

Platelet shape change and protein phosphorylation induced by ADP and thrombin are not sensitive to short periods of microgravity

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SUMMARY

Recent experiments have shown that the stimulation of animal cells *in vitro* by direct protein kinase C (PKC) activators is significantly reduced under microgravity (μ g). Platelets undergo protein phosphorylation and morphological changes a few seconds after stimulation by agonists such as phorbol esters which activate PKC. Therefore, taking advantage of parabolic plane flight to obtain short periods of microgravity, we studied phosphorylation of myosin light chain (20K), specific PKC-dependent phosphorylation of a 40,000 M_r protein,

pleckstrin (40K) and platelet shape change. SDS-PAGE analysis and electron microscopy were performed on platelets subjected to 20 seconds microgravity as compared to normal gravity (1 g) conditions. These investigations showed that neither Ca^{2+} -calmodulin-mediated activation nor the PKC-dependent pathways are inhibited during short periods of microgravity.

Key words: microgravity, parabolic flight, platelets, signal transduction, PKC

INTRODUCTION

Hypergravity can be obtained using centrifuges in ground-based laboratories, but reduced gravity can only be obtained under free-fall conditions and for microgravity periods exceeding 5 s, space flight, sounding rocket or parabolic plane flight must be used. Animal cells in culture are sensitive to physicochemical factors like temperature, pH and osmotic pressure. Are these cells likewise sensitive to a factor which has not changed since the beginning of life on earth, such as gravity? For resting eucaryotic cells the answer is still contradictory (Montgomery et al. 1978), but gravity seems to have an influence on agonist-stimulated cells. In fact, mitogen-induced lymphocyte proliferation and differentiation were almost completely inhibited during a spaceshuttle flight (Cogoli et al. 1984), and after a second such experiment, using an on-board earth gravity (1 g) control, this inhibition was attributed to microgravity (Bechler et al. 1986). In contrast, hypergravity enhances lymphocyte differentiation (Lorenzy et al. 1986). More recently, it has been shown in an epidermoid carcinoma cell line that the stimulated expression of proto-oncogenes is very significantly decreased under microgravity obtained during sounding rocket flight (De Groot and Kruijer, 1990). Interestingly the proto-oncogenes, which are involved in cell proliferation, are stimulated under hypergravity (De Groot et al. 1990). It has also been shown that this gene expression sensitivity to microgravity might be due to inhibition of the PKC-activation pathway but not to decreased Ca^{2+} -calmod-

ulin pathway activation (De Groot et al. 1991). The same conclusions were drawn from an experiment using lymphocyte and monocyte cell lines aboard a satellite flight (Limouse et al. 1991). During these investigations, the production of interleukins was dramatically reduced or undetectable when the cells were stimulated by phorbol 12-myristate 13-acetate (PMA), a PKC activator, whereas interleukin production was unchanged when the Ca^{2+} -ionophore (A23187) or other agonists were used. Nevertheless, during parabolic flight, PMA induction of O_2^- in peritoneal inflammatory cells was shown to be four times higher under microgravity than to earth gravity (Fleming et al. 1991).

Blood platelets undergo shape change and aggregation within a few seconds when activated by thrombin, ADP (Carty et al. 1986, 1987) or the Ca^{2+} -ionophore A23187 (Siess, 1989). The morphological changes are essentially mediated by Ca^{2+} -calmodulin-dependent mechanisms. PKC-dependent phosphorylation of pleckstrin, a 40,000 M_r protein (40K), occurring within a few seconds and the appearance of pseudopodia in less than one minute are also observed after stimulation by thrombin or phorbol esters like PMA (Siess, 1989). Therefore, platelets may be used to study cell activation during parabolic plane flight in which 20 s periods of microgravity are obtained. With such experiments we were able to investigate Ca^{2+} -calmodulin-dependent platelet shape change, myosin light chain (20K) phosphorylation and PKC-dependent 40K phosphorylation within these short periods of microgravity.

