Inhibition of Ca^{2+} inflow causes an abrupt cessation of growth-factor-induced repetitive free Ca^{2+} transients in single NIH-3T3 cells

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In single NIH-3T3 fibroblasts loaded with fura-2, bombesin induced one of three patterns of increase in the concentration of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$): a single transient increase, a sustained increase, or repetitive transient increases in $[\text{Ca}^{2+}]_i$. Foetal-calf serum and ATP also gave these three patterns of response, although a lower proportion of cells gave repetitive Ca^{2+} transients in response to ATP. An increase in the concentration of bombesin from 1 to 25 nM increased the proportion of cells which exhibited repetitive Ca^{2+} transients. At 25 nM-bombesin, the proportion of cells which exhibited repetitive Ca^{2+} transients increased as the extracellular Ca^{2+} (Ca^{2+}_o) concentration was increased from 1 to 5 mM. Removal of Ca^{2+}_o by addition of EGTA, or inhibition of Ca^{2+} inflow by treatment of cells incubated in the presence of Ca^{2+}_o with verapamil or an activator of protein kinase C, abruptly terminated repetitive Ca^{2+} transients, with only one transient observed after the cessation of Ca^{2+} inflow. Repetitive Ca^{2+} transients were not observed in cells incubated in the absence of Ca^{2+}_o and in the presence of EGTA. Addition of Ca^{2+}_o to cells previously incubated in the presence of EGTA caused a resumption of repetitive Ca^{2+} transients. Addition of thapsigargin alone induced a large transient increase in $[\text{Ca}^{2+}]_i$, whereas much smaller transient increases in $[\text{Ca}^{2+}]_i$ were induced in about 30 % of cells tested by caffeine or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) plus oligomycin. Thapsigargin or the combination of CCCP plus oligomycin completely inhibited bombesin-induced repetitive Ca^{2+} transients, whereas caffeine had no effect. It is concluded from the studies of the role of Ca^{2+}_o that NIH-3T3 cells differ from other cell types in the anatomical or chemical links between extracellular Ca^{2+} and the intracellular stores involved in the generation of Ca^{2+} transients, whereas the results of the experiments with inhibitors indicate that the generation of repetitive Ca^{2+} transients in NIH-3T3 cells is unlikely to involve Ca^{2+} -induced Ca^{2+} release from caffeine-sensitive stores.

INTRODUCTION

Growth factors, such as bombesin, foetal-calf serum (FCS) and platelet-derived growth factor, induce increases in the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in fibroblasts (Moolenaar *et al.*, 1984; McNeil *et al.*, 1985; Hesketh *et al.*, 1988; Blakeley *et al.*, 1989; Polverino *et al.*, 1990). These changes in $[\text{Ca}^{2+}]_i$ are responsible, in part, for transmitting signals from growth-factor receptors to induce cell proliferation (Rana & Hokin, 1990). The increase in $[\text{Ca}^{2+}]_i$ is due to the release of Ca^{2+} from an intracellular store, probably the endoplasmic reticulum, via the action of inositol 1,4,5-trisphosphate (InsP_3) and an increased inflow of Ca^{2+} across the plasma membrane (reviewed in Berridge & Irvine, 1989; Putney *et al.*, 1989). Knowledge of the changes in $[\text{Ca}^{2+}]_i$ which are induced by growth factors has been obtained from the measurement of $[\text{Ca}^{2+}]_i$ in cell suspensions (reviewed in Berridge & Irvine, 1984). However, other studies have shown that, when investigated in single cells, agonist-induced changes in $[\text{Ca}^{2+}]_i$ are much more complex (reviewed by Berridge *et al.*, 1988; Cobbold *et al.*, 1990; Berridge, 1990). The experiments are beginning to reveal the important role played by intracellular structure, the movement of Ca^{2+} across regions of the cytoplasmic space, and interactions between intracellular Ca^{2+} stores, in the regulation of $[\text{Ca}^{2+}]_i$ (Berridge, 1990).

Although some studies of the effects of growth factors on $[\text{Ca}^{2+}]_i$ in single fibroblasts have been reported, including the observation of repetitive Ca^{2+} transients (Hesketh *et al.*, 1988; Harootunian *et al.*, 1988; Byron & Villereal, 1989; Peres &

Giovannardi, 1990), relatively little is known about the different patterns of responses induced by agonists in single fibroblasts and about the characteristics of repetitive Ca^{2+} transients. The aim of the present experiments was to investigate the nature of the changes in $[\text{Ca}^{2+}]_i$ induced by the growth factors bombesin, FCS and ATP in single NIH-3T3 fibroblasts. Each of these agonists has been shown to stimulate the formation of inositol trisphosphates in fibroblasts (Wakelam *et al.*, 1986; Harootunian *et al.*, 1991b). In particular, the characteristics of the repetitive Ca^{2+} transients and how they depend on plasma-membrane Ca^{2+} inflow, the release of Ca^{2+} from the InsP_3 -sensitive store, a caffeine-sensitive store and the mitochondria were examined. The results indicate that, when studied individually, NIH-3T3 fibroblasts exhibit a variety of responses, one of which is a series of repetitive Ca^{2+} transients. These growth-factor-induced repetitive Ca^{2+} transients cease very rapidly upon removal of extracellular free Ca^{2+} (Ca^{2+}_o) or inhibition of Ca^{2+} inflow.

