Amplification of fluoroaluminate-stimulated inositol phosphate generation in a cell line overexpressing the p21^{N-ras} gene

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The stimulation of inositol phosphate generation in NIH-3T3 cells and a derived transformant overexpressing the p21^{N-ras} gene (T15+ cells) was examined. Incubation with NaF in the presence of Al³⁺ leads to the generation of inositol phosphates in each cell type, though the response in the T15+ cells is significantly amplified. The effect of fluoroaluminate is dose- and time-dependent. No differences were observed in fluoroaluminate-stimulated cyclic AMP accumulation among the cell types. In another NIH-3T3-derived cell line that expresses the transforming lys61 mutant of N-ras, no amplification of fluoroaluminate-stimulated inositol phosphate generation is observed. These results provide support for the proposal that, in the T15 cell line, p21^{N-ras} can act in a guanine nucleotide-binding regulatory protein (G-protein)-like manner.

INTRODUCTION

A range of growth factors stimulate the breakdown of PtdIns(4,5)P₂ upon binding to their appropriate receptors [see Berridge (1987) for a review]. The products of this breakdown, Ins(1,4,5)P₃ and *sn*-1,2-diacylglycerol, are both second-messenger molecules which appear to be important in the stimulation of mitogenesis in response to some growth factors (Berridge, 1987). Since activation of the inositol phospholipid response is thought, therefore, to be involved in the stimulation of proliferation, it is a potential target for the action of tumour-promoting agents, and certain oncogene products may exert their action by interacting with the pathway (Michell, 1984).

In order to investigate such a proposal, we recently examined the effect of transformation of NIH-3T3 cells, by either the overproduction of normal N-ras (McKay *et al.*, 1986; Wakelam *et al.*, 1986a), or by the expression of mutated N-ras genes (Hancock *et al.*, 1988), upon inositol phospholipid metabolism. Although we found an amplification of the turnover of inositol phospholipids in both cases, the apparent mechanism involved differed between the two types of *ras* transformation (Hancock *et al.*, 1988). In the T15+ cell line transformation is induced by overexpression of the normal N-ras proto-oncogene, whereas, in the pLHT5-11+ cell, transformation is induced by the expression of an N-ras gene with the lys61 mutation (Wakelam *et al.*, 1986a; Hancock *et al.*, 1988). Experiments with the T15+ cell demonstrated an amplification of inositol phosphate generation in response to certain growth factors, but in particular to bombesin, as compared with control cells, in the absence of any change in receptor number or affinity (Wakelam *et al.* 1986a). By contrast, in the pLHT5-11+ cell we found an amplification in the agonist-independent generation of inositol phosphate (Hancock *et al.*, 1988). We have interpreted these data as being support for the hypothesis that p21^{N-ras} can in certain situations interact with the inositol

phospholipid pathway and function as a guanine nucleotide-binding regulatory protein (G-protein).

A characteristic of G-proteins is that they can be activated directly by NaF in the presence of Al³⁺ (fluoroaluminate ions) (see Sternweis & Gilman, 1982). If the hypothesis that p21^{N-ras} is functioning as a G-protein in the T15+ cells is correct, then the stimulation of PtdIns(4,5)P₂ breakdown in this cell by fluoroaluminate ions should be amplified compared with the control cells. In the present paper this is shown to be the case, and no such amplification is observed in cells transformed by the N-ras oncogene (lys61).

METHODS

NIH-3T3, pLHT5.11+, T15– and T15+ cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Renfrewshire, Scotland, U.K.), containing 10% (v/v) donor calf serum (Northumbria Biologicals, Cramlington, Northd., U.K.) and dexamethasone as appropriate; the N-866 cell line was cultured in DMEM containing 5% (v/v) donor calf serum. They were maintained at 37 °C in an air/CO₂ (1:19) 95%–humidity atmosphere.

The cells were labelled for inositol phosphate experiments by changing the medium to inositol-free DMEM (Gibco) containing dialysed serum, dexamethasone as appropriate and 1 µCi of [³H]Ins (Amersham International, Amersham, Bucks., U.K.)/ml. In experiments to determine the changes in individual inositol phosphates, the radiolabel concentration was increased to 5 µCi/ml.

