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Bombesin stimulation of inositol 1,4,5-trisphosphate generation and intracellular calcium release is amplified in a cell line overexpressing the N-*ras* proto-oncogene

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Bombesin stimulation of T15 cells in which the inducible N-*ras* oncogene is overexpressed caused elevated production of inositol phosphates compared to uninduced cells [Wakelam, Davies, Houslay, McKay, Marshall & Hall (1986) Nature (London) **323**, 173–176]. This elevated response is shown here to result from increased generation of inositol 1,4,5-trisphosphate leading to an elevated release of intracellular stored Ca^{2+}. Single-cell analysis of Ca^{2+} release showed that the elevated response is not a consequence of an increased fraction of responding cells. These amplifications are consistent with $p21^{N\text{-}ras}$ acting like a guanine nucleotide coupling protein in this cell line.

INTRODUCTION

There are three functional *ras* genes in the mammalian genome, C-Ha-*ras*, C-Ki-*ras*2 and N-*ras* which appear to be expressed at low levels in most normal cells. These genes have been implicated in human tumours, yet the function of the gene products in the normal cell remains unclear (for review see Barbacid, 1987). Each gene encodes a similar polypeptide $p21^{ras}$, which is found associated with the plasma membrane, binds guanine nucleotide and expresses an intrinsic GTPase activity (Willingham *et al.*, 1983; McGrath *et al.*, 1984). These properties have led to the speculation that $p21^{ras}$ proteins are regulatory G-proteins which mediate the effects of external growth factors (Tanabe *et al.*, 1985).

To study this question the T15 cell line was constructed. This is an NIH-3T3-derived clone which contains the N-*ras* proto-oncogene under the control of the MMTV, glucocorticoid inducible, promoter. In the presence of the inducer dexamethasone, $p21^{N\text{-}ras}$ is overexpressed and the cells ($T15^+$) become transformed (McKay *et al.*, 1986). Using this cell line, we (Wakelam *et al.*, 1986) have demonstrated that the $T15^+$ cells exhibited an increased response to the ligand bombesin, in terms of total inositol phosphate production compared to the cells grown in the absence of the inducer ($T15^-$). As the number and affinity of the bombesin receptors on each cell remained unchanged, it was postulated that the overproduction of the N-*ras* proto-oncogene can increase the coupling of the bombesin receptor to the breakdown of inositol phospholipids. Further support for such a contention came from our recent experiments which showed that cells transformed by activated, point-mutated *ras* oncogenes appeared to express a constituitively activated phospholipase C activity, which was seen as an enhanced basal rate of production of total inositol phosphates (Hancock *et al.*, 1988).

The T15 cell presents an unusual situation in that the size of the response to a ligand is changed by increasing the levels of normal $p21^{N\text{-}ras}$ protein without any change in the ligand's receptor number or affinity. The agonist-stimulated generation of inositol phosphates is generally a consequence of the G-protein-mediated, phospholipase C-catalysed, hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to generate inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and *sn*-1,2-diacylglycerol (DAG) (see Downes & Michell, 1985, for review). If $p21^{N\text{-}ras}$ is acting in the T15 cell line to amplify the bombesin-stimulated breakdown of PtdIns(4,5)P_2 then there should be amplified Ins(1,4,5)P_3 generation and a corresponding amplification of agonist-stimulated intracellular Ca^{2+} release. In this paper we show that both of these responses are amplified, which is consistent with $p21^{N\text{-}ras}$ acting like a guanine nucleotide coupling protein in this cell line.

MATERIALS AND METHODS

Cell culture

T15 cells were cultured in DMEM (Gibco) containing antibiotics and 10% (v/v) donor calf serum (Sera Lab) in a 5% CO_2, 95% humidity atmosphere at 37 °C with ($T15^+$) or without ($T15^-$) dexamethasone at 80 nM or 2 μM; no differences were observed between cells treated with the two steroid concentrations.

Inositol phosphate experiments

The cells were plated at a density of 10^4 cells/ml in 24-well culture plates and used when confluent. The $T15^-$

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; PG, prostaglandin.

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cells were cultured in serum which had been treated with dextran-coated charcoal to remove contaminating steroids.

The medium was changed to inositol-free DMEM containing 10 μCi of [^{3}H]inositol/ml and dexamethasone as required 16 h before experiments were performed. Initial experiments demonstrated that the serum-free labelling of cells for 16 h had no effect upon the magnitude of responsiveness, but simply increased the incorporation of [^{3}H]inositol into inositol phospholipids and therefore into the generated inositol phosphates.

