Effects of fluorinated inositols on the proliferation of Swiss 3T3 fibroblasts

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The six monodeoxyfluoro-*myo*-inositols (*n*FIns) have previously been synthesized as potential inhibitors of signalling pathways mediated by phosphoinositides and their derivatives. Each of the six *n*FIns isomers was introduced into Swiss 3T3 fibroblasts by the techniques of microinjection or scrape loading at intracellular concentrations of approx. 2–4 mM. Of the six *n*FIns analogues, only 3FIns and 5FIns inhibited the serum-stimulated proliferation of 3T3 fibroblasts assayed by cell counting. Proliferation was inhibited to a similar extent by 3FIns or 5FIns, irrespective of which technique was used to introduce the *n*FIns analogues into the cells. Proliferation of cells 35 h after serum stimulation (i.e. when the first cell cycle was completed in control cells) was

inhibited by approx. 50 % by both 3FIns and 5FIns, and entry into S phase in the first cell cycle was inhibited to the same extent. This indicated that the *n*FIns analogues were inhibiting proliferation in the G₁ phase of the cell cycle. Proliferation during the second cell cycle (35–60 h after stimulation) was inhibited by 75–85 %. The inhibitory *n*FIns analogues were not toxic to the cells, nor did they affect the cellular ATP/ADP ratio. The effectiveness of the *n*FIns analogues in inhibiting proliferation was directly correlated with their ability to be incorporated into phosphatidylinositol analogues, suggesting that they may act by modulating phosphoinositide signalling pathways or other functions essential for DNA synthesis.

INTRODUCTION

Receptor-mediated stimulation of PtdIns metabolism has been implicated in the transduction of a variety of mitogenic signals. There are two known pathways involving PtdIns metabolism which may be important in mitogenesis. The first involves the hydrolysis of PtdIns(4,5)P₂ by phospholipase C- γ (PLC- γ), generating diacylglycerol and inositol phosphates, which activate protein kinase C and increase intracellular Ca²⁺ respectively (Nishizuka, 1984; Berridge, 1984). PLC- γ associates with activated receptor tyrosine kinases and tyrosine phosphorylation is thought to be essential for PLC- γ activity. The second pathway involves the formation of phosphoinositides phosphorylated at the 3-position by PtdIns 3-kinase, activated by tyrosine kinase receptors either by direct tyrosine phosphorylation (Auger et al., 1989; Coughlin et al., 1989; Traynor-Kaplan et al., 1989; Varticovski et al., 1991; Hawkins et al., 1992) or indirectly by binding to tyrosine-phosphorylated proteins (Backer et al., 1992).

At present, there is conflicting evidence concerning whether PtdIns(4,5)P₂ breakdown is essential for progression through G₁ phase to DNA synthesis. Microinjection of antibodies to PtdIns(4,5)P₂ in NIH 3T3 fibroblasts inhibits PtdIns(4,5)P₂ breakdown and the mitogenic effects of platelet-derived growth factor and bombesin, but not of epidermal growth factor, fibroblast growth factor (FGF) or insulin, which do not cause phosphoinositide breakdown in fibroblasts (Furakami et al., 1988; Matuoka et al., 1988). In rat L6 myoblasts, in which FGF causes PtdIns(4,5)P₂ breakdown, mutant FGF receptors which fail to associate with PLC- γ nevertheless mediate DNA synthesis in response to FGF (Mohammadi et al., 1992; Peters et al., 1992). The role of PtdIns 3-kinase in signal transduction has not been elucidated. The ligand-stimulated synthesis of 3-phosphorylated phospholipids has been shown to occur in a variety of cell types (Auger et al., 1989; Traynor-Kaplan et al., 1989; Hawkins et al., 1992), but it is still not clear which, if any, of the 3-phosphorylated phosphoinositide intermediates play essential roles in mitogenic signalling.

