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# Temporal relationship between inositol polyphosphate formation and increases in cytosolic $Ca^{2+}$ in quiescent 3T3 cells stimulated by platelet-derived growth factor, bombesin and vasopressin

**Eewa Nånberg and Enrique Rozengurt**

Imperial Cancer Research Fund, PO Box 123, Lincoln's Inn Fields, London WC2A 3PX, UK

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**We determined the temporal relationship between the formation of inositol phosphates and increase in cytosolic $[Ca^{2+}]$ elicited by bombesin, vasopressin and platelet-derived growth factor (PDGF) in quiescent Swiss 3T3 cells. These responses were measured under identical conditions. Bombesin caused a rapid increase in inositol 1,4,5-trisphosphate which coincided with the increase in cytosolic $[Ca^{2+}]$. This was followed by a slower but marked increase in inositol 1,3,4-trisphosphate and inositol-bisphosphate. Vasopressin elicited a similar sequence of events. In sharp contrast, highly purified porcine PDGF induced increases in cytosolic $[Ca^{2+}]$ and inositol 1,4,5-trisphosphate that were temporally uncoupled: detectable inositol polyphosphate formation occurred after $Ca^{2+}$ mobilization from intracellular stores. The same temporal dissociation was observed when a recombinant v-*sis* product was used instead of porcine PDGF. However, PDGF was as effective as bombesin in stimulating the formation of inositol phosphates after 5–10 min of incubation. The data suggest that PDGF increases cytosolic $[Ca^{2+}]$ via a different signal transduction pathway from that utilized by bombesin and vasopressin. These findings have important implications for understanding the signal transduction pathway activated by PDGF.**

*Key words:* Swiss 3T3 cells/platelet-derived growth factor/bombesin/inositol phosphate/$Ca^{2+}$

## Introduction

Quiescent cultures of cells reversibly arrested in the $G_0$ phase of the cell cycle have provided a useful model system to identify the signal transduction pathways activated by growth factors and mitogenic peptides (Rozengurt, 1986). One of the earliest responses elicited by addition of the mitogens platelet-derived growth factor (PDGF) (Ross and Vogel, 1978), bombesin (Rozengurt and Sinnett-Smith, 1983) and vasopressin (Rozengurt *et al.*, 1979) to quiescent 3T3 cells is mobilization of $Ca^{2+}$ from intracellular stores (Lopez-Rivas and Rozengurt, 1983, 1984; Owen and Villereal, 1983; Moolenaar *et al.*, 1984; Hesketh *et al.*, 1985; McNeil *et al.*, 1985; Rozengurt and Mendoza, 1985; Mendoza *et al.*, 1986a,b). The addition of these mitogens causes a transient rise of cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_{cyt}$), increases the rate of $Ca^{2+}$ efflux from radiolabelled cells and markedly decreases total $Ca^{2+}$ content of the cell. Although these mitogens bind to different receptors in Swiss 3T3 cells (Rozengurt, 1986; Collins and Rozengurt, 1983; Zachary and Rozengurt, 1985, 1987a,b) the mobilization of intracellular $Ca^{2+}$ may be mediated by inositol 1,4,5-trisphosphate [Ins(1,4,5)$P_3$] which has been proposed as a second messenger in the action of many ligands that induce receptor-mediated inositol lipid turnover and $Ca^{2+}$ mobilization (Berridge and Irvine, 1984; Majerus *et al.*, 1986) including PDGF, bombesin and vasopressin (Berridge *et al.*, 1984; Hasegawa-Sasaki, 1985; Heslop *et al.*, 1986; Takuwa *et al.*, 1987).

Recently, we found that $Ca^{2+}$ signals generated in response to bombesin and vasopressin can be distinguished from those elicited by addition of PDGF by several criteria including the kinetics and magnitude of the increase in $[Ca^{2+}]_{cyt}$, the sensitivity of this response to phorbol esters and the ability of these ligands to increase the production of Ins(1,4,5)$P_3$ (Lopez-Rivas *et al.*, 1987). These results suggest that the signal transduction pathways activated by PDGF that lead to a transient increase in $[Ca^{2+}]_{cyt}$ differ from those utilized by bombesin and vasopressin. These findings assume an added importance in view of the putative function of bombesin-like peptides as autocrine growth factors for human small cell carcinoma (reviewed by Zachary *et al.*, 1987) and of the increasing evidence implicating PDGF in mediating normal and abnormal cell proliferation (Ross *et al.*, 1986).

A crucial step for establishing that Ins(1,4,5)$P_3$ acts as a second messenger in $Ca^{2+}$ mobilization is to demonstrate that an increase in Ins(1,4,5)$P_3$ synthesis precedes or coincides with the rise in $[Ca^{2+}]_{cyt}$. Thus, we determined the temporal relationship between the formation of inositol phosphates and the increase in $[Ca^{2+}]_{cyt}$ elicited by addition of bombesin or PDGF to quiescent Swiss 3T3 cells. The formation of Ins(1,4,5)$P_3$ and of Ins(1,3,4)$P_3$ was measured under identical conditions to those used to monitor the changes in $[Ca^{2+}]_{cyt}$. We found that bombesin elicited a rapid increase in Ins(1,4,5)$P_3$ which coincided with the increase in $[Ca^{2+}]_{cyt}$. Vasopressin evoked a similar sequence of responses. In sharp contrast, the increase in inositol phosphates induced by PDGF was temporally dissociated from the rise in $[Ca^{2+}]_{cyt}$ promoted by this factor: the $Ca^{2+}$ response preceded the formation of inositol phosphates. These findings have important implications for understanding the signal transduction pathways activated by PDGF.

