Volume 228, number 1, 182–186 FEB 05556 February 1988

Inhibition of the amplified bombesin-stimulated inositol phosphate response in N-*ras* transformed cells by high density culturing

Michael J.O. Wakelam

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

Received 10 December 1987

The bombesin-stimulated inositol phosphate response is only increased in cells transformed by the overexpression of N-*ras* when they are cultured under sub-confluent conditions. The inhibition of the amplified bombesin stimulation can be partially reversed by either, incubation of the cells for 5 h with 1 mM suramin, or by a 30 min preincubation of cells in excess buffer. The inhibitory effects are probably caused by the effects of autocrines and may explain some of the different published observations concerning the effects of *ras* gene products upon inositol phospholipid metabolism.

N-*ras* gene; Inositol phosphate; Autocrine; Bombesin

1. INTRODUCTION

The three *ras* genes, N, Ha and Ki each code for 21 kDa molecular mass proteins termed p21. Each of these proteins has been shown to bind guanine nucleotides and to possess an intrinsic GTPase activity, which has led to the suggestion that they function as G-proteins in cells [1]. Whilst the protein sequences of the three p21 proteins show a greater than 85% homology, it is by no means clear if the functions of the proteins are the same in all cells. We have recently demonstrated that in cells transformed by overexpression of the N-*ras* proto-oncogene, under the control of a steroid inducible promotor, there is an increase in the breakdown of inositol phospholipids in response to a range of growth factors, in particular to bombesin, despite no change in receptor number [2]. This observation led us to propose that $p21^{N\text{-}ras}$ acted in this cell line to couple the receptor for bombesin to phospholipase C and was thus functioning in a Gp-like manner.

This suggestion of a function for the *ras* proteins is supported by similar observations by Chiarugi et al. [3] who demonstrated an increased muscarinic receptor stimulated inositol phospholipid response in Balb/c 3T3 fibroblasts transformed by the EJ-Ha-*ras* oncogene in the absence of a change in receptor number. This increase could be inhibited by incubation of permeabilised cells with an anti-*ras* antibody, Y-13259. Further support for the proposal has been provided by studies demonstrating an increase in inositol phospholipid metabolism in cells transformed by various *ras* genes [4,5].

However, recent work from Paries et al. [6] has shown a contrary picture, in that in cells transformed by overexpression of the Ha-*ras* proto-oncogene, also under the control of a steroid inducible promotor, the inositol phospholipid response to one growth factor, PDGF, was reduced whilst the response to another, bradykinin, was increased. These authors also show that the number of bradykinin receptors is increased in these cells in contrast to the previously reported

Correspondence address: M.J.O. Wakelam, Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

observations for bombesin and for muscarinic receptors. These and other unpublished data have led us to examine the T15 cell system in greater depth and here we report that the amplified bombesin stimulated inositol phospholipid response is only observed in sub-confluent cells and is attenuated by the known production of autocrines by *ras* transformed cells.

2. MATERIALS AND METHODS

NIH-3T3 cells were cultured in 25 cm^2 tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) donor calf serum (Gibco) and antibiotics as described in [2]. The T15+ cell line was cultured similarly except that the medium contained 2 μM dexamethasone (Sigma) to continually activate the MMTV-LTR promotor and thus stimulate the transcription of the inserted human fetal N-*ras* gene [7]. For inositol phospholipid experiments the medium was changed to inositol-free DMEM containing 10% (v/v) dialysed donor calf serum, dexamethasone as appropriate and 1 mCi/ml [^{3}H]-inositol (Amersham). 18 h later the medium was aspirated, the monolayers were washed with pre-warmed (37°C) Hank's modified saline and scraped with a teflon coated spatula into the same buffer containing 10 mM glucose and 1% (w/v) bovine serum albumin (HBG). The cells were collected by centrifugation washed in the same buffer and resuspended in HBG containing 10 mM LiCl. Following a 10 min incubation at 37°C, the cell suspension was dispensed into plastic insert vials containing agonist or buffer as described in section 3 and incubated in a shaking water bath at 37°C. Incubations were terminated after 20 min by the addition of chloroform/methanol (1:2). The vials were vortex-mixed and left to extract for 20 min at room temperature. The phases were split by the addition of chloroform and water and the accumulation of radiolabeled inositol phosphates determined upon an aliquot of the upper aqueous phase by batch chromatography on Dowex 1 × 8-formate resins [8] as described [2]. The radioactivity associated with the inositol phospholipids was determined by scintillation counting of an aliquot of the lower chloroform phase following air drying.

