Single-cell analysis of the mitogen-induced calcium responses of normal and protein kinase C-depleted Swiss 3T3 cells

A. N. Corps,* T. R. Cheek,† R. B. Moreton,†
M. J. Berridge,† and K. D. Brown*

*Department of Biochemistry
AFRC Institute of Animal Physiology
and Genetics Research
Babraham Hall

Cambridge CB2 4AT, U.K.

†AFRC Unit of Insect Neurophysiology
and Pharmacology

University of Cambridge

Department of Zoology

Downing Street, Cambridge CB2 3EJ, U.K.

Single-cell fluorescence image analysis has been used to characterize the mitogen-induced increases in intracellular free $[Ca^{2+}]_i$ ($[Ca^{2+}]_i$) in control and protein kinase C-depleted Swiss 3T3 cells. More than 80% of the control cells exhibited fast, transient responses to bombesin, vasopressin, or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In contrast, the $[Ca^{2+}]_i$ responses induced by platelet-derived growth factor (PDGF) were markedly more heterogeneous, slower, and often biphasic, with fewer cells (60–70%) responding. The peak $[Ca^{2+}]_i$ values obtained in response to each mitogen showed substantial variation between cells. Brief pretreatment of the cells with 12-O-tetradecanoyl phorbol 13-acetate (TPA) reduced the $[Ca^{2+}]_i$ responses to bombesin, but did not affect the responses to PDGF. Long-term pretreatment of the cells with TPA to down-modulate protein kinase C resulted in substantially prolonged $[Ca^{2+}]_i$ responses to bombesin, vasopressin, and $PGF_{2\alpha}$, but had no such effect on the responses to PDGF. We conclude that differences between the $[Ca^{2+}]_i$ responses to bombesin and PDGF, previously reported using cell populations, reflect differences occurring in individual cells, and that the $[Ca^{2+}]_i$ responses to bombesin, vasopressin, and $PGF_{2\alpha}$ (but not PDGF) are subject to feedback inhibition via protein kinase C.

Introduction

An increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$)¹ is one of the earliest responses

of mammalian cells to many hormones, neurotransmitters, or growth factors. In Swiss mouse 3T3 cells, a widely-used model system for studying cell growth control (Rozengurt, 1986), increased $[Ca^{2+}]_i$ and/or $^{45}Ca^{2+}$ release have been observed in response to several mitogens, including platelet-derived growth factor (PDGF), bombesin, vasopressin, and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Berridge *et al.*, 1984; Morris *et al.*, 1984; Hesketh *et al.*, 1985, 1988; Brown *et al.*, 1987; Ives and Daniel, 1987; Lopez-Rivas *et al.*, 1987). Each of these mitogens stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to inositol 1,4,5-trisphosphate ($1,4,5-IP_3$), which could mediate the mobilization of Ca^{2+} from intracellular stores, but recent evidence has suggested that there may be important differences between the mechanisms by which the mitogens induce calcium responses (Lopez-Rivas *et al.*, 1987; Hasegawa-Sasaki *et al.*, 1988; Blakeley *et al.*, 1989). Thus PDGF causes a substantially slower onset of inositol phosphate accumulation than do bombesin or vasopressin, and the PDGF-induced calcium responses, although also slower than those induced by bombesin and vasopressin, appear to precede the stimulated formation of inositol phosphates. Furthermore, the inositol phosphate formation and calcium mobilization responses to bombesin or vasopressin are sensitive to inhibition by phorbol esters, whereas the responses to PDGF are not affected by these compounds (Sturani *et al.*, 1986; Lopez-Rivas *et al.*, 1987; Hasegawa-Sasaki *et al.*, 1988; Hesketh *et al.*, 1988; Blakeley *et al.*, 1989). Lastly, in cells pretreated with high concentrations of phorbol esters to down-modulate protein kinase C, the accumulation of inositol phosphates in response to bombesin or vasopressin is enhanced, while the response to PDGF is unchanged or diminished (Blakeley *et al.*, 1989).

Such differences in the responses to different

¹ Abbreviations: $[Ca^{2+}]_i$, intracellular concentration of free

Ca^{2+} ; IP, inositol phosphate (with subscripts and locants as appropriate); PDGF, platelet-derived growth factor; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PIP_2 , phosphatidylinositol 4,5-bisphosphate; TPA, 12-O-tetradecanoylphorbol 13-acetate.

ligands, measured as the average of populations consisting of many thousands of cells, might be explained by heterogeneity of the cell population; e.g., different cells may respond preferentially to the different ligands, the size or synchrony of the responses may differ between cells, and, superimposed on these, there may be variable sensitivity of different cells to agents such as the phorbol esters. Recently, digital fluorescence image analysis microscopy using the Ca^{2+} -sensitive fluorophore fura-2 has made possible the quantitative analysis of $[\text{Ca}^{2+}]_i$ in single cells (Poenie *et al.*, 1986; Ambler *et al.*, 1988; Gonzalez *et al.*, 1988; Millard *et al.*, 1988; Monck *et al.*, 1988; O'Sullivan *et al.*, 1989). To clarify the cellular basis of the differences outlined above, we have examined the $[\text{Ca}^{2+}]_i$ responses of individual Swiss 3T3 cells to bombesin, vasopressin, $\text{PGF}_{2\alpha}$, and PDGF. We also report that the down-modulation of protein kinase C prolongs the $[\text{Ca}^{2+}]_i$ responses to bombesin, vasopressin, and $\text{PGF}_{2\alpha}$.

Results

$[\text{Ca}^{2+}]_i$ responses to bombesin

The mean basal $[\text{Ca}^{2+}]_i$ of control Swiss 3T3 cells was 75 ± 1 nM (mean $\pm$ SE; 371 cells from 35 fields). More than 75% of the cells gave values between 60 and 90 nM, and <5% gave values <50 nM or >100 nM. Control experiments showed that, in the absence of experimental additions, the basal $[\text{Ca}^{2+}]_i$ remained unchanged over the period of the experiments described. Within individual cells, $[\text{Ca}^{2+}]_i$ was not uniformly distributed and was often lower in a central region (see Figure 4, basal); this corresponded approximately to the nuclear region, as identified by staining with ethidium bromide after image analysis (T. R. Cheek and A. N. Corps, unpublished observations). Comparable data have been obtained using NIH-3T3 cells (Benjamin *et al.*, 1988; Chandra *et al.*, 1989). In addition, Chandra *et al.* (1989) showed regions of lower $[\text{Ca}^{2+}]_i$ in the cell periphery, which may correspond to the low $[\text{Ca}^{2+}]_i$ that is indicated by the pink color present at the edges of the cells in Figure 4.