Specific inhibitors of phosphoinositide metabolism are therefore potentially useful in establishing the role of different phosphoinositide pathways in mitogenesis. The modulating action of fluorinated *myo*-inositol analogues (*n*FIns) depends on a variety of factors. These include their uptake by the cells and the extent of any inhibition of *myo*-inositol uptake, the capacity of *n*FIns analogues to act as substrates or inhibitors of PtdIns synthase, and their consequent effects on the formation of polyphosphorylated phosphoinositides and their fluorinated analogues. We have recently shown that 1D-3-deoxy-3-fluoro-*myo*-inositol (3FIns) and 1D-5-deoxy-5-fluoro-*myo*-inositol (5FIns) were both incorporated rapidly into phosphoinositides after addition to permeabilized thymocytes. In contrast, 1D-1-deoxy-1-fluoro-*myo*-inositol (1FIns), 1D-2-deoxy-2-fluoro-*myo*-inositol (2FIns) and 1D-4-deoxy-4-fluoro-*myo*-inositol (4FIns) were not incorporated into phospholipid under the same conditions, and 1D-6-deoxy-6-fluoro-*myo*-inositol (6FIns) was very poorly incorporated (0.5 % of the amount of *myo*-inositol incorporated; Offer et al., 1993). The 3FIns, 5FIns and 6FIns all formed phospholipids with chromatographic properties which corresponded to those of substituted PtdIns and PtdIns mono-phosphates.

Each of the six *n*FIns analogues were introduced into fibroblasts to compare their effects on proliferation with their incorporation into phosphoinositides (Offer et al., 1993). Quiescent Swiss 3T3 fibroblasts were loaded with *n*FIns analogues by the techniques of microinjection or scrape loading to achieve high intracellular concentrations of the compounds and to by-pass their slow uptake into the intact cells, which we have previously demonstrated in intact murine thymocytes.

MATERIALS AND METHODS

*n*FIns analogues

The mono-fluorinated analogues of *myo*-inositol were synthesized by procedures described in Offer et al. (1992).

Abbreviations used: *n*FIns, *n*-fluoro-*n*-deoxy-*myo*-inositols; PLC, phospholipase C; FGF, fibroblast growth factor; DMEM, Dulbecco's modified Eagle medium; FCS, fetal-calf serum.

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Cell culture and [^3H]thymidine incorporation

Swiss 3T3 fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM; Flow Laboratories) containing 10% foetal-calf serum (FCS; Flow Laboratories), 44 mM NaHCO_3 , 8 mM D-glucose, 35 units/ml penicillin G and 80 m-units/ml streptomycin in a humidified atmosphere of CO_2 /air (1:9), at 37 °C. For microinjection experiments cells were seeded at approx. 50% confluence on to plastic Petri dishes deeply scored with grids (approx. 1 mm²) to prevent cell migration beyond the grid lines. For scrape loading, cells were seeded on plastic Petri dishes and allowed to reach 70% confluence before loading with *n*FIns.

To assay DNA synthesis, 1 $\mu\text{Ci/ml}$ [^3H]thymidine was added to the cells at the time of serum stimulation. Cells were lysed in 1% SDS, and lysates were precipitated with 15% trichloroacetic acid and collected by filtration through Whatman GF/C filters. Filters were dried and immersed in Optiphase HiSafe II scintillant for scintillation counting.

Scrape loading

Cells in 30 mm Petri dishes were washed twice in scrape-loading buffer (114 mM KCl, 15 mM NaCl, 5.5 mM MgCl_2 , 10 mM Tris/HCl, pH 7.2) at 37 °C, before adding 30 μl (180 μl for re-stimulation) of reagent or buffer. The cells were gently lifted off the Petri dishes with a rubber policeman and resuspended after 1–2 min in 600 μl of DMEM containing 10% serum. The cells were allowed to re-attach for 2 h on 30 mm dishes coated with rat tail type I collagen (Sigma) before serum deprivation for 22 h and subsequent restimulation with 10% FCS. Cells were scrape loaded in the presence of 200 mM *n*FIns analogue, and a concentration of 5 mM fluorinated inositols was maintained in the external medium throughout the experiment unless otherwise indicated. Cells (200–300 per sample) were counted by using a microscope grid (0.5–1 mm²).

Microinjection

Cells were grown on gridded Petri dishes, and 200 mM *n*FIns in scrape loading buffer was microinjected. Eppendorf 'Femtotips' were loaded with 2 μl of sample (scrape-loading buffer or *n*FIns) by using Eppendorf micro-loaders. Microinjections of *n*FIns isomers into the cytosol of 3T3 fibroblasts to give an estimated intracellular concentration of 2–4 mM were performed on a Zeiss Axiovert 35M microscope by using an Eppendorf micro-manipulator in a stage compartment in a humidified atmosphere of CO_2 /air (1:9) at 37 °C. Intracellular concentrations of the *n*FIns analogues were estimated as 2–4 mM by control experiments in which several hundred cells were co-injected with a dextran (3 kDa) labelled with fluorescein (FITC-dextran) or the fluorescent indicator, Quene 1 (Rogers et al., 1983). The amount of FITC-dextran was estimated by lysing the cells and comparing the fluorescence intensity of the released FITC-dextran with that of samples of the stock FITC-dextran solution added to the lysed cell suspension. The intracellular concentration of FITC-dextran (or Quene 1) was calculated by assuming an average intracellular volume for 3T3 fibroblasts of 2.0 pl.

