NIH-3T3 cells transformed with a *ras* oncogene exhibit a protein kinase C-mediated inhibition of agonist-stimulated Ca^{2+} inflow

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1. The ability of bombesin or platelet-derived growth factor (PDGF) to stimulate Ca^{2+} inflow (assessed by measuring changes in the intracellular free Ca^{2+} concentration in cells loaded with fura-2) in NIH-3T3 cells transformed with the EJ/T24-Ha-*ras*-1 oncogene is inhibited when compared with the action of the agonists on wild-type cells. 2. The effects of transformation with the *ras* oncogene are associated with complete inhibition of the ability of bombesin to release Ca^{2+} from intracellular stores, a substantial decrease in the number of bombesin receptors, no change in the ability of foetal calf serum or ionomycin to release Ca^{2+} from intracellular stores and the activation of protein kinase C. 3. The effects of transformation with the H-*ras* oncogene on the ability of bombesin or PDGF to stimulate Ca^{2+} inflow were mimicked by a 30 min exposure of wild-type cells to phorbol dibutyrate. This action of phorbol dibutyrate was completely blocked by prior treatment of wild-type cells for 24 h with the phorbol ester. 4. It is concluded that one of the actions of the H-*ras* oncogene in fibroblasts is to inhibit agonist-stimulated Ca^{2+} inflow by a mechanism which involves the activation of protein kinase C.

INTRODUCTION

Mature *ras* proteins (expressed by *ras* genes) are located at the cytoplasmic face of the plasma membrane and are necessary components of the process by which certain extracellular signals, such as serum and nerve growth factor, and certain intracellular signals, such as those induced by oncogenic protein-tyrosine kinases, stimulate cell proliferation and differentiation (reviewed in Marshall, 1988). Many established cell lines transfected with a *ras* oncogene exhibit an activation of protein kinase C (Wolfman & Macara, 1987; Huang *et al.*, 1988). This is most probably due to a constitutive increase in diacylglycerol (Wolfman & Macara, 1987), possibly as a result of increased hydrolysis of non-inositol phospholipids (Lacal *et al.*, 1987; Wolfman & Macara, 1987; but see Macara, 1989) or enhanced synthesis of diacylglycerol *de novo* (Chiarugi *et al.*, 1989). Evidence has been obtained which indicates that the activation of protein kinase C is part of the mechanism by which *ras* oncogenes induce cell transformation (reviewed in Marshall, 1988).

The role of normal *ras* proteins in intracellular signal transduction is not known. It has been proposed that *ras* proteins couple receptors in the plasma membrane to effector enzymes, such as phospholipases C specific for phosphatidylinositol 4,5-bisphosphate or phosphatidylcholine, by a mechanism analogous to that of oligomeric GTP-binding proteins (reviewed in Marshall, 1988). Since Ca^{2+} plays an important role as an intracellular messenger in the actions of mitogens, it has also been proposed that one of the functions of normal *ras* proteins is to alter intracellular free Ca^{2+} concentrations either through changes in the concentration of inositol polyphosphates (Chiarugi *et al.*, 1986; Benjamin *et al.*, 1988) or by other mechanisms (Schmitt *et al.*, 1988). It has recently been shown that 3T3 fibroblasts transformed with *ras* oncogenes exhibit an inhibition of T-type voltage-operated Ca^{2+} channels (Chen *et al.*, 1988).

The aim of the present study was to determine whether one of the functions of the normal *ras* proteins is to inhibit agonist-

stimulated plasma membrane Ca^{2+} inflow. The experiments have been conducted by comparing the ability of agonists to stimulate Ca^{2+} inflow in wild-type NIH-3T3 fibroblasts with their effects on fibroblasts transformed with the v-Ki-*ras* and EJ/T24-Ha-*ras*-1 oncogenes or on wild-type cells pre-treated with phorbol dibutyrate, an activator of protein kinase C (Kikkawa *et al.*, 1983). The results indicate that cells transformed with the *ras* oncogene exhibit a substantial inhibition of the ability of bombesin and platelet-derived growth factor (PDGF) to stimulate plasma membrane Ca^{2+} inflow. This inhibition is mimicked by the treatment of wild-type cells with phorbol dibutyrate.

MATERIALS AND METHODS

Cell culture

Wild-type NIH-3T3, K-*ras*-transfected (v-Ki-*ras*) and H-*ras*-transfected (EJ/T24-Ha-*ras*-1) cells were obtained from Dr. S. Aaronson, National Cancer Institute, Washington, DC, U.S.A. Portions of each cell line were stored in liquid N_2 . The frozen cells were removed, one portion at a time, and the cells were thawed and subcultured for a maximum of 20 passages. Except where indicated otherwise, the cells were grown at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 mg of gentamycin/ml, 2 mM-glutamine, 10 mM-Hepes/KOH (pH 7.4) and 10% (v/v) foetal calf serum (FCS) (culture medium). The concentration of total Ca^{2+} in this medium was 1.8 mM.

Fura-2 loading

Cells were grown to near-confluence in 90 mm Petri dishes then incubated for 24 h in culture medium in the absence of FCS. The cells were detached by treatment with 0.02% (w/v) trypsin/0.05% (w/v) EDTA, washed with fresh DMEM, and resuspended at a concentration of 2×10^6 cells/ml in DMEM. The cell suspension was divided into two samples (2 ml volume)

Abbreviations used: $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} concentration; Ca^{2+}_o , extracellular free Ca^{2+} ; DTPA, diethylenetriaminepenta-acetic acid; PDGF, platelet-derived growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum.