The $[\text{Ca}^{2+}]_i$ responses of individual Swiss 3T3 cells to bombesin are summarized in Figure 1 (see also Table 1). Very few cells responded to 10 or 30 pM bombesin, concentrations which give little or no stimulation of DNA synthesis, and those responses which were observed did not generally raise the $[\text{Ca}^{2+}]_i$ above the range recorded in untreated cells. About 65% of the cells responded to 100 pM bombesin, and >80% responded to higher concentrations. At all concentrations the responses were fast and transient (Figure 1a), but

the analysis revealed that there was significant variation between cells in the timing (Figure 1d–e) and magnitude (Figure 1f) of the responses. The first changes were detected after a short lag period that ranged from 10 ± 1 s at 100 pM bombesin to only 2 ± 1 s at 100 nM bombesin, and peak $[\text{Ca}^{2+}]_i$ was reached after 26 ± 2 and 10 ± 1 s, respectively, at these two concentrations. Previous single-cell studies using other cell types have shown a similar relationship between ligand concentration and the latency of the response (Millard *et al.*, 1988; Monck *et al.*, 1988). After the peak $[\text{Ca}^{2+}]_i$ was achieved, there was a rapid decline in $[\text{Ca}^{2+}]_i$ such that within 3 min of continuous application of bombesin, $[\text{Ca}^{2+}]_i$ was similar to or only slightly above basal (see also below).

Three features of the responses in individual cells merit further comment. First, a small number (<10%) of the cells treated with 100 pM bombesin exhibited additional transient elevations of $[\text{Ca}^{2+}]_i$, producing an oscillatory pattern (Figure 1a) similar to that observed in other cell types (see Berridge and Galione [1988] and Rink and Jacob [1989] for reviews). No oscillations were observed at higher doses of bombesin. Second, the values recorded for individual pixels suggested that localized $[\text{Ca}^{2+}]_i$ increases to over 400 nM occurred within those cells that showed the largest responses (data not shown). The highest $[\text{Ca}^{2+}]_i$ values frequently appeared to be located in the central region around the nucleus that had shown lower basal $[\text{Ca}^{2+}]_i$ (see Figure 4) as reported for NIH-3T3 cells responding to PDGF (Benjamin *et al.*, 1988). Third, some experiments were performed on cells that had been switched to 0.2% (v/v) serum to arrest their growth at a low density, thus reducing the possibility of intercellular contacts. The basal $[\text{Ca}^{2+}]_i$ in these sparse cells and the time-course and magnitude of the $[\text{Ca}^{2+}]_i$ changes induced by bombesin were not markedly different from those described above for confluent cell cultures (data not shown). The early $[\text{Ca}^{2+}]_i$ responses to bombesin therefore appear to be independent responses of individual cells. A similar conclusion also applies to the responses to PDGF.

$[\text{Ca}^{2+}]_i$ responses to vasopressin, $\text{PGF}_{2\alpha}$, and PDGF

At concentrations of vasopressin and $\text{PGF}_{2\alpha}$ which are optimal for the stimulation of DNA synthesis or accumulation of inositol phosphates, >80% of the cells exhibited rapid, transient rises in $[\text{Ca}^{2+}]_i$ that were similar to those induced by bombesin (Figures 2 and 3; note the extended time-scales of Figure 3 compared with Figure 1).

Figure 1. Bombesin-induced [Ca²⁺]_i responses in individual Swiss 3T3 cells. Cells were loaded with fura-2 and incubated as described in Materials and Methods, and bombesin was added at the concentrations indicated for 3 min as shown by the bar. (a–c) Photodiode recordings from typical cells; in (b) 2.5 mM EGTA (E) was present in the perfusion medium starting from 2 min before the addition of bombesin; in (c) 20 nM TPA (T) was present in the perfusion medium, starting from 6 min before the addition of bombesin. (d–f) Bombesin concentration dependence of the following parameters: (d) lag time before the observed rise in [Ca²⁺]_i; (e) time at which peak [Ca²⁺]_i was attained; and (f) peak [Ca²⁺]_i. Each dot represents a separate cell, and the results show all of the responsive cells in 3–7 experiments at each concentration of bombesin.

After lag periods of 2 ± 1 s and 4 ± 1 s for 1 μ M vasopressin and 1 μ M PGF_{2 α} , respectively, peak [Ca²⁺]_i values were reached after 12 ± 1 and 13 ± 1 s, respectively (Figure 3). The range of peak [Ca²⁺]_i occurring in different cells in response to vasopressin and PGF_{2 α} showed substantial overlap with that to bombesin (compare Figure 3c with Figure 1f); however, the mean peak value of the response to PGF_{2 α} (145 nM) was lower than that to vasopressin (175 nM), which was lower than

the response to an optimal concentration of bombesin (220 nM). As observed for the bombesin-induced responses, the responses to vasopressin and PGF_{2 α} declined rapidly from the peak [Ca²⁺]_i (Figure 2).

