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α-Thrombin-induced early mitogenic signalling events and G0 to S-phase transition of fibroblasts require continual external stimulation

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In resting Chinese hamster fibroblasts (CCL39) α-thrombin rapidly stimulates several biochemical events implicated in the mitogenic response, including the breakdown of inositol phospholipids, activation of a plasma membrane Na^+/H^+ antiporter, phosphorylation of ribosomal protein S6 and increased expression of the proto-oncogene c-*myc*. Complete removal of the growth factor during cellular G0/G1 transit precludes the re-initiation of DNA synthesis. The present study was designed to examine the fate of α-thrombin-activated early events following growth factor inactivation. In cells stimulated for 30 min with α-thrombin, neutralization of the growth factor results in: (i) immediate arrest of inositol phosphate formation, (ii) rapid inactivation of Na^+/H^+ exchange, (iii) deactivation of the S6 phosphorylating system and (iv) strong reduction of c-*myc* mRNA level. Our findings that commitment for DNA synthesis as well as persistent activation of 'early' cellular events requires continual growth factor stimulation suggest that: (i) growth factor-induced transmembrane signals have a short life and (ii) the generation of these signals during the 8 h of the pre-replicative phase is required for G0-arrested cells to enter the S phase.

Key words: growth factors/phosphoinositide breakdown/Na^+/H^+ antiporter/S6 phosphorylation/c-*myc* expression

Introduction

Studies involving the dissection of various biochemical events triggered by mitogens in resting cells have advanced our understanding of the control of cell proliferation. Such ubiquitous events as stimulation of phosphoinositide hydrolysis (Berridge, 1984), ion fluxes (Rozengurt, 1981; Leffert and Koch, 1985) and protein phosphorylation (Cooper and Hunter, 1984), which rapidly occur following mitogen addition to quiescent cells, are thought to be implicated in the signalling pathway leading to re-initiation of DNA synthesis. Recently, progress has been made in the characterization of a membrane-bound Na^+/H^+ antiporter whose activation by growth factors results in a rise in intracellular pH (Schuldiner and Rozengurt, 1982; Moolenaar *et al.*, 1983; Cassel *et al.*, 1983; Paris and Pouysségur, 1984; L'Allemain *et al.*, 1984). Using a line of Chinese hamster fibroblasts (CCL39) and a mutant derived from these cells which specifically lacks Na^+/H^+ exchange activity, we have demonstrated that pHi exerts a strict control on the rate of cell progression into the S phase (Pouysségur *et al.*, 1984, 1985).

Other mitogen-stimulated early events appear to be tightly linked to DNA synthesis re-initiation in CCL39 cells: activation of polyphosphoinositide breakdown (L'Allemain *et al.*, in preparation), phosphorylation of ribosomal protein S6 (Chambard *et al.*, 1983; Pouysségur *et al.*, 1982) and expression of c-*myc* proto-oncogene mRNA (Blanchard *et al.*, 1985). It is still not known whether all of these activated events are required to set in motion the G0/G1 → S transition and for how long their activation should persist to induce irrevocable commitment to DNA synthesis.

α-Thrombin is one of the most potent growth factors for CCL39 cells capable of stimulating the pleiotypic program (Pérez-Rodriguez *et al.*, 1981). DNA synthesis occurs after a constant lag of 8 h following α-thrombin addition to G0-arrested cultures (Van Obberghen-Schilling *et al.*, 1983). Previously, using an α-thrombin inhibitor, hirudin which permits immediate inactivation and complete removal of α-thrombin from the cell surface, we have determined that the presence of active α-thrombin is required throughout the entire pre-replicative phase (>8 h) for commitment to DNA synthesis to occur (Van Obberghen-Schilling *et al.*, 1982; Pouysségur *et al.*, 1983).

We demonstrate here that the continual presence of α-thrombin is required for persistent activation of phosphatidylinositol turnover, activation of Na^+/H^+ antiport and maintenance of intracellular alkalinization, ribosomal protein S6 phosphorylation and c-*myc* mRNA expression.

Results

Effect of α-thrombin removal on DNA synthesis re-initiation

α-Thrombin binds rapidly to CCL39 cells at 37°C and remains associated for prolonged periods of time at or near the cell surface (Van Obberghen-Schilling and Pouysségur, 1986). Dissociation of [^{125}I]α-thrombin bound to cells occurs slowly in buffer alone (not shown). However, as shown in Figure 1 (inset) addition of the potent thrombin inhibitor, hirudin (Markwardt, 1970), rapidly induces the removal of [^{125}I]α-thrombin bound to cells ($t_{1/2}$ ~5 min) by forming an essentially non-dissociable complex with the protease thereby blocking its enzymatic (and mitogenic) activity.

