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GTPγS activation of proto-oncogene expression in transiently permeabilised Swiss 3T3 fibroblasts

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A technique of transient permeabilisation has been used to show that the introduction of guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS), a non-hydrolysable analogue of GTP, into intact Swiss 3T3 fibroblasts stimulates phosphoinositide hydrolysis, cyclic AMP accumulation and the activation of c-*fos* and c-*myc* proto-oncogenes. Of a number of nucleotide triphosphates introduced into the cells, only GTP and its non-hydrolysable analogues activated inositol phosphate release, suggesting that this response is mediated by guanine nucleotide regulatory (G) protein(s). The data demonstrate that transient permeabilisation provides a method of examining the involvement of G-proteins in nuclear activation.

c-*fos*; c-*myc*; Guanine nucleotide; Phosphoinositide hydrolysis; cyclic AMP

1. INTRODUCTION

Swiss 3T3 fibroblasts in the quiescent state (G_0) can be stimulated to enter G_1 and progress to DNA synthesis in S phase by the appropriate combinations of mitogens [1–3]. In particular, a wide variety of mitogens which cause either hydrolysis of $PtdInsP_2$ to release $Ins(1,4,5)P_3$ [4–7] or activation of adenylate cyclase to produce cyclic AMP [1,8], cause the stimulation of DNA synthesis in combination with a second mitogen, for which insulin is generally the most effective. $PtdInsP_2$ breakdown and activation of adenylate cyclase are believed to be mediated by distinct guanine nucleotide regulatory proteins (G_p [9–11] and G_s [12,13]), as demonstrated, in part, by the stimulation of these responses in a variety of plasma membrane preparations by non-hydrolysable analogues of GTP. Thus G-proteins are strongly implicated in the mitogenic pathway from G_0 to S phase for at least two major classes of co-mitogens. However, it has not as yet been demonstrated that GTP analogues can activate proto-oncogenes (e.g. c-*fos* and c-*myc*) which are associated with the action of all mitogen combinations which stimulate DNA synthesis in Swiss 3T3 fibroblasts [1–3]. This requires access of the impermeant GTP analogues to the interior of the cell under conditions which retain functional signalling mechanisms for nuclear activation.

As a first stage in analysing more directly the role of G-proteins in the mitogenic pathway, we have characterised the use of a transient permeabilisation technique [14,15] to introduce GTPγS into the cytosol of quiescent 3T3 fibroblasts under conditions where the cells retain the capacity to progress to DNA synthesis in response to mitogenic stimulation. It is shown that GTPγS stimulates the accumulation of inositol phosphates and cyclic AMP and also activates c-*fos* and c-*myc* gene expression in intact Swiss 3T3 fibroblasts.

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Abbreviations: EGF, epidermal growth factor; GTPγS, guanosine 5'-*O*-(3-thiotriphosphate); IBMX, 3-isobutyl 1-methylxanthine; $Ins(1,4,5)P_3$, D-*myo*-inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline; $PtdIns(4,5)P_2$, 1-(3-*sn*-phosphatidyl)-D-*myo*-inositol 4,5-bisphosphate

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2. EXPERIMENTAL

2.1. *Cell culture and DNA synthesis*

Swiss 3T3 fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (Gibco), 44 mM $NaHCO_3$, 25 mM glucose, penicillin G (35 U/ml) and streptomycin (80 mU/ml) in a humidified atmosphere of air/CO_2 (90:10) at 37°C. Cells were seeded onto 30 or 90 mm plastic petri dishes (4×10^4 or 3×10^5 cells, respectively) and grown to confluence (5–7 days). To measure DNA synthesis in non-permeabilised or transiently permeabilised fibroblasts the cells (approx. 3×10^5/dish) were incubated for 40 h with [6-^{3}H]thymidine (1.0 μCi/ml) in serum-free DMEM with additions of either EGF (10 ng/ml) and insulin (1 μg/ml) or 10% fetal calf serum. For determination of DNA synthesis by incorporation of radioactivity into acid-precipitable material, the cell monolayer was washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1.5 mM KH_2PO_4; pH 7.2) and the cells were extracted with 1% SDS (0.5 ml). The SDS extract was transferred with rinsing (1×1.0 ml PBS) to ice-cold 15% trichloroacetic acid. After 20 min on ice the mixture was filtered under suction through a Whatman GF/C filter. Radioactivity bound to the air-dried filter was determined by liquid scintillation counting. Alternatively, to quantitate radiolabelled nuclei the cell monolayer was washed twice with ice-cold PBS (1.0 ml) and once with PBS:methanol (1:1, v/v; 1.0 ml). The cells were fixed in methanol, dried overnight, covered with a thin layer of melted photographic emulsion (Ilford D2) and autoradiographed for 9 days at 4°C. Photographs were taken on a Leitz-Diavert microscope (312.5 × magnification).