Following the labelling period the medium was removed and the monolayers washed three times with Hank's buffered saline, pH 7.4, containing 1% (w/v) bovine serum albumin and 10 mM-glucose (HBG). They were then incubated at 37 °C for 5 min in HBG containing 10 mM-LiCl. This medium was removed and replaced by fresh HBG containing 10 mM-LiCl and bombesin, as stated in the Results section; this solution had been prewarmed to 37 °C. Incubations were terminated by rapid aspiration followed by the addition of 50 μl of ice-cold 10% (v/v) perchloric acid. The contents of the wells were removed by scraping and the wells washed by the addition of 50 μl water. Following centrifugation the supernatants were neutralized by the addition of 1.5 M-KOH/60 mM-Hepes. The precipitated potassium perchlorate was removed by centrifugation.

The supernatants were analysed by ion-exchange chromatography either on Dowex 1 X8 formate columns or by h.p.l.c. Before application to 1 ml Dowex columns the samples were added to 1 ml of 5 mM-sodium tetraborate/0.5 mM-EDTA, pH 6.7. The columns were washed with 12 ml of water and 12 ml of 60 mM-ammonium formate/5 mM-sodium tetraborate and then inositol mono-, bis-, tris- and tetrakisphosphates were eluted with 0.1 M-formic acid containing 0.2 M-, 0.4 M-, 0.8 M- and 1.2 M-ammonium formate respectively. H.p.l.c. analysis was performed as described by Irvine *et al.* (1985) using a Partisil 10 SAX column (Whatman International). A stepped gradient from 0 to 1.7 M-ammonium formate, pH 3.7 (adjusted with orthophosphoric acid) was employed with a flow rate of 1 ml/min and samples collected every 15 s. The radioactivity associated with each sample generated by each method was determined by scintillation counting following the addition of either Ecoscint or Liquiscint (National Diagnostics).

Fura 2AM loading

The T15 cells were removed from dishes by trypsinization and resuspended in RPMI medium containing 10 mM-Hepes, pH 7.5, and 10% calf serum, spun down and resuspended in serum-free RPMI/Hepes at a cell concentration of 5×10^6/ml. They were loaded by incubating with 10 μM-Fura 2 AM (Molecular Probes, Junction City, OR, U.S.A.) at 37 °C for 15 min. The cells were spun down, resuspended in fresh Hepes-buffered RPMI and incubated for a further 45 min at 37 °C. Fluorimetric measurements demonstrated that this time was adequate for complete intracellular hydrolysis of the Fura 2 acetoxymethylester. The cells were then washed in HBS (140 mM-NaCl/5 mM-KCl/1.8 mM-$MgCl_2$/10 mM-glucose/10 mM-Hepes, pH 7.4) and resuspended in HBS at a concentration of 5×10^6 cells/ml.

For single measurements the cells were loaded as above except the cells remained on cover slips during the procedure. Under these conditions the intracellular concentration of Fura 2 was calculated to be of the order of 25 μM.

Fluorescence measurements

For each measurement 700 μl of the cell suspension was incubated in a thermostated cuvette in a Perkin–Elmer MP44A spectrophotometer. Following equilibration at 37 °C agonists were added as indicated; triplicate determinations were performed in each experiment. Under these experimental conditions very little dye leakage occurred, as determined by Mn^{2+} addition. Fura 2 fluorescence was monitored continuously using monochromator settings of 340 nm (excitation) and 500 nm (emission) using a 5 nm slit width. The $[Ca^{2+}]_i$ levels were calculated using the general formula:

$$[Ca^{2+}]_i = K_d\,(F - F_{min.})/(F_{max.} - F)$$

where K_d is the dissociation constant for Ca^{2+} binding to the indicator and F is arbitrary fluorescent units. For Fura 2 the K_d is 224 nm (Tsien, 1980). $F_{max.}$ was determined by lysing the cells with 1% Triton X-100, thus exposing the cells to 2 mM-Ca^{2+}. $F_{min.}$ was determined by the addition of 10 mM-EDTA at pH 8.5 (Pollock *et al.*, 1986).

Measurement of calcium in single cells

Single cells chosen for their healthy appearance (an absence of blebs and a good adherence to the coverslip) were observed on a Leitz Diavert microscope, with a Ploempak epifluorescence attachment, using a Leitz 100× oil immersion objective (numerical aperture 1.25). The Ca^{2+} concentrations are represented as the ratio of emission at 500 nm with excitation at 350 and 380 nm ('350/380') which is a monotonic function of $[Ca^{2+}]_i$ as previously described (Swann & Whittaker, 1986).