PDGF is the most effective single mitogen for Swiss 3T3 cells (Rozengurt, 1986), and the recombinant PDGF (B-B-chain homodimer) used in this study gives half-maximal and maximal stimulation of DNA synthesis at ~ 2 and 10 ng/ml,

Table 1. $[Ca^{2+}]_i$ responses to bombesin and PDGF: effects of TPA and EGTA*

Ligand	Condition	No. of experiments	% of cells responding	Lag time (s)		Time to peak (s)		Peak $[Ca^{2+}]_i$ (nM)		
				Range	Mean	Range	Mean	Range	Mean	% Basal
Bombesin (100 pM)	Control	4	66	5–16	10	10–44	26	76–311	153	210
	TPA†	4	22	6–14	8	18–36	26	92–147	108	140
Bombesin (10 nM)	Control	9	82	1–6	3	7–26	13	99–347	240	328
	EGTA‡	3	78	2–6	4	8–16	11	140–329	220	332
PDGF (10 ng/ml)	Control	11	60	8–100	40	17–145	80	84–176	115	157
	TPA†	3	55	25–70	42	42–104	79	84–142	105	148
PDGF (30 ng/ml)	Control	4	66	23–60	38	50–135	95	86–179	130	181
	EGTA‡	3	70	10–50	34	40–120	80	76–161	120	188

* Summary of data from a series of experiments similar to those shown in Figures 1–3.

† TPA (20 nM) was added from 6 min before the addition of mitogen.

‡ EGTA (2.5 mM, reducing extracellular $[Ca^{2+}]$ to $<0.1 \mu M$) was added from 2 min before the addition of mitogen.

respectively (Blakeley *et al.*, 1989). Sub-optimal doses of PDGF (1–3 ng/ml) induced an increase in $[Ca^{2+}]_i$ in 10–30% of Swiss 3T3 cells, while optimal concentrations (10–30 ng/ml) induced responses in 60–70% of the cells. In comparison with the $[Ca^{2+}]_i$ responses induced by bombesin, vasopressin, and $PGF_{2\alpha}$, those induced by PDGF were considerably slower and more variable (Figures 2 and 3 and Table 1). Very few PDGF-treated cells showed any increase in $[Ca^{2+}]_i$ before 10 s and, although a few cells reached peak $[Ca^{2+}]_i$ within 30 s, the peak was not generally attained

until between 1 and 2 min (Figure 3b). Indeed, in some cells the $[Ca^{2+}]_i$ response to PDGF appeared to be biphasic (Figure 2d), with a shoulder at ~45 s before a rise to a maximal level between 1 and 2 min. Whether the two phases represent different pools of Ca^{2+} and/or different triggering mechanisms remains to be established. The highest peak $[Ca^{2+}]_i$ values were ~100 nM in cells stimulated by 1 ng/ml PDGF (not shown) and ~180 nM in cells stimulated by 10–30 ng/ml PDGF (Figure 3c, Table 1, and data not shown). As well as being slower in onset than the $[Ca^{2+}]_i$

Figure 2. $[Ca^{2+}]_i$ responses of individual Swiss 3T3 cells to vasopressin, $PGF_{2\alpha}$, and PDGF. Cells were loaded with fura-2 and incubated as described in Materials and Methods. Vasopressin (VP), $PGF_{2\alpha}$, and PDGF were added at the concentrations indicated, for 3 or 5 min as shown by the bar. The results show photodiode recordings from typical cells: the distributions of lag times, peak times, and peak $[Ca^{2+}]_i$ are shown in Figure 3. In the third recording in (c), 20 nM TPA (T) was present in the perfusion medium, starting from 6 min before the addition of PDGF; in the third recording in (d), 2.5 mM EGTA (E) was present in the perfusion medium, starting from 2 min before the addition of PDGF.

Figure 3. Characteristics of the $[Ca^{2+}]_i$ responses of individual Swiss 3T3 cells to vasopressin, $PGF_{2\alpha}$, and PDGF. Cells were loaded with fura-2 and incubated as described in Materials and Methods. Vasopressin (VP, 1 μ M), $PGF_{2\alpha}$ (1 μ M), and PDGF (10 ng/ml) were added for 3, 3, and 5 min, respectively (see Figure 2). Photodiode recordings were made of all the cells in the field, and the results show all of the responsive cells in 3–8 experiments with each mitogen, each dot representing a separate cell. (a) Lag times before the observed rise in $[Ca^{2+}]_i$; (b) time at which peak $[Ca^{2+}]_i$ was attained; (c) peak $[Ca^{2+}]_i$. Note the extension of the time-scales in (a) and (b) compared with those for bombesin-induced responses in Figure 1.

responses to the other mitogens, the responses to PDGF were markedly less transient, many cells maintaining near-maximal $[Ca^{2+}]_i$ for over 1 min (Figure 2).

Modulation of the $[Ca^{2+}]_i$ responses to bombesin and PDGF

When EGTA was added to the medium to reduce extracellular free $[Ca^{2+}]$, the basal $[Ca^{2+}]_i$ of Swiss 3T3 cells decreased slowly in most experiments, from an average of 76 ± 2 to 65 ± 2 nM over 2 min (51 cells from 6 fields). Nevertheless, the addition of bombesin resulted in increases in $[Ca^{2+}]_i$ that were of similar magnitude and timing to those obtained in medium containing the normal level of Ca^{2+} (Figure 1b and Table 1). The number of cells responding was also unaffected. Similarly, the $[Ca^{2+}]_i$ responses to 30 ng/ml PDGF were not affected by the inclusion of EGTA in the medium (Figure 2d and Table 1). However, with concentrations of PDGF up to 10 ng/ml, the responses obtained in the presence of EGTA were more variable, with fewer cells responding and/or diminished responses in some experiments. The

reason for this variability is not clear, although the smaller, slower responses to PDGF may be more susceptible to the declining basal $[Ca^{2+}]_i$ caused by EGTA. Whatever the reason, the variability may explain some of the discrepancy between previous studies of the extracellular $[Ca^{2+}]$ dependence of PDGF-induced calcium responses: Hesketh *et al.* (1988) found no dependence of the rise in $[Ca^{2+}]_i$ on extracellular $[Ca^{2+}]$ whereas Gonzalez *et al.* (1988) found a partial dependence, and we have previously noted that EGTA gave a substantial inhibition of PDGF-induced $^{45}Ca^{2+}$ release (Blakeley *et al.*, 1989).

