Desensitization of prostaglandin $F_{2\alpha}$ -stimulated inositol phosphate generation in NIH-3T3 fibroblasts transformed by overexpression of normal c-Ha-ras-1, c-Ki-ras-2 and c-N-ras genes

Fiona M. BLACK and Michael J. O. WAKELAM*

Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland, U.K.

The stimulation of inositol phosphate generation in control and *ras*-gene-transformed NIH-3T3 cells by prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$) was investigated. Compared with the control cells, a desensitization of the response was observed in cells transformed by the overexpression of N-, Ha-, or Ki-*ras* genes. This desensitization was without effect upon the concentration causing half-maximal effect (EC_{50}), dissociation constant (K_d) or number of PGF $_{2\alpha}$ receptors. Inhibition of PG synthesis was without effect upon desensitization, demonstrating that the effect was not agonist-induced. Desensitization could be induced in NIH-3T3 cells by culturing under conditions where the cells were all in the exponential growth phase, or by a 12 h exposure to a C-kinase-activating phorbol ester. These results suggest that desensitization of certain agonist-induced inositol phospholipid responses in *ras*-transformed cells is a consequence of increased cell proliferation and associated amplification in C-kinase activity and is an indirect consequence of transformation by *ras*.

INTRODUCTION

The human *ras*-gene family comprises three similar genes: c-Ha-*ras*-1, c-Ki-*ras*-2 and c-N-*ras*. Each gene encodes a 21000-*M_r* protein termed 'p21^{ras}', which show approx. 85% sequence similarity and are expressed at low levels in most cells (Hall *et al.*, 1983). The *ras* proteins bind guanine nucleotides, express an intrinsic GTPase activity and have been suggested to function as G (guanine-nucleotide-binding)-proteins coupling the receptors for certain growth factors to the stimulation of phospholipase C (Barbacid, 1987).

Evidence in favour of this proposition came from the finding that in the T15 cell line, where the normal N-*ras* gene was overexpressed, the coupling of the bombesin receptor to phospholipase C was amplified (Wakelam *et al.*, 1986; Lloyd *et al.*, 1989). In addition, in cells transformed by an activated N- or Ha-*ras* gene, where the GTPase activities are significantly reduced, the agonist-independent stimulation of phospholipase C was found to be increased (Hancock *et al.*, 1988). An increase in the coupling of an agonist–receptor complex to phospholipase C has been detected in one other study (Chiarrugi *et al.*, 1986), but has not been observed in other NIH-3T3 cells transformed by the overexpression of normal N-*ras* (Lloyd *et al.*, 1989). It has also been demonstrated that the stimulation of inositol phosphate generation in response to some agonists, e.g. platelet-derived growth factor, is reduced as a consequence of the expression of EJ-Ha-*ras* (Parries *et al.*, 1987).

Since it is unclear what, if any, general effect upon inositol phospholipid metabolism occurs after transformation of cells by *ras*, we have studied agonist-induced effects upon inositol phosphate generation in a range of NIH-3T3 cell lines transformed by the overexpression of normal N-, Ha-, or Ki-*ras* genes. The results presented here demonstrate that the prostaglandin $F_{2\alpha}$ (PGF $_{2\alpha}$)-stimulated inositol phosphate response is desensitized in all the *ras*-transformed cells examined, but that this effect can be mimicked by culturing the parent NIH-3T3 cells under conditions where all the cells are in the exponential growth phase. The desensitized state observed in transformed cells is

thus probably a proliferation-related and not an oncogene-associated phenomenon.

MATERIALS AND METHODS

Cell lines

All cells were provided by Dr. C. J. Marshall, Institute of Cancer Research, Fulham, London, U.K. They were maintained in monolayer culture in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) donor-calf serum, except for NIH-3T3 and T15 cells, where the serum concentration was raised to 10%. The expression of the p21^{ras} proteins in T15 and H8-22 cells was induced by the inclusion in the medium of 2 μ M- or 100 nM-dexamethasone respectively. Cells in which expression of the genes was induced are denoted '+ '.