## Results

### *Bombesin induces concomitant increases in $[Ca^{2+}]_{cyt}$ and Ins(1,4,5)$P_3$*

The experiments presented here were designed to compare the abilities of bombesin and highly purified PDGF to elicit formation of inositol phosphates and increases in $[Ca^{2+}]_{cyt}$ in quiescent Swiss 3T3 cells. To examine the kinetics and magnitude of these responses under strictly comparable conditions, 3T3 cells grown on Cytodex 2 microbeads were

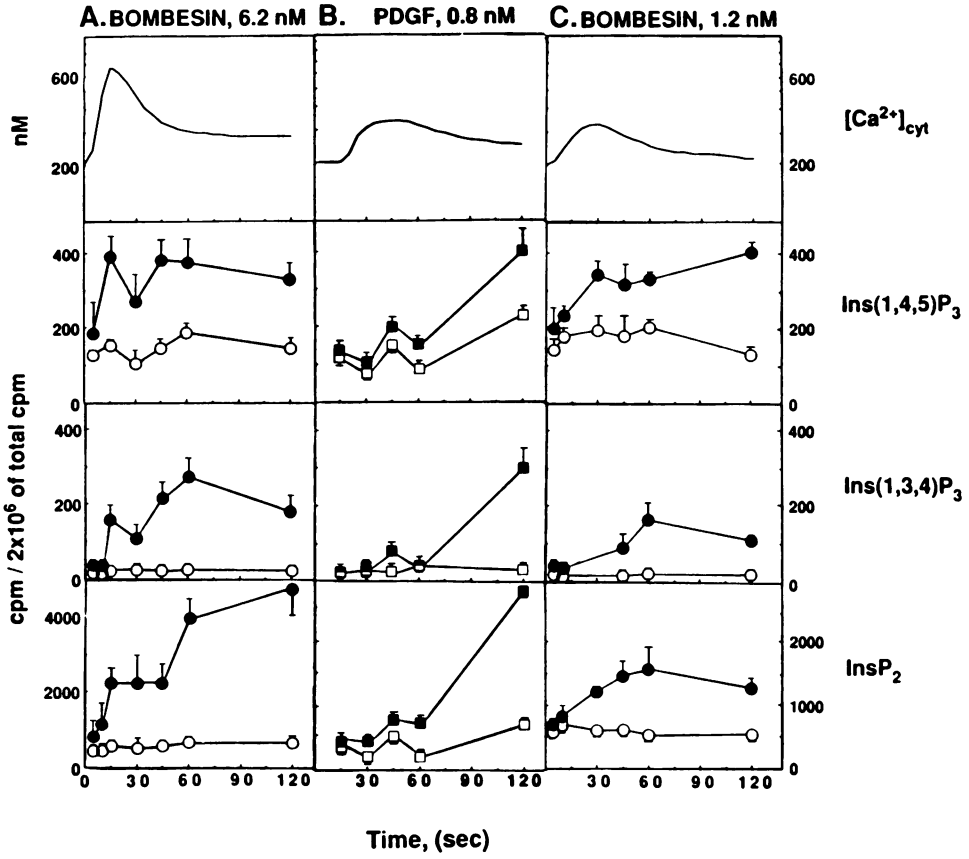


**Fig. 1.** Time-course of increased formation of inositol polyphosphates and $[Ca^{2+}]_{cyt}$ after addition of bombesin or highly purified PDGF to quiescent Swiss 3T3 cells. Swiss 3T3 fibroblasts were cultured on Cytodex 2 microbeads for 6–7 days to become quiescent. The cells were then loaded with myo-[$^3$H]inositol and fura 2/AME as described in Materials and methods. The basal $[Ca^{2+}]_{cyt}$ was recorded for 1 min before the cells received 6.2 nM bombesin (**A**, closed circles), 0.8 nM PDGF (**B**, closed squares), 1.2 nM bombesin (**C**, closed circles) or $H_2O$ (open symbols). The incubations were stopped 5–120 s later by addition of 0.5 vol ice-cold 30% trichloroacetic acid. The samples were analysed for their composition of inositol phosphates by anion-exchange chromatography on a Mono-Q column. The number of c.p.m. recovered in the different inositol phosphate fractions were calculated per $2 \times 10^6$ of total c.p.m. in each sample. The data represent means ± SEM from two to twelve independent experiments (one to three separate samples in each experiment). The SE bars that are not apparent fall within the size of the symbol. The upper part of the figure which describes the $[Ca^{2+}]_{cyt}$ levels was obtained from cells monitored in parallel with the inositol phosphate determinations.

labelled with myo-[$^3$H]inositol and also loaded with fura 2. Thus, the effect of the mitogens on the formation of inositol phosphates can be directly compared with the $Ca^{2+}$ signal monitored in the same culture.

Figure 1A shows that addition of bombesin at 6 nM, a concentration which is maximal for inducing mitogenesis (Rozengurt and Sinnett-Smith, 1983), increased $[Ca^{2+}]_{cyt}$ without any measurable delay. $[Ca^{2+}]_{cyt}$ reached peak values at 15 s and subsequently declined towards the basal level. Bombesin also provoked a sequential increase in the formation of inositol polyphosphates (Figure 1A). A rapid increase in Ins(1,4,5)$P_3$ which coincided with the elevation in $[Ca^{2+}]_{cyt}$ was followed by a marked and slower increase in Ins(1,3,4)$P_3$ and inositol-bisphosphate (Ins$P_2$). While the inositol-triphosphate (Ins$P_3$) and Ins$P_2$ pools reached an equilibrium after 1–2 min (Figure 1A), the inositol-monophosphate (InsP) fraction continued to accumulate over a period of 5–10 min (Figure 2). These results are consistent with reports using 3T3 cells attached to dishes and incubated in the absence of fura 2 (Heslop *et al.*, 1986; Takuwa *et al.*, 1987). The concomitant increase in Ins(1,4,5)$P_3$ and $[Ca^{2+}]_{cyt}$ shown in Figure 1 is consistent with the role of Ins(1,4,5)$P_3$ as a second messenger mediating the $Ca^{2+}$ mobilization induced by bombesin.

### *PDGF increases $[Ca^{2+}]_{cyt}$ prior to inositol polyphosphate formation*

In sharp contrast to the results obtained with bombesin, the increases in $[Ca^{2+}]_{cyt}$ and Ins(1,4,5)$P_3$ stimulated by PDGF were temporally uncoupled. Addition of highly purified porcine PDGF at 0.8 nM increased $[Ca^{2+}]_{cyt}$ after a lag of 15 s; peak $[Ca^{2+}]_{cyt}$ was observed after 35–45 s (Figure 1B). Dose–response studies revealed that the preparations of PDGF used in these studies promoted a maximal increase in DNA synthesis and induced a maximal increase in $[Ca^{2+}]_{cyt}$ when added at 0.8 nM (see also Figure 3). A salient feature of the results presented in Figure 1B is that PDGF failed to induce a significant increase in Ins(1,4,5)$P_3$, Ins(1,3,4)$P_3$ and Ins$P_2$ even 45 s after its addition. PDGF subsequently caused a marked increase in the formation of inositol phosphates (Figures 1B and 2). Indeed, PDGF was as effective as bombesin in stimulating InsP accumulation after 10 min of incubation (Figure 2).