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which were placed in two separate vials. The cells in each vial were loaded with fura-2, or served as controls for the determination of autofluorescence. Cells were loaded with fura-2 by the addition of the acetoxymethyl ester of fura-2 (3 μ M final concentration) in dimethyl sulphoxide [0.1 % (w/v) final concentration]. The cell suspension was incubated in a water bath at 37 °C and shaken at 90 cycles/min. Unloaded cells were treated in a similar manner with dimethyl sulphoxide alone. After 30 min the cells were diluted by the addition of 2 ml of DMEM and centrifuged at 150 g at room temperature for 4 min to pellet the cells. The cells were washed with DMEM and resuspended in 2 ml of DMEM.

Fluorescence of cells loaded with fura-2

Cells loaded with fura-2 (2 ml, 4×10^6 cells) were placed in a quartz cuvette (10 mm $\times$ 10 mm $\times$ 45 mm internal dimensions). Fluorescence measurements were performed in an Aminco-Bowman spectrofluorimeter (SPF 74; American Instrument Co., Silver Spring, MD, U.S.A.) coupled to a J10-280 microphotometer (American Instrument Co.) and potentiometric recorder (Beckman Instruments, Fullerton, CA, U.S.A.), or in an LS-50 spectrofluorimeter (Perkin-Elmer Ltd., Beaconsfield, Bucks., U.K.) equipped with a magnetic stirring device and a thermostatically controlled cell housing set at 37 °C. The cells were incubated for a period of 2 min before addition of agonist. Fluorescence was continuously recorded at an excitation wavelength of 340 nm or, in some experiments, 380 nm, and at an emission wavelength of 500 nm.

In some experiments, four traces were obtained for each experimental condition: one at each of the excitation wavelengths of 340 nm and 380 nm for two fura-2-loaded samples and one at each of the excitation wavelengths of 340 and 380 nm for two unloaded samples. The interval between each set of additions was kept the same within each experiment so that the traces obtained at the 340 nm and 380 nm excitation wavelengths could be directly compared. The values of fluorescence as a function of time were converted from analogue to digital form using a HIPAD digitizer (model DT 11; Bausch and Lomb, Houston Instrument Division, Austin, TX, U.S.A.).

Calculation of intracellular free Ca^{2+} concentration

Except where indicated otherwise, values of the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) were calculated from the values of fluorescence recorded at an excitation wavelength of 340 nm, as described by Pollock *et al.* (1986). In each series of experiments the fluorescence of unloaded cells was subtracted from the fluorescence of cells loaded with fura-2. The former was normally less than 15 % of the latter. The fluorescence signal caused by extracellular fura-2 was estimated using Mn^{2+} and diethylenetriaminepenta-acetic acid (DTPA), as described by Rink & Pozzan (1985). This was subtracted from the observed fluorescence of cells loaded with fura-2. The values of maximum (F_{max}) and minimum (F_{min}) fluorescence were determined by treatment of the cells with digitonin (50 μ g/ml) and 20 mM-Tris base (pH 10)/10 mM-EGTA (pH 8.0) respectively. A value of 224 nm was used for K_d , the dissociation constant for the interaction of Ca^{2+} with fura-2 at 37 °C (Gryniewicz *et al.*, 1985).

In some experiments, values of $[\text{Ca}^{2+}]_i$ were calculated from the ratio (R) of the fluorescence values obtained at 340 and 380 nm excitation wavelengths, using the following equation (Gryniewicz *et al.*, 1985):

$$[\text{Ca}^{2+}]_i = K_d \cdot \frac{(F_o)(R - R_{\text{min}})}{(F_s)(R_{\text{max}} - R)} \quad (1)$$

where R_{max} and R_{min} are the ratios of the values of fluorescence obtained at a saturating concentration of Ca^{2+} (1.8 mM- Ca^{2+}) and

at very low free $[\text{Ca}^{2+}]$ [1.8 mM- Ca^{2+} /20 mM-Tris base (pH 10.0)/10 mM-EGTA (pH 8.0)] respectively. F_o is the fluorescence at 380 nm excitation at very low free Ca^{2+} , and F_s is the fluorescence at 380 nm excitation at saturating Ca^{2+} .

The addition of CaCl_2 (1.8 mM) to cells loaded with fura-2 and incubated in the presence of EGTA caused a very rapid increase in fluorescence, followed by a slower second-phase increase. The nature of the initial rapid increase in fluorescence was investigated using supernatants obtained from cell suspensions incubated in the presence of EGTA but centrifuged 4 min after the addition of the EGTA [before the addition of external Ca^{2+} (Ca^{2+}_o)]. The addition of 1.8 mM- Ca^{2+} to the supernatant caused an increase in fluorescence similar in magnitude to the first-phase increase observed after the addition of Ca^{2+} to cell suspensions. No second-phase increase was observed. Furthermore, the addition of MnCl_2 (50 μ M) in the absence of added Ca^{2+} caused a decrease in fluorescence. This was reversed by the subsequent addition of DTPA (100 μ M). Another decrease in fluorescence, similar in magnitude to that induced by Mn^{2+} , was caused by the further addition of 2 mM-EGTA. No increase in fluorescence was observed upon treatment of the supernatant with digitonin. These observations indicate that the initial rapid first-phase increase in fluorescence observed after the addition of Ca^{2+} to cells incubated in the presence of EGTA represents the binding of Ca^{2+} to extracellular fura-2. On the basis of these results, the value of the first-phase increase in fluorescence was subtracted from all values of fluorescence obtained after the addition of CaCl_2 .

