Microgravity decreases c-*fos* induction and serum response element activity

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Summary

Several studies have shown that altered gravity conditions influence mammalian cell growth and differentiation. The molecular mechanisms underlying these effects, however, remain relatively obscure. In this paper we show that microgravity reached in a sounding rocket strongly decreases epidermal growth factor (EGF)-induced expression of the proto-oncogenes c-*fos* and c-*jun*, which are both implicated in the regulation of proliferation and differentiation. Decreased activity of the serum response element (SRE), present in the c-*fos* promoter–enhancer region, is probably responsible for the decrease in EGF-induced c-*fos* expression. In addition, we show that gravity alterations differentially modulate distinctive signal transduction pathways, indicating that gravity-dependent modulations of mammalian cell proliferation are unlikely to be caused by a non-specific stress response of the cell.

Key words: microgravity, c-*fos*, signal transduction, SRE.

Introduction

During the last decade life science research under altered gravity conditions has become a field of increasing interest. Although the mechanism of gravity sensation in plant cells is relatively well understood (Halstead and Dutcher, 1987), the knowledge of effects of altered gravity on animal cells is still rather limited. Extensive studies of gravity effects on mammalian cells were performed by Cogoli and coworkers, who showed that mitogenic stimulation of human lymphocyte cultures by the plant lectin concanavalin A (Con A) is almost completely depressed under microgravity (Cogoli *et al.* 1984; Bechler *et al.* 1986), and that hypergravity enhances the stimulation of lymphocytes by concanavalin A (Con A) (Lorenzi *et al.* 1986) as well as the proliferation of other mammalian cells (Tschopp and Cogoli, 1983).

How gravity alterations affect cell proliferation remains to be established. Possible clues for the underlying molecular mechanisms have been obtained from recent studies indicating that gravity may exert its effect by modulating the expression of growth regulatory genes, such as proto-oncogenes. Hypergravity appears to stimulate the proliferation of human HeLa cells through a reduction of the G_1 phase duration, and enhances the expression in these cells of the c-*myc* proto-oncogene (Kumei *et al.* 1989), whose product is known to play an important role in cellular proliferation (Kelly and Siebenlist, 1986). We have demonstrated that in human A431 cells epidermal growth factor (EGF)-induced expression of the c-*fos* proto-oncogene is significantly decreased under simulated weightlessness conditions, whereas it is enhanced by hypergravity (de Groot *et al.* 1990*a*). In addition, we found that EGF-induced cell rounding is modulated by gravity changes (Rijken *et al.* 1990). Taken together these observations suggest that gravity may affect the intracellular signalling pathways activated by mitogenic stimuli such as growth factors, ultimately resulting in the modulation of proto-oncogene expression.

The gene products of the c-*fos* and c-*jun* proto-oncogene family are known for their prominent role in cell proliferation (reviewed by Imler and Wasylyk, 1989) and differentiation (Müller and Wagner, 1984; de Groot *et al.* 1990*b*). Their expression is usually rapidly induced by growth factors and can be induced also by a variety of agents that bypass the receptor and mimic the partial activation of signal transduction pathways (for a review, see Verma and Sassone-Corsi, 1987). Examples of these latter agents are phorbol esters (e.g. 12-*O*-tetradecanoyl-phorbol-13-acetate, TPA), Ca^{2+} (e.g. A23187) and agents that raise the intracellular concentration of cyclic AMP (e.g. forskolin).

In this study we demonstrate that in A431 cells EGF-induced c-*fos* and c-*jun* expression is strongly repressed in real microgravity, probably as a result of changes in promoter–enhancer activity. In addition, we show that simulated microgravity differentially modulates distinctive signal-transduction pathways, since under these conditions EGF- and TPA-induced c-*fos* expression is decreased whereas A23187- and forskolin-induced c-*fos* expression is not.