2.2. *Transient permeabilisation*

Swiss 3T3 fibroblasts were transiently permeabilised by a modification of the scraping procedure first described by McNeil et al. [14]. Confluent monolayers of fibroblasts were washed once with serum-free DMEM (37°C, pH 7.2) followed by two washes with 'intracellular medium' at 4°C (114 mM KCl, 5 mM NaCl, 5.5 mM $MgCl_2$, 0.3 mM Na_2HPO_4, 5 mM Na_2ATP, 0.3 mM $CaCl_2$, 0.5 mM EGTA, 10 mM Pipes; pH 7.05). Intracellular medium with or without the indicated agents was added to the dishes and the cells were transiently permeabilised by removal from their substratum by gentle scraping with a rubber policeman [14]. The resulting cell suspension was transferred to a plastic centrifuge tube containing serum-free DMEM (pH 7.2, 37°C), centrifuged ($120 \times g$, 5 min) and the cell pellet resuspended in serum-free DMEM. The cells were re-seeded onto 30 mm petri dishes (3×10^5 cells/dish) or 90 mm petri dishes (2×10^6 cells/dish).

2.3. *Inositol phosphates accumulation*

Swiss 3T3 fibroblasts were incubated with *myo*-[2-^{3}H]inositol (10 μCi/ml) for 96 h to label the phosphoinositides and then washed twice with serum-free DMEM without *myo*-[2-^{3}H]-inositol. Cells were transiently permeabilised in the presence or absence of the indicated agents, resuspended in serum-free DMEM containing 10 mM LiCl and incubated for the times shown before the addition of chloroform/methanol (1:2, v/v) to terminate the reactions. Non-permeabilised cells were washed twice with intracellular medium and incubated in serum-free DMEM with 10 mM LiCl either with or without bombesin (10 nM). The incubations were terminated by removing the medium and adding chloroform/methanol (1:2, v/v) directly to the petri dishes. The ^{3}H-labelled inositol phosphates were recovered from the chloroform/methanol extracts and measured by anion-exchange chromatography on Dowex 1-X8 or Biorad AG1-X8 (formate forms) [16,17] and liquid scintillation counting of the eluates.

2.4. *Cyclic AMP assay*

Swiss 3T3 fibroblasts were transiently permeabilised in the presence or absence of GTPγS and resuspended at 10^6 cells/ml in DMEM containing 20 mM Hepes and 0.1 mM IBMX. After incubation for 15 min at 37°C aliquots (0.5 ml) of the cell suspension were pelleted by brief centrifugation ($13000 \times g$, 5 s), the supernatant solution removed and the cells resuspended in water (150 μl). The cell samples were boiled for 5 min, centrifuged ($13000 \times g$, 3 min) and an aliquot of the supernatant assayed for cyclic AMP by the method of Brown et al. [18].

2.5. *c*-fos *and c*-myc *mRNA*

Non-permeabilised or transiently permeabilised fibroblasts were incubated in serum-free DMEM for the times shown before terminating the incubations by removing the serum-free DMEM and harvesting the cells by scraping in ice-cold PBS, pH 7.2. The cell suspension was centrifuged ($120 \times g$, 6 min) and the pellet resuspended in 1.0 ml of 4 M guanidinium isothiocyanate solution containing 25 mM sodium citrate, 100 mM 2-mercaptoethanol, 0.5% *N*-lauryl sarcosine and 0.1% antifoam. Total cellular RNA, isolated by the method of Chirgwin et al. [19], was quantitated by absorbance at 260 nm and aliquots containing equal amounts of RNA were separated on 1.2% agarose, 6.6% formaldehyde gels. Equal loading was confirmed by staining with ethidium bromide before the RNA was transferred to nitrocellulose and hybridised with c-*fos* and c-*myc* probes (radiolabelled by nick translation) as described by Moore et al. [20]. After washing to remove non-specifically bound probe the filters were dried and exposed to pre-sensitised Fuji RX X-ray film for 3 h to 7 days at −70°C. The intensity of the bands was estimated by densitometry. The amounts of c-*fos* and c-*myc* mRNAs in non-permeabilised, unstimulated cells were undetectable. The c-*fos* probe was the large *Bgl*II fragment of v-*fos* cloned in pBR322 and the v-*myc* probe was a *Hin*dIII/*Bam*HI fragment of exons 2 and 3 of mouse c-*myc* cloned in pUC8. Both were provided by Dr S.M. Neuberger (MRC Laboratory of Molecular Biology, Hills Road, Cambridge, England).