Ca^{2+} inflow

Rates of Ca^{2+} inflow were calculated from plots of the initial rate of increase in fluorescence at 340 nm as a function of time after the addition of Ca^{2+}_o to cells incubated in the presence of EGTA. Values for the rate of change of fluorescence were converted to rates of change of $[\text{Ca}^{2+}]_i$, expressed as nmol/min per 10^7 cells.

Binding of bombesin to bombesin receptors and measurement of protein kinase C activity

The binding of bombesin to bombesin receptors on NIH-3T3 cells was measured using [3- ^{125}I]iodotyrosine 15 gastrin-releasing peptide (^{125}I -gastrin-releasing peptide) as a tracer for bombesin (Kris *et al.*, 1987). The amounts of ^{125}I -gastrin-releasing peptide bound to NIH-3T3 fibroblasts in the presence of a given concentration of bombesin were measured at 37 °C as described by Kris *et al.* (1987), with the following modifications. The experiments were performed in 6-well plates, all cell washes were performed using 2 ml of buffer, ^{125}I -gastrin-releasing peptide or a mixture of ^{125}I -gastrin-releasing peptide and bombesin was presented to the cells in a volume of 1.0 ml, and the cells were removed from the well by treatment with 1 ml of solution which contained 0.02 % (w/v) trypsin and 0.05 % (w/v) EDTA.

The preparation of particulate and cytosolic cell extracts and the assay of protein kinase C using histone and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ were conducted as described by Tapley & Murray (1984), with the following modifications. The cells were disrupted by aspiration (ten times using a 10 ml syringe fitted with a 25 gauge needle) and protein kinase C was eluted from the DEAE-cellulose columns in one batch using 10 ml of 0.4 M-NaCl in elution buffer. All experiments were conducted using cells quiesced for 24 h in culture medium in the absence of FCS.

Materials

The acetoxymethyl ester of fura-2 was obtained from Molecular Probes, Eugene, OR, U.S.A.; PDGF, $[\gamma\text{-}^{32}\text{P}]\text{P}_i$ and ^{125}I -gastrin-releasing peptide were from Amersham Australia,

Sydney, N.S.W., Australia; DMEM, FCS and trypsin were from Flow Laboratories, Rickmansworth, Herts., U.K.; DMEM and DMEM without Ca²⁺ were from Cytosystems, Castle Hill, N.S.W., Australia; bombesin was from Auspep, South Melbourne, Victoria, Australia; and digitonin, EGTA, histone HHS (lysine-rich) and phorbol dibutyrate were from Sigma Chemical Co., St. Louis, MO., U.S.A. [γ -³²P]ATP was prepared using [³²P]P_i, as described by Johnson & Walseth (1979). All other chemicals were of the highest grade available.

RESULTS

Ca²⁺ inflow in wild-type and transformed cells

Rates of Ca²⁺ inflow across the plasma membrane of NIH-3T3 fibroblasts treated with an agonist were assessed either by the prolonged increase in [Ca²⁺]_i which follows the transient increase in [Ca²⁺]_i immediately after addition of an agonist (Moolenaar *et al.*, 1984; Hesketh *et al.*, 1985, 1988; Pandiella *et al.*, 1989) or by the rate of increase in [Ca²⁺]_i after the addition of Ca²⁺_o to cells previously incubated in the absence of added Ca²⁺_o, as employed for the measurement of Ca²⁺ inflow in other cell types (Schilling *et al.*, 1988; Crofts & Barritt, 1989). Before the measurement of Ca²⁺ inflow, the cells were grown to near-confluence, then incubated for 24 h in the absence of FCS. Incubation in the absence of FCS was performed in order to reduce desensitization of plasma membrane receptors and to allow the concentrations of intracellular messengers to return to basal values. Incubation of cells for several hours in the absence of serum has been employed in the majority of other studies in which the properties of cells transformed with a *ras* oncogene have been compared

with those of wild-type cells (e.g. Chiarugi *et al.*, 1986; Parries *et al.*, 1987; Alonso *et al.*, 1988; Seuwen *et al.*, 1988; Benjamin *et al.*, 1988).

As shown previously for other types of fibroblasts (McNeil *et al.*, 1985; Mendoza *et al.*, 1986; Lopez-Rivas *et al.*, 1987; Ives & Daniel, 1987), the addition of bombesin (Fig. 1a) or PDGF (Fig. 1e) to wild-type NIH-3T3 cells incubated in the presence of 1.8 mM free Ca²⁺_o caused a rapid increase in [Ca²⁺]_i which, after reaching a maximum value, decreased to a value above the basal value (Figs. 1a and e cf. Fig. 1c, and Table 1). The increased value of [Ca²⁺]_i was maintained for several minutes. In contrast with the actions of bombesin and PDGF, 50 mM-KCl, added in order to depolarize the plasma membrane, induced no detectable increase in [Ca²⁺]_i (results not shown). As shown by others for other types of fibroblasts (Moolenaar *et al.*, 1984; Metcalfe *et al.*, 1985; Hesketh *et al.*, 1985, 1988) the plateau in [Ca²⁺]_i induced by bombesin or PDGF was abolished when the experiment was performed in the presence of excess EGTA (Figs. 1b and 1f and Table 1). Under these conditions (1.8 mM added Ca²⁺_o and 2.7 mM-EGTA), the concentration of free extracellular Ca²⁺ is less than 0.1 μ M (Blackmore *et al.*, 1982).