Materials and methods

Cells and plasmids

Human A431 epidermoid carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% foetal calf serum (FCS). One to four hours prior to stimulation, the medium was replaced with DMEM–Hepes without serum. As probe for Northern hybridization studies a 0.8 kb

(kilobase) *PstI* fragment of v-*fos* (Curran *et al.* 1982) was used. Chloroamphenicol acetyltransferase (CAT) reporter plasmids including pSV2CAT have already been described (Gorman *et al.* 1982). The thymidine kinase promoter from herpes virus (positions −109/+57) was fused to the CAT structural gene and used as a background for the analysis of SRE sequences in the transfection experiments. SRE/DSE$_3$tkCAT is described elsewhere (de Groot and Kruijer, 1990).

RNA isolation and Northern blotting

Total cellular RNA was isolated by the guanidine isothiocyanate–caesium chloride method (Chirgwin *et al.* 1979). A 15 μg sample of total RNA was denatured for 10 min at 68°C in 50% (v/v) formamide, 2.2 M formaldehyde, 20 mM 3-(*N*-morpholino)propane sulfonic acid (Mops), pH 7.0, 5 mM sodium acetate, 1 mM ethylenediamine tetraacetic acid (EDTA), separated through 0.8% agarose/2.2 M formaldehyde gels, and subsequently transferred to nitrocellulose filters (BA 85, Schleicher & Schuell) in 20× standard sodium citrate (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). RNA was immobilized by heating at 80°C for 2 h under vacuum. Hybridization was performed in 50% formamide, 5×SSC, 50 mM sodium phosphate, pH 6.8, 10 mM EDTA, 0.1% SDS, 0.1 mg ml^{-1} of sonicated salmon sperm DNA, 2×Denhardt's solution (1×Denhardt's solution contains 0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone) at 42°C overnight. ^{32}P-labeled probes were generated using a multiprime DNA-labeling kit (Amersham). After hybridization and washing, filters were exposed to Kodak XAR-5 film at −70°C using intensifying screens.

RNase protection analysis

RNase protection analysis was performed according to the method of Melton *et al.* (1984). A 1–2 μg sample of total cellular RNA was hybridized to ^{32}P-labeled complementary RNA probes derived from the human c-*fos* gene (Van Straaten *et al.* 1983), the human c-*jun* gene (Angel *et al.* 1988) and the human β-2-microglobulin gene (Suggs *et al.* 1981). After RNase digestion of the single-strand transcripts, protected fragments of 110, 155 and 80 nucleotides indicate expression of c-*fos*, c-*jun* and β-2-microglobulin, respectively.

DNA transfection and transient expression assays

A431 cells were plated in DMEM-Bic/7.5% FCS at 4×10^6 cells per 75 cm^2 tissue culture flask 24 h prior to transfection. Two hours before transfection, the flasks received fresh medium. Cells were incubated for 20–24 h with calcium phosphate-precipitated DNAs (10–20 μg plasmid per flask), followed by trypsinization of the cells and replating on clinostat coverslips. After 24 h, the medium was replaced by DMEM-Bic without FCS. After incubation for 2 h, the cells were mounted in clinostat tubes, rotated and stimulated with or without EGF. After harvesting the cells and measuring protein concentrations, CAT activity was determined. A 20 μg sample of cell extract was used for CAT assays (2 μg for pSV2CAT-transfected cells). A 1 μg sample of pSV2Apap DNA was always included to serve as internal control to correct for possible variations in the transfection efficiency. PAP assays were performed as described by Henthorn *et al.* (1988). CAT activity was determined as described by Gorman *et al.* (1982) and was quantified by liquid scintillation counting of TLC plate ^{14}C spots.

Clinostat experiments

For clinostat experiments, a portable fast-rotating clinostat developed in cooperation with CCM (Centre for Construction and Mechanization, Nuenen, The Netherlands) was used. All experiments were performed at 60 rotations min^{-1} at 37°C.