After a 24 h labelling period, the medium was removed, the monolayers washed with Hanks buffer and the cells scraped into Hanks buffer containing 1% (w/v) bovine serum albumin and 10 mM-glucose (HBG). The cells were collected by centrifugation (800 g; 5 min), washed twice more in HBG and then incubated for 10 min

Abbreviations used: G-protein, guanine nucleotide-binding regulatory protein; DMEM, Dulbecco's modified Eagle's medium; IBMX, isobutylmethylxanthine; EC₅₀, concentration producing 50% of maximal stimulation; G_i, inhibitory G-protein; G_p, G-protein activating phosphoinositidase C.

in HBG containing 10 mM-LiCl at 37 °C for the stated times. Incubations were terminated by the addition of chloroform/methanol (1:2, v/v) and, after allowing time for extraction, the phases were split by the addition of chloroform and water. Total inositol phosphates were determined by subjecting an aliquot of the upper phase to batch elution from Dowex 1 (X8; formate form) columns by the method of Berridge *et al.* (1982), as previously described (Wakelam *et al.*, 1986a).

In some cases incubations were performed with cells attached to 24-well tissue-culture dishes. In these experiments incubations were terminated by rapid aspiration of the incubation medium, followed by the addition of ice-cold HClO₄ (final conc. 10%, v/v). After neutralization with KOH and the removal of precipitated KClO₄ by centrifugation, the samples were loaded on to Dowex 1 (X8; formate form) columns and the individual phosphates separated by sequential batch elution as previously described (Wakelam *et al.*, 1986b). No differences in responsiveness were found between the two different incubation methods.

In experiments to determine the accumulation of cyclic AMP, cells were grown on 24-well tissue-culture dishes. The cells were used at a density of 5×10^4 per well as determined by Coulter counting. The cells were labelled with 2 μ Ci of [³H]adenine (Amersham International)/well in serum-free DMEM for 90 min. Preliminary experiments demonstrated that this period of time led to maximum [³H]adenine incorporation into the acid-soluble fraction. After the labelling period the cells were extensively washed with phosphate-buffered saline (8 mM - Na₂HPO₄ / 1.5 mM - KH₂PO₄ / 5 mM - KCl / 145 mM-NaCl, pH 7.4), at 37 °C and then incubated in DMEM containing 1 mM-isobutylmethylxanthine (IBMX) and the concentrations of fluoroaluminate stated in the Results and discussion section for 20 min. Incubations were terminated by the addition of 0.5 ml of 10% (w/v) trichloroacetic acid. The acid-soluble fraction was analysed for cyclic AMP content after chromatography upon Dowex (H⁺ form) and alumina columns as described by Salomon *et al.* (1974). Recovery from the columns was assessed by the addition of approx. 2500 d.p.m. of cyclic [¹⁴C]AMP to each sample.

Materials were obtained from stated sources, or previously given suppliers (Wakelam *et al.*, 1986a).

RESULTS AND DISCUSSION

Treatment of NIH-3T3, T15- and T15+ cells with 30 mM-NaF in the presence of 10 μ M-Al₂(SO₄)₃ resulted in the accumulation of inositol phosphates when 10 mM-LiCl was present. Table 1 shows that a greater generation was observed in the T15+ cells than in either the untransformed control cell, T15-, or parental cell line, NIH-3T3. NaF alone was only moderately effective in stimulating inositol phosphate generation, demonstrating that it was the fluoroaluminate ion which was activating phosphoinositide hydrolysis (results not shown). The response to bombesin in the cells, as we have previously reported (Wakelam *et al.*, 1986), was only significant in the T15+ cells. Although not being statistically significant, the stimulation by bombesin may have been additive with that to fluoroaluminate in each cell type (Table 1).

The response to fluoroaluminate was dose-dependent. Fig. 1 shows NaF dose-response curves in the presence of 10 μ M-Al₂(SO₄)₃ for the T15+ and NIH-3T3 cells. The dose-dependence of the stimulation of inositol phosphate generation followed similar patterns with an apparent EC₅₀ of about 25 mM. It is clear, however, from Fig. 1 that, at each concentration tested, the response in the N-ras-transformed cells was greater than in the untransformed control. This amplification is further demonstrated in Fig. 2, which shows the time-dependence of fluoroaluminate-stimulated inositol phosphate generation. Whereas significant generation of inositol phosphates can be detected in the T15+ cells after 2 min, they are only detected in NIH-3T3 cells after a 5 min incubation. This later onset was, however, probably due to the lower amount of inositol phosphate generation, rather than to any change in kinetics. The increase in inositol phosphate generation observed in Figs. 1 and 2 was a consequence of PtdIns(4,5)P₂ breakdown, since an increase in the generation of InsP₃ was observed in T15+ cells incubated with fluoroaluminate ions (results not shown).