3. RESULTS AND DISCUSSION

When confluent monolayers of fibroblasts were permeabilised in the presence of Lucifer yellow (1.8 mM), more than 95% of the cells took up the dye as observed by fluorescence microscopy (fig.1). To determine how long the fibroblasts remained permeable following removal from their substratum, cells were scraped in the absence of Lucifer yellow and transferred within 2 min to

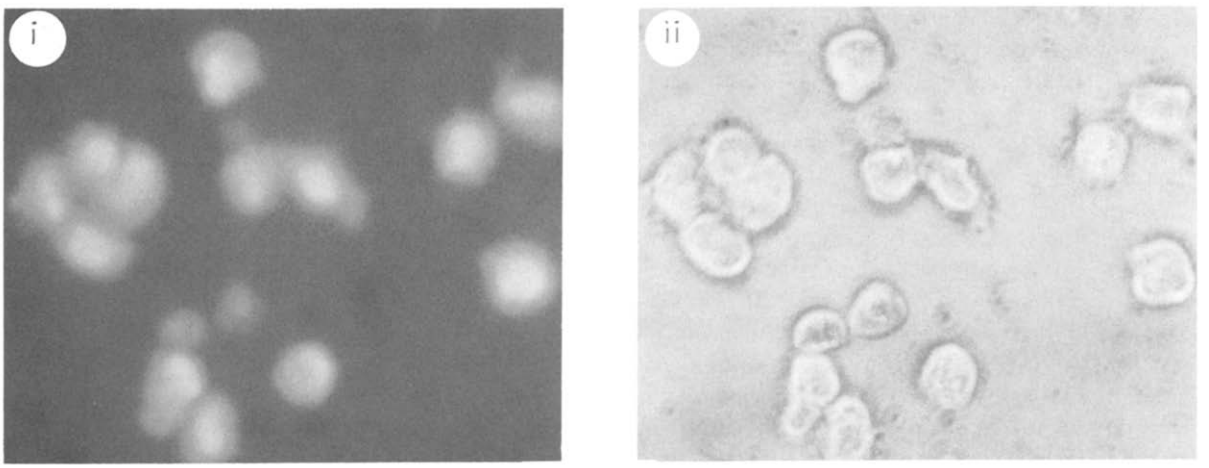

Fig.1. Lucifer yellow uptake in transiently permeabilised fibroblasts. Localisation of Lucifer yellow in fibroblasts 30 min after transient permeabilisation in the presence of the dye (1.8 mM). Panel (i) shows the image (284 × magnification) under violet/blue illumination and panel (ii) under both violet/blue and tungsten lamp illumination.

medium containing the dye. Less than 2% of the cells incorporated the dye, indicating that resealing occurs rapidly (i.e. in <2 min) in more than 98% of the cells. It was confirmed that molecules of up to at least 40 kDa were incorporated into a similar proportion of the cells by permeabilising the cells in the presence of fluorescein-labelled dextrans of various molecular mass ranges [14]. When the transiently permeabilised cells were re-seeded onto plastic petri dishes they initially displayed a rounded morphology and tended to form small aggregates of unattached cells (fig.1). After 60 min, however, more than 80% of the cells had adhered to the surface of the dishes sufficiently strongly to resist removal by repeated washing. Over a period of several hours the cells reverted to the morphology characteristic of adherent fibroblasts.