Treatment of Swiss 3T3 cells with TPA (20 nM for 6 min) did not affect basal $[Ca^{2+}]_i$, which was constant at 73 ± 2 nM (50 cells from 6 fields). However, TPA reduced the $[Ca^{2+}]_i$ response to bombesin, particularly when low concentrations of the peptide were used (Figure 1c and Table 1). Thus, only 20–25% of the cells responded to 100 pM bombesin in the presence of TPA, and these responses were mostly <50% of those in control cells (Table 1). At 1 nM bombesin 72% of the cells responded in the presence of TPA, but the

responses obtained were reduced in amplitude compared with those in control cells. However, at 10 nM bombesin the peak [Ca²⁺]_i values and number of cells responding in the presence of TPA were not significantly different from those in control cells (Figure 1c and data not shown). TPA did not affect the time-course of the [Ca²⁺]_i responses to any concentration of bombesin (Table 1 and results not shown). In contrast to the inhibitory effect of TPA on bombesin-induced [Ca²⁺]_i responses, TPA did not alter any of the parameters of the responses to PDGF (3–30 ng/ml), despite the fact that the magnitude of these responses was similar to that of the TPA-sensitive responses induced by low doses of bombesin (Figure 2c, Table 1, and results not shown).

[Ca²⁺]_i responses in protein kinase C-depleted cells

The long-term pretreatment of Swiss 3T3 cells with phorbol esters leads to a down-modulation of protein kinase C (for references see Materials and Methods). The hydrolysis of PIP₂ in response to bombesin, vasopressin, and PGF_{2α} (but not PDGF) is potentiated in these protein kinase C-depleted cells (Blakeley *et al.* [1989] and Table 2). The results presented in Figures 4 and 5 show that the [Ca²⁺]_i responses to bombesin, vasopressin, and PGF_{2α} are also potentiated after the depletion of protein kinase C.

The principal feature of this potentiation is a maintenance of the [Ca²⁺]_i signal above basal for a prolonged period compared with control cells; e.g., when protein kinase C-depleted cells had been incubated with 1 nM bombesin for 150 s the average [Ca²⁺]_i had decreased only to a level similar to that reached in control cells by 60 s (Figures 4, a and b, and 5a). This effect was even more pronounced in vasopressin- and PGF_{2α}-stimulated cells, with [Ca²⁺]_i in TPA-pretreated cells remaining significantly higher at 150 s than that in control cells at 60 s (Figures 4, c and d and 5, b and c). Taken together, the elevation of [Ca²⁺]_i above basal remaining after 150 s incubation of protein kinase C-depleted cells with each mitogen represented 35–40% of the peak response, compared with only 10–15% in control cells. Thus, the time for the decay of the [Ca²⁺]_i transient was increased by at least two- to three-fold in the protein kinase C-depleted cells.

Table 2. Mitogen-stimulated accumulation of inositol phosphates in fura-2-loaded Swiss 3T3 cells*

	[³ H]-inositol phosphates (cpm/well)†	
	Control cells	TPA-pretreated cells
Control	261 ± 55	321 ± 60
Bombesin (1 nM)	833 ± 136	1724 ± 386
Vasopressin (1 μM)	538 ± 41	986 ± 20
PGF _{2α} (1 μM)	327 ± 25	777 ± 73
PDGF (100 ng/ml)	538 ± 41	675 ± 114

* Stimulation by bombesin in non-fura-2-loaded cells in the same experiment was not significantly different from that shown, and similar results have been obtained in other experiments with loaded and nonloaded cells.

† Values shown are means ± SE of 3 determinations from separate dishes of cells stimulated for 5 min at 37°C with the indicated concentrations of mitogens. The labeling of the cells with myo-[2-³H]-inositol and the extraction and measurement of total [³H]-inositol phosphates were performed as described previously (Blakeley *et al.*, 1989).

In addition to the prolonged [Ca²⁺]_i responses to the three mitogens, the 48-h pretreatment with TPA also resulted in a significantly increased peak [Ca²⁺]_i response to PGF_{2α} (Figure 5c; 180 ± 5 vs. 144 ± 6 nM for TPA-pretreated vs. control cells; *p* < 0.001). However, the peak [Ca²⁺]_i values obtained in response to bombesin and vasopressin were not significantly different in the protein kinase C-depleted cells compared to the control cells. The 48-h pretreatment with TPA also had no effect on the basal [Ca²⁺]_i, the lag times of the responses to any of the mitogens, or the times at which peak [Ca²⁺]_i were obtained.

In contrast to the potentiated responses to the other three mitogens, the responses to PDGF showed no apparent changes after the depletion of protein kinase C (data not shown). Interestingly, however, prolonged [Ca²⁺]_i responses after protein kinase C depletion have also been noted in A431 cells stimulated by EGF (Pandiella *et al.*, 1987) and a T cell clone stimulated by Concanavalin A (Valge *et al.*, 1988).

Discussion

Single cell analysis has allowed the determination of the extent to which the population [Ca²⁺]_i response (i.e., the averaged response of several

Figure 4. [Ca²⁺]_i responses of control Swiss 3T3 cells and protein kinase C-depleted cells to bombesin and PGF_{2α}. Cells were pretreated with 300 nM TPA to down-modulate protein kinase C (b and d), loaded with fura-2 and incubated as described in Materials and Methods; (a and b) 1 nM bombesin and (c and d) 1 μM PGF_{2α} were added for 3 min. The false-color images show [Ca²⁺]_i in each field (a–d) at 4 times: basal, 15 s before mitogen addition; max, between 15 and 25 s after mitogen addition, such that most cells were at or near their peak [Ca²⁺]_i; and 60 s and 150 s after mitogen addition.