Table 1. Stimulation of inositol phosphate generation in T15+, T15- and NIH-3T3 cells by bombesin and fluoroaluminate ions

The Table shows the stimulation of inositol phosphate generation, in each cell type, in response to a 15 min treatment with the additions listed. The fold stimulation is determined by comparing the radioactivity (d.p.m.) in total inositol phosphates in incubations with stimulant to those incubations in the absence of any stimulant, are expressed as means $\pm$ S.E.M. and are pooled from four separate experiments. The radioactivity (d.p.m.) values shown are from a single typical experiment and are means $\pm$ S.D. ($n = 4$ in each case). The bombesin concentration was 0.1 μ M, that of NaF 30 mM and that of Al₂(SO₄)₃ 10 μ M. All other experimental details are given in the Methods section.

Cells	Parameter	Stimulant ...	Bombesin	Fluoro-aluminate	Bombesin + fluoroaluminate
NIH-3T3	Control d.p.m.		406 $\pm$ 23	—	—
	Stimulated d.p.m.		428 $\pm$ 20	1249 $\pm$ 256	1153 $\pm$ 31
	Mean fold stimulation		1.09 $\pm$ 0.04	1.84 $\pm$ 0.15	1.95 $\pm$ 0.10
T15	Control d.p.m.		436 $\pm$ 24	—	—
	Stimulated d.p.m.		485 $\pm$ 34	956 $\pm$ 26	1000 $\pm$ 108
	Mean fold stimulation		1.07 $\pm$ 0.03	1.92 $\pm$ 0.12	2.00 $\pm$ 0.18
T15+	Control d.p.m.		525 $\pm$ 10	—	—
	Stimulated d.p.m.		841 $\pm$ 7	2731 $\pm$ 142	3085 $\pm$ 224
	Mean fold stimulation		1.94 $\pm$ 0.10	3.44 $\pm$ 0.18	4.12 $\pm$ 0.20

Fig. 1. Dose-response curves for the effect of fluoroaluminate stimulation upon inositol phosphate generation in T15+ and NIH-3T3 cells

[³H]Ins-labelled cells were incubated with the stated concentration of NaF in the presence of 10 μ M- $\text{Al}_2(\text{SO}_4)_3$ for 20 min. The stimulated generation of inositol phosphates compared with the d.p.m. measured in the absence of stimulant is plotted against concentration; the results are means $\pm$ s.d. and are pooled from three experiments, $n = 9$ in each case. Other details are in the Methods section or the legend to Table 1. $\blacklozenge$, T15+ cells; $\square$, NIH-3T3 cells.

Fluoroaluminate is believed to activate G-proteins by interacting with the GTP-binding site and therefore stimulating their interaction with effector molecules in the absence of input from an agonist-receptor complex (Gilman, 1984; Kanaho *et al.*, 1985). The complex ion is thus only capable of activating a G-protein when it is in its GDP-bound form. NIH-3T3 cells can be transformed by the expression of mutated *ras* genes which produce a

Fig. 2. Time-dependence of fluoroaluminate-stimulated inositol phosphate generation

[³H]Ins-labelled cells were stimulated for the stated times with 30 mM-NaF/10 μ M- $\text{Al}_2(\text{SO}_4)_3$ and the d.p.m. associated with inositol phosphates were determined as described in the Methods section. Results are means $\pm$ s.d. and are pooled from two experiments, $n = 8$ in each case. $\blacklozenge$, T15+ cells; $\square$, NIH-3T3 cells.

p21 product which has a GTPase activity three orders of magnitude lower than that of the normal unmutated protein (Trahey & McCormick, 1987). Table 2 shows that fluoroaluminate increased the production of inositol phosphates in the pLHT5-11+ cell (which expresses the lys61 *N-ras* mutant gene), but that the amplification after a 20 min incubation compared with the 'unstimulated' incubation was the same as that observed in the control untransformed cell. It should be noted, however, that the production of inositol phosphates in this cell, both in the absence of any stimulant and also in the

Table 2. Effect of fluoroaluminate ion stimulation upon cells transformed by normal or mutated *N-ras* genes

The Table shows the d.p.m. associated with the inositol phosphates in NIH-3T3, T15+ and pLHT5.11+ cells at zero time and after a 20 min incubation in the absence of fluoroaluminate (-F/Al) and after a 20 min stimulation with 30 mM-NaF and 10 μ M- $\text{Al}_2(\text{SO}_4)_3$ (+F/Al). The fold stimulation in response to fluoroaluminate compared with the unstimulated control at $t = 20$ min is also shown. The results are expressed as means $\pm$ s.d. ($n = 4$) and are from a single experiment typical of three others which differed numerically because of differences in the control d.p.m. level. All other experimental details are as in the legend to Table 1 or the Methods section.