RESULTS AND DISCUSSION

Changes in inositol phosphates in T15 cells measured following chromatography upon Dowex columns

Stimulation of NIH-3T3, T15$^-$ and T15$^+$ cells with 1.5 μM-bombesin leads to maximal inositol phosphate generation in each cell type (Wakelam *et al.*, 1986). The resting levels of Ins(1,4,5)P_3 were found to be the same in the T15$^-$ and T15$^+$ cells, when measured with a specific binding assay (Palmer *et al.*, 1989) (results not shown). The time course of changes in the radioactivity associated with the separated inositol phosphates following bombesin stimulation of T15 cells is shown in Fig. 1. It is clear from the graphs in Figs. 1(*d*)–1(*e*) that the primary product of bombesin-stimulated inositol phospholipid breakdown in T15$^+$ cells was InsP_3, which reached a peak value afer 10 s of approx. 240% of the basal value. In six separate experiments this value varied from 160 to 390%, a variation which may be due to the stage in the cell cycle since the experiments were performed using non-synchronous cells. The radioactivity associated with the InsP_3 fraction then declined to a new steady state level (Fig. 1*d*). The InsP_2 fraction peaked at about 10 s following stimulation and appeared to plateau at a new steady state level (Fig. 1*e*). The radioactivity associated with the Ins$P$ fraction, however, was only significantly raised after 60 s. It then rose in a linear fashion, since the stimulation was performed in the presence of lithium ions (Fig. 1*f*). Stimulation of the T15$^+$ cells with bombesin

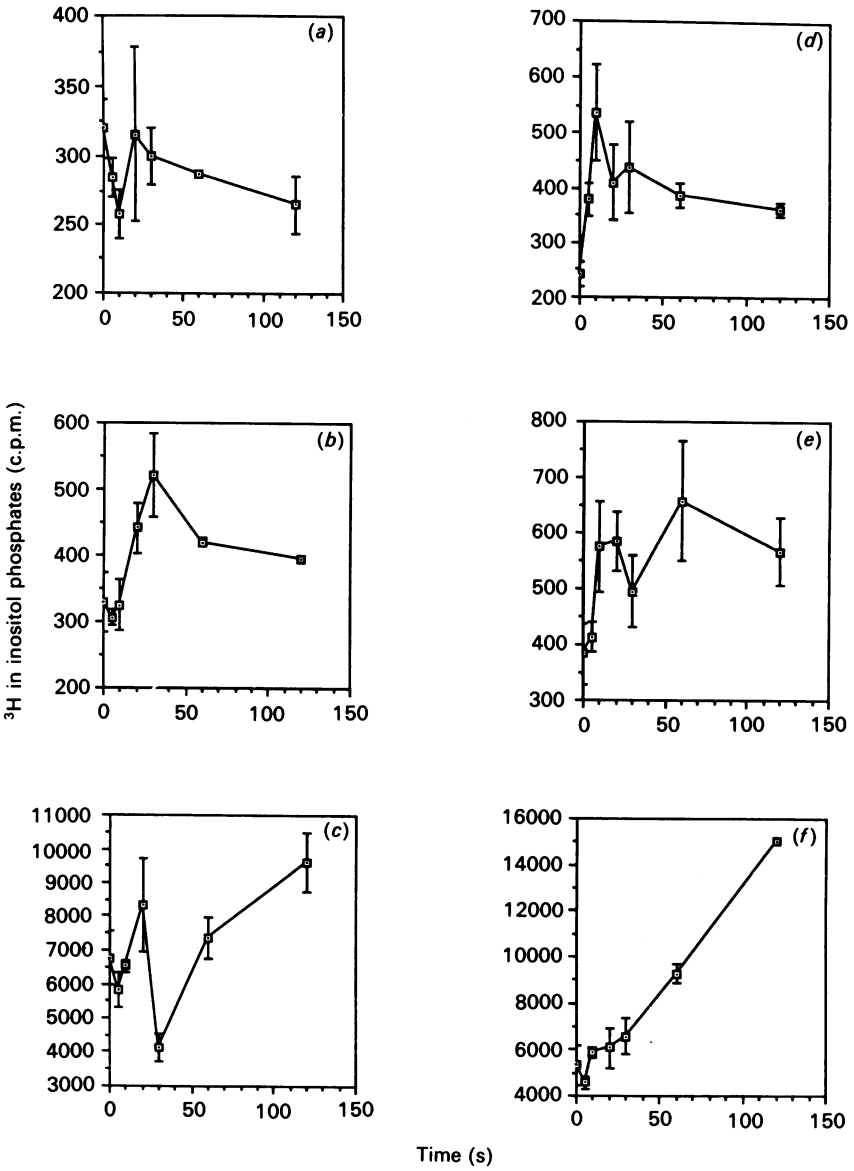

Fig. 1. Separation of inositol phosphates from T15$^+$ and T15$^-$ cells upon Dowex formate columns

T15$^+$ and T15$^-$ cells (approx. 10^5 cells in each case), prelabelled with [^{3}H]inositol as described in the Materials and methods section, were stimulated with 2.5 μM-bombesin for the stated times. Following neutralization the acid soluble extracts were separated on Dowex 1 formate columns as described. Results are means $\pm$ S.D. where $n = 4$ from one typical experiment. (*a*)–(*c*) T15$^-$ cells; (*d*)–(*f*), T15$^+$ cells; (*a*,*d*), InsP_3; (*b*,*e*), InsP_2; (*c*,*f*), Ins$P$.

caused a dose-dependent increase in the radioactivity associated with the InsP_3 fraction (Fig. 2). The Figure shows that the half-maximal stimulation (EC_{50}) of approx. 5 nm is similar to that obtained previously for the stimulation of total inositol phosphates generation (Wakelam *et al.*, 1986).