Figure 5. $[Ca^{2+}]_i$ responses of individual control Swiss 3T3 cells (left) and protein kinase C-depleted cells (right) to bombesin, vasopressin, and $PGF_{2\alpha}$. Cells were treated with 300 nM TPA for 48 h to down-modulate protein kinase C, loaded with fura-2, and incubated as described in Materials and Methods. (a) 1 nM bombesin, (b) 1 μ M vasopressin, and (c) 1 μ M $PGF_{2\alpha}$ were added for 3 min as indicated by the bar.

thousands of cells in a fluorimeter cuvette) reflects the behavior of individual cells. The data presented show that the $[Ca^{2+}]_i$ responses measured previously using populations of Swiss 3T3 cells do indeed reflect events occurring in the majority of cells within the population. However, they also reveal that there is considerable variation between cells in both the magnitude and timing of the responses. Our experimental system, in which ligands are delivered locally in <1 s and the video image is updated every 200 ms, allows us to detect the extremely rapid onset of the $[Ca^{2+}]_i$ rise in response to bombesin, vasopressin, and $PGF_{2\alpha}$: in several cells, this appeared to occur within 1 s. We have also observed that a small number of cells showed further $[Ca^{2+}]_i$ transients in response to low concentrations of bombesin, and that PDGF frequently induced a biphasic response. In addition, the fluorescence imaging technique has revealed that $[Ca^{2+}]_i$ in resting and stimulated Swiss 3T3 cells is not homogeneous. The lower basal and higher stimulated values of $[Ca^{2+}]_i$ in the nuclear region of the cell may indicate the presence of a calcium storage organelle close to the nucleus, as suggested for NIH-3T3 cells (Chandra *et al.*, 1989).

Comparison of the $[Ca^{2+}]_i$ responses to bombesin and PDGF

Several previous studies have shown that the inositol phosphate and calcium responses of populations of Swiss 3T3 cells to PDGF are slower than those to bombesin and other mitogens and insensitive to inhibition by phorbol esters such as TPA (Sturani *et al.*, 1986; Ives and Daniel, 1987; Lopez-Rivas *et al.*, 1987; Hasegawa-Sasaki *et al.*, 1988; Hesketh *et al.*, 1988; Blakeley *et al.*, 1989). Taken together, our results indicate that these differences cannot be attributed to different subpopulations of cells that are differentially responsive to the different mitogens and/or differentially sensitive to TPA. First, >80% of the Swiss 3T3 cells in our study responded to bombesin (or vasopressin or $PGF_{2\alpha}$) and $\leq 70\%$ to PDGF. Similarly, Hesketh *et al.* (1988) found that the majority of Swiss 3T3 cells responded to all ligands tested. Second, the responses to PDGF in individual cells are substantially slower and more prolonged than those induced by the other mitogens and show

The tracings show photodiode recordings from typical cells. The distribution diagrams below the tracings show $[Ca^{2+}]_i$ in all of the responsive cells in 3 experiments with each mitogen: basal, 15 s before mitogen addition; max, peak response (15–25 s) after mitogen addition; 60 s after mitogen addition; and 150 s after mitogen addition.

a heterogeneity of delay similar to that noted in a recent single-cell study of homologous and heterologous desensitization of PDGF-, ATP-, and bradykinin-induced [Ca²⁺]_i responses (Gonzalez *et al.*, 1988; see also Benjamin *et al.*, 1988). Third, the inhibitory action of phorbol esters on bombesin-stimulated increases in [Ca²⁺]_i apparently represents a reduction in the response in each cell, the fraction of cells responding only being reduced at low concentrations of bombesin where the proportional inhibition by TPA was greatest. In contrast, the [Ca²⁺]_i responses to PDGF were not affected by TPA even though the PDGF-induced responses were of similar amplitude to the TPA-susceptible responses to low doses of bombesin. The results therefore show that the differences between bombesin- and PDGF-induced responses occur within individual cells. It is possible that these differences are related to the presence or absence of receptor protein-tyrosine kinase activity, since the phosphorylation of phospholipase C- γ (C-II) by the PDGF and EGF receptor tyrosine kinases has recently been reported (Margolis *et al.*, 1989; Meisenhelder *et al.*, 1989); in contrast, tyrosine kinase activity has not reproducibly been found associated with the bombesin receptor (Isacke *et al.* [1986], but see also Cirillo *et al.* [1986]).

Potentiation of [Ca²⁺]_i responses after down-modulation of protein kinase C

The increase in [Ca²⁺]_i stimulated by bombesin, vasopressin, or PGF_{2 α} is markedly prolonged in cells pretreated with TPA to down-modulate protein kinase C, whereas that stimulated by PDGF remains unchanged. Combined with the respective sensitivities of the [Ca²⁺]_i responses to inhibition by TPA, this suggests that protein kinase C might mediate a feedback inhibition of the [Ca²⁺]_i responses to the former group of mitogens, as previously proposed for their inositol phosphate responses (Brown *et al.*, 1987; Hepler *et al.*, 1988; Blakeley *et al.*, 1989).

A feedback action of protein kinase C that limits the hydrolysis of PIP₂ to 1,4,5-IP₃ is consistent with the increase in the total inositol phosphates stimulated in the protein kinase C-depleted cells and may also be consistent with the increased maximal [Ca²⁺]_i responses to PGF_{2 α} . However, additional feedback effects may be required to explain why the [Ca²⁺]_i responses to the three mitogens are prolonged in the protein kinase C-depleted cells. These additional effects could also be mediated through the inositol phosphates: e.g., protein kinase C might promote the conversion of the active 1,4,5-IP₃ to 1,3,4,5-IP₄ (Imboden

and Pattison, 1987; Biden *et al.*, 1988) or IP₂ (Connolly *et al.*, 1986), such that the depletion of protein kinase C would allow a longer lifetime of the active isomer. Consistent with this, it has recently been shown that the depletion of protein kinase C leads to higher and more persistent 1,4,5-IP₃ levels in stimulated neuroblastoma cells (Fu *et al.*, 1988). Alternatively, although we find no effect of acute treatment with TPA on basal [Ca²⁺]_i or ⁴⁵Ca²⁺ efflux, protein kinase C might promote Ca²⁺ efflux from the raised [Ca²⁺]_i in stimulated cells: the depletion of protein kinase C might then permit a prolonged elevation of [Ca²⁺]_i independently of effects on inositol phosphates. However, the finding that PDGF, which activates protein kinase C (Rozengurt *et al.*, 1983; Hesketh *et al.*, 1988), does not induce a prolonged [Ca²⁺]_i response in the protein kinase C-depleted cells argues against this possibility.