Our earlier experiments revealed that when CCL39 cells are transiently exposed to α-thrombin then incubated in α-thrombin-free medium a significant commitment to DNA synthesis is observed if precautions are not taken to remove all residual growth factor from cells (Van Obberghen-Schilling *et al.*, 1982). However, as shown in Figure 1, when α-thrombin is completely removed from the cultures using hirudin, after a 3-h exposure, a rapid decline in the α-thrombin-induced re-initiation of DNA synthesis is observed which parallels hirudin-induced α-thrombin dissociation from cells. It should be noted that hirudin does not perturb the binding of other growth factors to CCL39 cells (e.g., platelet-derived growth factor) nor does it interfere with the mitogenic response of CCL39 cells to other purified growth factors (not shown).

More recently, we have employed the tripeptidyl chloromethyl ketone (PPACK) to inhibit specifically α-thrombin action (Van Obberghen-Schilling and Pouysségur, 1986). This α-thrombin inhibitor rapidly and irreversibly blocks the active center of α-thrombin (Kettner and Shaw, 1979), and consequently its biological activity. Like hirudin, PPACK is not toxic to cells,

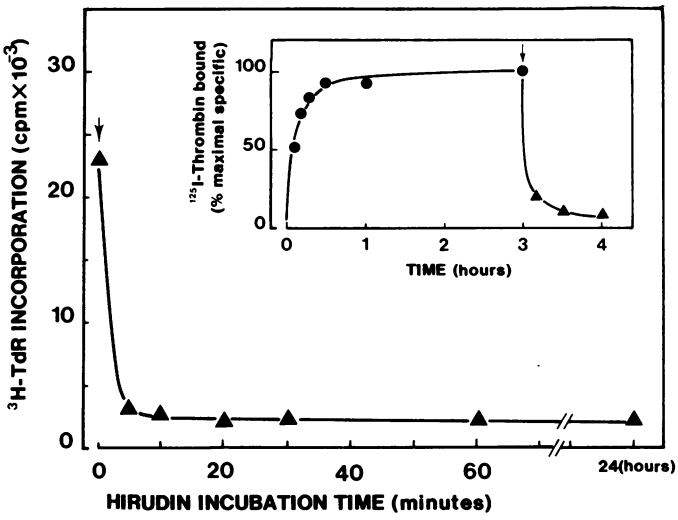

Fig. 1. Effect of α-thrombin removal on mitogenic stimulation of CCL39 cells. **Inset:** [^{125}I]α-thrombin association at 37°C and hirudin-induced dissociation. Specific association of [^{125}I]α-thrombin (0.15 U/ml = 55 ng/ml) to CCL39 cells was measured on duplicate wells as a function of time (●). Dissociation (▲) was achieved by rinsing monolayers rapidly twice with binding buffer followed by addition of buffer containing hirudin (arrow) at 10 α-thrombin-inactiving units/ml. Maximal specific binding represented 11% of total tracer added; non-specific binding accounted for 8.1% of specific binding. DNA synthesis re-initiation. G0-arrested cells were incubated for 3 h (time = 0−3 h) in serum-free culture medium (1:1 of DME:Ham's F12) 0.5 U/ml (180 ng/ml) α-thrombin. At time = 3 h (arrow) cultures were rapidly rinsed twice and medium containing hirudin (10 α-thrombin-inactivating units per ml) was added for the indicated times. Afterwards, cells were rinsed twice and incubation was continued in the absence of α-thrombin and hirudin. At time = 5 h [^{3}H]TdR was added and incorporation continued until time = 24 h.