EXPERIMENTAL

Cell culture and loading of cells with fura-2

The source of wild-type NIH-3T3 cells and conditions for cell growth were as described previously (Polverino *et al.*, 1990). The loading of suspensions of cells with fura-2, the measurement of fluorescence and the calculation of $[\text{Ca}^{2+}]_i$ were performed as described previously (Polverino *et al.*, 1990).

For studies of changes in $[\text{Ca}^{2+}]_i$ in single NIH-3T3 cells, the

Abbreviations used: FCS, foetal-calf serum; InsP_3 , inositol 1,4,5-trisphosphate; $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_o$, intracellular free Ca^{2+} and extracellular Ca^{2+} concentrations respectively; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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cells were grown in 35 mm-diam. plastic Petri dishes in which the centre of the dish was replaced by a 22 mm $\times$ 22 mm glass coverslip coated with poly-D-lysine (100 μ g/ml) (Kruskal *et al.*, 1984). Cells were washed three times with 150 mM-NaCl/5 mM-KCl/2 mM-CaCl₂/1 mM-MgCl₂/10 mM-glucose/20 mM-Hepes/KOH, pH 7.4 (Fluorescence Buffer; Kruskal & Maxfield, 1987), and then incubated in Fluorescence Buffer containing 3 μ M of the acetoxymethyl ester of fura-2 and 0.02 % (w/v) Pluronic F-127, at 37 °C under air/CO₂ (19:1). After 30 min the cells were washed three times with Fluorescence Buffer.

In order to verify that fura-2 in dye-loaded cells is located in the cytoplasmic space, the cells were treated with stepwise additions of digitonin (Streb *et al.*, 1983; Prentki *et al.*, 1984) to give final concentrations of 10, 20, 40, 80 and 100 μ M-digitonin, followed by 0.1 % (w/v) Triton X-100. Some 90 % of the fluorescence (measured at 360 nm) of cellular fura-2 was released within 30 s at 40 μ M-digitonin. The remaining fluorescence was released at 0.1 % Triton X-100. Cell morphology, as judged by phase-contrast microscopy, remained unaltered after treatment with 40 μ M-digitonin. Since it has been shown that concentrations of digitonin below 40 μ M selectively render the plasma membrane of cells permeable to small molecules, such as fura-2, and have little effect on intracellular membranes (Streb *et al.*, 1983; Prentki *et al.*, 1984), it is concluded that most of the cellular fura-2 is located in the cytoplasmic space.

Measurement of [Ca²⁺]_i in single cells

Cell fluorescence was measured by using an inverted TMD-EF microscope (Nikon Corp., Tokyo, Japan) fitted with a 100 W mercury vapour lamp and a rotating filter changer (UMANS, P.O. Box 361, Urbana, IL, U.S.A.). All illumination optics were quartz in order to maximize transmission of 345 nm light. The objective lens was a 40 $\times$ Nikon UV-fluor (UV-F series, 1.3 numerical aperture). Excitation filters were 5 nm bandpass quartz filters centred at 345 and 385 nm (Omega Optical Co., Brattleboro, VT, U.S.A.). Images of fluorescent cells were obtained by using a DM400 dichroic mirror (Nikon) and a BA510/20 long-pass barrier filter (Nikon). A variable-pinhole diaphragm (Nikon) was placed in the image plane in order to exclude fluorescence from any other source except the cell under study, and the emitted light was passed to a Nikon P1 photometer. The output of the photometer was coupled to an IBM-style AT computer. Filter changing and recording of fluorescence data were controlled by the UMANS software system.

To decrease the photobleaching of fura-2, neutral-density filters (Nikon) were placed in the excitation pathway. This allowed images to be collected for up to 15 min with minimal photobleaching or photodamage to the cells. A BA-BG18 i.r. filter (Nikon) was also included in the excitation light path, to prevent light of wavelength greater than 750 nm (transmitted by the 385 nm filter) from interfering with the measurement of [Ca²⁺]_i.