Stimulation of T15$^-$ cells with 2.5 μM-bombesin generated extremely small changes in the individual inositol phosphates with no significant rise in InsP_3 being detected upon stimulation of T15$^-$ cells as shown in Fig. 1*a*. A small rise in both Ins$P$ and InsP_2 was detectable, suggesting that there was InsP_3 generation though it was below detectable levels (Figs. 1*b* and *c*). In some experiments an aprox. 20 % increase in InsP_3 levels was detected when T15$^-$ cells were stimulated with 1 or 10 μM-bombesin; thus the rise in InsP_2 and Ins$P$ levels (Figs. 1*b* and 1*c*) is probably due to InsP_3 degradation, though the possibility of bombesin-stimulated PtdIns$P$ and PtdIns breakdown cannot be ruled out.

Analysis of inositol phosphates by h.p.l.c.

Separation of inositol phosphates by ion exchange chromatography on Dowex columns cannot discriminate between isomeric forms of the different phosphates. Such analysis requires separation on h.p.l.c. The time course of bombesin-stimulated Ins(1,4,5)P_3 generation in T15$^+$ cells is shown in Fig. 3(*a*). The data are from one experiment which was typical of three. Fig. 3(*b*) shows h.p.l.c. traces obtained in a typical experiment in which both T15$^-$ and T15$^+$ cells were stimulated with 2.5 μM-bombesin for 10 s. Whilst the generation of Ins(1,4,5)P_3 is clearly seen in the T15$^+$ cells it is so low as to be undetectable in the non-p21$^{N\text{-}ras}$ expressing T15$^-$ cells. Surprisingly, we were unable to detect the generation of

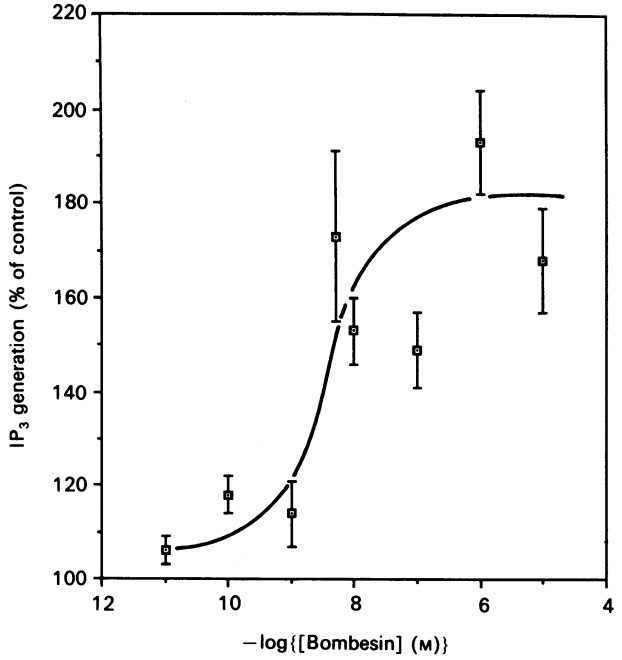

Fig. 2. Bombesin dose-dependence of InsP_3 generation in T15$^+$ cells

The generation of InsP_3 was determined in T15$^+$ cells stimulated with differing doses of bombesin for 10 s as described in the legend to Fig. 1. The results are means ± S.E.M. where $n = 9$ and are pooled from three separate experiments. The unstimulated control values of radioactivity associated with the InsP_3 fraction varied from 1000 to 3000 d.p.m. betwen experiments due to differences in cell number.