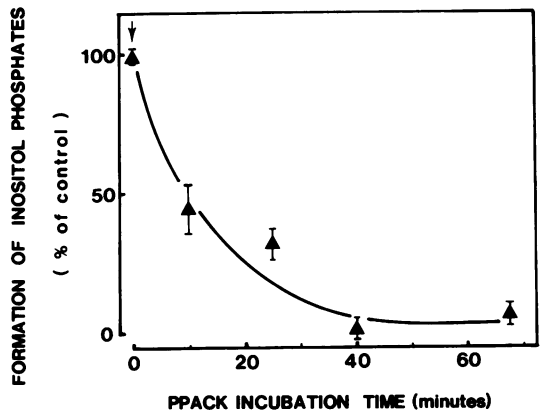

Fig. 2. Effect of α-thrombin inactivation on the rate of inositol phosphate formation. α-Thrombin was added at 0.1 U/ml (36 ng/ml) to G0-arrested cells labeled with [^{3}H]inositol. After 30 min (time 0 on the figure) a large excess of PPACK (10 α-thrombin-inactiving units/ml) was added to half of the cultures. LiCl (20 mM) was also added either at time 0, 15, 30 or 60 min, and [^{3}H]inositol phosphates accumulated after 10 min were determined as described in Materials and methods in both control and PPACK-treated cells. The incubation time indicated on the figure refers to the total incubation with PPACK, including the 10 min Li^+-treatment. Results are expressed at each time as the percentage of the rate of inositol phosphate accumulation in stimulated cells. At time 0 of the figure, 100% corresponds to 8500 d.p.m./10 min/dish. This rate slowly decreased with time and stabilized at 5500 d.p.m./10 min/dish after 30 min of additional incubation with α-thrombin. The basal inositol phosphate pool had the same value in non-stimulated cells and α-thrombin-stimulated cells in the absence of Li^+. This value averaged 6500 d.p.m./dish.

and is without effect on the action of other growth factors.

Therefore, using hirudin and PPACK as tools to remove and/or inactivate α-thrombin, we investigated the fate of early mitogen-stimulated events in CCL39 cells following removal of the mitogenic stimulus.

Rapid inactivation of polyphosphoinositide breakdown occurs following α-thrombin inactivation

One of the first detectable responses of G0-arrested CCL39 cells to α-thrombin is the stimulation of membrane polyphosphoinositide breakdown, which follows the activation of a wide variety of cell surface receptors (for review, see Berridge, 1984). Lithium has been used to disrupt the inositol phosphate cycle through its inhibitory action on the inositol 1-phosphatase (Berridge *et al.*, 1982). In the presence of Li^+, the rate of accumulation of the water-soluble inositol phosphates reflects the activity of the phospholipase C responsible for hydrolysis of phosphatidylinositol 4,5-bisphosphate into diacylglycerol (DAG) and inositol trisphosphate. In G0-arrested CCL39 cells pre-labeled with [^{3}H]inositol this phosphodiesterase activity is undetectable. In contrast, addition of α-thrombin triggers a very rapid accumulation of inositol phosphates, in a dose-dependent manner. At maximal α-thrombin concentrations this accumulation is detectable within 5 s; the phosphodiesterase remains activated at least during 9 h of the pre-replicative phase (L'Allemain *et al.*, in preparation). To monitor how fast the inactivation of phosphoinositide breakdown occurs after growth factor neutralization, quiescent cells were stimulated for 30 min with α-thrombin and subsequently an excess of the inhibitor PPACK was added to inactivate the protease. Lithium was then added at varying times, to allow [^{3}H]-inositol phosphate accumulation. As shown in Figure 2, the amount of inositol phosphates accumulating during a 10-min Li^+ treatment was already greatly reduced (~50%) when PPACK was added together with Li^+. This indicates a rapid inactivation of the phosphodiesterase activity. Complete inactivation of inositol phosphate formation was achieved after 40 min of incubation with the α-thrombin inhibitor.

Rapid inactivation of the Na^+/H^+ antiporter occurs following growth factor removal

Activation by growth factors of the amiloride-sensitive Na^+/H^+ antiporter leads, in CCL39 cells, to a rapid cytoplasmic alkalinization, of 0.2−0.3 pH units under optimal conditions (L'Allemain *et al.*, 1984). Therefore, to investigate the persistence of Na^+/H^+ antiporter activation after growth factor withdrawal, we first examined the variation of cytoplasmic pH. G0/G1-arrested cells were stimulated with α-thrombin for 30 min, which resulted in a pHi increase of ~0.15 pH units. Then, free α-thrombin was eliminated by washing and adding an excess of hirudin. Measurement of intracellular pH (Figure 3, lower panel) shows that pHi remains stable for ~10 min after hirudin addition. Thereafter, it progressively returns to the control level, with a $t_{1/2}$ ~20 min. If hirudin is eliminated after 30 min, and α-thrombin added back to the cells, cytoplasmic alkalinization occurs again, which demonstrates the reversibility of the hirudin effect. Thus, the Na^+/H^+ antiporter appears to be completely inactivated within 1 h after α-thrombin removal.

However, variation of the steady-state pHi might not reflect the time-course of Na^+/H^+ antiporter inactivation precisely since pHi also depends on H^+ production inside the cell. Even if the antiporter was quickly inactivated upon growth factor withdrawal, and consequently H^+ extrusion slowed down, it might take some time to accumulate metabolic H^+ in sufficient amounts to significantly decrease pHi.