Fluorescence measurements were performed at 37 °C by using a hair-drier heater to maintain the microscope stage at this temperature. Cells loaded with fura-2 and covered by 1 ml of Fluorescence Buffer were incubated for 2 min before addition of the first agent being tested. Fluorescence Buffer (1 ml) containing this agent at twice its final concentration was added to the dish, and the medium was mixed rapidly. All subsequent additions involved removal of 1 ml of solution from the dish, mixing with the agent under test, and re-addition to the dish. The ratio of fluorescence intensities at 345 nm and 385 nm was recorded at a rate of two ratios per second. After subtraction of background intensities, obtained from an adjacent cell-free portion of the dish, the ratio of fluorescence intensities at the two wavelengths was calculated and converted into values of [Ca²⁺]_i by comparison

with calibration solutions, in accordance with Grynkiewicz *et al.* (1985). Except where indicated otherwise, all data are reported as means $\pm$ S.E.M. for the numbers of experiments indicated in parentheses.

Cellular ATP concentrations

Cells grown to near-confluence in 90 mm Petri dishes were detached by treatment with 0.02 % (w/v) trypsin/0.05 % (w/v) EDTA. The cells were washed and resuspended at a concentration of 3×10^6 cells/ml in Fluorescence Buffer. A 2 ml portion of this suspension (6×10^6 cells) was placed into a vial and incubated in a shaking water bath (90 cycles/min) at 37 °C for 5 min before addition of dimethyl sulphoxide (control) or CCCP plus oligomycin. At the appropriate time the cells were centrifuged at 150 *g* for 2 min in order to pellet the cells. Then 1 ml of ice-cold 0.5 M-HClO₄ was added, and the tube was immediately mixed with a vortex mixer and placed on ice. After 30 min, during which the samples were occasionally mixed, the suspension was centrifuged at 10000 *g* for 5 min. The acid-soluble material was neutralized (pH 7.0) with 2 M-KOH, containing 0.3 M-Mops and 0.2 M-NaCl. The amounts of ATP present in the neutralized extracts were estimated by measuring the change in *A*₃₄₀ of NADPH in the coupled reaction using hexokinase and glucose-6-phosphate dehydrogenase (Berry *et al.*, 1987).

Materials

FCS and trypsin were obtained from Cytosystems, Castle Hill, N.S.W., Australia; carbonyl cyanide *m*-chlorophenylhydrazine (CCCP), oligomycin, ATP, Mops and caffeine were from Sigma Chemical Co., St. Louis, MO, U.S.A.; thapsigargin was from LC Services Corp., Woburn, MA, U.S.A.; and the acetoxymethyl ester of fura-2 was from Molecular Probes, Eugene, OR, U.S.A. Other chemicals were obtained from the sources described previously (Polverino *et al.*, 1990). CCCP and oligomycin were dissolved in dimethyl sulphoxide.

RESULTS

Effects of bombesin, FCS and ATP

In single NIH-3T3 cells incubated in the presence of 2 mM-Ca²⁺_o, bombesin (25 nM) caused repetitive transient increases in [Ca²⁺]_i (repetitive Ca²⁺ transients) (Fig. 1a), a single transient increase in [Ca²⁺]_i (Fig. 1c), a sustained increase in [Ca²⁺]_i (Fig. 1d) or no change in [Ca²⁺]_i. Addition of buffer alone caused no change in [Ca²⁺]_i (results not shown). The proportions of cells which gave each of these types of responses are shown in Table 1. The values of [Ca²⁺]_i at the initial peak after the addition of bombesin was 690 ± 90 nM (*n* = 16), 935 ± 9 nM (27) and 1215 ± 105 nM (17) for cells which displayed a single transient increase in [Ca²⁺]_i, repetitive Ca²⁺ transients and a sustained increase in [Ca²⁺]_i, respectively.

Each repetitive increase in [Ca²⁺]_i induced by bombesin coincided with an increase in fluorescence at 345 nm and a decrease at 385 nm (Fig. 1b; cf. Fig. 1a), indicating that the observed repetitive Ca²⁺ transients are not likely to be an artifact of the experimental system. The bombesin-induced repetitive Ca²⁺ transients had a period of 70 ± 5 s (*n* = 35) and a magnitude ('amplitude') of 425 ± 25 nM. The magnitude of the transients was 45 % of the initial transient increase in [Ca²⁺]_i (Fig. 1a). Between transients [Ca²⁺]_i returned to the basal value.

Most of the cells which did not respond to the addition of bombesin did give a transient increase in [Ca²⁺]_i after the addition of FCS (1 %, v/v). The few cells which gave no response to either agonist responded to the addition of 100 nM-ionomycin with a sustained increase in [Ca²⁺]_i. The increase in [Ca²⁺]_i was

Fig. 1. Patterns of increase in $[\text{Ca}^{2+}]_i$ observed in single NIH-3T3 cells after addition of bombesin

NIH-3T3 cells were loaded with fura-2, incubated in the presence of 2 mM- Ca^{2+} and 25 nM-bombesin, and the fluorescence at 345 and 385 nm was measured as a function of time and values of $[\text{Ca}^{2+}]_i$ were calculated as described in the Experimental section. (a) Repetitive transient increases in $[\text{Ca}^{2+}]_i$; (b) traces of fluorescence with excitation at 345 and 385 nm for the data shown in (a); (c) a single transient increase in $[\text{Ca}^{2+}]_i$; (d) a sustained increase in $[\text{Ca}^{2+}]_i$. Each plot is representative of the plots obtained for 39 (a), 21 (c), and 22 (d) similar experiments.

observed within 1–5 s after the addition of ionomycin (results not shown).