In contrast with the results obtained for wild-type cells, bombesin had no effect on [Ca²⁺]_i in H-*ras*- and K-*ras*-transformed cells (Fig. 2a and Table 1). In H-*ras*-transformed cells the rate of increase in [Ca²⁺]_i induced by PDGF (Fig. 2e) was much lower than that observed in wild-type cells (Fig. 1e). Moreover, in H-*ras*-transformed cells the [Ca²⁺]_i returned towards the basal value after addition of PDGF (Fig. 2e and Table 1), whereas [Ca²⁺]_i remained elevated for a period of time in wild-type cells (Fig. 1e and Table 1). The magnitude of the

Fig. 1. Effects of growth factors on [Ca²⁺]_i in wild-type cells incubated in the presence and absence of Ca²⁺_o.

Wild-type NIH-3T3 cells loaded with fura-2 were incubated in the presence of 1.8 mM-Ca²⁺_o (a, c and e) or in the presence of 1.8 mM-Ca²⁺_o and 2.7 mM-EGTA (b, d and f). Values of [Ca²⁺]_i were determined from the measurement of changes in fluorescence at 340 nm as described in the Materials and methods section. The growth factors employed were 25 nM-bombesin (a, b), no agonists (c, d) and 2 nM-PDGF (e, f). The times of addition of growth factor and of 3.0 mM-Ca²⁺_o (to cells incubated in the presence of EGTA) are indicated by the arrows. Each plot is a representative of the plots obtained for between four and 14 similar experiments (separate loadings with fura-2).

Fig. 2. Effects of growth factors on [Ca²⁺]_i in H-*ras*-transformed cells incubated in the presence and absence of Ca²⁺_o.

H-*ras*-transformed NIH-3T3 cells loaded with fura-2 were incubated in the presence of 1.8 mM-Ca²⁺_o (a, c and e) or in the presence of 1.8 mM-Ca²⁺_o and 2.7 mM-EGTA (b, d and f). The fluorescence of the cell suspension was measured at 340 nm excitation, and values of [Ca²⁺]_i were calculated as described in the Materials and methods section. The growth factors employed were: 25 nM-bombesin (a, b), no agonist (c, d) and 2 nM-PDGF (e, f). The times of addition of the growth factors and Ca²⁺_o (3.0 mM-CaCl₂ to cells incubated in the presence of EGTA) are indicated by the arrows. Each plot shown is a representative of the plots obtained for six similar experiments (e, f) or between eight and 19 similar experiments (a-d), each conducted with a separate loading of fura-2.

Table 1. Effects of agonists on $[Ca^{2+}]_i$ in NIH-3T3 cells

Values of $[Ca^{2+}]_i$ were calculated from the values of fluorescence obtained at 340 nm excitation for cells loaded with fura-2, as described in the Materials and methods section. The final concentrations of agonists were: bombesin, 25 nM; FCS, 1 % (v/v); PDGF, 2 nM; ionomycin, 0.1 μ M. Plots which are representative of those from which these values of $[Ca^{2+}]_i$ were calculated are shown in Figs. 1–3. Each value of $[Ca^{2+}]_i$ represents the results obtained from the number of experiments (separate loadings with fura-2) indicated in parentheses. The degrees of significance for comparison of the value of $[Ca^{2+}]_i$ obtained at the plateau 2 min after addition of an agonist with that obtained before addition of agonist, determined using Student's *t* test for paired samples, are: **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. The degrees of significance for comparison of the value obtained for $[Ca^{2+}]_i$ at the peak after addition of agonist to H-*ras*- or K-*ras*-transformed cells with that for wild-type cells, determined using Student's *t* test for unpaired samples, are: †*P* < 0.05, and ††*P* < 0.001.

Cell type	Ca^{2+}_o concentration (mM)	Agonist	$[Ca^{2+}]_i$ (nM)		
			Before addition of agonist	Peak after addition of agonist	Plateau 2 min after addition of agonist
Wild-type	1.8	Bombesin	230 ± 14 (43)	1150 ± 96 (43)	320 ± 14 (43)***
		FCS	170 ± 15 (14)	880 ± 110 (14)	234 ± 15 (14)***
		PDGF	190 ± 7 (9)	554 ± 40 (9)	275 ± 7 (9)***
	< 0.0001	Bombesin	200 ± 10 (32)	540 ± 25 (32)	164 ± 11 (32)***
		FCS	215 ± 27 (14)	1095 ± 310 (14)	183 ± 23 (14)**
		PDGF	180 ± 10 (5)	385 ± 28 (5)	157 ± 2 (5)
Wild-type treated for 30 min with phorbol dibutyrate	1.8	Bombesin	185 ± 20 (3)	547 ± 110 (3)	157 ± 16 (3)*
		Bombesin	227 ± 37 (7)	518 ± 70 (7)	272 ± 31 (7)
		Bombesin	163 ± 10 (5)	286 ± 50 (5)	141 ± 7 (5)*
	< 0.001	PDGF	300 ± 10 (8)	660 ± 45 (8)	300 ± 10 (8)
		Bombesin	280 ± 30 (12)	374 ± 47 (12)††	326 ± 25 (12)*
		FCS	228 ± 28 (19)	1302 ± 140 (19)†	228 ± 14 (19)
H- <i>ras</i> -transformed	1.8	PDGF	235 ± 26 (7)	496 ± 51 (7)	295 ± 26 (7)**
		Bombesin	260 ± 29 (16)	274 ± 33 (16)††	219 ± 28 (16)***
		FCS	222 ± 16 (18)	1060 ± 295 (18)	183 ± 16 (18)***
	< 0.0001	PDGF	234 ± 37 (6)	346 ± 48 (6)	212 ± 33 (6)**
		Ionomycin	203 ± 38 (5)	680 ± 202 (5)	182 ± 37 (5)
		Bombesin	266 ± 24 (13)	280 ± 23 (13)††	348 ± 28 (13)**
K- <i>ras</i> -transformed	1.8	FCS	330 ± 46 (5)	1150 ± 327 (5)	394 ± 50 (5)
		Bombesin	245 ± 40 (10)	250 ± 45 (10)††	205 ± 29 (10)*
		FCS	335 ± 47 (5)	990 ± 208 (7)	279 ± 33 (5)
	< 0.001	Ionomycin	220 ± 30 (4)	579 ± 112 (4)	118 ± 27 (4)**