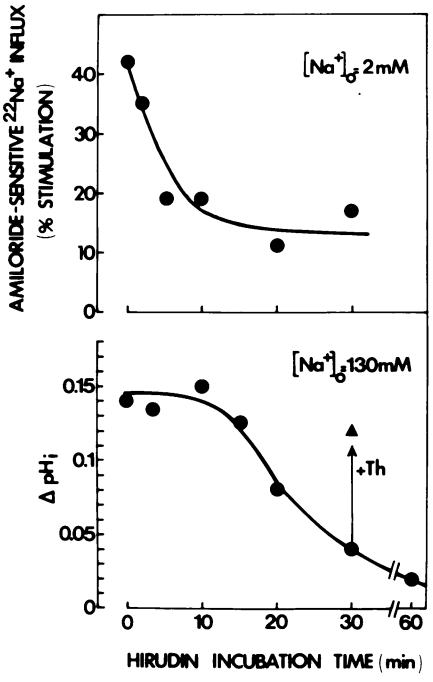

Fig. 3. Effect of α-thrombin removal on amiloride-sensitive $^{22}Na^+$ influx and on intracellular pH. **Lower panel.** Cytoplasmic pH was measured as described under Materials and methods at the times indicated after hirudin addition. [^{14}C]Benzoic acid was present in the incubation medium for the last 5 min. Results are expressed as the increase in pH over unstimulated cells. Hirudin had no effect on pHi of control cells (7.35 ± 0.03). After 30 min of incubation with hirudin, some cultures were washed three times and received 2 U/ml (0.72 μg/ml) of α-thrombin. pHi was measured after 15 min (●). Each point is the average of duplicates. **Upper panel.** Initial rates of amiloride-sensitive $^{22}Na^+$ uptake were determined over 3 min, as described under Materials and methods, following varying incubation times with hirudin. Results are expressed as % stimulation over control cells, pre-incubated in the absence of α-thrombin. Amiloride-sensitive and insensitive uptake rates in the unstimulated cells were 32 500 and 430 c.p.m./3 min, respectively; these rates were not affected by incubation with hirudin. At the end of the pre-incubation with or without α-thrombin, pHi, measured as described under Materials and methods, was 7.21 and 7.18 in stimulated and unstimulated cells, respectively. Each point is the average of duplicates.

Therefore, we addressed the question of the persistence of Na^+/H^+ antiporter activation more directly, by measuring the amiloride-sensitive $^{22}Na^+$ influx rate. Addition of α-thrombin to quiescent CCL39 cells results in stimulation of amiloride-sensitive $^{22}Na^+$ influx, due to an increased affinity of the Na^+/H^+ antiporter for internal H^+ (Paris and Pouysségur, 1984). However, under physiological conditions, the growth factor-induced pHi increase exerts a 'feedback' inhibitory effect on $^{22}Na^+$ influx, so that, after 30 min, $^{22}Na^+$ uptake rate is only slightly higher in stimulated cells than in control cells (Paris and Pouysségur, 1984). Conversely, the observed decrease of pHi upon α-thrombin removal (Figure 3) can be expected to stimulate $^{22}Na^+$ influx, due to the high dependence of $^{22}Na^+$ influx rate on pHi (Paris and Pouysségur, 1984), thereby possibly masking the inactivation of the transport system. Accordingly, to correlate directly the amiloride-sensitive $^{22}Na^+$ influx with the degree of growth factor-induced activation of the antiporter, it was necessary to set up experimental conditions under which pHi would not significantly vary upon α-thrombin addition or removal. This was achieved by decreasing the Na^+ concentration in the medium, since the magnitude of the cytoplasmic alkalinization was shown to be linked with Na^+_o concentration, in a dose-dependent manner (L'Allemain *et al.*, 1984). We observed that after equilibration in a 2 mM Na^+-containing medium, pHi increased by only 0.03 pH units upon α-thrombin stimulation (see legend to Figure 3). Therefore, we chose these conditions for studying the persistence of $^{22}Na^+$ influx stimulation after α-thrombin removal with hirudin. As clearly seen in Figure 3 (upper panel), the amiloride-sensitive $^{22}Na^+$ influx was immediately attenuated upon growth factor withdrawal, with no detectable lag. The reason why the uptake rate did not completely return to the basal level within 30 min is not clear. It might reflect some small difference in intracellular Na^+ concentration between stimulated and unstimulated cells, since $[Na^+]_{in}$ has also been suggested to influence $^{22}Na^+$ influx, in addition to pHi (Grinstein *et al.*, 1984).