MATERIALS AND METHODS

Parabolic flight

The experiments under reduced gravity were conducted aboard the French "Zero G" Caravelle. In each flight 31 parabolas are flown, one parabola corresponding to two 20 s hypergravity periods (1.8 *g*) separated by a 20 s microgravity (approximately 10^{-2} *g*) period (Fig. 1). For technical reasons, such as the amount of radioactive samples that could be stored in the plane and the time of preparation between two experiments, only the first, 15th and 30th parabolas could be used for performing our experiments. The 1 *g* controls were performed in flight and on the ground just before the corresponding microgravity experiments. In some flights the 1.8 *g* periods were also used as an additional control.

Apparatus

Platelet transportation from the laboratory to the plane and the experiment on board were performed in a specially designed apparatus (Microlab) described elsewhere (Schmitt, 1992). Briefly, Microlab is a spherical container built like a glove box with a double access. It is safe for low or low energy radiation and for hazardous chemical handling, is thermostated and has four openings hermetically fitted with gloves. The Microlab was fixed in the plane on a table at which two scientists could sit and work under the variable gravity conditions encountered during the flight.

Chemicals and drugs

ADP sodium salt, prostaglandin I₂ (PGI₂), staurosporin, phorbol 12-myristate 13-acetate (PMA) and diisopropylfluorophosphate (DFP) were from Sigma (St Louis, MO). Heparin was from Hoffman La Roche (Basel, Switzerland). Dimethyl sulfoxide (DMSO), glutaraldehyde and leupeptin were from Fluka (Buchs, Switzerland) and perchloric acid (HClO₄) was from Merck (Darmstadt, Germany). Ca²⁺-ionophore A23187 was from Boehringer (Ingelheim, Germany), aprotinin from Sanofi-Choay (Paris, France) and human fibrinogen from Kabi (Stockolm, Sweden). Human serum albumin (HSA), thrombin (3000 i.u./mg HIH) and apyrase (ATP diphosphohydrolase, EC 3.6.1.5) were from the Centre Régional de Transfusion Sanguine (Strasbourg, France). Carrier-free ³²P (PO₄³⁻, 370 MBq/ml) was from Amersham (Bucks, UK).

Preparation of washed human platelets

Blood was taken from healthy donors who had not taken any medication for at least 8 days. Approximately 250 ml of blood was collected into an anticoagulant solution of sodium citrate/citric acid/dextrose and the platelets were isolated by differential centrifugation and washed at 37°C, according to a modification (Cazenave et al, 1983) of the method described by Kinlough-Rathbone et al (1977). Unless otherwise stated, platelets were washed

in Tyrode's buffer, pH 7.3, 295 mosmol/l, containing 5 mM HEPES (Tyrode-HEPES buffer), 0.35% HSA and 1 μM PGI₂. The platelets were finally suspended in Tyrode-HEPES buffer containing 0.35% HSA and apyrase (2 μg/ml, a concentration that converts 0.25 μM ATP into AMP within 2 min at 37°C), in order to avoid ADP receptor desensitization. Cell count was adjusted to 300,000 platelets/μl.

Radioactive labeling of platelet proteins

For protein phosphorylation measurements, the platelet preparation procedure was modified as follows (Lanza et al, 1988). Platelets were first washed with 30 ml of phosphate-free, Tyrode-HEPES buffer, pH 7.3, 295 mosmol/l containing 0.35% HSA, 2 μl/ml heparin and 1 μM PGI₂. The cells were then resuspended in 5 ml of the same buffer in the presence of 64 MBq carrier-free ³²PO₄³⁻, incubated for 60 min at 37°C and washed in Tyrode-HEPES buffer containing phosphate, 0.35% HSA and 1 μM PGI₂. Platelets were finally resuspended at 300,000/μl in Tyrode-HEPES buffer with 0.1% HSA and 2 μg/ml apyrase.