It was important to establish whether the transiently permeabilised cells remained functionally

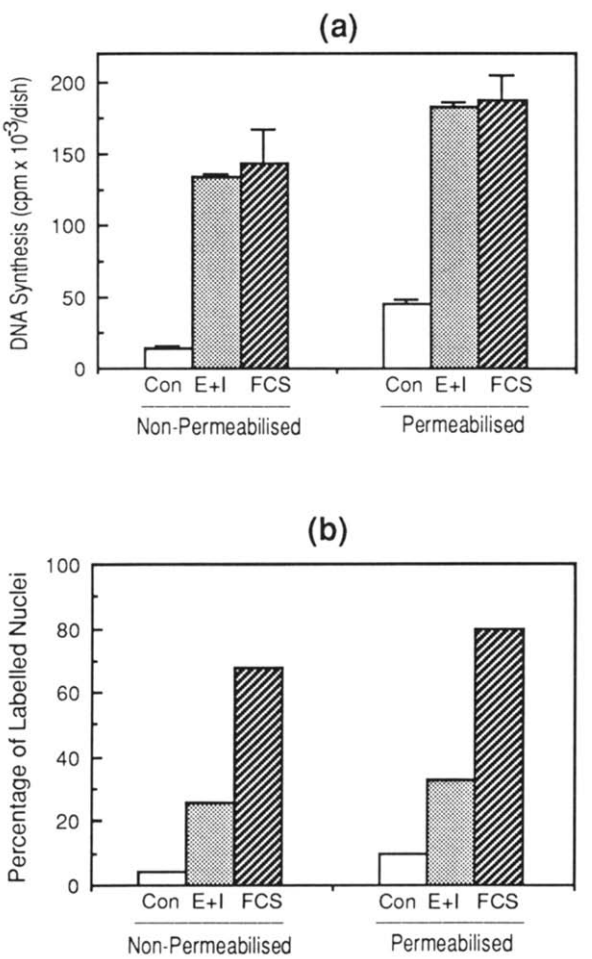

Fig.2. Effect of transient permeabilisation on DNA synthesis. DNA synthesis, measured by (a) incorporation of [6-^{3}H]thymidine into acid-precipitable material and (b) percentage of labelled nuclei, in non-permeabilised and transiently permeabilised cells. Cells were incubated for 40 h with [6-^{3}H]thymidine with EGF and insulin (E + I), fetal calf serum (FCS) or no addition (Con). To estimate the percentage of labelled nuclei approx. 250 nuclei were counted in duplicate for each sample. Data are from a single experiment representative of three.

intact. We therefore investigated the effect of transient permeabilisation on the ability of the cells to progress to DNA synthesis in S phase. Transient permeabilisation of quiescent fibroblasts did not stimulate the cells to enter S phase as assayed either by incorporation of [^{3}H]thymidine into acid-precipitable material from cells incubated for 40 h with [^{3}H]thymidine (fig.2a) or by autoradiography of ^{3}H-labelled nuclei (fig.2b). Furthermore, the transiently permeabilised cells retained the ability to progress to S phase in response to stimulation by a combination of EGF and insulin or by fetal calf serum and the percentages of permeabilised and non-permeabilised cells that responded to mitogenic stimulation were similar (fig.2b). The data show that very high proportions of the cells were permeabilised (>95%) and responded to mitogenic stimulation (up to 80%). This precludes the possibility that only cells that were not permeabilised by scraping (<5%) progressed to S phase.

The introduction of GTPγS into intact cells may be expected to activate all G-proteins, including G_p and G_s, as it has been demonstrated that GTPγS activates inositol phosphate formation and cyclic AMP accumulation in a number of different plasma membrane preparations [9,12]. Transient permeabilisation of Swiss 3T3 fibroblasts in the absence of GTPγS evoked a small but significant increase in inositol phosphate accumulation (fig.3). The presence of GTPγS during transient permeabilisation, however, caused a large and rapid stimulation of phosphoinositide hydrolysis which was similar to the response elicited by bombesin on non-permeabilised cells (fig.3). The