Immediately after microinjection with *n*FIns, cells were serum-deprived for 24 h before re-stimulation. In some experiments the same *n*FIns (5 mM) was added to the external medium immediately after microinjection and was maintained in the medium throughout the experiment. Control cell samples were injected with scrape-loading buffer under the same conditions as used for the *n*FIns analogues, and further control cell samples were not injected. Cells were counted on grids as described for the scrape-loading experiments.

Measurement of ATP/ADP ratios

Cells (10³/sample in 200 μl) in microtitre-plate wells were incubated with [^3H]adenine (50 $\mu\text{Ci/ml}$) for 4 h at 37 °C under the same conditions as for cell culture. The cells were washed 5 times with medium and incubated for a further 2 h. Lysis was carried out without removing the cells from the incubator by removing the medium and adding 50 μl of 5% acetic acid to the cells. The lysate was loaded on to a Polygram Cel 300 PEI t.l.c. plate (CamLab). Chromatography was carried out in 0.75 M KH_2PO_4 , pH 3.5. Separated nucleotides were revealed under u.v. light at 254 nm. The bands were cut out, placed in scintillation vials and wetted with a solution of 0.7 mM MgCl_2 and 20 mM Tris, pH 7.4, before addition of 4 ml of Optiphase scintillant for counting.

RESULTS

Effect of microinjected 3FIns and 5FIns on cell division

Sub-confluent proliferating Swiss 3T3 fibroblasts were incubated in serum-free DMEM in the presence of 5 mM 3FIns or 5FIns for 24 h. The quiescent cells were re-stimulated by addition of DMEM containing 10% FCS and 5 mM *n*FIns, and cell division was assayed by cell counting 35 h later. At this time, control cells in the absence of *n*FIns had completed one cell cycle (Table 1). There was no significant decrease in the proportion of cells (> 89%) which had divided by 35 h after addition of serum in the presence or absence of either *n*FIns in the medium (Table 1).

Since addition of extracellular *n*FIns had no effect on cell proliferation, microinjection was used to introduce the *n*FIns analogues into the cells. Each analogue was microinjected into sub-confluent proliferating fibroblasts in DMEM containing 10% FCS. The stock concentration of the microinjected *n*FIns was 200 mM and the final intracellular concentration was estimated at 2–4 mM (see the Materials and methods section). Immediately after injection, the cells were incubated in serum-free DMEM for 24 h before stimulation with medium containing 10% FCS. After 35 h, 60 $\pm$ 5% of the cells microinjected with

Table 1 Effect of *n*FIns on cell division

Extracellular *n*FIns: sub-confluent proliferating Swiss 3T3 fibroblasts were incubated in serum-free DMEM containing 5 mM *n*FIns (25 μl of 200 mM *n*FIns stock solution/ml) or 25 μl of scrape-loading buffer/ml. After 24 h, cells were stimulated with DMEM containing 10% FCS and 5 mM *n*FIns or scrape-loading buffer and counted 35 h later. A minimum of 200 cells were counted for each sample (values are means $\pm$ S.D., $n = 3$). Microinjected *n*FIns: sub-confluent proliferating Swiss 3T3 fibroblasts were microinjected with 200 mM *n*FIns or an equal volume of scrape-loading buffer. Immediately after injection, cells were incubated in serum-free DMEM for 24 h. Cells were then stimulated with DMEM containing 10% FCS and counted as described above (means $\pm$ S.D., $n = 3$). Microinjected and extracellular *n*FIns: sub-confluent proliferating Swiss 3T3 fibroblasts were microinjected with 200 mM *n*FIns or an equal volume of scrape-loading buffer. Immediately after injection, cells were incubated in serum-free DMEM in the presence of 5 mM *n*FIns (25 μl of 200 mM *n*FIns stock solution/ml) or 25 μl of scrape-loading buffer/ml for 24 h. Cells were then stimulated with DMEM containing 10% FCS and 5 mM *n*FIns (25 μl of 200 mM *n*FIns stock solution/ml) or 25 μl of scrape-loading buffer/ml, before counting as described above (means $\pm$ S.D., $n = 3$).