Inactivation of the S6 phosphorylating system occurs rapidly following α-thrombin removal

The phosphorylation of ribosomal protein S6 is another early event (5 min) associated with growth factor stimulation of G0-arrested CCL39 cells (Chambard *et al.*, 1983). We have previously characterized the phosphorylation of S6 by two-dimensional gel electrophoresis (Chambard and Pouysségur, in preparation); here we followed its phosphorylation after separation of acid-soluble proteins by one-dimensional PAGE. As shown in Figure 4 (lanes a and b), addition of a mitogenic concentration of α-thrombin to cells results in increased incorporation of ^{32}P into bands which correspond to the different phosphorylated forms of S6 (Martin-Perez and Thomas, 1983). Following 30 min of stimulation, the addition of hirudin (10-fold α-thrombin inactivating units) (lanes c – h) rapidly induced a decrease of ^{32}P labeling in S6; quantification by densitometry indicated that total S6 phosphorylation reached the basal level found in quiescent cells within 45 min (data not shown). It should be noted that the maximally phosphorylated forms of S6 (upper band) disappeared considerably faster (5 – 10 min) than the less phosphorylated forms of the protein (lower band). Since ^{32}P was added during a 10-min pulse at the end of the incubation period, labeling of S6 reflects the activation state of its phosphorylating system at the time of the pulse.

As shown in lane i, when hirudin was washed from the cell monolayer, S6 phosphorylation could once again be induced by α-thrombin, thus demonstrating the reversibility of hirudin inhibition. Incubation of hirudin together with serum did not prevent S6 phosphorylation (lane k), indicating the specificity of hirudin towards α-thrombin inactivation.

Persistent stimulation by α-thrombin is required for maximal c-myc expression

In accordance with reports of growth factor-stimulated increase in c-*myc* mRNA expression in quiescent fibroblasts (Kelly *et al.*, 1983), a rise in c-*myc* mRNA levels in CCL39 cells is observed following mitogenic stimulation (Blanchard *et al.*, 1985). The time course of this effect, determined by Northern analysis of equal amounts of poly(A)$^+$ RNA, is shown in Figure 5; hybridization of the filters to a probe for GAPDH, an enzyme whose expression does not significantly vary over the same time course, was routinely performed as a control for quantitation purposes. Whereas c-*myc* mRNA is barely detectable in resting cells, a rapid increase is seen upon α-thrombin stimulation, which reaches maximal levels after ~90 min. Subsequently, expression declines to a steady-state level which remains stable for at least several hours (5 h; Figure 5A).

We chose to examine the persistence of α-thrombin-induced c-*myc* expression after 30 min of growth factor stimulation. For these experiments, α-thrombin was neutralized after 30 min by