Tissue culture media and materials were obtained from Gibco, Paisley, Scotland; bombesin was from Cambridge Research Biochemicals, Cambridge, England; other materials were of the highest grade from previously reported sources.

3. RESULTS AND DISCUSSION

We have previously demonstrated that in T15 cells, induced to overexpress $p21^{N\text{-}ras}$ (T15+), bombesin elicits a marked stimulation in the generation of inositol phosphates [2]. These studies were performed, however, using cells that were never more than 50–60% confluent. When similar experiments were performed using T15+ cells at higher levels of confluency we found (table 1) that the magnitude of the inositol phosphate response to bombesin was progressively reduced as the number of cells per culture was increased.

Table 1

The effect of cell culture density upon the responsiveness of T15+ cells to bombesin

Cell density at plating	dpm in control	% increase in inositol phosphate generation by 0.1 μM bombesin
10^5/ml	224.5 ± 31.8	9 ± 6
5×10^4/ml	115.8 ± 9.4	22 ± 9
2.5×10^4/ml	88.0 ± 10.6	41 ± 8

T15+ cells were plated at the stated density, 48 h later the medium was changed to one containing [^{3}H]inositol and 18 h later bombesin stimulated inositol phosphate generation was determined as described in section 2. Results are means ± SD, $n = 4$ in each case and the data are from one experiment which gave qualitatively the same result as two others

This reduction in response to bombesin was not due, however, to the number of cells per incubation, since if cells from the same culture dishes were diluted in the incubation medium then the magnitude by which bombesin stimulated the generation of inositol phosphates was unchanged (table 2).

These results suggest that there is a change in the receptor activation machinery in *ras* transformed cells when they are cultured at a high density. This change was at least partially reversible, since incubation of T15+ cells in fresh buffer for 30 min, prior to challenge with bombesin, led to a marked augmentation of the ability of bombesin to stimulate inositol phosphate generation as compared to that seen using cells which had not been incubated with fresh medium prior to stimulation with the peptide (table 3).

These changes appeared to be specific for the cells transformed by overexpression of N-*ras*, as no alterations were observed in the already diminutive ability of bombesin to stimulate the generation of inositol phosphates in the wild type NIH-3T3 cells in response to any treatment (not shown). This effect upon bombesin stimulated inositol phosphate generation therefore, is either generated by the *ras* gene product, p21, or is an in-

Table 2

The effect of cell number dilution upon bombesin stimulation of inositol phosphate generation

Dilution of cells	dpm in control	% increase
None	732.5 ± 60.5	49 ± 10
1 in 2	461.2 ± 51.3	41 ± 11
1 in 3	243.3 ± 34.0	56 ± 15
1 in 4	210.8 ± 19.2	39 ± 5

T15+ cells labelled with [^{3}H]inositol as described in section 2 were scraped from 3 flasks each containing approx. 5×10^5 cells. They were diluted as described in the table before dispensing into vials and bombesin (0.1 μM)-stimulated inositol phosphate generation was determined as described in section 2. Results are means ± SD, $n = 4$ in each case and the data are from one experiment which is typical of two others

Table 3

Reversibility of the inhibition of bombesin stimulated inositol phosphate generation

(A) Washing experiments

Treatment of cells	dpm in control	% increase
No preincubation	272.2 ± 30.1	21 ± 8
15 min preincubation	318.6 ± 20.9	32 ± 9
30 min preincubation	268.9 ± 19.9	61 ± 9

(B) Suramin experiments

Additions to cells	% increase in inositol phosphates	
	Expt 1	Expt 2
No addition	47 ± 10	3 ± 5
5 h 1 mM suramin in culture	118 ± 31	35 ± 8
1 mM suramin in incubation only	47 ± 8	4 ± 6