The patterns of changes in $[\text{Ca}^{2+}]_i$ induced by FCS were similar to those induced by bombesin. In the 27 cells investigated, 7 (26%) gave a single transient increase, 2 (7%) a sustained increase, 17 (63%) repetitive Ca^{2+} transients and 1 (4%) no response. The period of the FCS-induced repetitive Ca^{2+} transients was 115 ± 15 s ($n = 16$), which was significantly greater ($P < 0.001$) than the period of the repetitive Ca^{2+} transients induced by 25 nM-bombesin. The repetitive Ca^{2+} transients induced by FCS were more irregular than those induced by bombesin. The initial transient increase in $[\text{Ca}^{2+}]_i$ induced by FCS was 1015 ± 150 nM ($n = 16$), whereas the magnitude of the FCS-induced repetitive Ca^{2+} transients was 350 ± 45 nM ($n = 16$).

Table 1. Effects of $[\text{Ca}^{2+}]_o$ on changes in $[\text{Ca}^{2+}]_i$ induced by bombesin in single NIH-3T3 fibroblasts

Cells loaded with fura-2 were incubated at a given $[\text{Ca}^{2+}]_o$ as described in the Experimental section. Values of $[\text{Ca}^{2+}]_i$ were determined from the fluorescence intensity recorded at 345 and 385 nm as described in the Experimental section. The numbers of cells tested at 1 mM-, 2 mM-, 3 mM- and 5 mM- Ca^{2+}_o were 17, 94, 77 and 29 respectively.

Pattern of change in $[\text{Ca}^{2+}]_i$ induced by 25 nM-bombesin	Proportion of cells which responded in a given manner			
	$[\text{Ca}^{2+}]_o \dots$	1 mM	2 mM	3 mM 5 mM
Repetitive transient increases		42	42	47 58
Sustained increase		6	22	16 11
Single transient increase		29	23	28 17
No response		23	13	9 14

In the presence of ATP, repetitive transient increases in $[\text{Ca}^{2+}]_i$ were observed in some cells. The number of cells which exhibited a single transient increase in $[\text{Ca}^{2+}]_i$, a sustained increase, repetitive Ca^{2+} transients and no response were: 2, 2, 0 and 0 cells respectively at 500 μM ATP; 4, 1, 1 and 0 cells respectively at 100 μM-ATP; and 6, 2, 1 and 0 cells respectively at 10 μM-ATP. At 1 μM-ATP, 3 cells gave no response and 1 cell gave a single transient increase in $[\text{Ca}^{2+}]_i$. No response was observed at 0.1 μM-ATP (2 cells tested).

Effect of bombesin concentration

An increase in the concentration of bombesin from 1 to 25 nM caused an increase in the proportion of cells which gave repetitive Ca^{2+} transients and a decrease in the proportion of cells which gave no response to the agonist (Table 2). For cells which gave repetitive transient increases in $[\text{Ca}^{2+}]_i$ in response to bombesin, both the time elapsed between the addition of bombesin and the initial increase in $[\text{Ca}^{2+}]_i$ (time of onset) and the period of the repetitive Ca^{2+} transients decreased as the concentration of bombesin increased (Table 3). This range of bombesin concentrations (1–25 nM) corresponded to the central part of the dose–response curve for the bombesin-induced increase in $[\text{Ca}^{2+}]_i$ in suspensions of fibroblasts (Fig. 2). At 250 nM-bombesin, which was close to a saturating concentration in the dose–response curve for cell suspensions, the proportion of single cells which gave repetitive transient increases in $[\text{Ca}^{2+}]_i$ decreased, whereas the proportion which gave a sustained increase in $[\text{Ca}^{2+}]_i$ increased (Table 2).

Dependence of bombesin-induced repetitive increases in $[\text{Ca}^{2+}]_i$ on Ca^{2+} inflow

An increase in the proportion of cells which gave repetitive transient increases in $[\text{Ca}^{2+}]_i$ in response to bombesin was observed when the concentration of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$) was increased from 1 to 5 mM (Table 1). In all cells tested, addition of bombesin to cells incubated in the absence of added Ca^{2+}_o and for 2 min in the presence of 1 mM-EGTA caused a single transient increase in $[\text{Ca}^{2+}]_i$ but no repetitive increases in $[\text{Ca}^{2+}]_i$ (Fig. 3a). Subsequent addition of 3 mM- Ca^{2+}_o caused a rapid increase in $[\text{Ca}^{2+}]_i$. The patterns of response observed after addition of Ca^{2+}_o were: repetitive Ca^{2+} transients (Fig. 3a) (8/19 cells, 42%), a sustained increase in $[\text{Ca}^{2+}]_i$ (8 cells, 42%), a single transient increase in $[\text{Ca}^{2+}]_i$ (2 cells, 11%) and no response (1 cell, 5%). No increase in $[\text{Ca}^{2+}]_i$ was observed in cells treated in a similar manner but in the absence of bombesin.