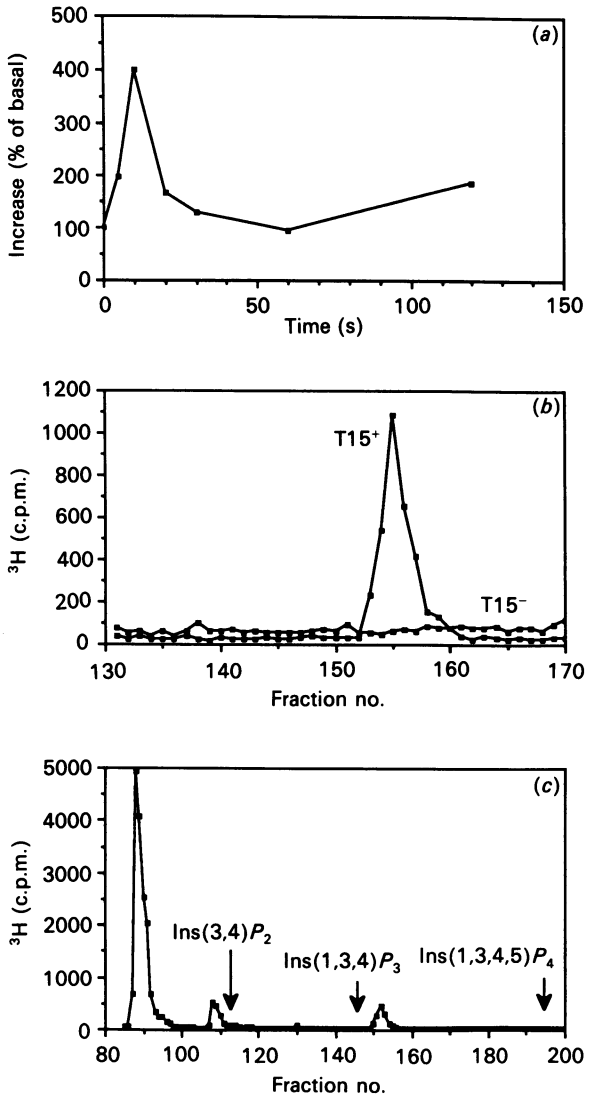

Fig. 3. Analysis of inositol phosphate generation in bombesin-stimulated T15$^+$ cells by h.p.l.c.

Extracts were prepared as described in the legend to Fig. 1 and separated on a Partisil 10 SAX column as described in the Materials and methods section. (*a*) shows the time course of bombesin-stimulated Ins(1,4,5)P_3 formation; (*b*) shows the h.p.l.c. trace following a 10 s stimulation with 1.5 μM-bombesin for T15$^+$ and T15$^-$ cells; (*c*) shows the trace obtained for an extract of T15$^+$ cells stimulated with 1.5 μM-bombesin for 120 s; the positions of migration of standard samples of Ins(3,4)P_2, Ins(1,3,4)P_3 and Ins(1,3,4,5)P_4 are shown.

Ins(1,3,4,5)P_4 at any time point tested (between 0 and 120 s). Fig. 3(*c*) shows the h.p.l.c. profile of [^{3}H]inositol-containing products following a 120 s bombesin stimulation of T15$^+$ cells. It is also clear from this trace that there was no split in the InsP_2 peak, which coeluted with an Ins(1,4)P_2 standard. This absence of an Ins(3,4,)P_2 peak supports the observation that no Ins(1,3,4,5)P_4 or Ins(1,3,4)P_3 were formed. This lack of InsP_4 formation was not due to the absence of InsP_3 kinase since preliminary experiments demonstrated that soluble extracts prepared from T15$^+$ and NIH-3T3 cells contain such an enzyme activity (results not shown).

Bombesin-stimulated intracellular Ca^{2+} changes

An important question to ask is whether the increased generation of Ins(1,4,5)P_3 is capable of eliciting a greater release of calcium from intracellular stores, raising cytoplasmic calcium levels to those capable of activating caclium-sensitive enzymes, some of which could have a role in *ras* transformation. To address this problem we measured bombesin-stimulated changes in intracellular free Ca^{2+} in the T15 cells.

Bombesin stimulated an increase in intracellular free Ca^{2+} as measured by changes in Fura 2 fluorescence in both T15$^+$ and T15$^-$ cells. However, the magnitude of the stimulated Ca^{2+} response was considerably greater in the T15$^+$ cells expressing p21$^{N\text{-}ras}$ (Figs. 4*a* and 4*c*). Indeed, this would be expected from the greatly increased stimulation of Ins(1,4,5)P_3 generation that we have observed in these cells (Fig. 1). The response was unaffected by the removal of extracellular calcium, demonstrating that the ion is being released from intracellular stores (results not shown).