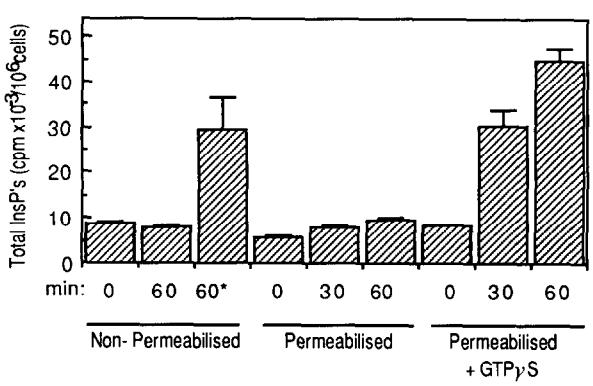

Fig.3. Effect of GTPγS on inositol phosphate accumulation in transiently permeabilised fibroblasts. Swiss 3T3 fibroblasts were pre-labelled for 96 h with *myo*-[2-^{3}H]inositol (10 μCi/ml) and then transiently permeabilised in the presence or absence of GTPγS (0.9 mM). Non-permeabilised cells were incubated either with no addition or in the presence of 10 nM bombesin (*). At the indicated times the reactions were terminated and the incorporation of [^{3}H]inositol into total inositol phosphates determined. Similar results were obtained in three other experiments.

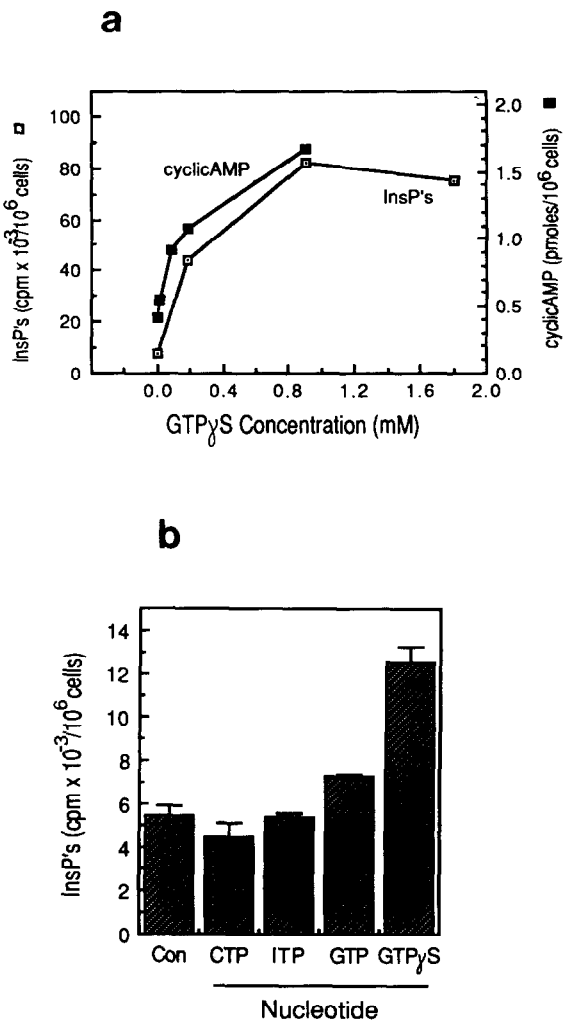

Fig.4. Effect of nucleotide triphosphates on inositol phosphate and cyclic AMP accumulation in transiently permeabilised fibroblasts. (a) Fibroblasts were transiently permeabilised in the presence of the indicated concentrations of GTPγS and either total inositol phosphates or cyclic AMP were determined after 30 or 15 min incubation, respectively. (b) Fibroblasts pre-labelled with *myo*-[2-^{3}H]inositol were permeabilised in the presence of ITP (0.9 mM), CTP (0.9 mM), GTP (0.9 mM), GTPγS (0.18 mM) or no additions (Con). Total inositol phosphates were measured after 30 min incubation.

effect of GTPγS on inositol phosphate accumulation increased as the concentration of the analogue in the permeabilisation medium was increased from 0.18 mM with maximal stimulation at 0.9 mM (fig.4a). In further experiments to examine the specificity of this response in transiently permeabilised cells, it was found that GTP stimulated a small increase in inositol phosphate accumulation whereas CTP and ITP were without effect (fig.4b). It was also found that guanylyl imidodiphosphate (a non-hydrolysable analogue of GTP) increased inositol phosphate accumulation with approximately the same efficacy as GTPγS (not shown). These data indicate that the stimulation of inositol phosphate accumulation in transiently permeabilised 3T3 fibroblasts by nucleotide triphosphates is specific to guanine nucleotides. This suggests, albeit indirectly, that phosphoinositide hydrolysis is coupled to G-protein(s) in these cells.