When cells are depleted of protein kinase C by pretreatment with TPA, the inositol phosphate and/or [Ca²⁺]_i responses to vasopressin and, in particular PGF_{2 α} , are potentiated more than the responses to bombesin (Blakeley *et al.*, 1989; Table 2; Figure 5). It therefore seems likely that the responses to vasopressin and PGF_{2 α} are more markedly affected by feedback inhibition through protein kinase C than are those to bombesin, e.g., because the receptors, G proteins and/or phospholipase C molecules involved have different sensitivities to protein kinase C. Neither the affinity of the bombesin receptor for bombesin, nor the number of bombesin receptors per cell, is affected by the pretreatment with TPA (K. D. Brown and C. J. Littlewood, unpublished results). However, we cannot exclude the possibility that part of the greater potentiation of the responses to vasopressin and PGF_{2 α} in the TPA-pretreated cells might be due to an increase in receptor number or affinity.

The stimulation of cell growth by mitogens can be mediated by multiple signaling pathways (Rozengurt, 1986), and regulatory cross-talk between the different pathways has been demonstrated in several studies. Thus, PIP₂ hydrolysis and calcium responses are inhibited via both protein kinase C-dependent and -independent pathways (Hesketh *et al.*, 1988; Gonzalez *et al.*, 1988). Furthermore, PIP₂ hydrolysis can be potentiated by growth factors which activate tyrosine kinases (Heslop *et al.*, 1986; Paris *et al.*, 1988), and protein kinase C can potentiate the stimulated accumulation of cyclic AMP (Rozengurt *et al.*, 1987). The depletion (or, indeed, inhibition) of protein kinase C may therefore have indirect effects that could complicate the analysis of experiments designed to investigate the causal involvement of the kinase

in cellular responses; e.g., the enhanced $[Ca^{2+}]_i$ signal demonstrated here may be able to compensate for the loss of protein kinase C activation.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), newborn-calf serum, antibiotics and trypsin for cell culture were obtained from Flow Laboratories. Nunc tissue culture dishes were purchased from GIBCO. Synthetic bombesin and vasopressin were from Bachem U.K. and Cambridge Research Biochemicals, respectively, and recombinant PDGF_{C- α} (B-B-chain homodimer) and *myo*-[2- 3 H]-inositol were from Amersham International. Phorbol esters and PGF_{2 α} were purchased from Sigma Chemical Co., and fura-2 was from Molecular Probes.

Cell culture

Stock cultures of Swiss 3T3 cells were maintained and passaged as described previously (Brown and Blakeley, 1983). For experimental use, cells were seeded into 35-mm dishes containing 22-mm-diameter glass cover slips. They were then incubated for 6–8 d at 37°C in a humidified atmosphere of CO₂/air (1:9), by which time they were confluent and quiescent at $\sim 2 \times 10^5$ cells/dish. Typically, such cultures gave between 8 and 12 cells per field when examined under the conditions routinely used for microscopy. In some experiments, sparse cultures were obtained by reducing the concentration of newborn calf serum from 10% (v/v) to 0.2% (v/v) 2 d after seeding; these cultures contained $\sim 5 \times 10^4$ cells/dish and gave 2–4 cells per microscopic field. Protein kinase C was down-modulated by adding 300 nM TPA to the culture medium for the final 48 h before use. This treatment, which substantially reduces the level of protein kinase C assayed by various methods (Rodriguez-Pena and Rozengurt, 1984; Blackshear *et al.*, 1985; Brown *et al.*, 1987; Adams and Gullick, 1989), did not significantly affect the number of cells per field.

Fura-2 fluorescence microscopy and image analysis

Cells were loaded with fura-2 and their $[Ca^{2+}]_i$ responses were analyzed in a Hepes-buffered salts solution (HBS) with minimal changes in cation composition, particularly Ca^{2+} , from those in DMEM. The solution contained (in mM): 150 NaCl, 5 KCl, 1 MgSO₄, 1.8 CaCl₂, 10 glucose, 10 HEPES pH 7.35. The cells were rinsed with HBS and then incubated with 2 ml HBS containing 1 μ M fura-2 acetoxymethylester for 45 min at 37°C, before rinsing again and transferring the cover slip into a 2 ml aluminium alloy chamber which was mounted in the heated stage of a Nikon Diaphot inverted epi-fluorescence microscope. The volume of solution in the chamber was maintained by balancing input from a reservoir and withdrawal through a suction line. Pre-addition of medium containing EGTA or TPA was made from the reservoir. Mitogens were added through a separate U-tube positioned within 1 mm of the field of view, such that within 1 s the cells in the field were exposed to the added mitogen at the required concentration; in practice, no significant differences were observed between the time-courses of responses of cells in different parts of the field, and very similar responses were obtained if the contents of the chamber were withdrawn by pipette and immediately replaced with medium containing mitogen.

The excitation, recording, and analysis of fura-2 fluorescence, using an Imagine image-processor (Synoptics Ltd, Cambridge, U.K.) has been described in detail previously (O'Sullivan *et al.*, 1989). In brief, fura-2 fluorescence was ex-

cited alternately at 340 and 380 nm (10 nm bandwidth), and emitted light was collected by a single-stage intensified CCD camera (Photonic Science, Tunbridge Wells, U.K.). The excitation source was switched in synchrony with the video time-base to give alternate TV frames at each of the two wavelengths; a "live" ratio image was formed every 200 ms from each successive pair of frames and stored on video tape (Sony Umatic) for subsequent processing. Formation of the ratio image was implemented in a look-up table which was computed from the formula and fura-2/ Ca^{2+} dissociation constant given by Grynkiewicz *et al.* (1985), calibrated under standard operating conditions using solutions of CaCl₂/10 mM EGTA with 5 μ M fura-2 as the penta-sodium salt. Output fluorescence intensities were proportional to $\log [Ca^{2+}]$ over the range 10 nM–1 μ M. Recorded video data were played back through Imagine, using a different program to give a false color representation of image intensities and to allow individual pictures to be captured on disc for calibration. Continuous traces of fluorescence intensity from individual cells were obtained by attaching photodiodes, each mounted behind a collecting lens of ~ 30 mm focal length, to the screen of a monochrome TV monitor. The response of the TV monitor/photodiode system was checked by using the computer to generate cell-sized spots of known digital intensity and was found to be linear, within 10%, over the full range used.