Table 2. Effect of bombesin concentration on the changes in $[Ca^{2+}]_i$ induced by this growth factor in single NIH-3T3 cells

NIH-3T3 cells were loaded with fura-2 as described in the Experimental section and incubated in the presence of 2 mM- Ca^{2+} . Values of $[Ca^{2+}]_i$ were determined from the measurement of changes in fluorescence at 345 and 385 nm after the addition of bombesin as described in the Experimental section. The numbers of cells tested at 1 nM-, 2.5 nM-, 10 nM-, 25 nM- and 250 nM-bombesin were 25, 25, 25, 31 and 14 respectively.

Pattern of change in $[Ca^{2+}]_i$ induced by bombesin	[Bombesin] ...	Proportion of cells which responded in a given manner (% of cells tested)				
		1.0 nM	2.5 nM	10 nM	25 nM	250 nM
Repetitive transient increases		32	32	47	49	21
Sustained increase		4	20	0	12	44
Single transient increase		8	8	36	9	28
No response		56	40	16	30	7

Table 3. Effect of bombesin concentration on the time of onset and period of repetitive transient increases in $[Ca^{2+}]_i$

The time of onset and period of the repetitive Ca^{2+} transients were determined for each of the cells which gave repetitive Ca^{2+} transients reported in Table 2. The numbers of cells at 1 nM-, 2.5 nM-, 10 nM-, 25 nM- and 250 nM-bombesin were 8, 8, 12, 14 and 3 respectively.

[Bombesin] (nM)	Time of onset of increase in $[Ca^{2+}]_i$ (s)	Period of repetitive Ca^{2+} transients (s)
1	33 ± 11	134 ± 27
2.5	21 ± 10	98 ± 23
10	8 ± 5	105 ± 17
25	4 ± 2	81 ± 10
250	0	45 ± 15

Fig. 2. Dose-response curve for the action of bombesin in inducing an increase in $[Ca^{2+}]_i$ in suspensions of NIH-3T3 cells

NIH-3T3 cells were loaded with fura-2, incubated in 2 mM- Ca^{2+} in the presence of the indicated concentration of bombesin, and values of $[Ca^{2+}]_i$ as a function of time were measured as described in the Experimental section. The data shown are the maximum values of $[Ca^{2+}]_i$ observed after addition of bombesin, plotted as a function of the bombesin concentration. Each data point represents the mean $\pm$ S.E.M. for the results obtained from 4–7 separate cell loadings with fura-2.

The addition of EGTA (Fig. 3b), verapamil (Fig. 3c), which at high concentrations has been shown to inhibit agonist-stimulated Ca^{2+} inflow in NIH-3T3 fibroblasts (Polverino *et al.*, 1990), or phorbol dibutyrate (Fig. 3d) to cells exhibiting repetitive Ca^{2+} transients completely prevented the repetitive transient increases in $[Ca^{2+}]_i$, or, in the case of phorbol dibutyrate, greatly increased the period of these transients. [It has previously been shown that phorbol dibutyrate, through the activation of protein kinase C, inhibits Ca^{2+} inflow in NIH-3T3 fibroblasts, most likely by phosphorylation of the putative Ca^{2+} channel or a protein involved in coupling receptors to the channel (Polverino *et al.*, 1990).] One further transient increase in $[Ca^{2+}]_i$ with a magnitude and period similar to those of the preceding transients was generally observed after addition of EGTA, verapamil or phorbol dibutyrate (Figs. 3b, 3c and 3d). This further transient is unlikely to be due to the time taken for the solution in the Petri dish to be thoroughly mixed, since, as described above, addition of ionomycin in a similar manner caused a very rapid response in $[Ca^{2+}]_i$. Addition of buffer alone to cells exhibiting bombesin-induced repetitive Ca^{2+} transients did not alter the transients (Fig. 3b).

Effects of thapsigargin, caffeine and inhibitors of mitochondrial oxidative phosphorylation

Thapsigargin caused a large single transient increase in $[Ca^{2+}]_i$ followed by a small maintained elevation of $[Ca^{2+}]_i$ above the basal value (Fig. 4a) [cf. results obtained for suspensions of other cell types (Thastrup *et al.*, 1987a,b, 1990)]. Subsequent addition of bombesin caused no further change in $[Ca^{2+}]_i$ (Fig. 4a) (15 out of 15 cells tested). The magnitude of the increase in $[Ca^{2+}]_i$ induced by thapsigargin was 554 ± 88 nM ($n = 17$).