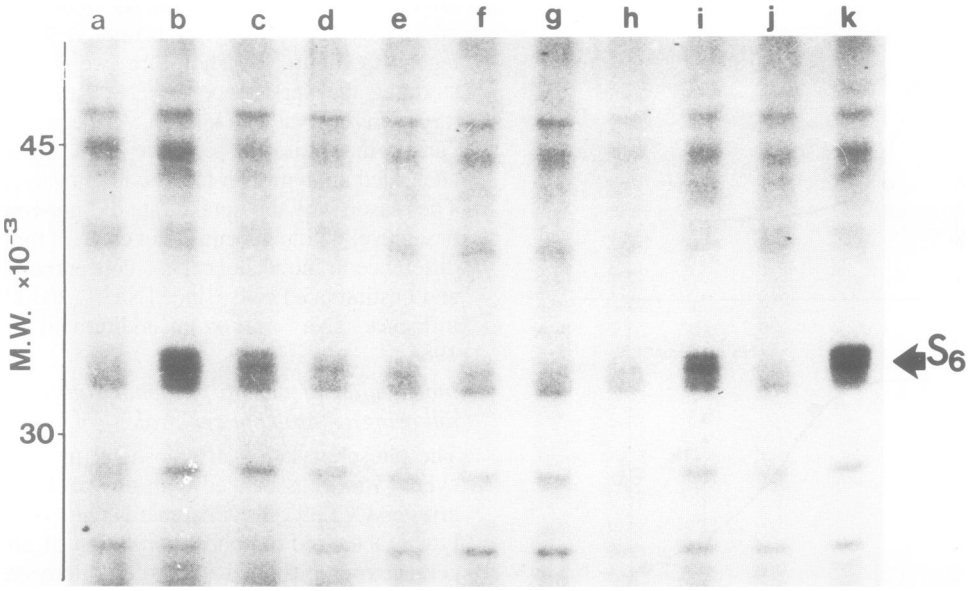

Fig. 4. Persistence of α-thrombin-stimulated ribosomal protein S6 phosphorylation. G0-arrested cultures of CCL39 cells in 12-well culture plates were incubated as follows. **Lane a:** (control) no addition, 30 min; **lane b:** α-thrombin 1 U/ml (0.36 μg/ml), 30 min; **lanes c – h:** α-thrombin 1 U/ml, 30 min, then hirudin (10 α-thrombin-inactivating units/ml) was added for: 5 min **(lane c)**, 10 min **(lane d)**, 15 min **(lane e)**, 30 min **(lane f)**, 45 min **(lane g)**, 60 min **(lane h). Lane i:** same conditions as in **lane h** (α-thrombin 30 min, hirudin 60 min) then the cells were washed with DMEM three times and α-thrombin 1 U/ml was added back for 30 min. **Lane j:** (control) no addition 90 min; **lane k:** 10% fetal calf serum 30 min, then hirudin (10 U/ml) was added for 60 min. At the end of the incubation period, cells were pulse-labeled with ^{32}P (500 μCi/ml, 10 min) and acid-soluble proteins were prepared for polyacrylamide gel electrophoresis as described in Materials and methods.

the addition of the α-thrombin inhibitor, PPACK, to cultures. As seen clearly by quantitation of normalized c-*myc* transcript levels (Figure 5B), after 30 min of α-thrombin stimulation, inactivation of the growth factor caused a rapid attenuation of c-*myc* mRNA induction. In PPACK-treated cultures maximal expression was prevented and c-*myc* transcript levels returned to the basal levels found in quiescent cells.

Discussion

A clear picture of the kinetics of activation and the persistence of mitogen-stimulated early events is necessary for elucidation of their molecular functioning and regulation during the mitogenic response. For such studies, the mitogen α-thrombin is particularly well suited since selective inhibitors exist which permit rapid and complete inactivation of the growth factor during the course of an experiment. The data we have presented here demonstrate that continual stimulation by α-thrombin of cells is required for persistent activation of four 'early' events of the mitogenic response. This finding sharply contrasts with the concept of 'competence' defined with platelet-derived growth factor (PDGF) in BALB/3T3 cells. Indeed, the salient feature of PDGF is induction of a long-term cellular memory termed 'competence' (Pledger *et al.*, 1977). This concept implies a rapid induction of a biochemical change relatively stable ($t_{1/2}$ 18 – 20 h) after a short exposure to growth factor (Singh *et al.*, 1983). Because α-thrombin and PDGF appear to initiate a common set of transmembrane signalling events, including rapid accumulation of c-*myc* mRNA, we think that the conceptual difference between the two growth factors reflects the difficulty in eliminating completely PDGF action after a brief exposure to cells.

Hydrolysis of phosphatidylinositol phosphates is one of the first detectable responses stimulated by α-thrombin in quiescent CCL39 cells. Our finding that the system subsides immediately following removal of the external stimulus from cells establishes a close association between the mitogen/cell interaction, and the breakdown of phosphatidylinositol.

This signal transduction system plays a central role in cellular activation processes. Phosphatidylinositol breakdown products, e.g., inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol, have been implicated as 'second messengers' capable of triggering a complex cascade of phosphorylation reactions *via* activation of calcium-dependent kinases (IP_3-mediated) and protein kinase C (DAG-mediated) (for reviews, see Berridge, 1984; Nishizuka, 1984). When this system subsides, what happens to other growth factor-stimulated events? Do stable intracellular messages exist?

Na^+/H^+ exchange

Arrest of Na^+/H^+ exchange occurs with no detectable lag following α-thrombin removal, similar to phosphoinositol lipid turnover. These results are consistent with reports suggesting that the Na^+/H^+ antiporter is set in motion through protein kinase C. Arguments in support of this model come from the demonstration that the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which binds to and directly activates protein kinase C (Castagna *et al.*, 1982), is capable of stimulating Na^+/H^+ exchange (Burns and Rozengurt, 1983; Moolenar *et al.*, 1984; Grinstein *et al.*, 1985). In CCL39 cells TPA has also been found to activate the Na^+/H^+ antiporter, yet more slowly and less efficiently than α-thrombin (S.Paris, unpublished observations) suggesting that stimulation of the exchanger by α-thrombin involves a more complex activation pathway; it remains to be elucidated to what extent this pathway is a consequence of phosphoinositide breakdown.