Inspection of Figure 1 reveals that the increase in $[Ca^{2+}]_{cyt}$ caused by PDGF starts and peaks before rather than concomitantly with the increase in the formation of inositol phosphate. This finding was substantiated in many other experiments using different preparations of PDGF and cell cultures. For example, in 12 independent experiments

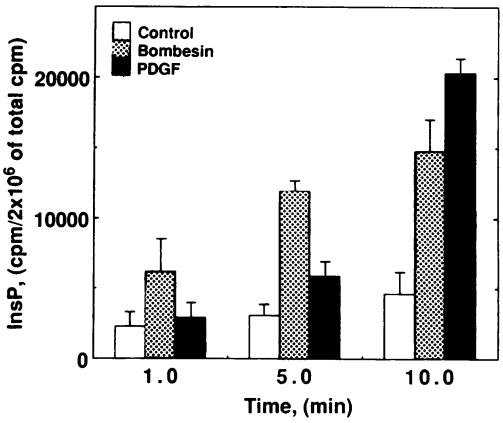


**Fig. 2.** Effect of highly purified porcine PDGF and bombesin on formation of InsP at various times in quiescent Swiss 3T3 cells. Quiescent cells grown on 33-mm culture dishes were loaded with myo-[$^3$H]inositol and pre-incubated as described in Materials and methods. At time zero the cells received vehicle (open bars), 6.2 nM bombesin (stippled bars) or 0.8 nM PDGF (closed bars). The incubations were stopped after 1 min, 5 min or 10 min. The water-soluble inositol phosphates were extracted and analysed on AG1-X8 (formate) columns as described in Materials and methods. The presented data show the number of c.p.m. recovered as inositol-monophosphate per 2 × $10^6$ of total c.p.m. in each sample (1.5 × $10^6$ cells). The data represent means ± SEM from three to six individual experiments.

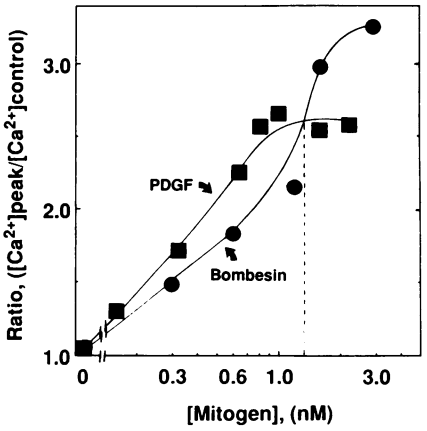


**Fig. 3.** Comparison between the dose-dependent effects of bombesin and highly purified porcine PDGF on $[Ca^{2+}]_{cyt}$ in Swiss 3T3 cells. Quiescent cells cultured on Cytodex 2 microbeads were loaded with fura 2/AME as described in Materials and methods. The cells were then treated with varying concentrations of bombesin (closed circles) or PDGF (closed squares) and the peak $[Ca^{2+}]_{cyt}$ was monitored. The $Ca^{2+}$ response was calculated as the ratio between peak $[Ca^{2+}]_{cyt}$ and $[Ca^{2+}]_{cyt}$ just prior to addition of the mitogen.

analysed at the peak of $[Ca^{2+}]_{cyt}$, $Ins(1,4,5)P_3$ rose from 187 ± 28 to 428 ± 68 c.p.m. in cells stimulated with bombesin. In contrast, the values of this second messenger were not significantly raised above the control levels in PDGF-treated cultures (143 ± 25 versus 195 ± 28 c.p.m.). Similar results were obtained under the following experimental conditions: (i) when the cells were labelled with myo-[$^3$H]inositol in an inositol-depleted medium thereby increasing 20-fold the specific activity of the labelled precursor; (ii) when the cultures were subjected to neutral extraction using ice-cold methanol containing 2.5 mM EDTA instead of 10% trichloroacetic acid; and (iii) when the concentration of PDGF was increased from 0.8 to 2.4 nM.

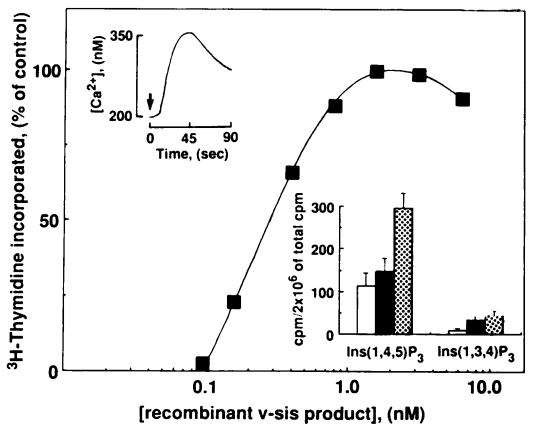


**Fig. 4.** Effect of a recombinant v-*sis* product on DNA synthesis, $[Ca^{2+}]_{cyt}$ and $InsP_3$ levels at peak $[Ca^{2+}]_{cyt}$ in quiescent Swiss 3T3 cells. Quiescent Swiss 3T3 cells on 33-mm culture dishes were incubated with [$^3$H]thymidine and varying concentrations of recombinant v-*sis* as described in Materials and methods. DNA was assessed as [$^3$H]thymidine incorporated in trichloroacetic-acid-insoluble material after 40 h. Values are expressed as a percentage of [$^3$H]thymidine incorporation obtained with 10% FBS (control). The data are means from three individual experiments. **Inset, left:** $[Ca^{2+}]_{cyt}$ response to 1.0 nM of recombinant v-*sis*. Quiescent cells cultured on Cytodex 2 microbeads were pre-incubated with fura 2/AME as described in Materials and methods. After recording the basal $[Ca^{2+}]_{cyt}$ for 1 min, 1.0 nM of recombinant v-*sis* was added at time zero and the $[Ca^{2+}]_{cyt}$ monitored for another 90 s. **Inset, right:** $Ins(1,4,5)P_3$ and $Ins(1,3,4)P_3$ levels at peak $[Ca^{2+}]_{cyt}$. Quiescent cells cultured on Cytodex 2 microbeads were loaded with myo-[$^3$H]inositol and fura 2/AME as described in Materials and methods. To the cells were added vehicle (open bars), 1.0 nM recombinant v-*sis* (filled bars) or 6.2 nM bombesin (stippled bars). The incubations were stopped by addition of trichloroacetic acid when the increase in $[Ca^{2+}]_{cyt}$ had reached peak levels, after 35 and 15 s for recombinant v-*sis* and bombesin respectively. The composition of the inositol phosphates was analysed on a Mono-Q column. The data represent means ± SEM from five individual experiments.