Inositol phosphate experiments

Cells were radiolabelled by incubation in inositol-free DMEM containing dialysed serum and 1 μ Ci of [3 H]inositol/ml for 24 h. The generation of inositol phosphates was determined by two methods, which gave identical results. The first method utilized cells scraped from 25 cm² flasks as previously described (Wakelam *et al.*, 1986). In the second method, incubations were performed with cells grown on 24-well plates. The monolayers were washed with pre-warmed Hanks buffered saline containing 10 mM-glucose and 2% (w/v) BSA (HBG) and incubated for 5 min in HBG containing 10 mM-LiCl before agonist addition. After a 30 min incubation with agonist at 37 °C, incubations were terminated by rapid aspiration of the medium followed by the addition of ice-cold 10% (v/v) HClO₄. The inositol phosphates in the acid-soluble extract were determined by the method of Berridge *et al.* (1982) after neutralization with Freon and tri-n-octylamine (Sharpes & McCarl, 1982).

Binding studies

Cells, grown to confluence on 24-well plates, were washed twice with DMEM and incubated for 1 h in DMEM containing 1% (w/v) BSA at 37 °C. They were then washed with Hanks

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium, PMA, phorbol 12-myristate 13-acetate ('TPA'); PGF $_{2\alpha}$, prostaglandin $F_{2\alpha}$; EC_{50} , concentration giving half-maximal response; K_d , dissociation constant; G-protein, guanine-nucleotide-binding protein.

* To whom correspondence and reprint requests should be addressed.

buffered saline containing 0.2% (w/v) BSA, 10 mM-Hepes, pH 7.3, and 0.01 mM-indomethacin, and incubated for 15 min at 4 °C in ligand-free binding medium. The binding medium consisted of Hanks modified saline supplemented with 0.05% (w/v) BSA, 10 mM-Hepes, pH 7.3, 0.01 mM-indomethacin, Gibco physiological amino acid mixture, 2 mM-glutamine and 2 mM-bacitracin. This medium was then replaced with fresh binding medium containing the stated concentration of [3 H]PGF_{2α}. After a 2 h incubation on ice, the monolayers were rapidly washed four times with ice-cold Hanks buffered saline containing 0.1% (w/v) BSA, 10 mM-Hepes, pH 7.3, and 0.01 mM-indomethacin. The washed cells were solubilized in 0.5 ml of 0.5 M-NaOH containing 2% (w/v) Na₂CO₃ and 1% (w/v) SDS. After neutralization with 1 M-HCl, the total cell-associated radioactivity was determined by liquid-scintillation counting. Non-specific binding was determined in the presence of 1 mM-PGF_{2α} and was generally about 35% of specific binding (37 ± 5; results mean from all experiments). Cell number was determined on cells incubated as described above, except in the absence of ligand, after treatment with trypsin, by Coulter counting.

RESULTS

Stimulation of confluent NIH-3T3 cells with PGF_{2α} (2.1 μM) for 30 min induced a 12-fold increase in the generation of inositol phosphates compared with unstimulated controls (Table 1). We have demonstrated elsewhere that this generation of inositol phosphates is a consequence of agonist-stimulated PtdIns(4,5)P₂ breakdown (Black & Wakelam, 1990). However, when NIH-3T3 cells which had been transformed by a several-fold overexpression of the normal Ha-, Ki-, or N-*ras* genes were stimulated with 2.1 μM-PGF_{2α}, only a 2–4-fold increase in inositol phosphate generation was observed (Table 1). This reduction in the stimulation of inositol phosphate generation in the *ras*-transformed cells was observed at all PGF_{2α} concentrations tested (Fig. 1). The Figure shows that the EC₅₀ (concentration causing half-maximal response) values obtained for all the cell lines tested were very similar (~10⁻⁷ M).