In (A) T15+ cells were preincubated for the stated time in 10 ml of HBG in a shaking water bath at 37°C, collected by centrifugation and inositol phosphate generation in response to bombesin was determined as described in section 2. In (B) some T15+ cells were exposed to 1 mM suramin for the final 5 h of the labelling period, suramin was added to some incubations as stated, and inositol phosphate generation in response to 0.1 μM bombesin determined as described in section 2. $n = 4$ in each case and results are expressed as means ± SD and are typical of at least 2 other experiments

direct effect as a consequence of transformation by *ras*. When cells are transformed by *ras* they begin to secrete a variety of autocrine growth factors which include TGFa, TGFb and PDGF-like molecules [9,10]. If the concentration of cells in a culture increases the concentration of such autocrines in the medium will also increase and it may be through their action that a desensitization of the bombesin response ensued. In an attempt to determine if these autocrines were responsible for the inhibitory effects observed two different types of experiments were performed. Firstly confluent, dense T15+ cells were exposed to 1 mM suramin for the last 5 h of the labelling period. This agent acts to inhibit the binding of a range of growth factors, including PDGF and EGF to their receptors [11]. Indeed such a treatment partially reversed the desensitization in bombesin responsiveness observed in high density cultures (table 3). In contrast, control experiments demonstrated that suramin had no effect upon bombesin-stimulated inositol phospholipid responses in either low density cultures or the wild type NIH-3T3 cells. Secondly, cells cultured at a low density were labelled for 24 h in a medium which had been conditioned by a high density culture for 24 h. Under such conditions the inositol phosphate response of the cells to bombesin was markedly attenuated by the presence of this medium as compared to cells exposed to a non-conditioned medium. Indeed, the magnitude of the bombesin-stimulated response in cells treated with 'high density' conditioned media dropped to a similar value to that observed in a dense culture of cells (fig.1). We feel that such experiments indicate that it is high concentrations of autocrine factors which are responsible for the inhibitory effects observed.

Since one of the known autocrines, PDGF, is able to stimulate inositol phospholipid breakdown in the T15+ cell [2], it is possible that persistent activation of protein kinase C, as a result of high concentrations of agonist (PDGF) in the high cell density medium, will lead to desensitization of the bombesin responsiveness. Alternatively, stimulation of tyrosine kinase activity by either PDGF or TGFa [12] could be inducing these effects. However, since suramin is equally effective at PDGF and EGF receptors it is not possible to discriminate between effects upon C- or tyrosine-kinase activities.

The relevance of the desensitization of bombesin-stimulated inositol phospholipid re-