The elevation in $[Ca^{2+}]_{cyt}$ induced by bombesin differed from that induced by PDGF in both the value of the peak $[Ca^{2+}]_{cyt}$ and the rate at which this peak was reached (Figure 1A and B). In view of the above results, it was important to assess the effect of bombesin on inositol phosphates when added at a concentration that induces a $Ca^{2+}$ response comparable to that promoted by PDGF. A dose–response study with bombesin revealed that the neuropeptide at 1.2 nM promoted a transient increase in $[Ca^{2+}]_{cyt}$ equivalent to that induced by PDGF at 0.8 nM (Figure 3). Figure 1C shows that bombesin at this lower concentration induced smaller but coincident increases in $[Ca^{2+}]_{cyt}$ and $Ins(1,4,5)P_3$. Thus the unusual temporal pattern of inositol phosphate formation and increase in $[Ca^{2+}]_{cyt}$ seen with PDGF was not merely due to the magnitude and rate of the $Ca^{2+}$ response induced by this growth factor.

Finally, a similar pattern of responses was observed when a recombinant v-*sis* preparation was added instead of porcine PDGF. The recombinant v-*sis* was a potent mitogen for Swiss 3T3 cells, with a maximum effect obtained at 1 nM (Figure 4). The recombinant v-*sis* induced an increase in $[Ca^{2+}]_{cyt}$ after a lag phase of 14–17 s and reached a peak value after 32–35 s (Figure 4). Thus, the amplitude and kinetics of the $Ca^{2+}$ response due to recombinant v-*sis* were similar to those seen with 0.8 mM porcine PDGF (Figure 1B). When measured under identical conditions, as described above, the $Ins(1,4,5)P_3$ levels at peak $[Ca^{2+}]_{cyt}$ were not

significantly higher in cells treated with 1.0 nM recombinant v-*sis* than in untreated cells (147 ± 37 versus 113 ± 40 c.p.m.) (Figure 4). Again, an increase in the inositol phosphates was seen after 1 min.

### *PDGF and bombesin release $Ca^{2+}$ from intracellular store(s)*

A possible explanation of the findings shown in Figure 1 is that while bombesin triggers Ins(1,4,5)$P_3$ formation leading to $Ca^{2+}$ release from intracellular stores, PDGF stimulates an early $Ca^{2+}$ influx across the plasma membrane, which is not linked to increased Ins(1,4,5)$P_3$ production. This possibility was tested in two different ways. Firstly, the effect of bombesin and recombinant v-*sis* on $[Ca^{2+}]_{cyt}$ was determined in 3T3 cells incubated in the presence of varying concentrations of extracellular $Ca^{2+}$. Stimulation of an influx pathway should be severely depressed by reducing the concentration of $Ca^{2+}$ in the medium. Figure 5A shows that a reduction in the extracellular $Ca^{2+}$ from 1.8 mM to 0.2 μM did not prevent the increase in $[Ca^{2+}]_{cyt}$ caused by addition of either bombesin or recombinant v-*sis*. Secondly, the effect of these mitogens on $^{45}Ca^{2+}$ efflux from quiescent 3T3 cells previously labelled with $^{45}Ca^{2+}$ to equilibrium was measured in a buffer lacking $Ca^{2+}$ but containing 50 μM EGTA. In this manner, unidirectional $Ca^{2+}$ efflux can be distinguished from $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange across cellular membranes. Figure 5B shows that either bombesin or recombinant v-*sis* markedly increased the rate of $^{45}Ca^{2+}$ efflux in the $Ca^{2+}$-depleted medium. The effect was similar to that obtained in the presence of 1.8 mM Ca (not shown). Similar data were obtained when porcine PDGF was used instead of recombinant v-*sis*. Thus, the early $Ca^{2+}$ response evoked by both bombesin and PDGF is a release of $Ca^{2+}$ from intracellular store(s).

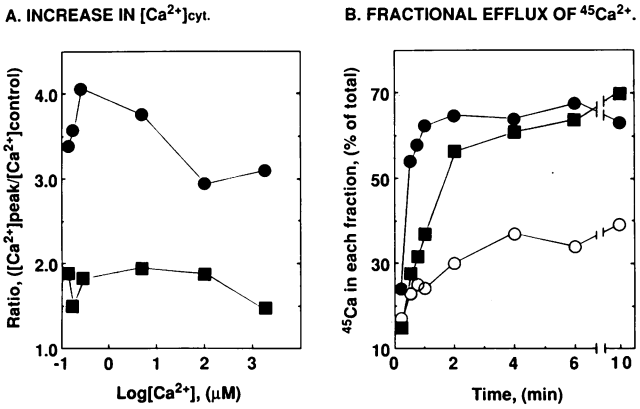


**Fig. 5.** Mobilization of intracellular $Ca^{2+}$ by bombesin and a recombinant v-*sis* product in Swiss 3T3 cells incubated in a low $Ca^{2+}$ medium. (**A**) Quiescent cells cultured on Cytodex 2 microbeads were pre-incubated and loaded with fura 2/AME as described in Materials and methods. Solution A was modified to contain 100 μM $Ca^{2+}$ and various amounts of EGTA to give concentrations of free $Ca^{2+}$ from 123 nM to 100 μM. Included in the figure also is the $Ca^{2+}$ response for cells incubated in normal solution A containing 1.8 mM $Ca^{2+}$. The increase in $[Ca^{2+}]_{cyt}$ after addition of 6.2 nM bombesin (closed circles) or 1.6 nM recombinant v-*sis* (closed squares) was expressed as the ratio between peak $[Ca^{2+}]_{cyt}$ in the presence of the mitogen and the $[Ca^{2+}]_{cyt}$ just prior to addition of the mitogen. The data represent means of duplicates from two individual experiments. (**B**) Quiescent Swiss 3T3 cells cultured on 33-mm Petri dishes were loaded with 8 μCi/ml of $^{45}Ca^{2+}$ for 12 h in conditioned media. The cells were then rapidly washed seven times with DMEM/3 mM EGTA and incubated for 10 min in 2 ml of solution A, modified to contain 50 μM EGTA and with no added $Ca^{2+}$. To the modified solution A was added vehicle (open circles), 6.2 nM bombesin (closed circles) or 1.6 nM recombinant v-*sis* (closed squares) at time zero. Samples were collected as described in Materials and methods. The extracellular distribution of $^{45}Ca^{2+}$ at a certain time point was expressed as a percentage of total $^{45}Ca^{2+}$ in each sample. The data represent means of duplicates from two individual experiments.