The data presented in the Figures show the values obtained for all of the responding cells in the microscopic fields of at least three coverslips for each experimental condition. The values quoted in the text are the mean $\pm$ SE for such data and generally refer to between 25 and 40 cells for each experimental condition.

Characteristics of fura-2-loaded Swiss 3T3 cells

When Swiss 3T3 cells were loaded as described above, essentially all of the cells retained hydrolyzed fura-2. The level of loading, assessed by the fluorescence brightness at a single excitation wavelength, varied somewhat between cells but this did not appear to affect the speed or magnitude of the $[Ca^{2+}]_i$ responses observed using the ratio image from alternate-wavelength excitation. Loading with fura-2 apparently caused no general disruption of cellular metabolism and signalling pathways; e.g., loaded cells showed no significant differences from untreated cells in either the magnitude or dose-response characteristics of both short-term (inhibition of ^{125}I -EGF binding) or long-term (3H -thymidine incorporation) responses to the mitogens (data not shown). Furthermore, loading with fura-2 did not prevent the mitogen-stimulated accumulation of inositol phosphates or its potentiation in protein kinase C-depleted cells (see Table 2).

Acknowledgments

We thank Diane Blakeley and Chris Littlewood for cell culture and for the inositol phosphate data, and Sue Porter for assistance with the preparation of the manuscript.

Received: July 28, 1989.

References

- Adams, J.C., and Gullick, W.J. (1989). Differences in phorbol-ester-induced down-regulation of protein kinase C between cell lines. *Biochem. J.* 257, 905–911.
- Ambler, S.K., Poenie, M., Tsien, R.Y., and Taylor, P. (1988). Agonist-stimulated oscillations and cycling of intracellular free

- calcium in individual cultured muscle cells. *J. Biol. Chem.* **263**, 1952–1959.
- Benjamin, C.W., Connor, J.A., Tarpley, W.G., and Gorman, R.R. (1988). NIH-3T3 cells transformed by the EJ-ras oncogene exhibit reduced platelet-derived growth factor-mediated Ca²⁺ mobilization. *Proc. Natl. Acad. Sci. USA* **85**, 4345–4349.
- Berridge, M.J., and Galione, A. (1988). Cytosolic calcium oscillators. *FASEB J.* **2**, 3074–3082.
- Berridge, M.J., Heslop, J.P., Irvine, R.F., and Brown, K.D. (1984). Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. *Biochem. J.* **222**, 195–201.
- Biden, T.J., Altin, J.G., Karjalainen, A., and Bygrave, F.L. (1988). Stimulation of hepatic inositol 1,4,5-trisphosphate kinase activity by Ca²⁺-dependent and -independent mechanisms. *Biochem. J.* **256**, 697–701.
- Blackshear, P.J., Witters, L.A., Girard, P.R., Kuo, J.F., and Quamo, S.N. (1985). Growth factor-stimulated protein phosphorylation in 3T3-L1 cells. Evidence for protein kinase C-dependent and -independent pathways. *J. Biol. Chem.* **260**, 13304–13315.
- Blakeley, D.M., Corps, A.N., and Brown, K.D. (1989). Bombesin and platelet-derived growth factor stimulate formation of inositol phosphates and Ca²⁺ mobilization in Swiss 3T3 cells by different mechanisms. *Biochem. J.* **258**, 177–185.
- Brown, K.D., and Blakeley, D.M. (1983). Inhibition of the binding of ¹²⁵I-labelled epidermal growth factor to mouse cells by a mitogen in goat mammary secretions. *Biochem. J.* **212**, 465–472.
- Brown, K.D., Blakeley, D.M., Hamon, M.H., Laurie, M.S., and Corps, A.N. (1987). Protein kinase C-mediated negative-feedback inhibition of unstimulated and bombesin-stimulated polyphosphoinositide hydrolysis in Swiss-mouse 3T3 cells. *Biochem. J.* **245**, 631–639.
- Chandra, S., Gross, D., Ling, Y.-C., and Morrison, G.H. (1989). Quantitative imaging of free and total intracellular calcium in cultured cells. *Proc. Natl. Acad. Sci. USA* **86**, 1870–1874.
- Cirillo, D.M., Gaudino, G., Naldini, L., and Comoglio, P.M. (1986). Receptor for bombesin with associated tyrosine kinase activity. *Mol. Cell. Biol.* **6**, 4641–4649.
- Connolly, T.M., Lawing, W.J., Jr., and Majerus, P.W. (1986). Protein kinase C phosphorylates human platelet inositol trisphosphate 5'-phosphomonoesterase, increasing the phosphatase activity. *Cell* **46**, 951–958.
- Fu, T., Okano, Y., and Nozawa, Y. (1988). Bradykinin-induced generation of inositol 1,4,5-trisphosphate in fibroblasts and neuroblastoma cells: effect of pertussis toxin, extracellular calcium, and down-regulation of protein kinase C. *Biochem. Biophys. Res. Commun.* **157**, 1429–1435.
- Gonzalez, F.A., Heppel, L.A., Gross, D.J., Webb, W.W., and Parries, G. (1988). The rapid desensitization of receptors for platelet derived growth factor, bradykinin and ATP: studies on individual cells using quantitative digital video fluorescence microscopy. *Biochem. Biophys. Res. Commun.* **151**, 1205–1212.
- Gryniewicz, G., Poenie, M., and Tsien, R.Y. (1985). A new generation of Ca²⁺ indicators with greatly improved fluorescent properties. *J. Biol. Chem.* **260**, 3440–3450.
- Hasegawa-Sasaki, H., Lutz, F., and Sasaki, T. (1988). Pathway of phospholipase C activation initiated with platelet-derived growth factor is different from that initiated with vasopressin and bombesin. *J. Biol. Chem.* **263**, 12 970–12 976.
- Hepler, J.R., Earp, H.S., and Harden, T.K. (1988). Long-term phorbol ester treatment down-regulates protein kinase C and sensitizes the phosphoinositide signaling pathway to hormone and growth factor stimulation. *J. Biol. Chem.* **263**, 7610–7619.
- Hesketh, T.R., Moore, J.P., Morris, J.D.H., Taylor, M.V., Rogers, J., Smith, G.A., and Metcalfe, J.C. (1985). A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. *Nature* **313**, 481–484.
- Hesketh, T.R., Morris, J.D.H., Moore, J.P., and Metcalfe, J.C. (1988). Ca²⁺ and pH responses to sequential additions of mitogens in single 3T3 fibroblasts: correlations with DNA synthesis. *J. Biol. Chem.* **263**, 11 879–11 886.
- Heslop, J.P., Blakeley, D.M., Brown, K.D., Irvine, R.F., and Berridge, M.J. (1986). Effects of bombesin and insulin on inositol (1,4,5) trisphosphate and inositol (1,3,4) trisphosphate formation in Swiss 3T3 cells. *Cell* **47**, 703–709.
- Imboden, J.B., and Pattison, G. (1987). Regulation of inositol 1,4,5-trisphosphate kinase activity after stimulation of human T cell antigen receptor. *J. Clin. Invest.* **79**, 1538–1541.
- Isacke, C.M., Meisenhelder, J., Brown, K.D., Gould, K.L., Gould, S.J., and Hunter, T. (1986). Early phosphorylation events following the treatment of Swiss 3T3 cells with bombesin and the mammalian bombesin-related peptide, gastrin-releasing peptide. *EMBO J.* **5**, 2889–2898.
- Ives, H.E., and Daniel, T.O. (1987). Interrelationship between growth factor-induced pH changes and intracellular Ca²⁺. *Proc. Natl. Acad. Sci. USA* **84**, 1950–1954.
- Lopez-Rivas, A., Mendoza, S.A., Nanberg, E., Sinnott-Smith, J., and Rozengurt, E. (1987). Ca²⁺-mobilizing actions of platelet-derived growth factor differ from those of bombesin and vasopressin in Swiss 3T3 mouse cells. *Proc. Natl. Acad. Sci. USA* **84**, 5768–5772.
- Margolis, B., Rhee, S.G., Felder, S., Mervic, M., Lyall, R., Levitzki, A., Ullrich, A., Zilberstein, A., and Schlessinger, J. (1989). EGF induces tyrosine phosphorylation of phospholipase C-II: a potential mechanism for EGF receptor signaling. *Cell* **57**, 1101–1107.
- Meisenhelder, J., Suh, P.-G., Rhee, S.G., and Hunter, T. (1989). Phospholipase C- γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. *Cell* **57**, 1109–1122.
- Millard, P.J., Gross, D., Webb, W.W., and Fewtrell, C. (1988). Imaging asynchronous changes in intracellular Ca²⁺ in individual stimulated tumor mast cells. *Proc. Natl. Acad. Sci. USA* **85**, 1854–1858.
- Monck, J.R., Reynolds, E.E., Thomas, A.P., and Williamson, J.R. (1988). Novel kinetics of single cell Ca²⁺ transients in stimulated hepatocytes and A10 cells measured using Fura-2 and fluorescent videomicroscopy. *J. Biol. Chem.* **263**, 4569–4575.
- Morris, J.D.H., Metcalfe, J.C., Smith, G.A., Hesketh, T.R., and Taylor, M.V. (1984). Some mitogens cause rapid increase in free calcium in fibroblasts. *FEBS Lett.* **169**, 189–194.
- O'Sullivan, A.J., Cheek, T.R., Moreton, R.B., Berridge, M.J., and Burgoyne, R.D. (1989). Localization and heterogeneity of agonist-induced changes in cytosolic calcium concentration in single bovine adrenal chromaffin cells from video imaging of fura-2. *EMBO J.* **8**, 401–412.
- Pandiella, A., Vicentini, L.M., and Meldolesi, J. (1987). Protein kinase C-mediated feed back inhibition of the Ca²⁺ response at the EGF receptor. *Biochem. Biophys. Res. Commun.* **149**, 145–151.
- Paris, S., Chambard, J.-C., and Pouyssegur, J. (1988). Tyrosine kinase-activating growth factors potentiate thrombin- and AIF α -