Cell line	t (min)...	Radioactivity (d.p.m.)		Fold stimulation at 20 min
		0	20	
			-F/Al +F/Al	
NIH-3T3		348 $\pm$ 27	413 $\pm$ 21 746 $\pm$ 42	1.86 $\pm$ 0.12
pLHT5.11+		267 $\pm$ 31	603 $\pm$ 51 1315 $\pm$ 188	2.18 $\pm$ 0.24
T15+		459 $\pm$ 53	506 $\pm$ 79 2161 $\pm$ 299	4.31 $\pm$ 0.20

Table 3. Effect of culture conditions upon fluoroaluminate-stimulated inositol phosphate generation

The Table shows representative results of a series of experiments designed to test the effect of culture conditions upon fluoroaluminate-stimulated inositol phosphate generation. The results are from single experiments, typical of two others, and $n = 4$ in each case. Cells were stimulated for 25 min in each case. Low-density refers to cells at 60–70% confluency and high density to cultures in which the cells were overconfluent, i.e. overgrown. The dexamethasone concentration was $2 \mu\text{M}$, and other details are as in Table 1.

Expt.	Cell line	Culture conditions	Radioactivity (d.p.m.)		Fold stimulation
			Control	Stimulated	
1	NIH-3T3	Normal	977 ± 66	3038 ± 154	3.11 ± 0.26
	NIH-3T3	Dexamethasone, 14 days	780 ± 73	2879 ± 163	3.89 ± 0.36
	T15+	Normal	464 ± 41	2451 ± 132	5.29 ± 0.24
2	T15+	Low density	322 ± 70	1268 ± 92	3.93 ± 0.18
	T15+	High density	275 ± 12	673 ± 58	2.44 ± 0.22
	NIH-3T3	Normal	240 ± 14	528 ± 52	2.21 ± 0.20

presence of fluoroaluminate, was greater than in the untransformed cell; it was only the amplification by the ion that was the same in the control and mutant *ras*-transformed cells. The similar magnitude of fluoroaluminate-stimulated inositol phosphate generation, in the NIH-3T3 and pLHT5-11+ cells, reflects activation of the normal G_p present in the cells.

The increase in fluoroaluminate-stimulated inositol phosphate generation was not caused by the presence of dexamethasone in the culture medium. This is clear not only from the results obtained with the pLHT5-11+ cells (Table 2), but also, when NIH-3T3 cells were treated with $2 \mu\text{M}$ -dexamethasone for 2 weeks, no significant effect upon fluoroaluminate-stimulated inositol phosphate generation was observed (Table 3). A small amplification was observed in the steroid-treated NIH-3T3 cells, but it was too small to explain that observed in the T15+ cells.

The results presented here demonstrate that the inositol phosphate response to fluoroaluminate is increased in the cell line when overexpressing the $p21^{N-ras}$ protein. Since the effect of this ion is to activate directly G-proteins in whole cells in the absence of a receptor stimulant, the data provides further support for our proposition that, in the T15 cell line, $p21^{N-ras}$ causes an increase in inositol phospholipid metabolism and that this effect is at the level of the G-protein to phospholipase C coupling.

The results in Table 2 also suggest that the fluoroaluminate ion can only activate $\text{PtdIns}(4,5)P_2$ breakdown when the appropriate G-protein is in the GDP-bound state. The lys61 mutation of *N-ras* has an extremely low GTPase activity and will thus be essentially always in a GTP-bound state; therefore fluoroaluminate, as shown in Table 2, will be unable to further activate inositol phosphate generation, and any stimulated inositol phosphate generation observed in response to the ion will have been due to the activation of the normal G_p within the cell.

The dose-dependence of the fluoroaluminate observed in the present work was similar to that observed in the stimulation of phospholipase C in turkey erythrocytes (Harden *et al.*, 1988); it is, though, different from that observed in stimulating $\text{PtdIns}(4,5)P_2$ breakdown in CCL39 cells (Paris & Pouyssegur, 1987). The higher apparent EC_{50} observed here (Fig. 1), however, is not an

effect of *ras* transformation, since we have observed an identical value in the parental NIH-3T3 line. This difference may be due to the fact that in CCL39 cells the inositol lipid response is pertussis-toxin-sensitive, whereas in NIH-3T3 cells it is not (Milligan *et al.*, 1989).