Table 1. Stimulation of inositol phosphate generation by PGF_{2α} in control, Ha-, Ki- and N-*ras* transformed NIH-3T3 cells

Cells were labelled with [3 H]inositol (1 μCi/ml) and, after a 30 min stimulation, inositol phosphate generation was determined as described in the Materials and methods section. Results are expressed as means ± S.E.M., pooled from several experiments with *n* = 6–51. N-866, N-872 and T15+ are NIH-3T3 cells transformed by overexpressed N-*ras*; EC-816, EC-806, EC-807 and H8-22+ are cells transformed by over-expressed Ha-*ras*; Ki-858 and Ki-860 are cells transformed by over-expressed Ki-*ras*. No differences were observed in the basal rate of inositol phosphate generation among any of the cell lines (see Hancock *et al.*, 1988).

Cell line	Inositol phosphate generation (% of untreated control)
NIH-3T3	1250 ± 78
N-866	263 ± 27
N-872	263 ± 49
T15+	380 ± 73
EC-816	413 ± 56
EC-807	261 ± 34
EC-806	355 ± 34
H8-22+	332 ± 22
Ki-858	181 ± 24
Ki-860	219 ± 10

Fig. 1. Dose-dependence of PGF_{2α}-stimulated inositol phosphate generation in normal and *ras*-transformed NIH-3T3 cells

Cells were labelled with 1 μCi of [3 H]inositol/ml for 24 h. Inositol phosphate generation was determined as described in the Materials and methods section. Results are expressed as means ± S.D., pooled from several experiments. (a) NIH-3T3 cells; (b) ○, T15+; △, N-866; ●, EC 816; ■, EC 806; □, H8-22+, ▢, Ki-858; ▲, Ki-860.

In order to determine whether the reduced stimulation of inositol phosphate generation was due to a decrease in receptor number, binding studies were performed. Fig. 2 shows that the specific binding of [3 H]PGF_{2α} to NIH-3T3 cells as a function of radiolabelled ligand concentration was saturable. Scatchard analysis of the binding data (Fig. 2, inset) demonstrated that NIH-3T3 cells possess a single class of cell-surface receptor of *K_d* (dissociation constant) 0.43 × 10⁻⁶ M, and that there are approx. 400 000 receptors per cell. Binding experiments performed upon the *ras*-transformed cells demonstrated that, with the exception of T15 and H8-22 cells, there was no significant difference either in the number of PGF_{2α} receptors per cell or in the *K_d* value

Fig. 2. Characteristics of [³H]PGF_{2α} binding to NIH-3T3 cells

The Figure shows the concentration-dependence of the binding of [³H]PGF_{2α} to intact quiescent NIH-3T3 cells at 4 °C determined as described in the Materials and methods section. The inset shows a Scatchard plot of the same data; bound ('Bound') radiolabelled ligand is expressed in fmol/per 10⁶ cells; the concentration of PGF_{2α} in the medium 'free' is in fmol.

of these receptors (Table 2). The number of PGF_{2α} receptors on the T15+ and H8-22+ cells was increased compared with the control NIH-3T3 line; however, there was no change in *K_d* (Table 2).

In order to determine if there was increased synthesis of PGF_{2α} in the *ras*-transformed cells, which could lead to an agonist-induced desensitization of the inositol phosphate response, normal and *ras*-transformed cells were incubated with the cyclooxygenase inhibitor indomethacin (0.1 mM) for 72 h. This treatment had no effect upon PGF_{2α}-stimulated inositol phosphate generation (results not shown).

It was noted that NIH-3T3 cells cultured to different densities gave differing responses to PGF_{2α}. Table 3 shows that, as the number of cells in a culture dish is increased, and consequently the number of actively dividing cells is diminished, the magnitude of the stimulation of inositol phosphate generation by PGF_{2α} is increased. This increase in response is not due to the number of cells per incubation, since when cells scraped from the same dishes were diluted in incubation medium, there was no effect upon the magnitude of PGF_{2α}-stimulated inositol phosphate generation (results not shown). No differences were found in receptor number between cells cultured at different cell densities (results not shown).

Removal of growth-factor-containing serum from proliferating NIH-3T3 cells, but not from their transformed derivatives,

Table 2. PGF_{2α} receptors on normal and *ras*-transformed cells

Binding studies were performed as described in the Materials and methods section. The results are pooled from the stated number of experiments (*n*) and are expressed as means ± s.d.