Caffeine (10 mM) caused a small single transient increase in $[Ca^{2+}]_i$ in 8/28 (29%) of cells (Fig. 4b) and no increase in $[Ca^{2+}]_i$ in 20/28 (71%) of cells (Fig. 4c). In those cells in which caffeine alone induced an increase in $[Ca^{2+}]_i$, the magnitude of the increase was 350 ± 57 nM ($n = 8$), which was 40% of the initial transient increase in $[Ca^{2+}]_i$ induced by bombesin.

Subsequent addition of bombesin to cells previously treated with caffeine induced repetitive Ca^{2+} transients (Figs. 4b and 4c) in 10/28 (36%) of the cells. There were no significant differences in the characteristics of the repetitive Ca^{2+} transients induced by bombesin when cells in which caffeine alone caused a transient increase in $[Ca^{2+}]_i$ were compared with those in which caffeine caused no change in $[Ca^{2+}]_i$. Under the former conditions (a caffeine-induced transient increase in $[Ca^{2+}]_i$), the subsequent addition of bombesin induced repetitive Ca^{2+} transients in 3/8 (37%) of cells. The magnitudes of the initial transient increase in $[Ca^{2+}]_i$ and the subsequent repetitive transients induced by bombesin were 950 ± 160 nM and 440 ± 120 nM respectively. The

Fig. 3. Effects of depletion of Ca^{2+}_o and inhibition of Ca^{2+} inflow on repetitive increases in $[\text{Ca}^{2+}]_i$ induced by bombesin

NIH-3T3 cells were loaded with fura-2, incubated, and values of $[\text{Ca}^{2+}]_i$ were determined as a function of time as described in the Experimental section. (a) Cells were incubated in the presence of 1 mM-EGTA and no added Ca^{2+}_o for 2 min before the addition of bombesin. The times of addition of bombesin (25 nM) and CaCl_2 (3 mM) are indicated by the arrows. (b) Cells were incubated in the presence of 2 mM- Ca^{2+}_o . Bombesin (25 nM), Fluorescence Buffer and EGTA (3 mM) were added at the times indicated. (c) and (d) Cells were incubated in the presence of 2 mM- Ca^{2+}_o , and bombesin (25 nM) and verapamil (100 μM) or phorbol dibutyrate (300 nM) were added at the times indicated. Each plot represents one of the plots obtained for 19 (a), 4 (b), 4 out of 5 (c) and 11 out of 12 (d) similar experiments each conducted with a separate loading of fura-2.

repetitive Ca^{2+} transients induced by bombesin had a period of 55 ± 3 s. Under the latter conditions (no caffeine-induced increase in $[\text{Ca}^{2+}]_i$) the subsequent addition of bombesin induced repetitive Ca^{2+} transients in 7/20 (35%) of cells. The magnitudes of the initial transient increase in $[\text{Ca}^{2+}]_i$ and the subsequent repetitive transients induced by bombesin were 640 ± 170 nM and 420 ± 55 nM respectively. The period of the bombesin-induced repetitive Ca^{2+} transients was 58 ± 5 s.

Addition of CCCP plus oligomycin, which inhibits the accumulation of Ca^{2+} in mitochondria in intact cells (Joseph *et al.*, 1983), caused a small single transient increase in $[\text{Ca}^{2+}]_i$ (Fig. 4d)

Fig. 4. Effects of thapsigargin (a), caffeine (b, c) and CCCP plus oligomycin (d) on bombesin-induced repetitive transient increases in $[\text{Ca}^{2+}]_i$

NIH-3T3 cells loaded with fura-2, incubated in the presence of 2 mM- Ca^{2+}_o , and values of $[\text{Ca}^{2+}]_i$ were determined as a function of time as described in the Experimental section. Bombesin (25 nM), thapsigargin (160 nM), caffeine (10 mM) and CCCP (10 μM) plus oligomycin (20 μM) were added at the times indicated by the arrows. Each plot is representative of the plots obtained for 17 (a), 3 (b), 7 (c) and 11 (d) similar experiments each conducted with a separate loading of fura-2. Addition of 0.5% (v/v) dimethyl sulphoxide alone in place of CCCP plus oligomycin had no effect on $[\text{Ca}^{2+}]_i$.

in 7/17 (40%) of cells tested. Subsequent addition of bombesin to cells previously treated with CCCP plus oligomycin resulted in a single transient increase in $[\text{Ca}^{2+}]_i$ (Fig. 4d) in 10/17 cells (60%), whereas the other cells tested (7/17) (40%) gave no response to bombesin. In those cells in which CCCP plus oligomycin induced an increase in $[\text{Ca}^{2+}]_i$, the magnitude of the increase was 170 ± 40 nM ($n = 7$). In a series of control experiments conducted in parallel in which 0.5% (v/v) dimethyl sulphoxide was added in place of CCCP plus oligomycin, the initial transient increase in $[\text{Ca}^{2+}]_i$ induced by bombesin was 611 ± 66 nM ($n = 20$).