MASER-3 sounding rocket experiment

A431 cells were cultured on coverslips and mounted into the CIS-1 plunger box experiment units (CCM, Nuenen; see Fig. 1). The experiment units were assembled in boxes and loaded in the CIS-1 module (Fokker Space and Systems, Amsterdam, The Netherlands) in the payload of the rocket. The temperature of the experiment units was kept at 37°C during the whole experiment by actively controlling the temperature of the experiment boxes. After microgravity was reached in the rocket, the cells were stimulated with EGF (100 ng ml^{-1}) or medium alone (−EGF) by activation of plunger A (see Fig. 1). After 6 min the cells were washed with phosphate-buffered saline (PBS) (plungers B and D) and lyzed in guanidine isothiocyanate (plunger E). After recovery of the payload, RNA was isolated as described above.

Results

Previously we have shown that EGF-induced expression of the c-*fos* proto-oncogene is significantly decreased under simulated hypogravity conditions (de Groot *et al.* 1990*a*). To investigate whether this effect also occurs in real microgravity, we performed an experiment in the CIS-1 module flown on the MASER-3 sounding rocket (Swedish Space Cooperation, April, 1989). A431 cells were cultured in CIS-1 plunger box experiment units (Fig. 1) and stimulated with EGF (100 ng ml^{-1}) or medium alone (−EGF) for 6 min after microgravity was reached in the rocket. Following recovery of the experiment units, RNA was isolated from the lyzed cells and analyzed for c-*fos* transcripts by RNase protection. As shown in Fig. 2, no c-*fos* transcripts were detected in unstimulated (−EGF) cells or in the 1 *g* control experiment (Ground), which was performed simultaneously with the flight experiment, or in the microgravity samples (Flight). By contrast, the 1 *g* samples that were stimulated with EGF for 6 min showed strong expression of the c-*fos* gene, which was decreased by about 50% in the cells that were treated with EGF in microgravity.

Since the product of the c-*fos* gene is known to form a functional heterodimeric complex with the product of the c-*jun* proto-oncogene (reviewed by Busch and Sassone-Corsi, 1990), the expression of the c-*jun* gene was studied. As shown in Fig. 2, c-*jun* expression was not detectable in

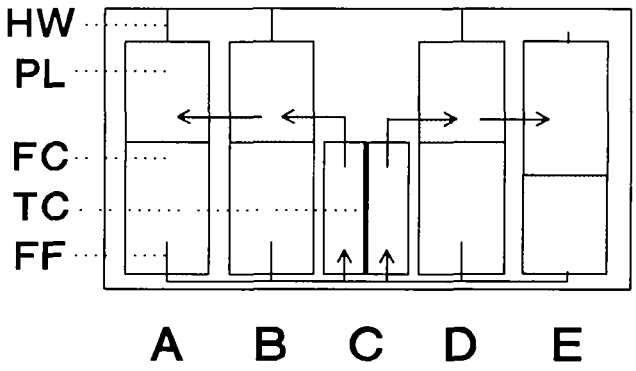


Fig. 1. Schematic representation of the CIS-1 plunger-box experiment unit. The CIS-1 plunger-box experiment unit is a slight modification of the unit used in the FROGS experiment flown on spacelab D1 (Ubbels, 1987) and was designed and constructed by CCM (Nuenen, The Netherlands). A transverse section of the module is shown. A431 cells are cultured on thermanox coverslips (TC) and mounted in the central chamber (C). After activation of plunger (PL) A by melting of a heater wire (HW), the cells are treated with EGF or medium alone, which is present in the fluid compartment (FC). Washing of the cells is performed by activation of plungers B and D, while the cells are lyzed by activation of plunger E. Plunger E is shown in the activated position. The route of the various solutions is indicated by arrows. After recovery, the lyzed cells are collected from chamber C and RNA is isolated.