Platelet activation conditions

1 ml samples of platelet suspension were pipetted into each of several 3 ml siliconized glass tubes fixed on a rack inside the Microlab. Two needles were stuck in the cap of each tube and were connected to syringes filled with either an agonist or the stopping solution. A third needle was used as an air outlet to equilibrate the pressure within the tube to that of the sphere and therefore to the cabin pressure. Six different agonist and control solutions (0.1 ml) were injected simultaneously into six tubes 5 s after the beginning of a microgravity period. The size and wetting properties of the glass tubes were designed to keep the platelet suspension on the bottom of the tubes during the microgravity periods without wetting the tube walls by capillary force. Mixing conditions were optimal, and were controlled by adding a colored solution (0.5 ml) to 1ml of platelet suspension in a siliconized glass tube (3 ml) with a syringe. The reaction volume also permitted a reduction of convection currents in the fluid during addition of an agonist solution, thereby minimizing fluid movements that could alter microgravity.

Protein phosphorylation studies

The ³²PO₄³⁻-labeled platelet suspension was dispensed into 3 ml siliconized glass tubes and placed at 37°C in the Microlab. Platelet activation was induced by 100 μM ADP, 0.5 i.u./ml thrombin, 1 μM or 10 μM calcium ionophore A23187 and 1 μM or 10 μM PMA, each activation condition being tested during two different flights. 5 s after the beginning of the microgravity period, 0.5 ml of the agonists and their corresponding control buffers were added to the platelets. After 15 s, just before the end of the microgravity period, platelet proteins were precipitated with 0.5 ml ice cold 1.5 M HClO₄ and were kept on ice until the end of the flight and then at -20°C until further processing. For SDS-PAGE analysis, precipitated proteins were centrifuged at 12,000*g* for 1 min, rinsed with Tyrode's buffer without albumin and recentrifuged. The pellet was dissolved in 100 μl of a solution containing 2% SDS, 40 mM Tris, 2.5% glycerol, 2 mM *N*-ethyl-maleimide, 5% 2-mercaptoethanol and protease inhibitors (50 μg/ml leupeptin, 1 mM DFP and 20 i.u./ml aprotinin). The samples were solubilized at 100°C for 5 min and the radioactivity of 1 ml samples were determined by liquid-scintillation counting. Proteins (300,000 cts/min) were subjected to discontinuous SDS-PAGE (7%-13% polyacrylamide gels) in 0.025 M Tris buffer, 0.192 M glycine, pH 8.35, according to Laemmli (1970). The gels were stained with Coomassie Blue-R and dried and labeled polypeptides were located by autoradiography on Kodak X-Omat film (Rochester, NY).

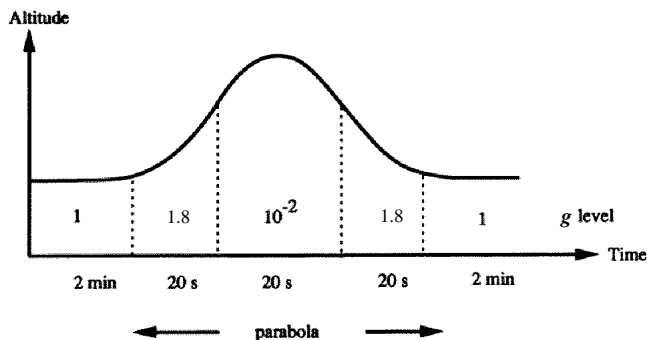


Fig. 1. Schematic representation of a parabolic plane flight profile.

Electron microscopy studies

Unlabeled platelets in suspension were activated as described above and fixed by addition of 0.5 ml of a solution containing 1% glutaraldehyde, 0.1 M cacodylate buffer and 0.1 M sucrose, pH 7.3. For cytoplasmic morphological studies, the cells were washed with cacodylate buffer and post-fixed for 2 h with 1% osmium tetroxide. Platelets were then washed twice, dehydrated with alcohol and embedded in Epon. After staining, ultrathin sections were observed under a Hitachi H300 transmission electron microscope. The number of intracytoplasmic granules was determined on 18 different platelet sections, together with the surface areas of the sections and of the granules. To detect external shape changes, platelets were washed with cacodylate buffer and dehydrated on glass slides, gold-coated and observed with a Cambridge Stereoscan 360 scanning electron microscope.

Image analysis

Autoradiographic film analysis was performed with a Biocom 200 instrument (Biocom, Paris, France). The radioactivity bands corresponding to the myosin light chain (20K) and the 40K polypeptide were normalised by comparison with a control band for which the incorporation of radioactivity was not modified during platelet activation. Platelet section micrographs were analysed with an AST analyser (CRIS, Toulouse, France).