### *Effect of vasopressin on Ins(1,4,5)$P_3$ and $[Ca^{2+}]_{cyt}$*

In view of the striking differences in the effects of bombesin and PDGF on $[Ca^{2+}]_{cyt}$ and inositol phosphates, it was of interest to determine the time course of these responses in cells stimulated by another growth-promoting peptide. Vasopressin binds to specific receptors in Swiss 3T3 cells (Collins and Rozengurt, 1983) and induces an array of early responses (Mendoza *et al.*, 1980; Burns and Rozengurt, 1983; Dicker and Rozengurt, 1980; Rozengurt *et al.*, 1981) including $Ca^{2+}$ mobilization (Lopez-Rivas and Rozengurt, 1984; Hesketh *et al.*, 1985; Rozengurt and Mendoza, 1985; Mendoza *et al.*, 1986a) and inositol polyphosphate accumulation (Brown *et al.*, 1984; Lopez-Rivas *et al.*, 1987). These responses were measured under similar conditions in 3T3 cells challenged with vasopressin at 18 nM, a concentration that elicits maximal mitogenesis (Rozengurt *et al.*, 1979) and $Ca^{2+}$ mobilization (Lopez-Rivas *et al.*, 1987). Figure 6 shows that the transient elevation in $[Ca^{2+}]_{cyt}$ in response to vasopressin coincided with the rapid increase in Ins(1,4,5)$P_3$ elicited by this peptide. Vasopressin also induced a slightly delayed increase in both Ins(1,3,4)$P_3$ and Ins$P_2$ (not shown). Clearly, vasopressin is like bombesin and differs from PDGF in its ability to elicit rapid and coincident increase in Ins(1,4,5)$P_3$ and $[Ca^{2+}]_{cyt}$ in 3T3 cells.

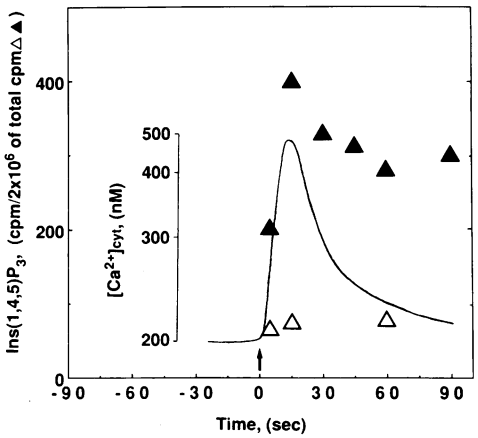


**Fig. 6.** Comparison of the time course of increase in $[Ca^{2+}]_{cyt}$ and Ins(1,4,5)$P_3$ formation in quiescent Swiss 3T3 cells exposed to vasopressin. Quiescent cells grown on Cytodex 2 microbeads were loaded with D-myo-[2-$^3$H]inositol and fura 2/AME as described in Materials and methods. The basal $[Ca^{2+}]_{cyt}$ was monitored for 1 min and at time 0, 18 nM vasopressin (closed triangles) or vehicle (open triangles) was added to the cells. Samples were collected 5, 15, 30, 45, 60 and 90 s thereafter and the composition of the inositol phosphates was subsequently analysed using a Mono-Q column. The data presented show the c.p.m. recovered as Ins(1,4,5)$P_3$ per 2 × $10^6$ c.p.m. of total c.p.m. in each sample. The values are means from two or three independent experiments. The insert shows the $[Ca^{2+}]_{cyt}$ level monitored in cells loaded with fura 2/AME and monitored in parallel with the inositol phosphate determinations.

## Discussion

The purpose of this study was to determine the temporal relationship between inositol polyphosphate formation and the increase in $[Ca^{2+}]_{cyt}$ in quiescent 3T3 cells treated with bombesin, vasopressin and PDGF. Responses were measured under identical conditions in cells grown on microbeads. Our results show a striking difference in the kinetics of inositol polyphosphate formation induced by bombesin and vasopressin compared with those of PDGF. Bombesin stimulated the formation of Ins(1,4,5)$P_3$ and subsequently of Ins(1,3,4)$P_3$ and Ins$P_2$ within seconds of their addition. These results agree with reports using 3T3 cells attached to dishes and incubated in the absence of fura 2 (Heslop *et al.*, 1986; Takuwa *et al.*, 1987). A similar result was obtained when the cells were challenged with vasopressin instead of bombesin. The concomitant increases in Ins(1,4,5)$P_3$ and $[Ca^{2+}]_{cyt}$ demonstrated here are entirely consistent with the proposed role of Ins(1,4,5)$P_3$ as a second messenger in the release of $Ca^{2+}$ from intracellular stores promoted by bombesin and vasopressin.

A salient aspect of the results presented here is that PDGF, in contrast to bombesin and vasopressin, stimulated inositol polyphosphate formation after a considerable lag period well beyond the $Ca^{2+}$ mobilization promoted by this factor. The temporal dissociation between these responses was confirmed in many independent experiments analysed at the time that $[Ca^{2+}]_{cyt}$ reached is maximum value (35−45 s) after addition of PDGF and under a variety of experimental conditions including stimulation with different preparations of homodimers of the B chain of PDGF. Previous studies in other laboratories have shown that phosphoinositide breakdown (Habenicht *et al.*, 1981) and enhanced InsP, Ins$P_2$ and Ins$P_3$ production (Berridge *et al.*, 1984; Hasegawa-Sasaki, 1985) occurred several minutes after the addition of PDGF. However, neither a precise temporal relationship between inositol polyphosphates and $Ca^{2+}$ mobilization was defined nor was Ins(1,4,5)$P_3$ separated from Ins(1,3,4)$P_3$ in these earlier studies. The present results with PDGF emphasize the need for comparing Ins(1,4,5)$P_3$ formation with $Ca^{2+}$ mobilization under identical conditions.

The results presented here with PDGF contrast with those obtained with many other ligands in a variety of target cells (Berridge, 1987). Interestingly, Ambler *et al.* (1987) reported that $\alpha_1$-adrenergic agonists induce a rapid rise in $[Ca^{2+}]_{cyt}$ in $BCH_3$ muscle cells which preceded the increase in Ins(1,4,5)$P_3$. Since $\alpha_1$-adrenergic agonists bind to two different receptor subtypes in this cell system, one mediating $Ca^{2+}$ influx and the other $Ca^{2+}$ mobilization via Ins(1,4,5)$P_3$ (Han *et al.*, 1987), it is possible that the early increase in $[Ca^{2+}]_{cyt}$ is mediated by the influx pathway. In the present studies with quiescent Swiss 3T3 cells we verified that PDGF (like bombesin) mobilizes $Ca^{2+}$ from an intracellular store.