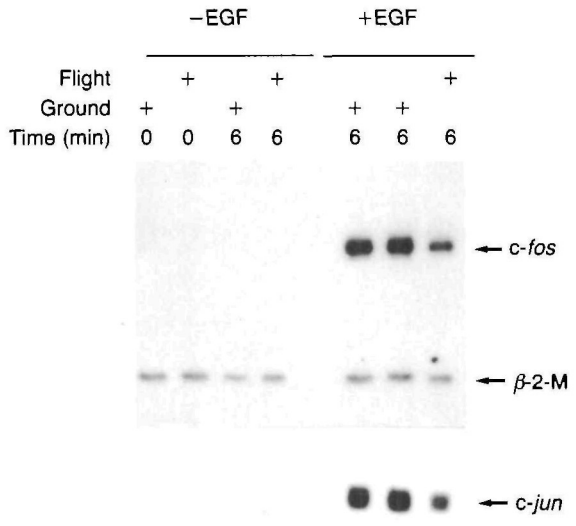
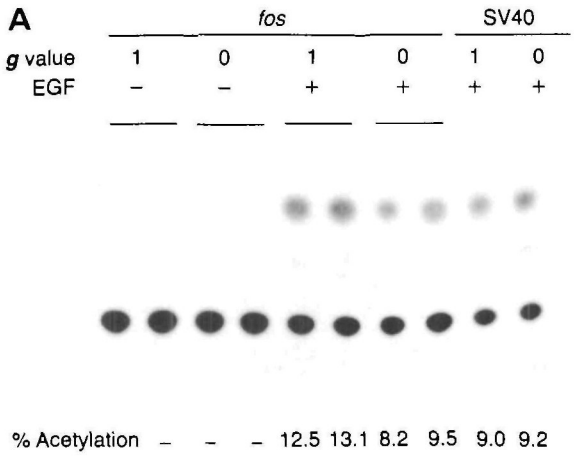


Fig. 2. Microgravity decreases EGF-induced c-*fos* and c-*jun* expression. A431 cells were cultured on Thermanox coverslips, mounted in the CIS-1 plunger box experiment units and assembled in the CIS-1 module on the MASER-3 sounding rocket. As soon as microgravity was reached, the cells were treated with EGF (+EGF, 6 min) or with medium alone (−EGF, 6 min). As a control one sample was lyzed immediately after launch (−EGF, 0 min) to study the effects of the high ***g*** values reached during the launch of the rocket. Simultaneously a 1 ***g*** reference experiment (Ground) was performed identically to the microgravity experiment (Flight). After all samples were washed and lyzed, RNA was isolated and analyzed for c-*fos*, c-*jun* and β-2-microglobulin (β-2-M) transcripts by RNase protection. Autoradiography was for 16 h.

unstimulated cells, whereas it was decreased by about 50 % in the EGF-treated flight sample as compared to EGF-treated 1 ***g*** control cells. Expression of the β-2-microglobulin gene, a gene that is not modulated by EGF treatment, was constant in all flight and ground samples, indicating that the observed decrease in expression of c-*fos* and c-*jun* is specific for EGF-induced signal transduction in these cells.