The agonist prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) stimulates a similar response in both T15$^-$ and T15$^+$ cells, showing that the pathways leading to the release of intracellular calcium can respond to a different agonist (Figs. 4*b* and 4*d*). The release of calcium ions in the T15$^-$ cells in response to this agonist is as great as that achievable in the T15$^+$ cells in response to either stimulant. The inositol phosphate response to $PGF_{2\alpha}$ in T15$^-$ cells (approx. 12-fold increase in total inositol phosphates in response to 1 μM-$PGF_{2\alpha}$) is much greater than that to bombesin and it is not amplified by the expression of p21$^{N\text{-}ras}$ (Black &

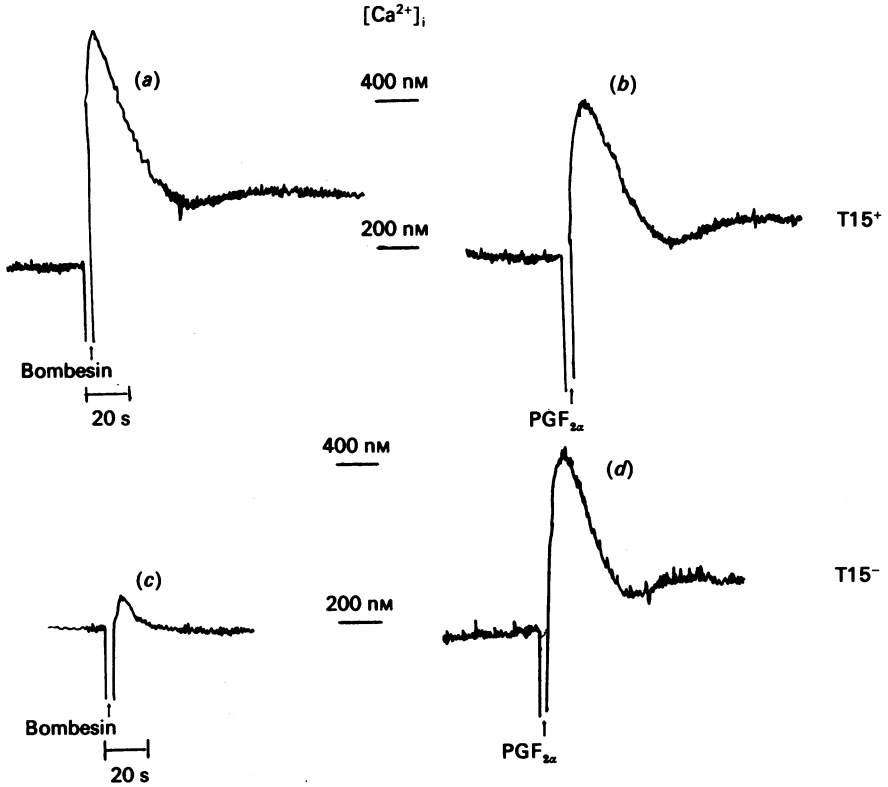

Fig. 4. Agonist-induced effects upon intracellular calcium in T15 cells

Changes in Fura 1 fluorescence were measured in $T15^+$ (*a*,*b*) and $T15^-$ (*c*,*d*) cells as described in the Materials and methods section, in response to 1.5 μM-bombesin (*a*,*c*) or 1 μM-$PGF_{2\alpha}$ (*b*,*d*). Each trace is representative of six separate determinations.

Wakelam, 1988). Indeed the inositol phosphate response is lower in the $T15^+$ cells compared to the $T15^-$ cells. However, its magnitude is still greater than the bombesin response in both cell lines (Black & Wakelam, 1988).

The increased Ca^{2+} response was seen at all the bombesin concentrations tested. Fig. 5 shows the dose–response curves for the stimulation of the increase in intracellular free Ca^{2+} in both $T15^+$ and $T15^-$ cells. The dose–response curves were very similar to those seen when measuring inositol phosphate generation (Wakelam *et al.*, 1986) and to the generation of InsP_3 (Fig. 2), which can be assumed to be Ins(1,4,5)P_3 (see Fig. 3). Thus the release of intracellular calcium appeared to be proportional to the generation of Ins(1,4,5)P_3. The bombesin dose–response curves for InsP_3 generation and the increase in intracellular free Ca^{2+} in the $T15^+$ cells mirrored one another; thus it would appear that the generation of Ins(1,4,5)P_3 at each bombesin concentration was limiting for Ca^{2+} release in this cell type. The amplification in bombesin-stimulated PtdIns(4,5)P_2 breakdown, when $p21^{N\text{-}ras}$ was expressed in the T15 cell line, was thus able to result in a significant increase in agonist-stimulated Ca^{2+} mobilization, an effect which would not occur if the inositol phosphate response being amplified was already capable of inducing maximal Ca^{2+} release. Therefore, in the $T15^+$ cell, bombesin will, as previously shown (Wakelam *et al.*, 1986), be able to induce a mitogenic effect. Since Ins(1,4,5)P_3 is being generated there will be concomitant production of *sn*-1,2-diacylglycerol which would activate protein kinase C. This suggests that both arms of this signalling pathway would be activated to a greater extent following bombesin stimulation in the $T15^+$ cells compared to the $T15^-$ cells. This may play a role in the transformation of this cell line.