Our findings have important implications regarding the mechanisms by which the signal generated in response to PDGF binding to its receptor is transduced to its site of action. At least two different models could account for the temporal relationship shown here. The first model assumes that Ins(1,4,5)$P_3$ mediates $Ca^{2+}$ mobilization but envisages that the PDGF−receptor complex stimulates inositol polyphosphate formation only after it has been translocated into an intracellular compartment. In contrast, bombesin could stimulate Ins(1,4,5)$P_3$ when the receptor is located in the plasma membrane. This possibility takes into account the fact that PDGF causes rapid and extensive down-regulation of its surface receptors (Heldin *et al.*, 1982) while bombesin does not (Zachary and Rozengurt, 1987a). This model could explain the lag period between addition of PDGF and the onset of $Ca^{2+}$ mobilization and inositol polyphosphate formation. Furthermore, by assuming that inositol polyphosphates are released in the cytosol adjacent to the putative $Ca^{2+}$ stores it is also possible to explain the apparent temporal dissociation of the responses: local concentrations of Ins(1,4,5)$P_3$ would suffice to promote $Ca^{2+}$ mobilization. Interestingly, recent reports suggest that a second phase of inositol lipid turnover elicited by angiotensin in adrenal glomerulosa cells depends on the internalization of the receptor (Delafontaine *et al.*, 1987).

The second model is that PDGF might stimulate the generation of a different second messenger from Ins(1,4,5)$P_3$ which either acts independently of this metabolite to mobilize $Ca^{2+}$ or increases the sensitivity of the $Ca^{2+}$ mobilizing system to Ins(1,4,5)$P_3$. Interestingly, a recent report indicates that the activation of the tyrosine kinase of the PDGF receptor by its ligand phosphorylates and thereby activates a phosphatidyl inositol kinase (Kaplan *et al.*, 1987). Activation of this enzyme could initiate a different pathway leading to the formation of inositol polyphosphates. Furthermore, recent reports have suggested that GTP (Lukacs *et al.*, 1987; Chueh *et al.*, 1987), NADPH (Clapper *et al.*, 1987) and/or arachidonic acid (Beaumier *et al.*, 1987) could play a role in $Ca^{2+}$ mobilization. These possibilities present a considerable challenge for future efforts to elucidate the signal transduction pathway in PDGF action.

## Materials and methods

### *Cell culture*

Swiss 3T3 cells (Todaro and Green, 1963) were maintained in 90-mm Nunc Petri dishes in DMEM, 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere of 10% $CO_2$ and 90% air at 37°C. The cells were subcultured to 33-mm dishes with medium containing 10% FBS. The cultures were used when they had become quiescent and confluent 7−8 days later. Cultures were shown to be quiescent by autoradiography after 40 h with [$^3$H]thymidine as described previously (Dicker and Rozengurt, 1980).

Most studies were carried out in quiescent Swiss 3T3 cells grown on Cytodex 2 microbeads. For these experiments, Cytodex 2 microbeads were allowed to swell in PBS for at least 4 h, rinsed twice with PBS and autoclaved in siliconized glass bottles containing PBS. Cells ($2.5 \times 10^7$) were seeded into a flask containing 750 mg of Cytodex 2 microbeads and 160 ml of DMEM supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS. The mixture of cells, beads and medium was gassed with 10% $CO_2$:90% air, maintained at 37°C and stirred intermittently (2 min on, 30 min off). On the following day, the volume of medium was increased to 500 ml and the mixture was re-gassed with 10% $CO_2$:90% air and stirred continuously at the slowest rate which prevented the beads from settling (~25 r.p.m.). Seven days later, the cells were quiescent as indicated by the increase in [$^3$H]thymidine incorporation following the addition of purified mitogens in serum-free medium (data not shown).

### *Measurement of $[Ca^{2+}]_i$*

$[Ca^{2+}]_i$ was measured with the fluorescent $Ca^{2+}$ indicator fura 2 using a modification of the procedure of Tsien *et al.* (1982). At the beginning of the experiment, quiescent cells on Cytodex 2 microbeads were washed twice with serum-free DMEM. The beads were then resuspended in 180 ml of DMEM by gentle shaking. Aliquots (10 ml) were transferred to plastic tubes and incubated for 10 min with 1 μM fura 2 tetra-acetoxymethyl ester (fura 2/AME). The stock solution of fura 2/AME (1 mM) was dissolved in dimethyl sulphoxide. After this incubation, the beads were washed three times with solution A which contained 120 mM NaCl, 20 mM LiCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM $MgCl_2$, 25 mM glucose, 16 mM Hepes,

6 mM Tris and a mixture of amino acids at the same concentrations as those in DMEM (pH 7.20). The beads were suspended in solution A (final volume 2.0 ml) and transferred to a 1-$cm^2$ quartz cuvette. The suspension was stirred continuously and maintained at 37°C. Fluorescence was monitored in a Perkin-Elmer LS-5 luminescence spectrometer with an excitation wavelength of 336 nm and an emission wavelength of 510. $[Ca^{2+}]_{cyt}$ was calculated using the formula of Tsien *et al.* (1982):

$$[Ca^{2+}]_{cyt} \text{ in nM} = \frac{K(F-F_{min})}{(F_{max}-F)}$$

where $F$ is the fluorescence at the unknown $[Ca^{2+}]_{cyt}$, $F_{max}$ is the fluorescence after the trapped fluorescence is released by the addition of 0.02% Triton X-100 and $F_{min}$ is the fluorescence remaining after the $Ca^{2+}$ in the solution is chelated with 10 mm EGTA. The value of $K$ was 220 for fura 2 (Mendoza *et al.*, 1986). The change in $[Ca^{2+}]_{cyt}$ following the addition of mitogens is presented both as the ratio between the peak $[Ca^{2+}]_{cyt}$ and the control $[Ca^{2+}]_{cyt}$ and as the increase in $[Ca^{2+}]_{cyt}$ expressed in nM.