The effect of GTPγS on the accumulation of cyclic AMP in transiently permeabilised fibroblasts was also examined. Transient permeabilisation of the cells in the presence of GTPγS caused a large increase in the intracellular concentration of cyclic AMP to approx. 1.6 pmol/10^6 cells, whereas the concentration of cyclic AMP in cells permeabilised in the absence of GTPγS was 0.4 pmol/10^6 cells (fig.4a). By comparison, non-permeabilised cells contained 0.16 pmol of cyclic AMP/10^6 cells. The stimulation of cyclic AMP accumulation by GTPγS occurred over the same concentration range as required for phosphoinositide hydrolysis (fig.4a).

To examine whether GTPγS could stimulate gene expression, the amounts of c-*fos* and c-*myc* mRNA were compared in cells permeabilised in the presence or absence of GTPγS (fig.5). Permeabilisation of cells in the absence of GTPγS caused a small increase in the amounts of both mRNAs whereas the presence of GTPγS during

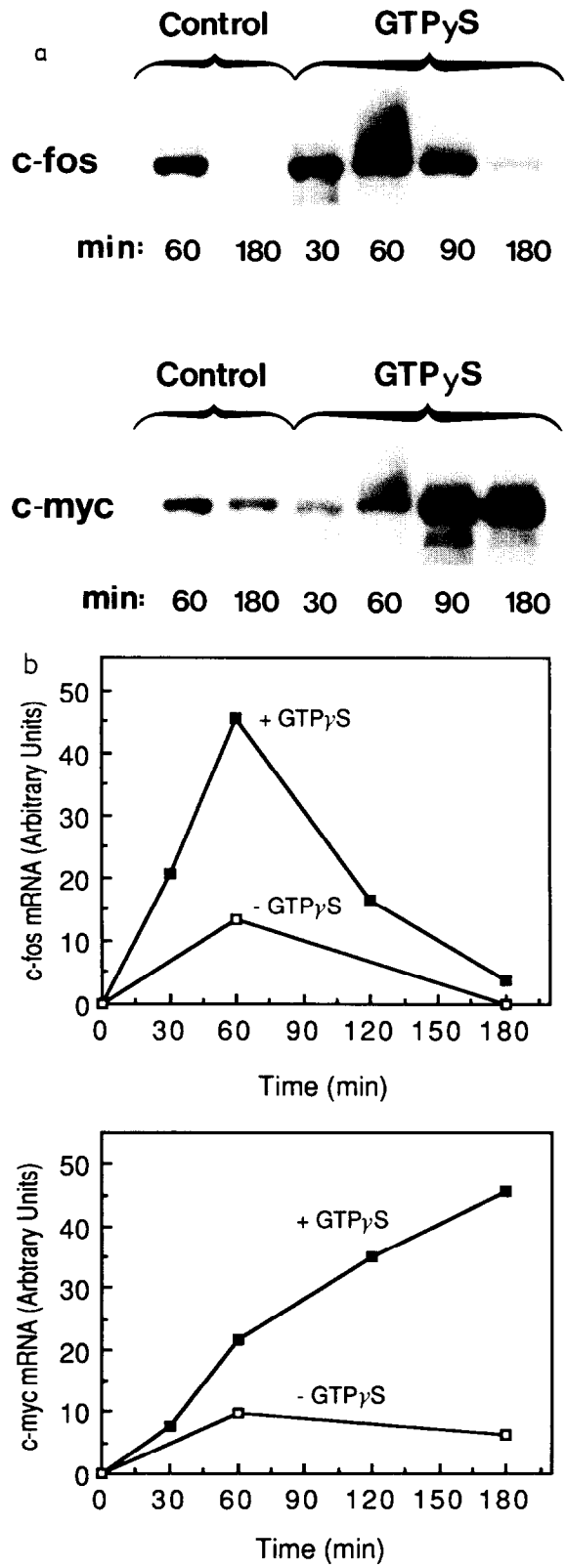

Fig.5. Time course of the amounts of c-*fos* and c-*myc* mRNA in fibroblasts transiently permeabilised in the presence of GTPγS. Northern blot analysis of c-*fos* and c-*myc* mRNA following transient permeabilisation of Swiss 3T3 fibroblasts in the absence (Control, □) or presence (■) of GTPγS (0.9 mM). 5 μg of total RNA were analysed in each lane. (a) Autoradiographs of nitrocellulose filters probed for c-*fos* or c-*myc*. (b) Data from densitometric scans of the bands in a expressed in arbitrary units.