Table 2. Effects of agonists on the rate of increase in $[Ca^{2+}]_i$ caused by the addition of Ca^{2+} to cells loaded with fura-2 and incubated in the presence of EGTA

Wild-type or H-*ras*-transformed cells were loaded with fura-2 and incubated in the presence of 1.8 mM- Ca^{2+} and 2.7 mM-EGTA in the spectrofluorimeter cuvette as described in the Materials and methods section and the legends of Figs. 1, 2 and 3. After addition of the indicated agonist(s), 3.0 mM- $CaCl_2$ was added and rates of increase in $[Ca^{2+}]_i$ were calculated from changes in fluorescence measured at 340 nm excitation as described in the Materials and methods section. The concentrations of agonist employed were: bombesin, 25 nM; PDGF, 2 nM; KCl, 50 mM. When present, verapamil (200 μ M) was added 1 min before the EGTA. When used, treatment with phorbol dibutyrate was for 30 min as described in the legend to Fig. 3. The results are the means ± S.E.M. of the number of experiments (separate loadings with fura-2) indicated in parentheses. N.D. indicates that the value was not determined. The degrees of significance for comparison of the rate of increase in $[Ca^{2+}]_i$ obtained in the presence of an agonist with that obtained in the absence of an agonist, determined using Student's *t* test for unpaired samples, are: **P* < 0.01, and ***P* < 0.001.

Agonist	Cell type...	Rate of increase in $[Ca^{2+}]_i$ (nmol/min per 10 ⁷ cells)	
		Wild-type	H- <i>ras</i> -transformed
None		0.14 ± 0.02 (10)	0.06 ± 0.03 (5)
Bombesin		1.17 ± 0.20 (11)**	0.07 ± 0.03 (4)
Bombesin + verapamil		0.25 ± 0.11 (9)	0.06 ± 0.03 (4)
KCl		0.16 ± 0.03 (3)	N.D.
Bombesin after 30 min treatment with phorbol dibutyrate		0.19 ± 0.08 (5)	N.D.
Bombesin after 24 h treatment with phorbol dibutyrate		1.18 ± 0.24 (5)	N.D.
Bombesin after 24 h and 30 min treatments with phorbol dibutyrate		1.18 ± 0.10 (3)	N.D.
PDGF		3.42 ± 1.17 (5)**	0.32 ± 0.02 (5)*
PDGF after 30 min treatment with phorbol dibutyrate		0.60 ± 0.001 (5)*	N.D.

transient increase in $[Ca^{2+}]_i$ induced by FCS (Table 1) and the shapes of the curves for the transient increase in $[Ca^{2+}]_i$ induced by FCS (results not shown) in H-*ras*- or K-*ras*-transformed cells were similar to those in wild-type cells.

The addition of 3.0 mM-Ca²⁺ to wild-type cells incubated in the presence of EGTA and bombesin or PDGF caused a rapid and extensive increase in $[Ca^{2+}]_i$ (Figs. 1b and 1f and Table 2) compared with the increase observed in wild-type cells incubated under similar conditions in the absence of an agonist (Fig. 1d and Table 2). Only a very small increase in $[Ca^{2+}]_i$ was observed when 50 mM-KCl was added in place of bombesin or PDGF (Table 2). Verapamil (200 μ M) almost completely inhibited Ca²⁺ inflow induced by bombesin (Table 2). Lower concentrations of verapamil (10 and 1 μ M) had no effect (results not shown).

In H-*ras*-transformed cells incubated in the presence of EGTA and bombesin or PDGF, subsequent addition of 3.0 mM-Ca²⁺ caused only a very slow increase in $[Ca^{2+}]_i$ (Figs. 2b and 2f, cf. Fig. 2d, and Table 2). This rate of increase is substantially lower than the increase observed in wild-type cells treated with bombesin or PDGF (Table 2).

Since wild-type but not H-*ras*- or K-*ras*-transformed cells can be quiesced (Seuwen *et al.*, 1988), it is possible that the observed inhibition of bombesin- or PDGF-stimulated Ca²⁺ inflow in H-*ras*-transformed cells reflects differences in the growth characteristics of the two cell types rather than an effect of the oncogenic *ras* proteins. In order to test this possibility, experiments were conducted using wild-type cells grown to a non-confluent state and in the presence of serum until the time of loading with fura-2 (i.e. cells in exponential growth phase). The rate of Ca²⁺ inflow observed following the addition of 2 mM-Ca²⁺ to these cells incubated in the absence of added Ca²⁺, and in the presence of bombesin was 2.15 ± 0.2 nmol/min per 10^7 cells ($n = 12$), compared with a value of 0.88 ± 0.1 nmol/min per 10^7 cells ($n = 8$) for cells grown to confluence and then quiesced by incubation for a further 24 h in the absence of serum before being loaded with fura-2.

In order to determine whether transformation with *ras*

oncogenes alters processes of intracellular Ca²⁺ movement other than plasma membrane Ca²⁺ inflow, experiments using the Ca²⁺ ionophore ionomycin were conducted. The addition of ionomycin (100 nM) to both wild-type and *ras*-transformed cells incubated in the presence of EGTA induced a transient increase in $[Ca^{2+}]_i$ (results not shown). Neither the maximum increase in $[Ca^{2+}]_i$ (Table 1) nor the shape of the plots of $[Ca^{2+}]_i$ as a function of time (results not shown) in H-*ras* or K-*ras*-transformed cells were significantly different compared with those observed in wild-type cells.