Cell line	<i>ras</i> Gene	10 ⁻⁶ × <i>K_d</i> (M)	Receptor no.	<i>n</i>
NIH-3T3	—	0.437 ± 0.06	391 889 ± 63 468	12
EC-806	Ha	0.459 ± 0.04	268 219 ± 73 830	2
EC-807		0.318	310 823 ± 3509	1
EC-816		0.501 ± 0.05	392 101 ± 68 767	2
H8-22+		0.211	502 144 ± 68 767	2
Ki-858	Ki	0.368 ± 0.04	456 028 ± 26 966	2
Ki-860		0.649	400 566 ± 6091	1
N-866	N	0.803 ± 0.72	625 213 ± 20 1308	2
N-872		0.835 ± 0.67	537 548 ± 78 021	2
T15+		0.435 ± 0.18	1731 335 ± 67 033	2

Table 3. Effect of cell culture density upon the responsiveness of NIH-3T3 cells to PGF_{2α}

NIH-3T3 cells were plated at the stated density; 16 h later the medium was changed to one containing 1 μCi of [³H]inositol/ml and, 24 h later, PGF_{2α} (2.1 μM)-stimulated inositol phosphate accumulation was determined as described in the Materials and methods section. Results are means ± s.d., *n* = 4 in each case, and the data are from one experiment which gave qualitatively the same results as three others.

Cell density at plating	Radioactivity (d.p.m.) in:		Increase in inositol phosphates (%)
	Control cells	PGF _{2α} -treated cells	
5 × 10 ⁵	620 ± 14	11 983 ± 108	1832 ± 27
2.5 × 10 ⁵	625 ± 6	11 185 ± 309	1690 ± 49
1 × 10 ⁵	288 ± 14	2565 ± 72	790 ± 25
7.5 × 10 ⁴	204 ± 19	1547 ± 17	659 ± 9
5 × 10 ⁴	143 ± 5	358 ± 12	151 ± 9

caused a cessation of proliferation. When NIH-3T3 cells were cultured under serum-free conditions and then stimulated with PGF_{2α}, an increased inositol phosphate response was observed compared with cells cultured in the presence of 10% calf serum (Table 4). However, no such change in response was observed in *ras*-transformed cells treated in the same way (Table 4).

Activation of protein kinase C has frequently been demonstrated to result in the desensitization of agonist-stimulated inositol phosphate generation. To determine if such a mechanism could be involved in the desensitization observed here, normal and *ras*-transformed NIH-3T3 cells were cultured in the presence of the C-kinase activator phorbol 12-myristate 13-acetate (PMA). Exposure of both control and *ras*-transformed cells to 100 nM-PMA for 12 h caused a significant decrease in PGF_{2α}-stimulated inositol phosphate generation (Table 5). Exposure of the cells to the phorbol ester for 24 or 48 h also reduced the PGF_{2α}-stimulated inositol phosphate generation, but to a lesser extent than that observed by the shorter treatment. Incubation of cells with the non-C-kinase-activating β-phorbol was without effect upon inositol phosphate responses (Table 5).

Table 4. Effect of different labelling conditions upon $\text{PGF}_{2\alpha}$ -stimulated inositol phosphate accumulation in NIH-3T3 and EC-807 cells

NIH-3T3 and EC807 cells were labelled for 24 h with 1 μCi of [^3H]inositol/ml in medium containing a defined growth-factor supplement (1 ng of insulin/ml, 10 ng of transferrin/ml and 10 pg of epidermal growth factor/ml) or medium supplemented with 10% (v/v) donor-calf serum. The cells were then stimulated with $\text{PGF}_{2\alpha}$ (2.1 μM). Results are means $\pm$ S.D. where $n = 4$ and are from one experiment typical of two others. Other details are as in the legend to Table 3.