permeabilisation resulted in a dramatic increase in both c-*fos* and c-*myc* mRNAs (fig.5). The stimulation of the c-*fos* gene by GTPγS was rapid and transient, reaching a maximum within 60 min and returning to a very low amount after 3 h. In contrast the amount of c-*myc* mRNA remained elevated for at least 3 h. Treatment of non-permeabilised cells with EGF for 30 min caused approx. 20% of the c-*fos* response elicited by GTPγS in transiently permeabilised cells (not shown). There was no activation of c-*fos* gene transcription when non-permeabilised cells were exposed to GTPγS in intracellular medium or in serum-free DMEM which eliminates the possibility that this agent acts on the outside of the cells to stimulate gene expression. These results show that in transiently permeabilised cells GTPγS activates the c-*fos* and c-*myc* genes with similar time courses to the responses in mitogen-stimulated fibroblasts [21,22].

The data presented illustrate the novel use of transient permeabilisation of fibroblasts to determine the effect of impermeant agents such as GTPγS on a range of cellular responses. Although transient permeabilisation causes a significant perturbation of the cells as demonstrated by its effect on the amount of c-*fos* and c-*myc* mRNA (fig.5) and the small increases in inositol phosphate formation (fig.3) and cyclic AMP accumulation, this is not sufficient either to stimulate mitogenesis or to compromise the mitogenic response to added growth factors. Furthermore, the results suggest that transient permeabilisation is a powerful technique for investigating the effects of impermeant reagents on intracellular responses, such as gene expression, in the mitogenic pathway.

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REFERENCES

[1] Rozengurt, E. (1986) Science 234, 161–166.
[2] Metcalfe, J.C., Hesketh, T.R., Smith, G.A., Morris, J.D.H., Corps, A.N. and Moore, J.P. (1985) J. Cell Sci. suppl.3, 199–228.
[3] Metcalfe, J.C., Moore, J.P., Smith, G.A. and Hesketh, T.R. (1986) Br. Med. Bull. 42, 405–412.
[4] Macphee, C.H., Drummond, A.H., Otto, A.M. and Jiminez de Asua, L. (1980) J. Cell Physiol. 119, 35–40.
[5] Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, C., Mitchell, C.D. and Ross, R. (1981) J. Biol. Chem. 256, 12329–12335.
[6] Berridge, M.J., Heslop, J.P., Irvine, R.F. and Brown, K.D. (1984) Biochem. J. 222, 195–201.
[7] Heslop, J.P., Blakeley, D.M., Brown, K.D., Irvine, R.F. and Berridge, M.J. (1986) Cell 47, 703–709.
[8] Rozengurt, E. (1981) Adv. Cycl. Nucleotide Res. 14, 429–442.
[9] Cockroft, S. and Gomperts, B.D. (1985) Nature 314, 534–536.
[10] Magnaldo, I., Talwar, H., Anderson, W.B. and Pouyssegur, J. (1987) FEBS Lett. 210, 6–10.
[11] Paris, S. and Pouyssegur, J. (1987) J. Biol. Chem. 262, 1970–1976.
[12] Northup, J.K. (1985) in: Molecular Mechanisms of Transmembrane Signalling (Cohen, P. and Houslay, M.D. eds) Mol. Aspects Cell. Regul. vol.4, pp.91–116, Elsevier, Amsterdam, New York.
[13] Gilman, A.G. (1984) Cell 36, 577–579.
[14] McNeil, P.L., Murphy, R.F., Lanni, F. and Taylor, D.L. (1984) J. Cell Biol. 98, 1556–1564.
[15] McNeil, P.L. and Taylor, D.L. (1985) Cell Calcium 6, 83–93.
[16] Downes, C.P. and Michell, R.H. (1981) Biochem. J. 198, 133–140.
[17] Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982) Biochem. J. 206, 587–595.
[18] Brown, B.L., Ekins, R.P. and Albano, J.D.M. (1972) Adv. Cycl. Nucleotide Res. 2, 25–40.
[19] Chirgwin, J.M., Przybyla, A.E., MacDonald, P.J. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[20] Moore, J.P., Todd, J.A., Hesketh, T.R. and Metcalfe, J.C. (1986) J. Biol. Chem. 261, 8158–8162.
[21] Greenberg, M.E. and Ziff, E.B. (1984) Nature 311, 433–438.
[22] Muller, R., Bravo, R., Burckhardt, J. and Curran, T. (1984) Nature 312, 716–720.