The possibility that the absence of an effect of bombesin in inducing a transient increase in $[Ca^{2+}]_i$ and stimulation of Ca²⁺ inflow in H-*ras* and K-*ras*-transformed cells was due to a decreased number of receptors for bombesin on these cells was tested by measuring the binding of a bombesin-related peptide, gastrin-releasing peptide, to the cells. The amounts of ¹²⁵I-gastrin-releasing peptide specifically bound to wild-type cells at 10^{-15} M-gastrin-releasing peptide and at 10^{-15} M-gastrin-releasing peptide in the presence of 10^{-13} M-bombesin were $(6.6 \pm 3.4) \times 10^{-18}$ ($n = 10$) and $(2.7 \pm 1.3) \times 10^{-15}$ ($n = 5$) mol/ 10^6 cells respectively, whereas the amounts of ¹²⁵I-gastrin-releasing peptide specifically bound to H-*ras*-transformed cells under these conditions were two orders of magnitude lower, 5.6×10^{-18} and 6.8×10^{-17} mol/ 10^6 cells respectively (means of two experiments). K-*ras*-transformed cells displayed no detectable specific binding of ¹²⁵I-gastrin-releasing peptide.

Role of protein kinase C

The treatment of wild-type cells loaded with fura-2 with phorbol dibutyrate for 30 min in order to activate protein kinase C (Kikkawa *et al.*, 1983) completely inhibited bombesin-stimulated Ca²⁺ inflow, as assessed by the re-addition of Ca²⁺ in the presence of EGTA (Fig. 3a compared with Fig. 3b, and Table 2) and substantially inhibited PDGF-stimulated Ca²⁺ inflow (Table 2). As shown by others for other types of fibroblasts (Hesketh *et al.*, 1988), the action of phorbol dibutyrate was associated with a decrease in the magnitude of the transient

Fig. 3. Effect of pre-treatment of wild-type cells with phorbol dibutyrate on the ability of bombesin to alter $[Ca^{2+}]_i$.

(a, b) Cells were incubated in the presence (a) or absence (b) of 300 nM-phorbol dibutyrate for 30 min during the period in which the cells were loaded with fura-2 (see the Materials and methods section). The cells were washed and resuspended in fresh DMEM. (c, d) Cells were incubated in the presence of 300 nM-phorbol dibutyrate for 24 h, washed and resuspended in fresh DMEM. The cells were then incubated in the absence (c) or presence (d) of 300 nM-phorbol dibutyrate for 30 min during which the cells were loaded with fura-2. The cells were washed and suspended in fresh DMEM. The fluorescence of the cell suspensions was measured at 340 nm in the presence of 1.8 mM-Ca²⁺ and 2.7 mM-EGTA, and values of $[Ca^{2+}]_i$ were calculated as described in the Materials and methods section. The times of addition of bombesin (25 nM) and CaCl₂ (3 mM) are indicated by the arrows. Each plot shown is a representative of the plots obtained for five similar experiments, each conducted with a separate loading of fura-2.

Table 3. Comparison of the activity of protein kinase C in H-*ras*- and K-*ras*-transformed cells with that in wild-type cells

The extraction of protein kinase C, separation of particulate and cytosolic fractions, and measurement of the rate of histone phosphorylation in the presence of calcium and phosphatidylserine (protein kinase C activity) or EGTA were performed as described in the Materials and methods section. The values are the means $\pm$ S.E.M. of three determinations, each made with separate cell preparations. The degrees of significance for a comparison of enzyme activity in *ras*-transformed cells with that in wild-type cells, determined using Student's *t* test for paired samples, are: **P* < 0.02, and ***P* < 0.01.

Cell type	Subcellular fraction	Rate of histone phosphorylation (pmol/min per 5 mg of protein)	
		Ca ²⁺ and phosphatidylserine	EGTA
Wild-type	Cytosolic	1.19 $\pm$ 0.14	0.36 $\pm$ 0.05
	Particulate	0.97 $\pm$ 0.14	0.29 $\pm$ 0.08
H- <i>ras</i> -transformed	Cytosolic	0.65 $\pm$ 0.10**	0.30 $\pm$ 0.08
	Particulate	1.19 $\pm$ 0.12	0.43 $\pm$ 0.05
K- <i>ras</i> -transformed	Cytosolic	0.72 $\pm$ 0.09*	0.36 $\pm$ 0.08
	Particulate	1.12 $\pm$ 0.08	0.33 $\pm$ 0.03

increase in [Ca²⁺]_i induced by bombesin in the absence (Fig. 3a and Table 1) and presence (Table 1) of added Ca²⁺_o. However, phorbol dibutyrate caused no change in the transient peaks in [Ca²⁺]_i induced by FCS in the absence or presence of Ca²⁺_o, or in the transient peak of [Ca²⁺]_i induced by PDGF in the absence of Ca²⁺_o (results not shown).

Inhibition by 30 min of treatment with phorbol dibutyrate of bombesin-stimulated Ca²⁺ inflow was not observed in wild-type cells pre-treated for 24 h with phorbol dibutyrate in order to deplete the cells of protein kinase C (Rodriguez-Pena & Rozengurt, 1984) (Fig. 3d compared with Fig. 3c, and Table 2). Moreover, the ability of a 30 min exposure to phorbol dibutyrate to inhibit the transient increase in [Ca²⁺]_i induced by bombesin was abolished by pre-treatment of the cells with phorbol dibutyrate for 24 h (Figs. 3c and 3d). Pre-treatment with phorbol dibutyrate for 24 h caused no substantial change in the transient increase in [Ca²⁺]_i or in the initial rate of Ca²⁺ inflow induced by bombesin (Figs. 3b and 3c, and Table 2).