The induction of c-*fos* by EGF is mediated by the serum response element (SRE) present in the 5′ regulatory region of the c-*fos* gene (Gilman *et al.* 1986; Treisman, 1986; Greenberg *et al.* 1987). This sequence binds at least three different regulatory proteins, of which p67-SRF (serum response factor) is probably mediating the effects of serum and EGF on c-*fos* expression (Treisman, 1987). To assess whether the observed effects of microgravity on EGF-induced c-*fos* expression are caused by a modulation of SRE activity, we coupled three copies of the c-*fos* SRE to a heterologous promoter (HSV (herpes simplex virus) thymidine kinase, TK) and the bacterial chloroamphenicol acetyl transferase (CAT) gene. A431 cells were transfected with this construct (named *fos* in Fig. 3) and replated on clinostat slides after 24 h. The slides were mounted on a fast-rotating clinostat after attachment of the cells for 24 h and rotated at 60 revs min^{-1} for another 2 h. Hereafter, the cells were either treated with EGF (100 ng ml^{-1}) or medium alone for 8 h. Collection of the cells and assaying for CAT activity was performed as described in Materials and methods. As shown in Fig. 3A, no CAT activity was detected in cells treated with medium alone, while treatment with EGF leads to a strong enhancement of CAT activity. Comparison of EGF-treated cells that were rotated on the clinostat (0 ***g***) with cells cultured in fixed clinostat tubes (1 ***g***) shows that simulated hypogravity decreased EGF-induced SRE activity. The activity of the simian virus 40 (SV40) promoter, that is transcriptionally active in these cells, was not modulated by simulated hypogravity, indicating that the decrease is specific for EGF-induced SRE activity. Quantification of CAT activity of four independent experiments shows that EGF-induced SRE activity (*fos* in Fig. 3B), but not the activity of the SV40 promoter, is decreased by about 30 % under simulated hypogravity conditions (Fig. 3B). These results show that the observed decrease in EGF-induced c-*fos* expression is most likely caused by a decrease in SRE activity.

A variety of different signal transduction pathways lead to rapid increase of c-*fos* expression (reviewed by Verma and Sassone-Corsi, 1987). To examine if the observed modulations of c-*fos* expression by gravity changes are specific for EGF-induced signal transduction, we studied



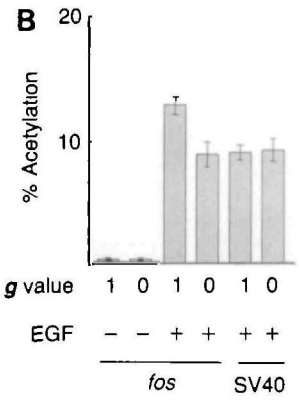


Fig. 3. Hypogravity decreases SRE activity. (A) A431 cells cultured in 75 cm^2 flasks were transfected with 10 μg of a construct containing three copies of the c-*fos* serum response element (SRE; Treisman, 1986) coupled to the HSV thymidine kinase promoter and the bacterial CAT gene (*fos* in the figure) by $Ca_3(PO_4)_2$ precipitation. Control cells were transfected with an SV40-driven CAT gene (pSV2CAT; Gorman *et al.* 1982). After 24 h the cells were trypsinized and replated on Thermanox coverslips for 24 h. The cells were mounted on a fast rotating clinostat and rotated for 2 h at 60 revs min^{-1} at 37 °C. EGF (+EGF) or medium alone (−EGF) was added and after rotation in the clinostat for 8 h the cells were collected and CAT activity was determined on TLC plates. A 20 μg sample of cell extract was routinely used for CAT assays (2 μg for pSV2CAT-transfected cells). Control cells were incubated identically in non-rotating fixed clinostat tubes (1 ***g*** in figure). B. The CAT activity of 4 independent experiments as in A was quantified by liquid scintillation counting of ^{14}C-labeled TLC spots. Error bars indicate standard deviation.

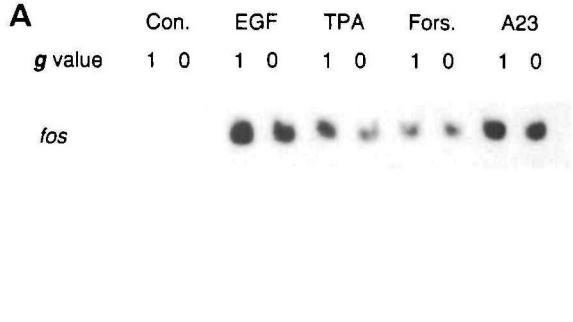


Fig. 4. Differential sensitivity of discrete signal transduction pathways for hypogravity. A. A431 cells were cultured on Thermanox coverslips for 48 h before mounting on a fast-rotating clinostat. Cells were rotated at 60 revs min^{-1} (0 *g* in figure) or cultured in fixed tubes for 2 h before addition of EGF (100 ng ml^{-1}), TPA (100 ng ml^{-1}), forskolin (Fors., 10 μM), A23187 (A23, 2.5 μM) or medium alone (Con.). After 15 min the cells were lyzed and RNA was isolated. c-*fos* expression was determined by Northern blotting as described in Materials and methods. Autoradiography was for 24 h. B. The results of 3 independent experiments, as in A, were quantified by scanning of the autoradiographs. Bars represent the ratio of c-*fos* expression in control cells (1 *g*) and rotated cells (0 *g*). Error bars represent the standard deviation.