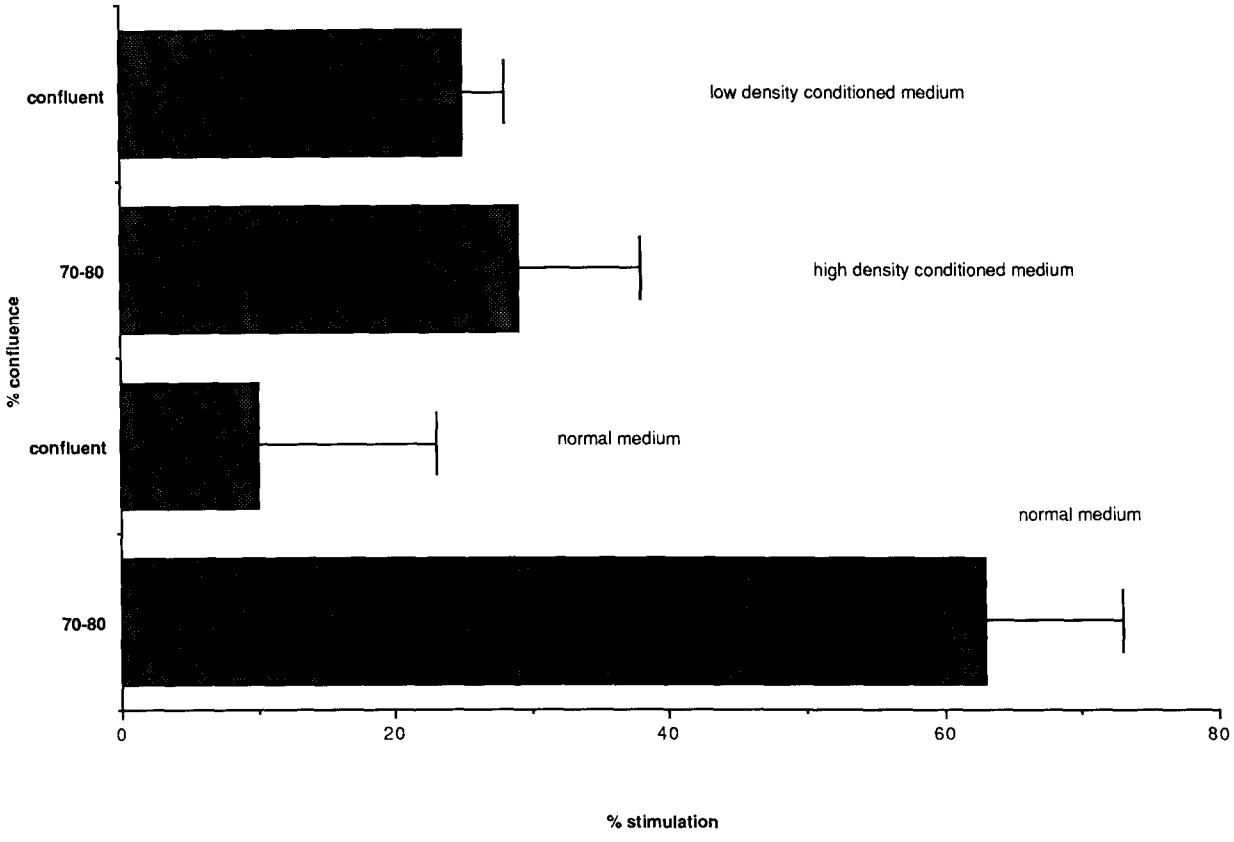

Fig.1. The effect of conditioned media upon the response of T15+ cells to bombesin. Inositol free medium was conditioned by the stated type of culture of T15+ cells by a 24 h exposure. For labelling purposes 1 mCi/ml [^{3}H]inositol was added before use. Bombesin (0.1 μM)-stimulated inositol phosphate generation was determined as described in section 2 and results are means ± SD of one experiment where $n = 4$. The experiment was repeated once and gave qualitatively similar results.

sponses by autocrines is unclear. Transformed cells lose contact inhibition of growth and the T15+ cell forms 'mounds' of cells in cultures of increasing density [7]. It is unknown if all the cells in such a culture are still dividing, but it is possible that it is only on the outside of such 'mounds' that cell division is still activated. If this is the case then inhibition of the agonist-stimulated inositol phospholipid response by a high local concentration of growth factors, such as would occur inside one of these 'mounds', would provide an ideal regulatory mechanism. However, whatever the mechanism, or role of this effect, the results in this paper make it clear that comparison of experimental data between different laboratories of the effects of *ras* gene products upon inositol phospholipid metabolism must be made with great care, and could go some way towards explaining the differing published observations.

Acknowledgements: This work was supported by grants from the Cancer Research Campaign and the Medical Research Council. I thank Sandra Gardner and Christine Holcroft for invaluable technical assistance and Professor M.D. Houslay for helpful discussions.

REFERENCES

[1] Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827.
[2] Wakelam, M.J.O., Davies, S.A., Houslay, M.D., McKay, I., Marshall, C.J. and Hall, A. (1986) Nature 323, 173–176.
[3] Chiarugi, V.P., Pasquali, F., Vannuchi, S. and Ruggiero, M. (1986) Biochem. Biophys. Res. Commun. 141, 591–599.

[4] Preiss, J., Loomis, C.R., Bishop, W.R., Stein, R., Neidel, J.E. and Bell, R.M. (1986) J. Biol. Chem. 261, 8597–8600.
[5] Fleischman, L.F., Chawala, S.B. and Cantley, L.C. (1986) Science 231, 407–410.
[6] Paries, G., Hoebel, R. and Racker, E. (1987) Proc. Natl. Acad. Sci. USA 84, 2648–2652.
[7] McKay, I.A., Marshall, C.J., Cales, C. and Hall, A. (1986) EMBO J. 5, 2617–2621.
[8] Berridge, M.J., Downes, C.P. and Hanley, M.R. (1982) Biochem. J. 206, 587–595.
[9] Marshall, C.J., Hall, A. and Ozanne, B. (1985) Proc. R. Soc. London B226, 99–106.
[10] Bowen-Pope, D., Vogel, A. and Ross, R. (1984) Proc. Natl. Acad. Sci. USA 81, 2396–2400.
[11] Bestholtz, C., Johnson, A., Heldin, C.-H. and Westermark, B. (1986) Proc. Natl. Acad. Sci. USA 83, 6440–6444.
[12] Foulkes, J.G. and Rosner, M.R. (1985) Mol. Asp. Cell. Reg. 4, 217–252.