The effects of CCCP plus oligomycin on $[\text{Ca}^{2+}]_i$ were associated with a decrease in the cellular concentration of ATP. The amounts of ATP in cells incubated in the presence of CCCP plus

oligomycin for 2 and 3 min were 6.59 ± 0.06 and 6.63 ± 0.01 nmol/ 10^6 cells (both $n = 4$) respectively. Each of these values is significantly less than the value of 8.94 ± 0.09 nmol/ 10^6 cells ($n = 3$) obtained for cells incubated in the presence of dimethyl sulphoxide ($P < 0.001$ and $P < 0.001$ respectively, by Student's t test for unpaired samples).

DISCUSSION

The results indicate that, as reported for a number of other cell types (Connor *et al.*, 1987; Ambler *et al.*, 1988; Jacob *et al.*, 1988; Winiger & Schlegel, 1988; Prentki *et al.*, 1988; Kawanishi *et al.*, 1989; Millard *et al.*, 1989; Rooney *et al.*, 1989; Byron & Villereal, 1989), several different patterns of increase in $[Ca^{2+}]_i$ are observed in single NIH-3T3 fibroblasts after the addition of bombesin, FCS or ATP. Although the proportion of cells giving each of these responses was influenced by changes in the concentration of bombesin or, at a fixed bombesin concentration, by changes in $[Ca^{2+}]_o$, three basic patterns of response were observed under each of the extracellular environments tested. A feature of the heterogeneous responses observed in NIH-3T3 cells is the high proportion of cells which exhibited a sustained increase in $[Ca^{2+}]_i$ in response to bombesin and other agonists, even at low agonist concentrations.

The results obtained in the present experiments with NIH-3T3 cells differ in two ways from the changes in $[Ca^{2+}]_i$ in single REF52 rat embryo fibroblasts reported by Harootunian *et al.* (1988). Firstly, the responses of NIH-3T3 cells are far more heterogeneous. Secondly, depolarization of the plasma membrane was not required in order to detect repetitive Ca^{2+} transients.

A correlation was observed between the magnitude of the first peak in $[Ca^{2+}]_i$ induced by bombesin and the nature of the subsequent response in $[Ca^{2+}]_i$. The highest initial increase in $[Ca^{2+}]_i$ was associated with a sustained increase in $[Ca^{2+}]_i$, whereas the lowest initial increase in $[Ca^{2+}]_i$ was associated with a single transient increase in $[Ca^{2+}]_i$. This correlation suggests that the subsequent response of a cell may be determined by the magnitude of the initial increase in $[Ca^{2+}]_i$. Variation in the initial increase in $[Ca^{2+}]_i$ may be due to differences in the Ca^{2+} content or capacity of the agonist-sensitive intracellular stores, in the degree of coupling between the receptor and the generation of $InsP_3$, or in the rate of Ca^{2+} inflow (cf. Kawanishi *et al.*, 1989).

Characteristic properties of bombesin-induced Ca^{2+} transients observed in NIH-3T3 fibroblasts include the much larger increase in $[Ca^{2+}]_i$ in the initial spike compared with subsequent Ca^{2+} transients, the decrease in the period of the transients when the concentration of bombesin is increased, and the return of $[Ca^{2+}]_i$ to the basal value between transients. Since a mechanism which involves repetitive changes in Ca^{2+} inflow across the plasma membrane coupled to changes in membrane potential (Gorman & Thomas, 1978; Wallen & Grillner, 1987; Schlegel *et al.*, 1987) is not consistent with these properties, it is concluded that generation of the transients does not involve this type of oscillatory Ca^{2+} movement across the plasma membrane. Therefore it is likely that the agonist-induced repetitive Ca^{2+} transients in NIH-3T3 fibroblasts are generated by a mechanism which involves changes in intracellular Ca^{2+} movement.

Three further characteristics of the bombesin-induced Ca^{2+} transients observed in the present experiments are the inhibition by pre-treatment with thapsigargin, inhibition by CCCP plus oligomycin and insensitivity to caffeine. The results obtained with thapsigargin, which releases Ca^{2+} from $InsP_3$ -sensitive Ca^{2+} stores (Thastrup *et al.*, 1990; Law *et al.*, 1990), indicate that $InsP_3$ -sensitive Ca^{2+} stores are required for the generation of Ca^{2+} transients.