RESULTS

Platelet shape change and protein phosphorylation were investigated under microgravity as compared to earth gravity. Microgravity periods of 20 to 22 s were obtained during parabolic plane flight and human blood platelets were activated at 37°C with thrombin, ADP, the Ca²⁺ ionophore A23187, the phorbol ester PMA or their respective control buffers.

Morphological studies showed that shape change (transformation of the discoid platelet into a spherocyte – Frojmovic and Milton, 1982) and the appearance of pseudopodia occurred within 15 s when platelets were stimulated with standard agonists like thrombin or ADP, but not when they were stimulated with PMA. Performing these activation experiments at earth gravity and microgravity over 15 s, no significant difference in shape change could be seen under the scanning electron microscope (Fig. 2). Using transmission electron microscopy (Fig. 3), the number and area of intracytoplasmic dense granules per unit platelet area were determined and found to be identical under earth gravity and microgravity after stimulation for 15 s by thrombin (Table 1).

Specifically to investigate PKC activity under reduced gravity, we studied phosphorylation of the 40K protein pleckstrin with ³²P-labeled platelets. SDS-PAGE analysis

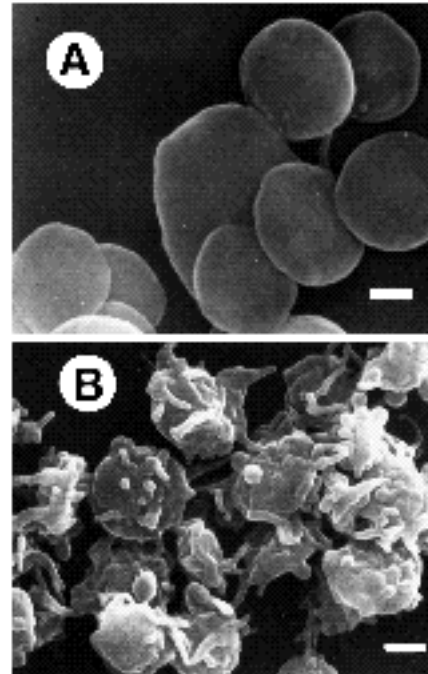


Fig. 2. Scanning electron micrograph representing platelets activated by PMA (10 µM) (A) or ADP (100 µM) (B) for 15 s. Unstimulated platelets have the same shape as in (A) and thrombin stimulated platelets the same shape as in (B). No difference in shape change could be seen between earth gravity and microgravity under the PMA, ADP, thrombin or control conditions. Bars, 1 µm.

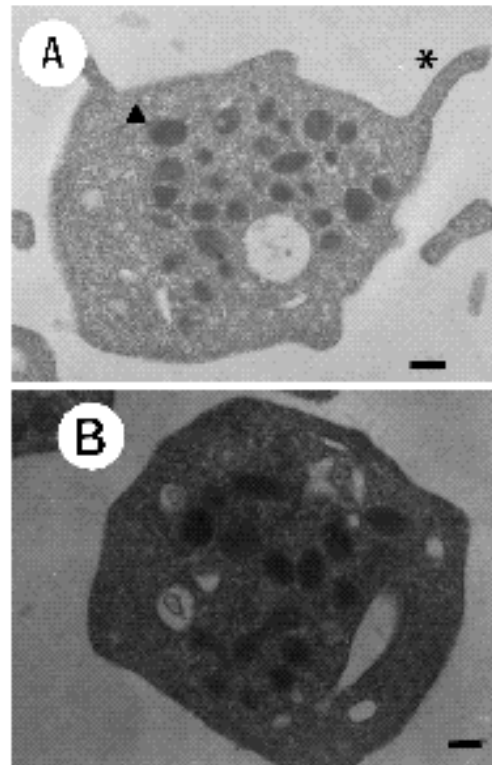


Fig. 3. Transmission electron micrograph showing a cross section of an unactivated platelet (A) and a platelet activated by 0.5 i.u./ml thrombin for 15 s (B). Note the presence of granules (triangle) and pseudopodia (star). Bar, 0.25 µm.