The presence of activated protein kinase C in *ras*-transformed cells in comparison with wild-type cells (Wolfman & Macara, 1987; Huang *et al.*, 1988) was confirmed by measuring the activity of protein kinase C (assayed in the presence of Ca²⁺ and phospholipid) in the cytosolic and particulate fractions prepared from these cells (Table 3). The amount of protein kinase C present in the cytosolic extract was significantly lower in H-*ras*- and K-*ras*-transformed cells compared with wild-type cells. No difference in the rate of histone phosphorylation measured in the absence of Ca²⁺ and phospholipid and in the presence of EGTA was observed in H-*ras*- or K-*ras*-transformed cells compared with wild-type cells (Table 3).

DISCUSSION

The results described have shown that, in NIH-3T3 wild-type cells, bombesin and PDGF each cause a substantial increase in Ca²⁺ inflow, as assessed by the prolonged increase in [Ca²⁺]_i above the basal value in experiments conducted in the presence of Ca²⁺_o, and by the enhanced rate of increase in [Ca²⁺]_i observed after the addition of Ca²⁺_o to cells incubated in the presence of EGTA and the agonist. The observations that (i) bombesin-

stimulated Ca²⁺ inflow is inhibited by high but not low concentrations of verapamil and (ii) addition of extracellular K⁺ in place of an agonist did not substantially stimulate Ca²⁺ inflow suggest that the agonist-stimulated inflow is mediated by a type of receptor-operated Ca²⁺ channel (Hurwitz, 1986; Rink, 1988).

The loss of the ability of bombesin to stimulate Ca²⁺ inflow in H-*ras*-transformed cells used in these experiments is most probably due to the decreased binding of bombesin to its receptors, since the amount of ¹²⁵I-gastrin-releasing peptide bound by H-*ras*-transformed cells was considerably lower than that which bound to wild-type cells [but see Wakelam *et al.* (1986) and Alonso *et al.* (1988), who reported no change in the number of bombesin receptors in H-*ras*-transformed cells]. Moreover, the ability of bombesin to release Ca²⁺ from intracellular stores, as well as to stimulate Ca²⁺ inflow, is lost in H-*ras*-transformed cells. It is possible that the loss of bombesin receptors and the lack of response of the *ras*-transformed cells to bombesin results from the activation of protein kinase C caused by the action of growth factors produced by the transformed cells (Bowen-Pope *et al.*, 1984; Marshall *et al.*, 1985) or by another mechanism (Black & Wakelam, 1990).

In contrast with the actions of bombesin, the ability of PDGF to release Ca²⁺ from intracellular stores was only slightly impaired in H-*ras*-transformed cells (cf. Wakelam *et al.*, 1986; Kamata & Kung, 1988; but see Parries *et al.*, 1987), whereas the ability of PDGF to stimulate Ca²⁺ inflow was substantially inhibited in the transformed cells. It is concluded that the impaired action of PDGF on Ca²⁺ inflow in *ras*-transformed cells is unlikely to be due to a decrease in PDGF receptors, and is most likely to be due to diminished coupling between PDGF receptors and receptor-operated Ca²⁺ channels, or to an inhibition of the receptor-operated channel itself.

It is considered unlikely that transformation by H-*ras* oncogenes leads to major alterations in the movement of Ca²⁺ through other channels and transporters in the cell because the patterns of the transient increases in [Ca²⁺]_i induced by FCS and ionomycin observed in the absence of added Ca²⁺_o were similar in wild-type and transformed cells. The shape of these curves would be expected to change if cell transformation altered the rates of Ca²⁺ outflow across the plasma membrane or of Ca²⁺ movement across the membranes of intracellular organelles.

Three observations provide evidence which indicates that the inhibition of the ability of bombesin and PDGF to stimulate Ca²⁺ inflow in H-*ras*-transformed cells is due to the activation of protein kinase C. Firstly, the effects of transformation with the H-*ras* oncogene on the ability of bombesin to stimulate Ca²⁺ inflow were mimicked by a short exposure of wild-type cells to phorbol dibutyrate, an activator of protein kinase C (Kikkawa *et al.*, 1983). Secondly, this ability of a short exposure to phorbol dibutyrate to mimic the action of transformation with the H-*ras* oncogene was not exhibited in wild-type cells pre-treated with phorbol dibutyrate for 24 h. This treatment has been shown to deplete the cells of protein kinase C (Rodriguez-Pena & Rozengurt, 1984). Thirdly, the present results, which confirm those of others (Wolfman & Macara, 1987; Huang *et al.*, 1988), have shown that protein kinase C is activated in the H-*ras*-transformed cells employed in the present experiments, as indicated by lower amounts of protein kinase C activity in the cytosolic fractions of transformed cells.

