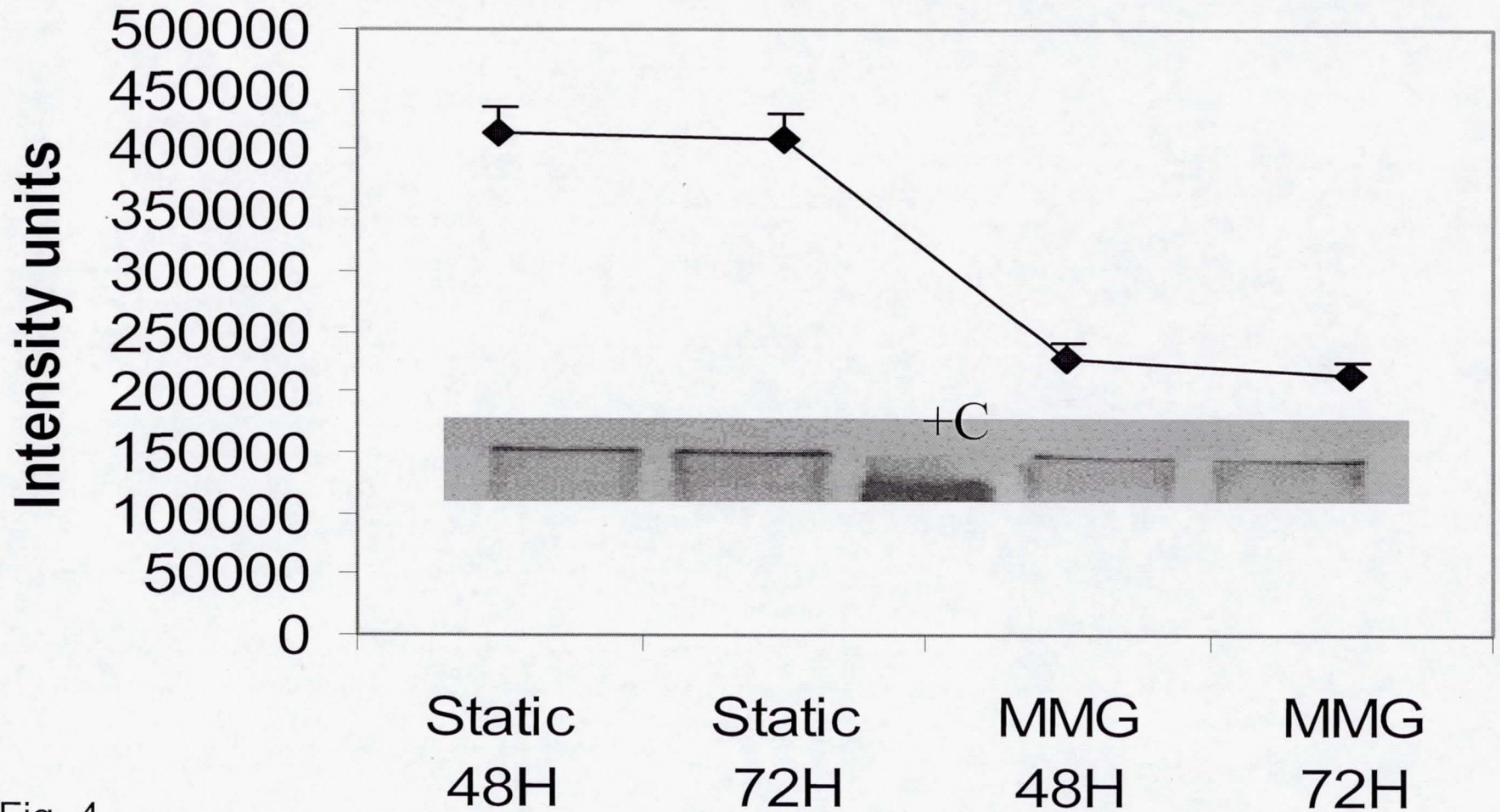


Fig. 4