	Increase in cell number (%) from 0 to 35 h		
	Extracellular <i>n</i> FIns	Microinjected <i>n</i> FIns	Microinjected and extracellular <i>n</i> FIns
Buffer	97 $\pm$ 2	89 $\pm$ 4	90 $\pm$ 4
5FIns	89 $\pm$ 4	60 $\pm$ 5	48 $\pm$ 4
3FIns	92 $\pm$ 5	74 $\pm$ 8	50 $\pm$ 7

Figure 1 Effect of microinjected *n*FIns on cell division

Sub-confluent proliferating Swiss 3T3 fibroblasts were microinjected on gridded plates with 200 mM 5FIns (□), 200 mM 3FIns (■), or buffer (○). Cells were immediately incubated in serum-free DMEM containing 5 mM *n*FIns (25 μ l of 200 mM *n*FIns stock solution/ml) or 25 μ l of scrape-loading buffer/ml. After 24 h, cells were stimulated with DMEM containing 10% FCS and 5 mM *n*FIns (25 μ l of 200 mM *n*FIns stock solution/ml) or 25 μ l of scrape-loading buffer/ml, and the cells were counted at the times indicated. At least 200 cells were counted for each sample (values are means $\pm$ S.D., $n = 3$).

5FIns and $74 \pm 8\%$ of cells injected with 3FIns had divided (Table 1). Under the same conditions, $89 \pm 4\%$ of cells microinjected with buffer alone had undergone one round of cell division.

For some samples in the same set of experiments, the *n*FIns analogue which had been injected into the cells was also added at 5 mM to the serum-free medium immediately after microinjection. The *n*FIns was left in the medium throughout the 24 h quiescence period and the subsequent 35 h of re-stimulation with serum. In the presence of 5 mM 5FIns or 3FIns in the medium, the increases in cell number were $48 \pm 4\%$ and $50 \pm 7\%$ respectively. Thus addition of extracellular *n*FIns to the injected cells enhanced the inhibition of cell proliferation (Table 1).

The data for 3FIns and 5FIns showed that the concentrations of the analogues used caused only partial inhibition of cell proliferation over the first cell cycle. To determine whether the maximal inhibitory effects of the *n*FIns analogues had been achieved, longer experiments were performed to assay cell division through a second cell cycle. Sub-confluent fibroblasts were microinjected with *n*FIns, and extracellular *n*FIns was added as in previous experiments. Cells were counted 35 h and 60 h after serum stimulation. The inhibition of proliferation by 3FIns and 5FIns 35 h after stimulation was very similar to that described above (compare data in Figure 1 and Table 1). However, between 35 and 60 h after serum stimulation, when the number of control cells increased by $77 \pm 32\%$, cells injected with 5FIns or 3FIns only increased in number by $9 \pm 22\%$ and $16 \pm 20\%$ respectively (Figure 1). These experiments demonstrate that there was much stronger inhibition of proliferation by the *n*FIns isomers over the period when control cells passed through a second cell cycle. However, the data do not allow a distinction to be made between a small proportion of the *n*FIns-treated cells undergoing a second round of division or a large increase in the first cell-cycle time of a small proportion of the cells.

Effect of scrape-loaded 3FIns and 5FIns on cell division

For biochemical analysis of the effects of *n*FIns analogues on the cell cycle, much larger numbers of cells are required than can be

Figure 2 Effect of scrape-loaded *n*FIns on cell division

Confluent Swiss 3T3 fibroblasts were scrape loaded in the presence of scrape-loading buffer (○), 200 mM 5FIns (□) or 200 mM 3FIns (■). The suspension of *n*FIns-loaded cells was diluted in medium containing 10% FCS to obtain a final extracellular *n*FIns concentration of 5 mM and re-plated on collagen-coated gridded dishes. For scrape buffer-loaded cells, scrape-loading buffer was added to the suspension in DMEM containing 10% FCS before re-plating on collagen-coated gridded dishes. After 2 h, the medium was replaced with serum-free DMEM containing 5 mM *n*FIns (25 μ l of 200 mM *n*FIns stock solution/ml) or 25 μ l of scrape-loading buffer/ml. After 24 h the cells were stimulated with DMEM containing 10% FCS and 5 mM *n*FIns (25 μ l of 200 mM *n*FIns stock solution/ml) or 25 μ l of scrape-loading buffer/ml. Cells were counted by using grids on the plates. At least 200 cells were counted for each sample (values are means $\pm$ S.D., $n = 3$).