The ability of CCCP plus oligomycin to inhibit bombesin-induced transient increases in $[Ca^{2+}]_i$ suggests that mitochondria play a role in the generation of these transients, although the amount of Ca^{2+} present in these organelles is much smaller than that present in thapsigargin-sensitive stores. (This conclusion assumes that the inhibitors do not substantially affect the processes by which Ca^{2+} moves across the plasma membrane.) The role of mitochondria may involve the provision of an essential Ca^{2+} store and/or the provision of ATP. The observations that CCCP plus oligomycin markedly decreases the cellular concentration of ATP and the magnitude of the single transient increase in $[Ca^{2+}]_i$ induced by bombesin and completely prevents a bombesin-induced sustained increase in $[Ca^{2+}]_i$ indicate that the most likely cause of the inhibition of bombesin-induced Ca^{2+} transients by CCCP plus oligomycin is a decrease in the cellular ATP concentration. This may lead to a decrease in the activity of enzymes which utilize ATP, including those involved in the provision of phosphatidylinositol 4,5-bisphosphate, GTP (and hence the activation of oligomeric GTP-binding regulatory proteins), phosphorylated proteins, and $(Ca^{2+} + Mg^{2+})$ -ATPase Ca^{2+} pumps in the plasma membrane and endoplasmic reticulum (cf. Millard *et al.*, 1989).

Several observations indicate that, in NIH-3T3 fibroblasts, Ca^{2+} -induced Ca^{2+} release from a caffeine-sensitive intracellular store is unlikely to be involved in the mechanism by which repetitive Ca^{2+} transients are generated. These include the inability of caffeine to potentiate the generation of Ca^{2+} transients, the relatively small amount of Ca^{2+} detected in caffeine-sensitive stores, and the failure of thapsigargin, caffeine or ionomycin to induce Ca^{2+} transients when added in the absence of agonists (cf. Harootunian *et al.*, 1988, 1991a).

In view of the results obtained with caffeine and the other properties of agonist-induced Ca^{2+} transients in NIH-3T3 fibroblasts, it is concluded that three possible mechanisms for the generation of Ca^{2+} transients are consistent with the present data. These are: (i) oscillations in $InsP_3$ concentration caused by the action of protein kinase C on the receptor or the GTP-binding regulatory protein which couples the receptor to phosphatidylinositol 4,5-bisphosphate-specific phospholipase C (Cobbold *et al.*, 1990); (ii) oscillations in $InsP_3$ caused by changes in Ca^{2+} -stimulated phospholipase C activity induced, in turn, by changes in $[Ca^{2+}]_i$ resulting from the uptake and release of Ca^{2+} by the $InsP_3$ -sensitive Ca^{2+} store (Meyer & Stryer, 1988); and (iii) inhibition of $InsP_3$ -mediated Ca^{2+} release caused by an increase in $[Ca^{2+}]_i$ (Parker & Ivorra, 1990). Although it is not possible at present to distinguish between these three mechanisms, the observation that the period of the transients induced by bombesin differs from that for transients induced by FCS suggests that the first mechanism may be the most likely (cf. Harootunian *et al.*, 1991b).

The abrupt cessation of repetitive Ca^{2+} transients which follows inhibition of Ca^{2+} inflow has not been observed in other cell types which exhibit repetitive Ca^{2+} transients of the type described here (Kruskal & Maxfield, 1987; Prentki *et al.*, 1988; Jacob *et al.*, 1988; Ambler *et al.*, 1988; Kawanishi *et al.*, 1989; Millard *et al.*, 1989; Rooney *et al.*, 1989), although it is observed in cells which exhibit oscillations in $[Ca^{2+}]_i$ linked to changes in membrane potential (Gorman & Thomas, 1978; Wallen & Grillner, 1987; Schlegel *et al.*, 1987). The observation that one normal transient increase in $[Ca^{2+}]_i$ is observed after inhibition of Ca^{2+} inflow indicates that Ca^{2+} inflow is not required directly to generate each Ca^{2+} transient (cf. Kruskal & Maxfield, 1987; Ambler *et al.*, 1988; Jacob *et al.*, 1988; Millard *et al.*, 1989; Rooney *et al.*, 1989). The observation that repetitive Ca^{2+} transients cease abruptly after inhibition of Ca^{2+} inflow indicates that, in comparison with other cell types (Berridge & Galione, 1988): (a)

agonist-sensitive intracellular Ca^{2+} stores in NIH-3T3 cells are very rapidly depleted during the generation of each Ca^{2+} transient; (b) Ca^{2+} released into the cytoplasmic space of NIH-3T3 cells is rapidly extruded from the cells; and/or (c) the anatomical relationship between the plasma membrane and intracellular stores in NIH-3T3 cells differs from the relationship present in many other types of cells.

We gratefully acknowledge Dr. Chester Regen, P.O. Box 361, Urbana, IL 61801, U.S.A., for advice in setting up the UMANS System for the measurement of $[\text{Ca}^{2+}]_i$ in single cells, and the laboratory of Professor Michael N. Berry and Dr. John Phillips of the Department of Medical Biochemistry, School of Medicine, Flinders University, for performing the ATP assays. This work was supported by grants from the Clive and Vera Ramaciotti Foundations and the Anticancer Foundation of the Universities of South Australia.

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Received 5 February 1991/8 May 1991; accepted 16 May 1991