S6 phosphorylating system

The system responsible for phosphorylation of the most highly phosphorylated forms of S6 undergoes rapid inactivation following α-thrombin removal. Highly phosphorylated S6 derivatives

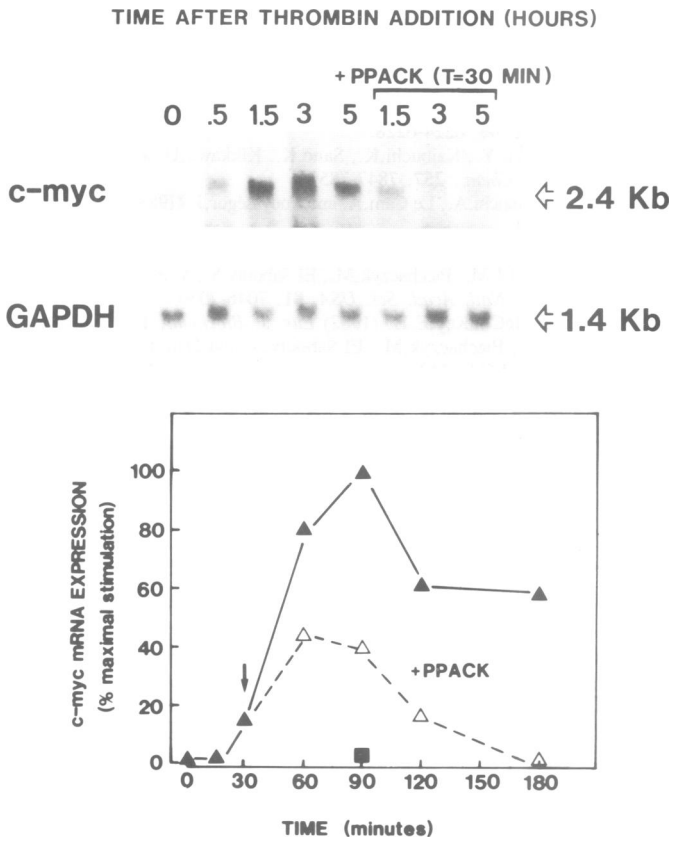

Fig. 5. Persistence of c-*myc* mRNA expression in α-thrombin-stimulated CCL39 cells. G0-arrested cultures were stimulated with 2 U/ml (0.72 μg/ml) α-thrombin for various times. After 30 min, the indicated cultures were rinsed once and received medium containing PPACK at a 10-fold excess of α-thrombin inactivating units. **(A)** Northern blots of the poly(A)$^+$ RNA from cultures were hybridized with probes specific for c-*myc* (above) or GAPDH (below) sequences as described in Materials and methods. **(B)** Results from a representative experiment were quantitated by densitometric scanning; % maximal c-*myc* mRNA levels (normalized against GAPDH) are expressed as a function of time with (△) or without (▲) PPACK addition after 30 min of α-thrombin exposure. Cultures which received PPACK at time 0 are shown (■).

are thought to be involved in protein synthesis activation since they have been found preferentially associated with newly formed polysomes after serum stimulation of quiescent cells (Thomas *et al.*, 1982; Duncan and McConKey, 1982). Our experiments do not allow us to determine whether the increase in S6 phosphorylation induced by α-thrombin can be accounted for by the stimulation of a kinase or by inhibition of a phosphatase. They do, however, emphasize the strict dependence of the activation system on some growth factor-regulated signalling event(s).

C-myc expression

The function of this cellular homolog to the avian retrovirus (MC29) oncogene is presently unknown. Nonetheless, the observations that mitogenic agents stimulate an increase in the expression of c-*myc* in quiescent fibroblasts and lymphocytes (Kelly *et al.*, 1983; Campisi *et al.*, 1984) have led to the proposal that the c-*myc* gene product may play an essential role in G0/G1 transit or, more precisely, in the transition from a G0-arrested state to a proliferative state (Thompson *et al.*, 1985).

Our findings that α-thrombin-stimulated induction of elevated c-*myc* mRNA levels in CCL39 cells can be reversed, as rapidly as it occurs, by neutralization of the growth factor would suggest the involvement of some growth factor-dependent event(s) with rapid turnover in the activation process. Accordingly, c-*myc* mRNA has been reported to be exceedingly unstable (half-life 15 min) in various cell types (Dani *et al.*, 1984). Recent studies to probe the regulatory mechanism of c-*myc* expression in CCL39 cells have revealed that the c-*myc* gene is constitutively transcribed at high rates in G0-arrested cells and that growth factors do not induce a significant change in this rate (Blanchard *et al.*, 1985). Altogether these data imply that c-*myc* expression is controlled by some finely-tuned post-transcriptional event(s) that affect the level of messenger degradation. Moreover, the putative regulatory event(s) is highly sensitive to growth factor regulation.