microinjected. The effects of scrape loading fibroblasts with *n*FIns analogues on cell proliferation were therefore compared with the effects of loading by microinjection. The scrape-loading experiments were designed to be as similar as possible to the microinjection experiments described above. Confluent Swiss 3T3 fibroblasts were washed three times in scrape-loading buffer before scraping in the presence of 200 mM 3FIns or 5FIns. The suspension of loaded cells was diluted 40-fold in medium containing 10% FCS to give an extracellular concentration of 5 mM *n*FIns. Cells were seeded and allowed to re-attach to collagen-coated plates for 2 h at similar cell densities (approx. 50% confluent) to those used in microinjection experiments. The cells were then incubated in serum-free DMEM in the presence of 5 mM *n*FIns for 24 h and stimulated with medium containing 10% FCS and 5 mM *n*FIns. Cells were counted at 40, 60 and 80 h. The increase in number of cells scrape-loaded with 5FIns or 3FIns at 40 h after serum stimulation was $41 \pm 1.4\%$ ($n = 3$) and $43 \pm 0.8\%$ ($n = 3$) respectively, compared with $100 \pm 22\%$ ($n = 3$) for cells scraped in buffer alone (Figure 2). At 80 h after serum stimulation there was a further increase in relative cell number of $17 \pm 2.4\%$ ($n = 3$) for cells loaded with 5FIns and $25 \pm 7\%$ ($n = 3$) for cells loaded with 3FIns. Comparison of the data from microinjection of *n*FIns shown in Figure 1 with the corresponding data for scrape-loaded cells (Figure 2) shows that the effects of introducing *n*FIns analogues into fibroblasts by the two techniques were very similar.

Isomer specificity of inhibition of cell proliferation

In similar experiments to those described above, each of the six *n*FIns isomers was introduced into cells by either microinjection or scrape loading and the cells were counted after one cell cycle in the presence of 5 mM extracellular *n*FIns analogue as described above. Under these conditions, microinjection of 1FIns, 4FIns or 6FIns or scrape loading of 2FIns had no significant effect on proliferation assayed 35 h after serum stimulation compared with cells injected or scraped with buffer alone (Table 2).

Table 2 Effect of fluorinated *myo*-inositol analogues on cell division

Sub-confluent proliferating Swiss 3T3 fibroblasts were microinjected on gridded dishes with scrape-loading buffer or a 200 mM solution of 1FIns, 3FIns, 4FIns, 5FIns or 6FIns and immediately incubated in serum-free DMEM for 24 h in the presence of 5 mM *n*FIns or buffer (see the Materials and methods section). 2FIns was scrape loaded into the cells as described in the legend to Figure 2. Cells were then stimulated with 10% FCS in the presence of 5 mM extracellular *n*FIns (25 μ l of 200 mM *n*FIns stock solution/ml) or 25 μ l of scrape-loading buffer/ml and counted 35 h later. A minimum of 200 cells was counted for each sample (values are means $\pm$ S.D., $n = 3$).

	Increase in cell number (%) from 0 to 35 h
Buffer	115 $\pm$ 15
1FIns	110 $\pm$ 10
2FIns	99 $\pm$ 9
3FIns	57 $\pm$ 9
4FIns	115 $\pm$ 8
5FIns	32 $\pm$ 8
6FIns	96 $\pm$ 12

Figure 3 Effect of scrape-loaded *n*FIns on cell proliferation

Confluent fibroblasts (10^4 cells/sample) were scrape loaded in the presence of scrape-loading buffer ($\circ$), 200 mM 5FIns ($\square$) or 200 mM 3FIns ($\blacksquare$) and allowed to re-attach to collagen-coated wells for 2 h. The cells were then incubated in serum-free DMEM in the presence of 5 mM *n*FIns (25 μ l of 200 mM *n*FIns stock solution/ml) or 25 μ l of scrape-loading buffer/ml for 24 h, before stimulation with 10% FCS with 1 μ Ci of [3 H]thymidine/ml. Cells were counted and then lysed at the times indicated and precipitated with trichloroacetic acid for scintillation counting (values are means $\pm$ S.D., $n = 3$).