It is concluded that the inhibition of agonist-stimulated Ca²⁺ inflow in H-*ras*-transformed cells is a consequence of the activation of protein kinase C. The activation of protein kinase C may also account for the decrease in the number of receptors for bombesin. Chen *et al.* (1988) have reported that the transformation of NIH-3T3 cells by the *ras* or *fms* oncogenes leads to suppression of the activation of T-type voltage-operated Ca²⁺

channels measured using the patch-clamp technique. The inhibition of these voltage-operated Ca²⁺ channels may also involve protein kinase C although, as discussed above, it is considered unlikely that the increase in Ca²⁺ inflow caused by bombesin and PDGF observed in the present study is through voltage-operated Ca²⁺ channels. The conclusion that the activation of protein kinase C leads to inhibition of bombesin- or PDGF-stimulated Ca²⁺ inflow in NIH-3T3 fibroblasts is similar to the observation that activation of protein kinase C in neutrophils inhibits bivalent-cation influx stimulated by formylmethionyl-leucyl-phenylalanine (McCarthy *et al.*, 1989).

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REFERENCES

- Alonso, T., Morgan, R. O., Marvizon, J. C., Zarbl, H. & Santos, E. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4271–4275
- Benjamin, C. W., Conner, J. A., Tarpley, W. G. & Gorman, R. R. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4345–4349
- Black, F. M. & Wakelam, M. J. O. (1990) *Biochem. J.* **267**, 809–813
- Blackmore, P. F., Hughes, B. P., Shuman, E. A. & Exton, J. H. (1982) *J. Biol. Chem.* **257**, 190–197
- Bowen-Pope, D., Vogel, A. & Ross, R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2396–2400
- Chen, C., Corbley, M. J., Roberts, T. M. & Hess, P. (1988) *Science* **239**, 1024–1026
- Chiarugi, V. P., Pasquali, F., Vannucchi, S. & Ruggiero, M. (1986) *Biochem. Biophys. Res. Commun.* **141**, 591–599
- Chiarugi, V. P., Bruni, P., Pasquali, F., Magnelli, L., Basi, G., Ruggiero, M. & Farnararo, M. (1989) *Biochem. Biophys. Res. Commun.* **164**, 816–823
- Crofts, J. N. & Barritt, G. J. (1989) *Biochem. J.* **264**, 61–70
- Gryniewicz, G., Poenie, M. & Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Hesketh, T. R., Moore, J. P., Morris, J. D. H., Taylor, M. V., Rogers, J., Smith, G. A. & Metcalfe, J. C. (1985) *Nature (London)* **313**, 481–484
- Hesketh, T. R., Morris, J. D. H., Moore, J. P. & Metcalfe, J. C. (1988) *J. Biol. Chem.* **263**, 11879–11886
- Huang, M., Chida, K., Kamata, N., Nose, K., Kato, M., Homma, Y., Takenawa, T. & Kuroki, T. (1988) *J. Biol. Chem.* **263**, 17975–17980
- Hurwitz, L. (1986) *Annu. Rev. Pharmacol. Toxicol.* **26**, 225–258
- Ives, H. E. & Daniel, T. O. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 1950–1954
- Johnson, R. A. & Walseth, J. F. (1979) *Adv. Cyclic Nucleotide Res.* **10**, 135–167
- Kamata, T. & Kung, H.-F. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5799–5803
- Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. & Nishizuka, Y. (1983) *J. Biol. Chem.* **258**, 11442–11445
- Kris, R. M., Hazan, R., Villines, J., Moody, T. W. & Schlessinger, J. (1987) *J. Biol. Chem.* **262**, 11215–11220
- Lacal, J. C., Moscat, J. & Aaronson, S. A. (1987) *Nature (London)* **330**, 269–272
- Lopez-Rivas, A., Mendoza, S. A., Nanberg, E., Sinnett-Smith, J. & Rozengurt, E. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5768–5772
- Macara, I. G. (1989) *Mol. Cell. Biol.* **9**, 325–328
- Marshall, C. J. (1988) *J. Cell Sci. Suppl.* **10**, 157–169
- Marshall, C. J., Voudsen, K. & Ozanne, B. (1985) *Proc. R. Soc. London B* **226**, 99–106
- McCarthy, S. A., Hallam, T. J. & Merritt, J. E. (1989) *Biochem. J.* **264**, 357–364
- McNeil, P. L., McKenna, M. P. & Taylor, D. L. (1985) *J. Cell Biol.* **101**, 372–379
- Mendoza, S. A., Schneider, J. A., Lopez-Rivas, A., Sinnett-Smith, J. W. & Rozengurt, E. (1986) *J. Cell Biol.* **102**, 2223–2233
- Metcalfe, J. C., Hesketh, T. R., Smith, G. A., Morris, J. D. H., Corps, A. N. & Moore, J. P. (1985) *J. Cell Sci. Suppl.* **3**, 199–228
- Moolenaar, W. H., Tertoolen, L. G. J. & de Laat, S. W. (1984) *J. Biol. Chem.* **259**, 8066–8069
- Pandiella, A., Magni, M. & Meldolesi, J. (1989) *Biochem. Biophys. Res. Commun.* **163**, 1325–1331
- Parries, G., Hoebel, R. & Racker, E. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2648–2652
- Pollock, W. K., Rink, T. J. & Irvine, R. F. (1986) *Biochem. J.* **235**, 869–877
- Rink, T. J. (1988) *Nature (London)* **334**, 649–650
- Rink, T. J. & Pozzan, T. (1985) *Cell Calcium* **6**, 133–144
- Rodriguez-Pena, A. & Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
- Schilling, W. P., Ritchie, A. K., Navarro, L. T. & Eskin, S. G. (1988) *Am. J. Physiol.* **255**, H219–H227
- Schmitt, H. D., Puzicha, M. & Gallwitz, D. (1988) *Cell* **53**, 635–647
- Seuwen, K., Lagarde, A. & Pouyssegur, J. (1988) *EMBO J.* **7**, 161–168
- Tapley, P. M. & Murray, A. W. (1984) *Biochem. Biophys. Res. Commun.* **122**, 158–164
- 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

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