Table 1. Platelet activation by thrombin under earth gravity and microgravity

	earth gravity	microgravity
Number of vesicles/platelet section	14 ± 2.5	16 ± 2.4
Vesicle area/platelet area	7.1 ± 1.5	7.0 ± 0.8

Results from platelet cross section image analysis: the number of vesicles per platelet cross section and the vesicle area divided by the area of the platelet section (mean ± s.e.m., n = 18) after activation by 0.5 i.u./ml thrombin.

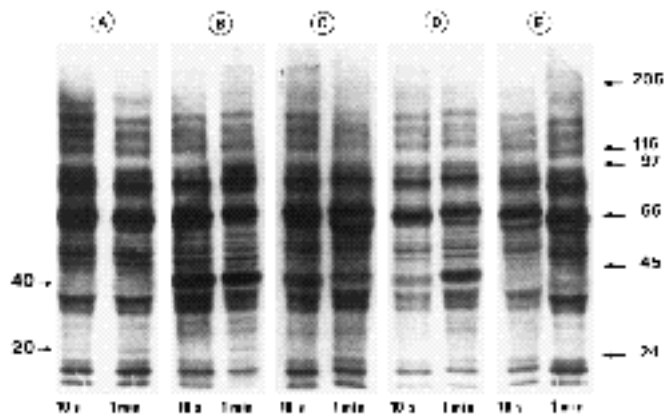


Fig. 4. Discontinuous SDS-PAGE autoradiographs of platelet proteins at earth gravity. Platelets were activated by different agonists for 10 s or 1 min in ground-based experiments. ADP (10 μ M) (A) and A23187 (1 μ M) (C) induced myosin light chain (20K) phosphorylation, clearly visible after 1 min and 10 s respectively. Thrombin (0.5 i.u./ml) (B) and PMA (1 μ M) (D) induced rapid phosphorylation of the 40K protein. The control showed only baseline phosphorylation (E). The position of marker proteins ($M_r \times 10^{-3}$) is indicated.

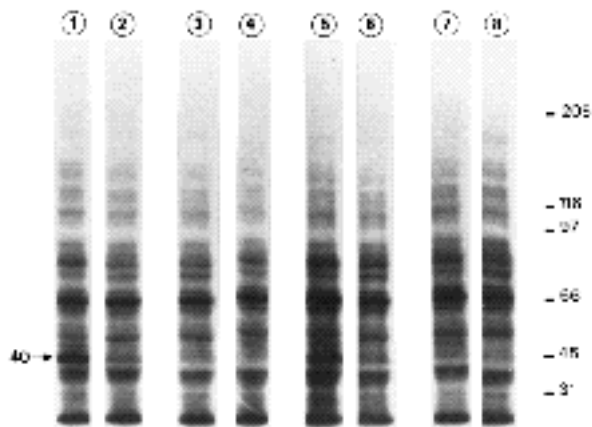


Fig. 5. Discontinuous SDS-PAGE autoradiographs at earth gravity showing PKC-dependent phosphorylation of the 40K protein. Lane 1, thrombin (0.5 i.u./ml); Lane 2, thrombin (0.5 i.u./ml) + staurosporin (0.2 μ M); Lane 3, thrombin control buffer; Lane 4, thrombin control buffer + staurosporin (0.2 μ M); Lane 5, PMA (1 μ M); Lane 6, PMA (1 μ M) + staurosporin (0.2 μ M); Lane 7, PMA control buffer; Lane 8, PMA control buffer + staurosporin (0.2 μ M). The position of marker proteins ($M_r \times 10^{-3}$) is indicated.

showed that the 40K band was clearly visible after only 10 s of PKC activation by either thrombin or PMA (Fig. 4). This band was further characterized as being specific for PKC activation by preincubating the platelets with staurosporin, a well known PKC inhibitor (Watson et al. 1988). Under these conditions the 40K band disappeared almost totally, following platelet stimulation with PMA (Fig. 5). Comparing the phosphorylation patterns obtained under the different gravity levels in flight (1 g, 1.8 g and μ g), no significant difference was observed in 40K radiolabeling after 15 s of activation (Fig. 6, Table 2).