Single cell calcium measurements

Since the determinations of calcium release and of inositol phosphate generation were carried out on a population of cells, the results shown could be due to a greater proportion of the $T15^+$ cells responding to bombesin with the same magnitude as a smaller proportion of the $T15^-$ cells. To test this single cell experiments were performed.

The resting intracellular calcium levels appear similar in the $T15^-$ and $T15^+$ cells. When stimulated with bombesin the increase in intracellular calcium was significantly greater in the $T15^+$ cells as compared to the $T15^-$ cells (Fig. 6). The $T15^-$ response to bombesin varied between a 35 and 42% increase in calcium levels with an average increase of 39% (S.D. 3.1), whereas the $T15^+$ response varied between an 87 and 260% increase over basal levels with an average of 155% (S.D. 66).

The reason for the variability in the $T15^+$ response is unclear although *ras* antibody staining of the cells did show a variable distribution of the *ras* protein (results not shown). The responses to prostaglandin $F_{2\alpha}$, however, were identical in $T15^-$ and $T15^+$ cells with little variability between individual cells. These results, thus, clearly show that each $T15^+$ cell tested had an amplified response to bombesin in terms of an increase in intracellular calcium release as compared to $T15^-$ cells and that the increased resonsiveness was not due to a greater proportion of the $T15^+$ cells responding to the agonist.

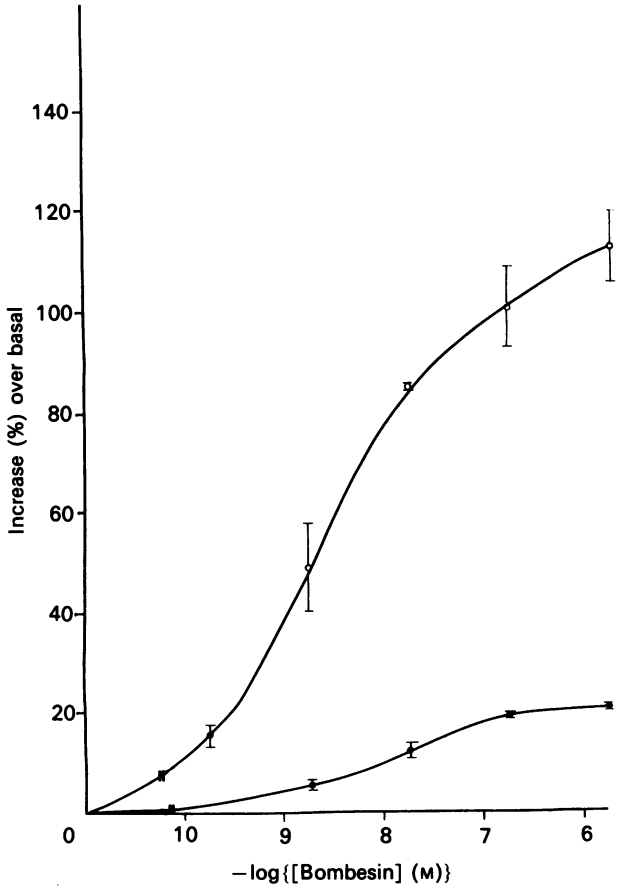

Fig. 5. Bombesin dose–response curves for intracellular calcium concentrations

Results are means ± S.E.M. and the data are pooled from three separate experiments ($n = 3$). The data are expressed as the % increase in $[Ca^{2+}]_i$ with respect to cells incubated in the absence of bombesin. ○, T15⁺ cells; ●, T15⁻ cells.

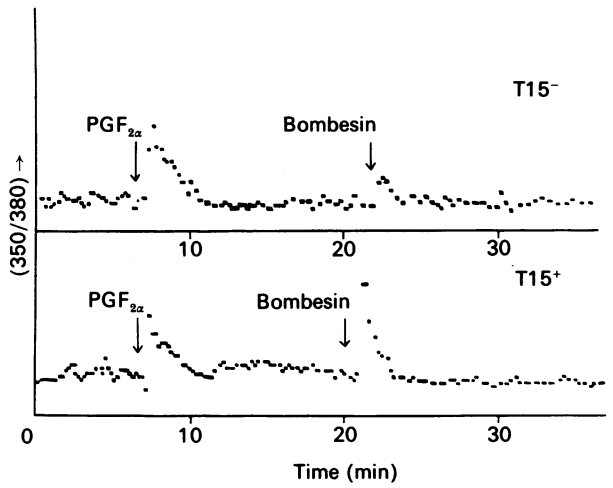

Fig. 6. Single-cell calcium measurements

Changes in Fura 2 fluorescence were measured in T15⁺ and T15⁻ cells as described in the Materials and methods section in response to 2.5 μM-bombesin or 1 μM-$PGF_{2\alpha}$. Each trace is representative of four separate determinations.