### $^{45}Ca^{2+}$ efflux

Quiescent cultures of Swiss 3T3 cells were loaded with $^{45}Ca^{2+}$ by incubating them for 12–24 h in conditioned medium to which 4–8 μCi/ml of $^{45}Ca^{2+}$ was added. The efflux of $^{45}Ca^{2+}$ was measured at 37°C in an atmosphere of 90% air:10% $CO_2$. The cultures were washed rapidly seven times with 2 ml of DMEM/3 mM EGTA. They were then incubated with 2.0 ml of solution A (140 mM NaCl, no LiCl). At the times indicated, 200-μl samples were removed and replaced with 200 μl of fresh medium. At the end of the efflux, medium was aspirated and the $^{45}Ca^{2+}$ remaining in the cells extracted with 0.1 M NaOH/2% $Na_2CO_3$/1% SDS. The sum of the $^{45}Ca^{2+}$ in each sample plus the $^{45}Ca^{2+}$ remaining in the cells was taken as the total $^{45}Ca^{2+}$ in the cells at the onset of the efflux period.

### Parallel measurements of intracellular $Ca^{2+}$-levels and formation of inositol phosphates in Swiss 3T3 cells: labelling and incubation of the cells

Swiss 3T3 cells were cultured on Cytodex 2 microbeads for 6–7 days as described above. Twenty-four hours prior to the experiments the cells were transferred to 100 ml fresh DMEM with 1% FBS and 5 μCi/ml of D-myo-[2-$^3$H]inositol added. At the beginning of the experiment the cell suspension was diluted 1:1 with DMEM. Aliquots (10 ml) of cells were transferred to plastic vials and the cells washed three times in DMEM, resuspended and incubated for 20 min in DMEM. Then 1 μM of fura 2/AME was added and the incubations continued for another 10 min. Remaining extracellular myo-[$^3$H]inositol and fura 2/AME were removed by three washes in solution A containing 120 mM NaCl and 20 mM LiCl. The cells were incubated in this buffer for 10 min before being transferred to a quartz cuvette (total volume 2 ml). The basal $Ca^{2+}$ signal was recorded for 1 min before the appropriate mitogen or vehicle was added. The incubations were stopped by addition of 1 ml ice-cold 30% trichloroacetic acid (final concentration 10%). The trichloroacetic acid extracts were collected and the beads rinsed with 2 × 5 ml $H_2O$ which were pooled with the acid.

For some experiments Swiss 3T3 fibroblasts were cultured in DMEM/10% FBS on 33-mm Petri dishes for 7 days to become confluent and quiescent (see above). During the final 24 h, 5 μCi/ml of D-myo-[2-$^3$H]inositol was present. At the beginning of the experiments remaining extracellular myo-[$^3$H]inositol was removed by repeated washes and a 30-min preincubation in DMEM. The cells were then incubated for another 10 min in DMEM containing 120 mM NaCl and 20 mM LiCl before addition of the factors. The incubations were continued for the time indicated and stopped by aspirating the medium and addition of 0.5 ml ice-cold 10% trichloroacetic acid per dish. The dishes were left at 4°C for 30 min before the acid-soluble extracts were collected and the dishes were rinsed with 2 × 1 ml of $H_2O$ which was added to the acid extracts. Extracts from six dishes were pooled and further processed.

The pooled samples were extracted with 4 × 14 ml of water-saturated diethylether to remove the acid. Remaining diethylether was evaporated, the pH was set to 8–9 with $NaHCO_3$ and the sample filtered through Millex-GS 0.22-μM filters and thereafter stored at −20°C.

### Analysis of the inositol phosphate composition

The protocol used was based on the method published by Meek (1986). The inositol phosphates were separated by anion-exchange chromatography using a Mono-Q column fitted in a Pharmacia FPLC system. The inositol phosphates were eluted by a linear gradient of $Na_2SO_4$ in 0.1 mM EDTA/10 mM Hepes reaching from 0 to 125 mM of $Na_2SO_4$ in 25 min. The gradient was halted at 125 mM $Na_2SO_4$ for 5 min and thereafter increased to 400 mM $Na_2SO_4$ in 18 min. The flow rate was set to 1 ml/min and 0.3-ml fractions were collected between 55 and 125 mM $Na_2SO_4$, otherwise 1-ml fractions were collected. The identity of the peaks of recovered radioactivity as determined by comparing the retention times for D-myo-[2-$^3$H]inositol 1-phosphate, D-myo-[2-$^3$H]inositol 1,4-bisphosphate and D-myo-[2-$^3$H]inositol 1,4,5-trisphosphate. As an internal standard 15 μg ATP was added to every sample. InsP eluted around 25 nM $Na_2SO_4$; $InsP_2$ eluted around 60 nM $Na_2SO_4$ and Ins(1,3,4)$P_3$ and Ins(1,4,5)$P_3$ at 100 nM and 115 nM $Na_2SO_4$ respectively. With this method occasionally some of the InsP co-eluted with the free inositol. Hence, we have focused on presenting data based on the c.p.m. recovered as $InsP_2$ and $InsP_3$ which showed very good resolution of the individual peaks of isomers. In the experiments presented in Figure 2, samples were analysed for their content of inositol phosphates by anion-exchange chromatography using a Bio-Rad AG1-X8 (formate form) resin and stepwise elution with increasing concentrations of ammonium formate as described by Berridge *et al.* (1983). The same [$^3$H]inositol phosphate standards were used for identification of the eluted material. The eluted peaks of some samples were further diluted and re-chromatographed to certify the elution pattern.

### DNA synthesis

Cultures of quiescent Swiss 3T3 cells in 33-mm dishes were washed twice with DMEM and incubated in 2 ml of a 1:1 mixture of DMEM and Waymouth media containing [$^3$H]thymidine at 1 μCi/ml. Acid-precipitable radioactivity was measured as described previously (Dicker and Rozengurt, 1980).

### Materials

Highly purified porcine PDGF was obtained from Bioprocessing Ltd. Bombesin and (Arg$^8$)-vasopressin were obtained from Sigma Chemical Co. (St Louis, MO). The recombinant v-*sis* product was obtained from the Radiochemical Centre (Amersham, UK). Fetal bovine serum was purchased from Gibco Europe (UK). AG1-X8 formate form (mesh size 200–400) was purchased from Bio-Rad. Cytodex 2 microbeads were purchased from Pharmacia, Uppsala, Sweden. $^{45}CaCl_2$, D-myo-[2-$^3$H]inositol, D-myo-[$^3$H]inositol 1-monophosphate, D-myo-[$^3$H]inositol 1,4-bisphosphate and D-myo-[$^3$H]inositol 1,4,5-trisphosphate were obtained from the Radiochemical Centre (Amersham, UK). Fura 2 tetra-acetoxymethyl ester was purchased from Calbiochem. All other chemicals were of reagent grade.