the effects of simulated hypogravity on c-*fos* expression induced by the phorbol ester TPA, the Ca^{2+} ionophore A23187 and the activator of protein kinase A, forskolin. Cells were cultured for 40 h on clinostat slides before mounting on a fast-rotating clinostat. After rotation at 60 revs min^{-1} for 2 h, the cells were treated with EGF (100 ng ml^{-1}), TPA (100 ng ml^{-1}), forskolin (10 μM), A23187 (2.5 μM) or medium alone (CON) for 15 min. RNA was isolated and analyzed for c-*fos* expression by Northern blotting. As shown in Fig. 4A, both EGF- and TPA-induced c-*fos* expression was decreased under hypogravity conditions (0 *g* in the figure), whereas forskolin- and A23187-induced c-*fos* expression remained constant. Scanning of the autoradiographs of four independent experiments shows that both EGF- and TPA-induced c-*fos* expression are significantly decreased in hypogravity (25 % and 30 % respectively), while no significant differences were found with A23187 or forskolin. These results clearly demonstrate that only a subset of signal transduction pathways that lead to c-*fos* induction is sensitive to gravity alterations, and they are encouraging for further exploration of the effects of altered gravity conditions on mammalian cell proliferation and intracellular signal transduction.

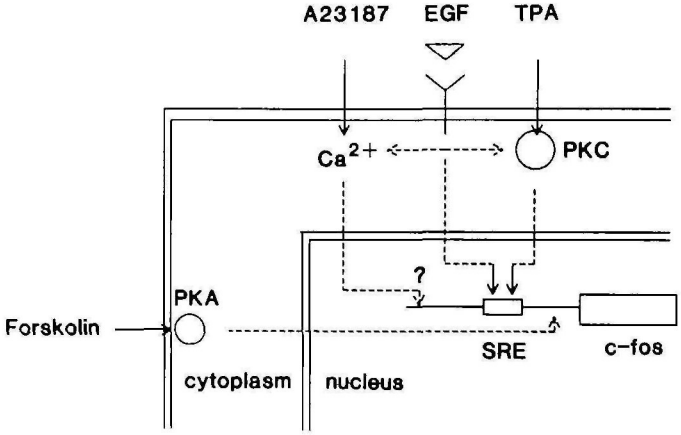


Fig. 5. Schematic representation of different signal transduction pathways leading to enhanced c-*fos* expression.

Discussion

Life science research under altered gravity conditions is important in determining the capacity of cells and organisms to cope with the problems of gravitational stress. Moreover, it might lead to clues on the functioning of cells and organisms under normal gravity conditions. In this report we show that EGF-induced expression of the c-*fos* and c-*jun* proto-oncogenes is severely decreased in real microgravity, extending our results from previous studies in simulated hypogravity (de Groot *et al.* 1990*a*). At least for the c-*fos* gene this effect is probably caused by decreased activity of the SRE located in its 5′ regulatory sequences. Studying other agents that induce c-*fos* expression clearly shows that only a subset of signal transduction pathways is sensitive to gravity changes.