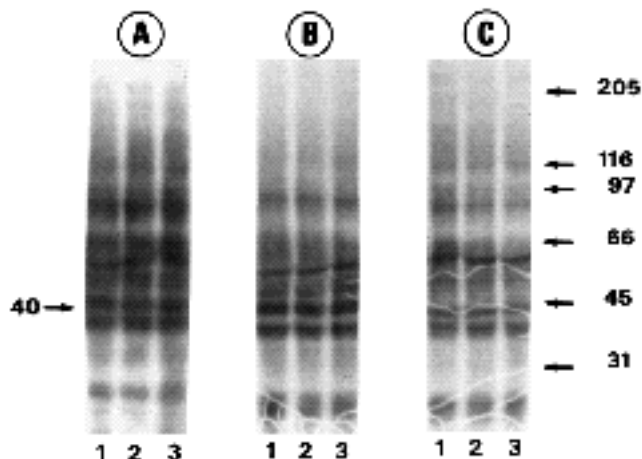


Fig. 6. Discontinuous SDS-PAGE autoradiographs of platelet proteins where platelets were activated by different agonists for 15 s during parabolic flight. (A) PMA (10 μ M); (B) thrombin (0.5 i.u./ml); (C) A23187 (10 μ M). Lane 1, earth gravity; lane 2, 1.8 g; lane 3, microgravity. The position of marker proteins ($M_r \times 10^{-3}$) is indicated.

Table 2. Phosphorylation of the 40K protein under different gravity levels in flight

Agonist	Gravity		
	1 g	1.8 g	μ g
Thrombin	1.22	1.21	1.26
A23187	1.06	1.15	1.10
PMA	1.04	1.05	1.03

Results from SDS-PAGE image analysis showing the ratio between phosphorylation intensity of the 40K protein and a standard unchanged band. Platelets were stimulated with various agonists under earth gravity and microgravity conditions. The accuracy of the ratio is ± 0.03 .

DISCUSSION

During space flight, resting eucaryotic cell cultures have not proven to be strongly affected by the unusual conditions essentially represented by microgravity (Montgomery et al. 1978). Nevertheless, Cogoli et al. (1984) and Bechler et al. (1986) showed that lymphocyte activation by concanavalin A (Con A) was almost completely inhibited under microgravity. More recently, two independent teams have looked at interleukin production in lymphocytes and monocytes (Limouse et al. 1991) and *c-fos* and *c-jun* proto-oncogene expression in an epidermoid carcinoma cell line (De Groot et al. 1991). In the interleukin production experiments, the cells were exposed for several hours to microgravity before stimulation by PMA, which reduced interleukin (IL)-1 secretion by 85% and totally abolished IL-2 production in comparison with ground controls. In experiments with an epidermoid cell line, the cells were activated for 6 min by epidermal growth factor or by the phorbol ester TPA immediately after reaching microgravity, leading to a 50% inhibition in proto-oncogene expression. These results strongly suggest that PKC, which is a Ca^{2+} - and phospholipid-dependent protein kinase, or a step of the PKC-modulated cell activation pathway might be sensitive to microgravity.

On the other hand, many studies using monocytes or lymphocytes have shown an enhancement of cytokine secretion under microgravity. In an early experiment by Talas et al. (1983) for example, lymphocytes were induced to produce about 5 times more interferon (INF)- during space flight than ground controls. More recent studies conducted on the space shuttle (Chapes et al. 1992) confirmed these data: INF- secretion was increased two-fold 14 h after stimulation by poly(I:C) and INF- secretion was increased three-fold 24 h after stimulation by Con A under microgravity. Likewise, a monocyte cell line secreted three times more IL-1 and 63% more Tumor Necrosis Factor- , 12 h after lipopolysaccharide stimulation under microgravity. On the contrary, in another experiment (Bechler et al. 1992), Con A-induced INF- synthesis was reduced under microgravity but increased three-fold when lymphocytes were in the presence of microcarriers. It is however not yet known whether poly (I:C), Con A or lipopolysaccharides induce activation of PKC.