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## References

Ambler,S.K., Thompson,B., Solski,P.A., Brown,J.H. and Taylor,P. (1987) *Mol. Pharmacol.*, **32**, 376–383.
Beaumier,L., Faucher,N. and Naccache,P.H. (1987) *FEBS Lett.*, **221**, 289–292.
Berridge,M.J. (1987) *Annu. Rev. Biochem.*, **56**, 159–193.
Berridge,M.J. and Irvine,R.F. (1984) *Nature*, **312**, 315–321.
Berridge,M.J., Dawson,R.M.C., Downes,C.P., Heslop,J.P. and Irvine,R.F. (1983) *Biochem. J.*, **212**, 473–482.
Berridge,M.J., Heslop,J.P., Irvine,R.F and Brown,K.D. (1984) *Biochem. J.*, **222**, 195–201.
Brown,K.D., Blay,J., Irvine,R.F., Heslop,J.P. and Berridge,M.J. (1984) *Biochem. Biophys. Res. Commun.*, **123**, 377–384.
Burns,C.P. and Rozengurt,E. (1983) *Biochem. Biophys. Res. Commun.*, **116**, 931–938.
Chueh,S.-H., Mullaney,J.M., Ghosh,T.K., Zachary,A.L. and Gill,D.L. (1987) *J. Biol. Chem.*, **262**, 13857–13864.
Clapper,D.L., Walseth,T.F., Dargie,P.J. and Lee,H.C. (1987) *J. Biol. Chem.*, **262**, 9561–9568.
Collins,M.K.L. and Rozengurt,E. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 1924–1928.
Delafontaine,P., Griendling,K.K., Gimbrone,M.A., Jr and Alexander,R.W. (1987) *J. Biol. Chem.*, **262**, 14549–14554.
Dicker,P. and Rozengurt,E. (1980) *Nature*, **287**, 607–612.
Habenicht,A.J.R., Glomset,J.A., King,W.C., Wist,C., Mitchell,C.D. and Ross,R. (1981) *J. Biol. Chem.*, **256**, 12329–12335.
Han,C., Abel,P.W. and Minneman,K.P. (1987) *Nature*, **329**, 333–335.
Hasegawa-Sasaki,H. (1985) *Biochem. J.*, **232**, 99–109.
Heldin,C.-H., Wasteson,Å. and Westermark,B. (1982) *J. Biol. Chem.*, **257**, 4216–4221.

Hesketh,T.R., Moore,J.P., Morris,J.D.H., Taylor,M.V., Rogers,J., Smith,G.A. and Metcalfe,J.C. (1985) *Nature*, **313**, 481–484.
Heslop,J.P., Blakely,D.M., Brown,K.D., Irvine,R.F. and Berridge,M.J. (1986) *Cell*, **47**, 703–709.
Kaplan,D.R., Whitman,M., Schaffhausen,B., Pallas,D.C., White,M., Cantley,L. and Roberts,T.M. (1987) *Cell*, **50**, 1021–1029.
Lopez-Rivas,A. and Rozengurt,E. (1983) *Biochem. Biophys. Res. Commun.*, **114**, 240–247.
Lopez-Rivas,A. and Rozengurt,E. (1984) *Am. J. Physiol.*, **247**, c156–c162.
Lopez-Rivas,A., Mendoza,S.A., Nånberg,E., Sinnett-Smith,J. and Rozengurt,E. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 5768–5772.
Lukacs,G.L., Hajnoczky,G., Hunyady,L. and Spät,A. (1987) *Biochim. Biophys. Acta*, **931**, 251–254.
Majerus,P.W., Connolly,T.M., Deckmyn,H., Ross,T.S., Bross,T.E., Ishii,H., Bansal,V.S. and Wilson,D.B. (1986) *Science*, **234**, 1519–1526.
McNeil,P.L., McKenna,M.P. and Taylor,D.L. (1985) *J. Cell Biol.*, **101**, 372–379.
Meek,J.L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4162–4166.
Mendoza,S.A., Wigglesworth,N.M. and Rozengurt,E. (1980) *J. Cell Physiol.*, **105**, 153–162.
Mendoza,S.A., Lopez-Rivas,A., Sinnett-Smith,J.W. and Rozengurt,E. (1986a) *Exp. Cell Res.*, **164**, 536–545.
Mendoza,S.A., Schneider,J.A., Lopez-Rivas,A., Sinnett-Smith,J.W. and Rozengurt,E. (1986b) *J. Cell Biol.*, **102**, 2223–2233.
Moolenaar,W.H., Tertoolen,L.G.J. and de Laat,S.W. (1984) *J. Biol. Chem.*, **269**, 8066–8069.
Owen,N.E. and Villereal,M.L. (1983) *J. Cell Physiol.*, **117**, 23–29.
Ross,R. and Vogel,A. (1978) *Cell*, **14**, 203–210.
Ross,R., Raines,E.W. and Bowen-Pope,D.F. (1986) *Cell*, **46**, 155–169.
Rozengurt,E. (1986) *Science*, **234**, 161–166.
Rozengurt,E. and Mendoza,S.A. (1985) *J. Cell Sci.*, Suppl. **3**, 229–242.
Rozengurt,E. and Sinnett-Smith,J.W. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 2936–2940.
Rozengurt,E., Legg,A. and Pettican,P. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 1284–1287.
Rozengurt,E., Brown,K.D. and Pettican,P. (1981) *J. Biol. Chem.*, **256**, 715–722.
Takuwa,N., Takuwa,Y., Bollag,W.E. and Rasmussen,H. (1987) *J. Biol. Chem.*, **262**, 182–188.
Todaro,G.J. and Green,H. (1963) *J. Cell Biol.*, **17**, 299–313.
Tsien,R.Y., Pozzan,T. and Rink,T.J. (1982) *Nature*, **295**, 68–71.
Zachary,I. and Rozengurt,E. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7616–7620.
Zachary,I. and Rozengurt,E. (1987a) *J. Biol. Chem.*, **262**, 3947–3950.
Zachary,I. and Rozengurt,E. (1987b) *EMBO J.*, **6**, 2233–2239.
Zachary,I., Woll,P.J. and Rozengurt,E. (1987) *Dev. Biol.*, **124**, 295–308.

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