Cell line	Labelling conditions	Radioactivity in:		Increase in inositol phosphates (%)
		Control cells	$\text{PGF}_{2\alpha}$ -treated cells	
NIH-3T3	Growth-factor supplement	868 $\pm$ 45	46 793 $\pm$ 437	5291 $\pm$ 389
NIH-3T3	10% Calf serum	543 $\pm$ 22	12 653 $\pm$ 805	2230 $\pm$ 148
EC-807	Growth-factor supplement	9218 $\pm$ 401	51 433 $\pm$ 693	457 $\pm$ 8
EC-807	10% Calf serum	10 231 $\pm$ 1385	54 345 $\pm$ 1705	431 $\pm$ 17

Table 5. Effect of a 48 h treatment with PMA upon $\text{PGF}_{2\alpha}$ -stimulated inositol phosphate generation in NIH-3T3, EC 807 (Ha-*ras*) and N-866 (N-*ras*) cells

Cells were labelled with 1 μCi of [^3H]inositol/ml in the presence or absence of PMA (100 nM) for the stated times, or β -phorbol (100 nM) for 24 h before stimulation with $\text{PGF}_{2\alpha}$ (2.1 μM). Results are means $\pm$ S.D., where $n = 12$ –16, and are pooled from three separate experiments.

Period of pretreatment with PMA (h)	Cell line...	Increase in inositol phosphates (% of control)		
		NIH-3T3	EC-807	N-866
0		575 $\pm$ 29	332 $\pm$ 31	360 $\pm$ 13
12		243 $\pm$ 36	36 $\pm$ 4	44 $\pm$ 8
24		334 $\pm$ 14	90 $\pm$ 7	73 $\pm$ 21
48		372 $\pm$ 14	73 $\pm$ 10	64 $\pm$ 2
β -Phorbol (24 h)		598 $\pm$ 15	321 $\pm$ 23	380 $\pm$ 6

DISCUSSION

The results presented here demonstrate that, in cells transformed by the overexpression of normal N-, Ha- or Ki-*ras* genes, the stimulation by $\text{PGF}_{2\alpha}$ of inositol phosphate generation is reduced (Table 1). This effect is not due to any diminution in receptor number, or to a change in EC_{50} or K_d (Fig. 1; Tables 1 and 2) and thus would appear to be due to a reduced coupling of the agonist-receptor complex to phospholipase C. The increase in receptor number in the T15+ and H8-22+ cells may be due to inhibition of $\text{PGF}_{2\alpha}$ synthesis in the cells by the presence in the culture medium of the steroid analogue dexamethasone. This could reduce agonist-induced receptor down-regulation, as has been observed in mouse fibrosarcoma cells (Tashjian *et al.*, 1975). The reduced inositol phosphate response is unlikely to be due to any reduction in phospholipase C activity, since we have previously demonstrated no change in the basal rate of inositol phosphate generation in cells transformed by an overexpression of normal *ras* genes (Hancock *et al.*, 1988). The site of the desensitization may thus be at either the level of coupling between the receptor and G-protein, or the G-protein and phospholipase C.

Desensitization of an agonist's second-messenger response can be induced both by exposure to the agonist, i.e. homologously, or after exposure to an alternative agonist, i.e. heterologously. It appears that the desensitization observed here is induced

heterologously, since inhibition of $\text{PGF}_{2\alpha}$ synthesis by treatment of the cells with indomethacin has no effect upon the stimulation of inositol phosphate generation in response to $\text{PGF}_{2\alpha}$.

Seuwen *et al.* (1988) detected an apparent desensitization of the stimulation of inositol phosphate generation in response to fluoroaluminate ions in CCL 39 fibroblasts transformed by activated Ha- and Ki-*ras* genes. Although these data suggest that the desensitization in *ras*-transformed cells is at the G-protein level, this is unlikely to be the sole site. In the T15+ cell, which overexpresses p21^{N-*ras*}, we have detected an amplification both in bombesin- (Wakelam *et al.*, 1986; Lloyd *et al.*, 1989) and in fluoroaluminate (Wakelam, 1989)-stimulated $\text{PtdIns}(4,5)\text{P}_2$ breakdown. However, $\text{PGF}_{2\alpha}$ -stimulated inositol phosphate generation is desensitized in this cell (Table 1), with no change in affinity of dissociation constant, or a decrease in receptor number (Figs. 1 and 2; Table 2). Therefore at least part of the desensitization mechanism must be at the level of receptor-to-G-protein coupling.