- induced phosphoinositide breakdown in hamster fibroblasts. *J. Biol. Chem.* **263**, 12 893–12 900.
- Poenie, M., Alderton, J., Steinhardt, R.A., and Tsien, R.Y. (1986). Calcium rises abruptly and briefly throughout the cell at the onset of anaphase. *Science* **233**, 886–889.
- Rink, T.J., and Jacob, R. (1989). Calcium oscillations in non-excitable cells. *Trends Neurosci.* **12**, 43–46.
- Rodriguez-Pena, A., and Rozengurt, E. (1984). Disappearance of Ca^{2+} -sensitive, phospholipid-dependent protein kinase C activity in phorbol ester-treated 3T3 cells. *Biochem. Biophys. Res. Commun.* **120**, 1053–1059.
- Rozengurt, E. (1986). Early signals in the mitogenic response. *Science* **234**, 161–166.
- Rozengurt, E., Murray, M., Zachary, I., and Collins, M. (1987). Protein kinase C activation enhances cAMP accumulation in Swiss 3T3 cells: inhibition by pertussis toxin. *Proc. Natl. Acad. Sci. USA* **84**, 2282–2286.
- Rozengurt, E., Rodriguez-Pena, M., and Smith, K.A. (1983). Phorbol esters, phospholipase C and growth factors stimulate the phosphorylation of a M, 80 000 protein in intact quiescent 3T3 cells. *Proc. Natl. Acad. Sci. USA* **80**, 7244–7248.
- Sturani, E., Vincentini, L.M., Zippel, R., Toschi, L., Pandiella-Alonso, A., Comoglio, P.M., and Meldolesi, J. (1986). PDGF-induced receptor phosphorylation and phosphoinositide hydrolysis are unaffected by protein kinase C activation in mouse Swiss 3T3 and human skin fibroblasts. *Biochem. Biophys. Res. Commun.* **137**, 343–350.
- Valge, V.E., Wong, J.G.P., Datlof, B.M., Sinskey, A.J., and Rao, A. (1988). Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. *Cell* **55**, 101–112.