Effect of *n*FIns on DNA synthesis

To determine whether the *n*FIns isomers inhibited entry into S phase after stimulation with serum, confluent fibroblasts were washed three times in scrape-loading buffer before scrape loading in the presence of 200 mM 5FIns or 3FIns. The suspension of scrape-loaded cells was diluted, seeded on to collagen-coated dishes and incubated in medium containing 5 mM *n*FIns and 10% FCS to allow the cells to re-attach. After 2 h the medium was replaced with serum-free DMEM containing 5 mM *n*FIns for 24 h before stimulation with medium containing 10% FCS, and [3 H]thymidine was added to the cells at the same time. Incorporation into DNA was measured at 20, 40, 60 and 80 h after stimulation (Figure 3). The data show that approx. 50% of the *n*FIns-loaded cells pass through S phase in the first cell cycle after serum stimulation, which accounts quantitatively for the

Figure 4 Effect of scrape-loaded *n*FIns on ATP/ADP ratios

Confluent Swiss 3T3 fibroblasts (10^4 cells/sample) were scrape loaded in the presence of 200 mM 5FIns ($\blacksquare$), 200 mM 3FIns ($\bullet$) or scrape-loading buffer ($\circ$) and allowed to re-attach on to collagen-coated dishes for 2 h. The suspension of cells loaded with *n*FIns was diluted in serum-free DMEM (1:40 dilution) to obtain a final *n*FIns extracellular concentration of 5 mM. In scrape-loading-buffer-loaded samples 25 μ l of scrape-loading buffer/ml was added to the extracellular serum-free DMEM before re-plating on to collagen-coated dishes. After 24 h, cells were stimulated with DMEM containing 10% FCS and 5 mM *n*FIns (25 μ l of 200 mM *n*FIns stock solution/ml) or 25 μ l of scrape-loading buffer/ml. [3 H]Adenine (50 μ Ci/ml) was added to the cells 4 h before lysis in 5% acetic acid at the times indicated. Lysates were spotted on t.l.c. plates and chromatographed in 0.75 M KH_2PO_4 , pH 3.5. Bands were revealed under u.v. light and excised for scintillation counting. For control non-scraped samples, quiescent 3T3 fibroblasts were stimulated with 10% FCS and 50 μ Ci [3 H]adenine was added 4 h before lysis at the times indicated ($\square$). Chromatography was carried out as described above.

decrease in cell number at 40 h when the first cell cycle is complete in the control cells (see Figure 2).

The lower cell numbers observed after microinjection or scrape loading of *n*FIns into fibroblasts compared with control cell samples might result from cells detaching from the substratum in response to the *n*FIns analogues. However, less than 6% of the trichloroacetic acid-precipitable [3 H]thymidine radioactivity was detectable in the supernatant medium of cells which had been scrape loaded with 5FIns, 3FIns or buffer at 40, 60 or 80 h after stimulation, indicating that the decrease in cell number was not due to loss of adherent *n*FIns-loaded cells.

Effect of *n*FIns on intracellular ATP/ADP ratios

The inhibition of cell division observed by scrape loading or microinjection of 5FIns or 3FIns might be due to non-specific toxic effects of the fluorinated *myo*-inositol analogues within the cells. Intracellular ATP/ADP ratios measurements can be used as an indication of the metabolic status of a population of cells. In Swiss 3T3 fibroblasts, the ATP/ADP ratios remain approximately constant throughout the cell cycle. Stimulation of quiescent fibroblasts with 10% FCS causes no change in the ATP/ADP ratios measured by incorporation of [3 H]adenine into nucleosides (ATP/ADP = approx. 4.4) 20, 40 or 60 h later. Scrape loading of 5FIns or 3FIns did not affect the intracellular ATP/ADP ratios measured 20, 40 or 60 h after serum stimulation compared with unloaded cells or cells loaded with buffer alone (Figure 4). The ratio values of $4.4 \pm 0.2:1$ obtained for fibroblasts may be compared with ratios of 4.9–7.1:1 reported for rat lymphocytes (Ardawi and Newsholme, 1983) and 2.5–5.6:1 for macrophages (Newsholme et al., 1987). These observations suggest that scrape loading of 3FIns or 5FIns does not cause sufficient metabolic stress to alter the ATP/ADP ratios.