Transformation of cells by a range of *ras* genes has been demonstrated to lead to an increase in the levels of diacylglycerol (Preiss *et al.*, 1986; Kamata *et al.*, 1987; Lacal *et al.*, 1987a; Wolfman & Macara, 1987) and to a corresponding increase in the activity of protein kinase C (Wolfman & Macara, 1987). Indeed, protein kinase C activity has been demonstrated to be essential for *ras*-induced mitogenesis (Lacal *et al.*, 1987b). The stimulation of mitogenesis in a range of normal fibroblast cell lines by growth factors has also been shown to be accompanied by an increase in diacylglycerol levels and in protein kinase C activity (Habernicht *et al.*, 1981; Cook *et al.*, 1990). An increase in protein kinase C activity induces the desensitization of agonist-stimulated $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis in a number of cell types (see, e.g., Brown *et al.*, 1987). Thus, if the desensitization of $\text{PGF}_{2\alpha}$ -stimulated inositol phospholipid breakdown is a consequence of a proliferation-related increase in protein kinase C activity, one would expect to observe desensitization in both the transformed cells and in rapidly proliferating non-contact-inhibited NIH-3T3 cells, as is indeed demonstrated in Tables 1 and 3.

Removal of serum from NIH-3T3 cells causes a cessation of proliferation, a decrease in C-kinase activity and consequently a reduction in the observed desensitization (Table 4). However, the removal of serum from *ras*-transformed cells did not result in resensitization. Since *ras*-transformed cells are known to secrete a range of autocrine growth factors, including transforming growth factor α and platelet-derived-growth-factor-like molecules (Owen & Ostrowski, 1987), it is not possible to remove from the culture medium factors which are able to stimulate a range of signalling pathways, including inositol phospholipid breakdown. Protein kinase C activity will therefore continue to

be stimulated in *ras*-transformed cells, even in the absence of serum. It is therefore possible that the desensitization of $\text{PGF}_{2\alpha}$ -stimulated inositol phosphate generation in both the *ras*-transformed and the proliferating NIH-3T3 cells is due to increased C-kinase activity. This suggestion is supported by the finding that, when normal and *ras*-transformed NIH-3T3 cells are cultured in the presence of the C-kinase activator PMA, their response to $\text{PGF}_{2\alpha}$ is significantly reduced as compared with untreated control cells (Table 5). Incubation of cells for 12 h with PMA caused a maximal decrease in response, which then increased after a 24 h incubation with the phorbol ester. However, after a 48 h incubation with PMA the $\text{PGF}_{2\alpha}$ -stimulated inositol phosphate response remained significantly lower than in control cells or those treated with the inactive β -phorbol (Table 5). In control and *ras*-transformed NIH-3T3 cells, prolonged exposure to PMA does not lead to a total down-regulation in C-kinase activity. After a 48 h exposure to the phorbol ester the binding of [^3H]phorbol dibutyrate was reduced to between 10 and 20% of the control (S. A. Davies, F. M. Black & M. J. O. Wakelam, unpublished work). Therefore activation of protein kinase C can still occur and induce desensitization.

The site of the possible action of protein kinase C is unclear, but may be at the receptor level. Desensitization of the β -adrenergic-receptor-stimulated adenylate cyclase activity has been shown to be due to receptor phosphorylation (Sibley *et al.*, 1987), a reaction which can be catalysed by protein kinase C (Bouvier *et al.*, 1987). However, direct demonstration of protein kinase C-catalysed $\text{PGF}_{2\alpha}$ receptor phosphorylation awaits receptor purification or the generation of specific antibodies.