A recent study has shown that hypergravity caused by centrifugation enhances the expression of the c-*myc* proto-oncogene in proliferating HeLa cells in the absence of EGF (Kumei *et al.* 1989). Although no changes in c-*fos* expression were found in these cells, our results are probably in agreement with this study, since we only detect changes in c-*fos* expression in cells stimulated with growth factors or phorbol esters and not in unstimulated resting (no FCS, see Fig. 2 and 4A) or proliferating cells (7.5 % FCS, unpublished data). In addition, we previously showed an enhancement of c-*fos* expression in hypergravity in EGF-treated, but not in untreated A431 cells (de Groot *et al.* 1990*a*). The molecular basis of the differences between c-*fos* and c-*myc* sensitivity to gravity changes remains to be determined, but may involve the high constitutive expression of c-*myc* as opposed to the very low basal level expression of c-*fos* in unstimulated cells.

EGF-induced c-*fos* expression is mediated by the SRE present in the c-*fos* promoter–enhancer region (see Fig. 5; and Gilman *et al.* 1986; Treisman, 1986; Greenberg *et al.* 1987). This sequence binds at least three different proteins, of which p67-SRF is thought to mediate the effects of serum and EGF on c-*fos* expression (Treisman, 1987). Treatment of cells with EGF or serum leads to rapid phosphorylation of SRF, a process likely to be important in the transcriptional activation of c-*fos* (Prywes *et al.* 1988). Our results demonstrate that activation of the SRE by EGF is decreased under simulated hypogravity conditions, and suggest that this decrease is the basis for the observed effects of microgravity on EGF-induced c-*fos* expression. In this respect it is worthwhile to mention that TPA-induced c-*fos* expression, a process that we show to be decreased in hypogravity, is also mediated by the SRE (see Fig. 5; and Gilman, 1988; Stumpo *et al.* 1988). By contrast, the effects of forskolin and A23187 on c-*fos* expression are not

sensitive to gravity changes, while these agents exert their effects on c-*fos* expression through regulatory sequences distinct from the SRE (see Fig. 5; and Fisch *et al.* 1987; Gilman, 1988). The mechanism by which SRE activity is decreased under hypogravity conditions remains to be determined, but one could speculate that EGF-induced phosphorylation of p67-SRF might somehow be modulated by gravity changes.

A variety of different agents induce expression of the c-*fos* proto-oncogene. Our results show that EGF and TPA, but not forskolin- and A23187-induced c-*fos* expression is decreased in hypogravity. EGF exerts its effects through binding to its plasma-membrane-located receptor, followed by the activation of an intracellular signal transduction cascade (reviewed by Carpenter, 1987). Interestingly, activation of protein kinase C (PKC), the receptor for the phorbol ester TPA, is a part of this signal transduction cascade (Fig. 5). This suggests that PKC activity, or the activity of a downstream target of PKC might be sensitive to gravity alterations. By contrast, treatment with either EGF or A23187 leads to a rise in intracellular Ca^{2+} (Fig. 5), suggesting that this process or its downstream targets are probably not influenced by gravity changes. Forskolin raises the intracellular cyclic AMP concentration *via* activation of protein kinase A (PKA). Until now, no second messengers have been identified that are shared by both EGF- and forskolin-induced signal transduction (Fig. 5), further implicating PKC or one of its targets in gravity-dependent modulations of mammalian signal transduction.

The products of the c-*fos* and c-*jun* genes are known to form functional heterodimeric complexes (reviewed by Busch and Sassone-Corsi, 1990), and are implicated in the control of proliferation (reviewed by Imler and Wasylyk, 1989) and differentiation (Müller and Wagner, 1984; de Groot *et al.* 1990*b*). Our study shows that EGF-induced expression of these genes is strongly reduced in microgravity. Since a number of studies have shown gravity-dependent modulations of mammalian cell proliferation and differentiation (Cogoli *et al.* 1984; Bechler *et al.* 1986; Lorenzi *et al.* 1986; Duke, 1983), our findings suggest that gravity-dependent effects on c-*fos* and c-*jun* expression are likely to be of major importance for the cellular reaction to gravitational stress. Further study of the regulation of these and other regulatory genes will hopefully lead to a more complete understanding of the way gravity exerts its effects on mammalian cells.

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