DISCUSSION

In a previous study 3FIns was shown to cause dose-dependent inhibition of cell proliferation in normal and *v-sis*-transformed NIH 3T3 fibroblasts stimulated by serum in *myo*-inositol-free medium. The estimated EC_{50} values for the inhibition of proliferation by extracellular 3FIns assayed by cell counting over 4 days were 13 mM for the normal cells and 2 mM for the *v-sis*-transformed cells (Kozikowski et al., 1990). In our experiments, 3 μ M inositol was the minimum concentration required for maximal stimulation of Swiss 3T3 fibroblasts by serum (S. C. Cosulich, unpublished work), consistent with previous data for a wide variety of cells (Eagle et al., 1957). The experiments were therefore performed in DMEM containing normal concentrations of inositol (40 μ M) to ensure that the cells were fully competent to proliferate. Addition of 5 mM of any *n*FIns analogue to the medium did not affect proliferation significantly. The intracellular concentration of *n*FIns achieved by uptake from the medium will depend, in part, on the transport mechanisms and the competition for uptake by *myo*-inositol. The *myo*-inositol concentration in the medium used (DMEM) was 40 μ M, and serum typically contains 65 μ M *myo*-inositol (Flow Laboratories). The total extracellular concentration of inositol in the presence or absence of serum was therefore less than approx. 50 μ M, compared with 5 mM *n*FIns. The data therefore imply that when there is a 100-fold excess of *n*FIns over *myo*-inositol in the medium, uptake of the *n*FIns analogues into the cells is not sufficient to load the cells to concentrations which inhibit proliferation.

Intracellular *n*FIns loaded by microinjection or scraping the cells presumably competes with endogenous *myo*-inositol for incorporation into PtdIns. The concentration of *myo*-inositol in mammalian cells has been estimated as 1–10 mM (Biden and Wollheim, 1986), whereas the estimated intracellular concentration of *n*FIns achieved by microinjection or scrape loading was 2–4 mM. It is therefore likely that the concentrations of *n*FIns were similar to the endogenous *myo*-inositol concentrations in the cells. If it is assumed that the incorporation of 3FIns and 5FIns into analogues of PtdIns is responsible for the inhibition of proliferation (see the discussion of mechanism below), it is clear that cytosolic concentrations of *n*FIns in the mM concentration range are required to synthesize sufficient amounts of fluorinated phosphatidylinositols to inhibit proliferation.

Effect of *n*FIns on cell division

When the uptake mechanisms for *n*FIns analogues into intact cells are by-passed by microinjection or scrape loading, only the 3FIns and 5FIns analogues would be expected to be incorporated into the *n*FIns PtdIns analogues at significant proportions of the total phosphoinositide pool, on the basis of the above indirect estimates. Consistent with this prediction, the inhibition of fibroblast proliferation by the *n*FIns analogues was shown to be specific for 3FIns and 5FIns. Introduction of any of the other fluorinated *myo*-inositol analogues (1FIns, 2FIns, 4FIns or 6FIns) did not affect cell division. This suggests that only 3FIns and 5FIns may act as substrates for PtdIns synthase in Swiss 3T3 fibroblasts, as in permeabilized thymocytes (Offer et al., 1993).

The inhibition of cell division 35 h after addition of serum to *n*FIns-loaded cells was approx. 50%, increasing to approx. 75–85% between 35 and 60 h. These data suggest that the 24 h incubation in serum-free medium to render the cells quiescent after microinjection or scrape loading of *n*FIns before serum stimulation is not sufficient to achieve maximal incorporation of

*n*FIns. As the turnover of phosphoinositides is stimulated by mitogens and growth factors (Taylor et al., 1984; Berridge, 1987), prolonged incubation in the presence of serum may be required to achieve maximal incorporation of *n*FIns and hence inhibition of proliferation.

Addition of 3FIns or 5FIns to the extracellular medium increased the inhibition of proliferation caused by microinjection of the same *n*FIns, although extracellular *n*FIns alone had no significant effect. This may indicate that efflux of microinjected *n*FIns occurs from the cells with time and would be consistent with findings by Moyer et al. (1988a) who reported that 80% of labelled *myo*-inositol leaked from L1210 leukaemia cells within 3 h. Slow uptake of *n*FIns from the medium may either sustain or increase the intracellular *n*FIns concentration and hence its incorporation into phospholipid.