The dose-dependence in the cells used in the present study was also much greater than that which has been demonstrated for increasing the activity of adenylate cyclase via the stimulation of stimulatory G-protein (G_s) (Harwood & Rodbell, 1973). At fluoroaluminate concentrations which activate the breakdown of $\text{PtdIns}(4,5)P_2$ (Fig. 1), the stimulation of adenylate cyclase in fat-cell membranes is inhibited (Harwood & Rodbell, 1973). This raises the possibility that fluoroaluminate will only stimulate the breakdown of $\text{PtdIns}(4,5)P_2$ when the activity of adenylate cyclase is not raised; it is perhaps pertinent to this point that we have observed an inhibition of agonist-stimulated $\text{PtdIns}(4,5)P_2$ hydrolysis, in both NIH-3T3 and T15+ cells, by agents which raise intracellular cyclic AMP (Milligan *et al.*, 1989). Table 4

Table 4. Effect of fluoroaluminate upon intracellular cyclic AMP accumulation in T15+ and NIH-3T3 cells

Cyclic AMP concentrations were determined in the cells in the presence of 1 mM-IBMX in order to inhibit intracellular cyclic AMP phosphodiesterase activity. The cells were stimulated for 20 min with the stated concentration of NaF in the presence of $10 \mu\text{M-Al}_2(\text{SO}_4)_3$. Results are means ± S.E.M. pooled from two separate experiments with $n = 8$ in each case. Other details are as in the Methods section.

[NaF] (mM)	Cyclic AMP accumulation (pmol/ 5×10^4 cells)	
	Cells... T15+	NIH-3T3
0	0.126 ± 0.014	0.120 ± 0.027
1	0.141 ± 0.026	0.137 ± 0.002
2	0.141 ± 0.013	0.127 ± 0.030
5	0.154 ± 0.034	0.160 ± 0.022
10	0.190 ± 0.032	0.163 ± 0.011
20	0.166 ± 0.020	0.199 ± 0.036
30	0.238 ± 0.071	0.261 ± 0.064

demonstrates that effect of fluoroaluminate upon intracellular cyclic AMP concentrations in NIH-3T3 and T15+ cells. These experiments were performed in the presence of 1 mM-IBMX so as to inhibit cyclic AMP phosphodiesterase activity. Therefore the increases in cyclic AMP concentrations measured are a reflection of changes in adenylate cyclase activity. No differences in either the basal level or in the magnitude of stimulation of cyclic AMP generation by fluoroaluminate were observed between the cell types (Table 4). The results in Table 4 also show that there is no inhibition of cyclic AMP accumulation at high concentrations of fluoroaluminate; this would suggest no stimulation by the ion of the G_i (inhibitory G) protein (which may be G_{i1}). Activation of G_i would be expected to reduce adenylate cyclase activity; in support of this observation we have been unable to detect the presence of this particular G-protein in either NIH-3T3 cells or their *ras*-transformed derivatives (Milligan *et al.*, 1989). The increased fluoroaluminate-stimulated inositol phosphate generation in the T15+ cell was therefore not due to a change in cyclic AMP level.

Seuwen *et al.* (1988) observed a desensitization of the inositol phospholipid response in CCL39 fibroblasts transformed by mutant Ha-*ras* both in response to agonists and to fluoroaluminate. Recent work from this laboratory has demonstrated that, when T15+ cells are grown at high density, there is a desensitization of the amplified bombesin-stimulated inositol phosphate response (Wakelam, 1988).

Experiments involving washing and inhibitors led me to propose that the inhibition observed was a consequence of autocrine action (Wakelam, 1988). I was unable to say from the experiments reported at what level the inhibitory effect was being manifested. However, since I have now observed an apparent desensitization of the amplified fluoroaluminate response in high-density cultured T15+ cells (Table 3), it is probable that the desensitizing effect is post receptor, being at the level of either G-protein or phospholipase C. The results in Table 3 and Wakelam (1988) suggest one reason for the discrepancy between my observations and those of Seuwen *et al.* (1988). The transformed cells used by these workers were cultured at a very high density, and the medium, which would have contained high levels of autocrines, was not washed away from the cells before stimulation. In the light of my

experiments (Table 3; Wakelam, 1988) I would expect to observe a desensitization under such conditions, as indeed was found by Seuwen *et al.* (1988). The results presented here thus provide further support for my proposition that, in the T15+ cell, $p21^{N-ras}$ can function to couple receptors to phospholipase C and therefore behaves in a G_p -like manner.

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