Together these results show that in the T15 cell line the induction of the N-*ras* proto-oncogene causes an amplified response in terms of Ins(1,4,5)P_3 production and intracellular calcium release following stimulation by the agonist bombesin. A good case, therefore, could be made for the N-*ras* proto-oncogene acting, either directly or indirectly, in a G_p-like manner in these cells. However, there are inconsistencies with this simple model. When these experiments have been repeated with cell lines constitutively overexpressing the N-*ras* proto-oncogene the amplification of the bombesin response is not seen. None of the N-*ras*-transformed NIH-3T3 cell lines tested showed an amplified response to bombesin in generating Ins(1,4,5)P_3 or in increasing intracellular calcium concentration (A. C. Lloyd & M. J. O. Wakelam, unpublished work). These cell lines have a similar number of bombesin receptors and so the explanation for the difference is not obvious.

There are several possible explanations for this effect. Among them is the possibility that long term growth leads to a down-regulation of the response in the constitutive cell lines. Whilst this is unlikely, as T15⁺ cells can be cultured in the presence of dexamethasone for several weeks without a loss in responsiveness, high density culturing can induce a desensitization of bombesin-stimulated inositol phosphate generation (Wakelam, 1988). A second possibility is the loss of a feedback mechanism in the T15 cells. In Swiss 3T3 cells it has been shown that activation of C-kinase, by tetradecanoylphorbol acetate, inhibited the stimulation of calcium release in response to bombesin (Brown *et al.*, 1987). This type of inhibition cannot be mimicked in the T15⁺ cells (A. C. Lloyd, unpublished work) which might point to a possible peculiarity of this cell line, allowing an amplified response to be measured under these conditions. A third possibility is that dexamethasone is causing the amplification of the response. Although this is a possibility it is difficult to conceive as dexamethasone addition had no effect on the response in NIH-3T3 cells or in NIH-3T3 cells constitutively overexpressing the N-*ras* proto-oncogene (A. C. Lloyd & M. J. O. Wakelam, unpublished work).

In conclusion, we have shown that, in the T15 cell line, the stimulation by bombesin of Ins(1,4,5)P_3 generation and of the release of intracellular stored calcium is amplified when the N-*ras* proto-oncogene is overexpressed. These results are consistent with the N-*ras* proto-oncogene, directly or indirectly, increasing the coupling of the bombesin receptor to phospholipase C.

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REFERENCES

Barbacid, M. (1987) Annu. Rev. Biochem. **56**, 779–827

Black, F. M. & Wakelam, M. J. O. (1988) Biochem. Soc. Trans. **16**, 990–991

Brown, K. D., Blakely, D. M., Hamon, M. H., Laurie, M. S. & Corps, A. N. (1987) Biochem. J. **245**, 631–639

Downes, C. P. & Michell, R. H. (1985) Mol. Asp. Cell. Regul. **4**, 2–56

Hancock, J. F., Marshall, C. J., McKay, I. A., Gardner, S., Houslay, M. D., Hall, A. & Wakelam, M. J. O. (1988) Oncogene **3**, 187–193

Irvine, R. F., Änngård, E. E., Letcher, A. J. & Downes, C. P. (1985) Biochem. J. **229**, 505–511

McGrath, J. P., Capon, D. J., Goeddel, D. V. & Levinson, A. D. (1984) Nature (London) **310**, 644–649

McKay, I. A., Marshall, C. J., Cales, C. & Hall, A. (1986) EMBO J. **5**, 2617–2621

Palmer, S., Hughes, K. T., Lee, D. Y. & Wakelam, M. J. O. (1989) Cell. Signal. **1**, 147–156

Pollock, W. K.,Rink, T. J. & Irvine, R. F. (1986) Biochem. J. **235**, 869–877

Swann, K. A. & Whittaker, M. J. (1986) J. Cell. Biol. 103, 2333–2342

Tanabe, T., Nukada, T., Nishikawa, Y., Sugimolo, K., Suzchi, H., Takahashi, H., Noda, M., Haga, T., Ichikawa, K., Minamino, N., Matsui, H. E. & Numa, S. (1985) Nature (London) **315**, 242–245

Tsien, R. Y. (1980) Biochemistry **19**, 2396–2404

Wakelam, M. J. O. (1988) FEBS Lett. **228**, 182–186

Wakelam, M. J. O., Davies, S. A., Houslay, M. D., McKay, I. A., Marshall, C. J. & Hall, A. (1986) Nature (London) **323**, 173–176

Willingham, M. C., Banks-Schiegal, S. P. & Pastan, I. E. (1983) Exp. Cell. Res. **149**, 141–149

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