Mechanism of inhibition of cell division by *n*FIns

The *n*FIns analogues were synthesized as potential inhibitors of signalling mediated by phosphoinositides and their derivatives, and all of the evidence is consistent with modulation of these pathways. Neither 5FIns nor 3FIns affects the ATP/ADP ratios measured at different times during the cell cycle, and the effect on cell number is not due to cell death induced by the *n*FIns analogues. The isomer specificity for inhibition of proliferation is correlated with the synthesis of PtdIns analogues and of their monophosphorylated derivatives in permeabilized thymocytes (Offer et al., 1993). The proportions of *n*FIns-loaded cells entering DNA synthesis and subsequently dividing in the first cell cycle after stimulation with serum are very similar, which implies that the inhibition of proliferation occurs in the G_1 phase of the cell cycle. This is consistent with inhibition of signalling mediated by early phosphoinositide responses. Furthermore, the high levels of inhibition of proliferation after prolonged serum stimulation (75–85%) suggest that the steps in the signalling pathway affected by the *n*FIns analogues are essential for proliferation.

Mechanisms of inhibition of proliferation by 3FIns and 5FIns may involve one or more of a wide range of possible effects of the analogues. The *n*FIns analogues may perturb the levels of PtdIns and the 3- and 4,5-phosphorylated inositol lipids, although no evidence for this has been obtained in the experiments described. The levels of these lipids may be decreased by direct competition of 3FIns or 5FIns with inositol. This would be similar to the effect of decreasing the concentration of inositol below that required to sustain maximal proliferation. The steady-state inositol phosphate concentrations in quiescent and/or stimulated cells may also be affected by the *n*FIns analogues if the analogues are direct inhibitors of inositol kinase (Stephens et al., 1990) or any of the inositol polyphosphate kinases or phosphatases.

The amounts of phosphorylated inositol lipids may also be perturbed if either 3FIns or 5FIns is incorporated into lipids, although this remains to be demonstrated under the experimental conditions described. The fluorine substituents will prevent phosphorylation by a 3-hydroxy or a 5-hydroxy kinase, and possibly a 4-hydroxy kinase. The intracellular signalling pathways regulated by PtdIns 3-kinase are not yet elucidated, but there is increasing evidence that PtdIns 3-kinase and 3-phosphorylated phospholipids play an important role in signalling and cell proliferation (Whitman and Cantley, 1988; Carpenter and Cantley, 1990; Hawkins et al., 1992). A function for the 3-phosphorylated derivatives of PtdIns in actin organization has also been suggested (Eberle et al., 1990). The presence of a fluorine atom on the 5-position will block formation of PtdIns(4,5) P_2 and inhibit the signalling pathways mediated by

this phosphoinositide. Incubation of electroporated thymocytes with 3FIns or 5FIns has been shown to produce two products: a major component with the chromatographic properties of a modified PtdIns and a minor product with the properties of a PtdIns4P analogue. However, there was no product corresponding to a modified PtdIns(4,5) P_2 (Offer et al., 1993). These data are consistent with studies of the phospholipids formed in L1210 cells incubated with 5FIns (Moyer et al., 1988b) and the role of *n*FIns analogues as substrates and inhibitors of PtdIns kinase in turkey erythrocyte membranes (McPhee et al., 1991).

If fluorinated inositol lipid analogues are formed, they may compete with the normal substrate(s) of PLC and affect the amounts of inositol polyphosphates and diacylglycerol formed. Fluorinated inositol lipid analogues may be substrates for PLC and produce fluorinated inositol phosphates. The lipid analogues may also modulate the function of PtdIns as a structural component. For example, PtdIns anchors a variety of proteins to the plasma membrane and has also been reported to have a role in microtubule assembly.

Some of the potential effects of the *n*FIns analogues are outlined above to indicate that it is not possible to offer a simple interpretation of their inhibitory effects on proliferation. However, the synthesis of radiolabelled *n*FIns analogues by scaling up the methods recently described (Offer et al., 1992) should allow any phospholipids which incorporate the *n*FIns analogues in intact cells to be detected and their metabolic products to be identified. These data will provide a starting point for analysing mechanisms by which cell proliferation and other biochemical responses to mitogenic stimulation are modulated by *n*FIns analogues.

Note added in proof (received 4 May 1993)

The authors wish to draw attention to an error in a previous paper from this laboratory [Offer, Metcalfe and Smith (1993) *Biochem. J.* **291**, 553–560]. Please refer to the Corrections section on p. 951 of this issue.

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