The experiments presented here also suggest a possible explanation for the observed variability of effects of transfecting *ras* genes into fibroblast cells upon inositol phospholipid metabolism. Since such cells are continuously proliferating and do not contact-inhibit, they do not quiesce. Therefore, any amplified agonist-stimulated inositol phosphate response may be desensitized and thus not be apparent in such a non-synchronous population of cells. It is thus only in cells such as T15, where some aspect of desensitization is perhaps non-functional, that an amplified agonist-stimulated inositol phospholipid response is detectable. However, even in this cell line we have observed that high-density culturing leads to autocrine-induced desensitization of bombesin-stimulated inositol phosphate generation (Wakelam, 1988).

We are grateful to Dr. C. J. Marshall for generously supplying the cell lines used in this work. This study was supported by a grant from the Cancer Research Campaign.

REFERENCES

- Barbacid, M. (1987) *Annu. Rev. Biochem.* **56**, 779–827
- Berridge, M. J., Downes, C. P. & Hanley, M. R. (1982) *Biochem. J.* **206**, 587–589
- Black, F. M. & Wakelam, M. J. O. (1990) *Biochem. J.* **266**, 661–667
- Bouvier, M., Leeb-Lundberg, L. M. F., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1987) *J. Biol. Chem.* **262**, 3106–3113
- Brown, K. D., Blakeley, D. M., Hamon, M. H., Laurie, M. S. & Corps, A. N. (1987) *Biochem. J.* **245**, 631–639
- Chiarrugi, V. P., Pasquali, F., Vannucchi, S. & Ruggiero, M. (1986) *Biochem. Biophys. Res. Commun.* **141**, 591–599
- Cook, S. J., Palmer, S., Plevin, R. & Wakelam, M. J. O. (1990) *Biochem. J.* **265**, 617–620
- Habenicht, A. J. R., Glomset, J. A., King, W. C., Nist, C., Mitchell, C. D. & Ross, R. (1981) *J. Biol. Chem.* **256**, 12329–12335
- Hall, A., Marshall, C. J., Spurr, N. & Weiss, R. A. (1983) *Nature (London)* **303**, 396–400
- Hancock, J. F., Marshall, C. J., McKay, I. A., Gardner, S. D., Houslay, M. D., Hall, A. & Wakelam, M. J. O. (1988) *Oncogene* **3**, 187–193
- Kamata, T., Sullivan, N. F. & Wooten, M. W. (1987) *Oncogene* **1**, 37–46
- Lacal, J. C., Moscat, J. & Aaronson, S. A. (1987a) *Nature (London)* **330**, 269–272
- Lacal, J. C., Fleming, T. P., Warren, B. S., Blumberg, P. M. & Aaronson, S. A. (1987b) *Mol. Cell. Biol.* **7**, 4146–4149
- Lloyd, A. C., Davies, S. A., Crossley, I., Whitaker, M., Houslay, M. D., Hall, A., Marshall, C. J. & Wakelam, M. J. O. (1989) *Biochem. J.* **260**, 813–819
- Owen, R. D. & Ostrowski, M. C. (1987) *Mol. Cell. Biol.* **7**, 2512–2520
- Parries, G., Hoebel, R. & Racker, E. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2648–2652
- Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E. & Bell, R. M. (1986) *J. Biol. Chem.* **261**, 8597–8600
- Seuwen, K., Lagard, A. & Pouyssegur, J. (1988) *EMBO J.* **7**, 161–168
- Sharpes, E. S. & McCarl, R. L. (1982) *Anal. Biochem.* **124**, 421–424
- Sibley, D. R., Daniel, K., Strader, C. D. & Lefkowitz, R. J. (1987) *Arch. Biochem. Biophys.* **258**, 24–32
- Tashjian, A. H., Voelkel, E. F., McDonough, J. & Levine, L. (1975) *Nature (London)* **280**, 408–410
- Wakelam, M. J. O. (1988) *FEBS Lett.* **228**, 182–186
- Wakelam, M. J. O. (1989) *Biochem. J.* **259**, 737–741
- Wakelam, M. J. O., Davies, S. A., Houslay, M. D., McKay, I., Marshall, C. J. & Hall, A. (1986) *Nature (London)* **323**, 173–176
- Wolfman, A. & Macara, I. G. (1987) *Nature (London)* **325**, 359–361

Received 31 July 1989/5 December 1989; accepted 11 December 1989