As space flights are rare, we adapted ground experiments using human blood platelets to parabolic plane flights which are more frequent. Platelets are discoid cell fragments lacking a nucleus. They can be activated by various agonists (Fig. 7), inducing stepwise shape-change, aggregation and the secretion of granule contents which include several platelet activators. Ca²⁺ and PKC activation pathways act synergistically during these steps. Independently, a rise in intracytoplasmic Ca²⁺ can induce all three steps, whereas direct PKC activation leads only to slight aggregation, ATP secretion and extension of pseudopodia without spheration of platelets (Siess, 1989). Shape change is extremely fast ($T_{\frac{1}{2}} = 2.5$ s for spheration) and is rapidly followed by the appearance of pseudopodia. The PKC-mediated phosphorylation of the 40K protein pleckstrin reaches a maximum within 30-60 s. In our experiments, having access to 20 s microgravity, we studied this rapid protein phosphorylation using induction by agonists like thrombin and PMA. When comparing platelet activation experiments under the different gravity levels, no significant difference in the 40K phosphorylation pattern could be observed. Therefore, PKC which mediates 40K phosphorylation directly, is not inhibited during short periods of microgravity. This experiment also indirectly confirms that PMA binds to its intracytoplasmic receptor (PKC) under microgravity, as previously demonstrated using radiolabeled PMA (Manié et al. 1990). When looking at Ca²⁺-mediated platelet activation by thrombin or A23187, no significant modifications in shape change, phosphorylation patterns or secretion were noticed. In addition, the effects of ADP on platelet shape-change and phosphorylation of the myosin light chain (20K) were not influenced.

Several hypotheses can explain why the PKC activation pathway is unaffected by microgravity in our experiments. The level of gravity is 10⁻⁶ g during satellite flight, 10⁻⁴ g in a sounding rocket but reaches only 10⁻² g during parabolic flight. Therefore, microgravity in parabolic flights might not be sufficiently low to induce an effect on platelets. Furthermore, the time lapse before stimulation (5 s) might be too short to induce an inhibition process. No difference was seen when activating platelets after 15 or 30 parabolas (respectively 5 or 10 min of cumulative micro-

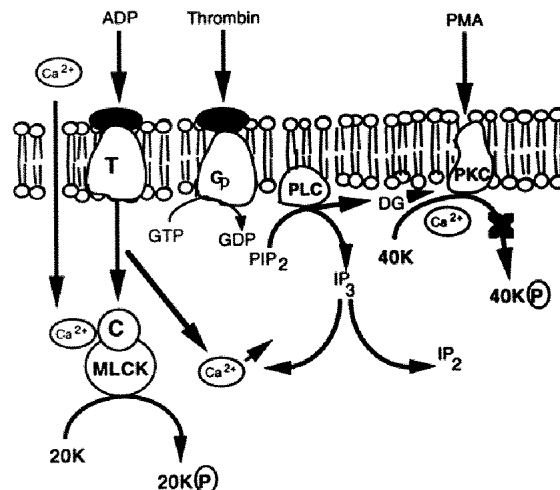


Fig. 7. Schematic representation of different signal transduction pathways leading to platelet activation. T, transduction; MLCK, myosin light chain kinase; C, calmodulin; PLC, phospholipase C; Gp, G protein which activates PLC; X, inhibition by staurosporin.

gravity), but the effect of microgravity might be reversed between parabolas. Finally, microgravity-induced inhibition could occur downstream of the PKC-phosphorylated proteins which are involved in cell activation. Interestingly, Chapes et al. (1992) showed very recently that PMA-induced O₂⁻ production by inflammatory cells during parabolic flight was four times greater under microgravity than at earth gravity. Since the reported decrease in cell activation by phorbol esters was reflected only by a decrease in protein secretion (Limouse et al. 1991) or gene expression (De Groot et al. 1991), it is likely that microgravity could inhibit cell activation at a step between PKC-dependent protein phosphorylation and gene expression. Phosphorylated 40K is thought to be involved in actin polymerisation, which could in turn be responsible for the secretion of ATP contained in intracytoplasmic granules and the emission of pseudopodia after PMA stimulation. However, these phenomena are observed only after some minutes of activation and cannot be studied during parabolic flight.

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