In conclusion, the molecular events described in these studies are four among many which make up the continuum of the pleiotypic response. It is clear that they are intimately associated with the signalling system triggered by α-thrombin at the cell surface. Their interdependency as well as their causal relation with G0→S transit remains to be clarified.

Materials and methods

Highly purified human α-thrombin (2660 N.I.H. Units/mg) was generously provided by Dr J.W.Fenton II (New York State Department of Health, Albany, NY). Bovine α-thrombin (≥300 N.I.H. Units/mg) and ouabain were purchased from Sigma (St. Louis, MO), and hirudin (~1333 α-thrombin inhibitory units per mg) from Diagnostica Stago (Asnières, France). Diethylamiloride was a gift from Dr E.Cragoe Jr. (Merck Sharp and Dohme Research Laboratory) and D-PPACK was from Dr E.Shaw (Brookhaven National Laboratory, Upton, NY).

[^{32}P]dCTP, ^{22}NaCl, Na^{125}I (carrier-free) and [^{32}P]orthophosphate (carrier-free) were products of the Radiochemical Centre (Amersham, France); [methyl-^{3}H]-thymidine [^{3}H]TdR, [7-^{14}C]benzoic acid and myo-[2-^{3}H(N)]inositol were from New England Nuclear, France.

Cell culture and DNA synthesis

The Chinese hamster fibroblast line CCL39 (American Type Culture Collection) was cultivated in Dulbecco's modified Eagle's medium (DME; Gibco) containing 5% fetal calf serum and antibiotics as previously described (Pouysségur *et al.*, 1982). For measurements of DNA synthesis re-initiation, cells were grown to confluence in 24-well culture plates then rendered quiescent by a 24-h incubation in serum-free medium (1:1 ratio of DME:Ham's F12). For the experiments described in this study, cultures received [^{3}H]TdR (4.5 μM at 1 μCi/ml) 5 h after growth factor addition; G0-arrested CCL39 cells begin to incorporate [^{3}H]TdR ~8 h post-stimulation (Van Obberghen-Schilling *et al.*, 1983). [^{3}H]TdR incorporation was terminated 24 h after mitogen addition and radioactivity incorporated into trichloroacetic acid (TCA)-precipitable material was assayed by liquid scintillation spectrometry.

[^{125}I]α-thrombin binding

Binding of biologically active [^{125}I]α-thrombin (50–70 μCi/μg) to confluent monolayers of CCL39 cells in 24-well culture plates (~10^6 cells/well) was performed as described (Van Obberghen-Schilling *et al.*, 1983). Incubations were carried out in the presence of 14 μg/ml bovine α-thrombin for determination of non-specific binding.

Measurement of phosphoinositide breakdown

Confluent cells in 35-mm dishes were arrested and labeled to equilibrium with [^{3}H]inositol (2 μCi/ml) for 20 h in serum-free DME medium. α-Thrombin and the inhibitor PPACK were added as indicated in the figure legend, without changing the medium. The accumulation of inositol phosphates was measured essentially as described by Bone *et al.* (1984). Briefly, 20 mM LiCl was added and after 10 min the cultures were washed twice with ice-cold phosphate-buffered saline (PBS) and extracted with 0.5 ml of 10% (w/v) $HClO_4$. The acid extract was neutralized with KOH and buffered with 50 mM Mops pH 7.0. $KClO_4$ was precipitated at 4°C for 30 min and eliminated by centrifugation. The supernatant was applied to a 0.6 ml column of Dowex 1 × 8, formate form. Free inositol was eluted with 5 ml of deionized water, and the inositol phosphate pool (inositol mono-, di- and tri-phosphates) with 5 ml of 0.1 M formic acid/1.5 M ammonium formate.

Measurement of intracellular pH

Intracellular pH was calculated from equilibrium distribution of [^{14}C]benzoic acid as previously described (L'Allemain *et al.*, 1984; Paris and Pouysségur, 1984). To measure the persistence of α-thrombin-induced cytoplasmic alkalinization, G0/G1-arrested cells were pre-incubated for 30 min with 2 U/ml α-thrombin in

an isotonic medium (130 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM glucose), buffered at pH 7.4 with 20 mM Hepes/Tris. Then the cultures were washed twice and received 1 ml of the same medium containing hirudin (10 α-thrombin-inactivating units/ml). After various times, [^{14}C]benzoic acid was added and pHi measured after 5 min.

Measurement of amiloride-sensitive $^{22}Na^+$ uptake

G0/G1-arrested cultures, in 35-mm dishes, were equilibrated for 15 min in a low Na^+ medium (2 mM NaCl, 128 mM choline Cl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 20 mM Hepes/Tris pH 7.4). Then α-thrombin was added (2 U/ml) and the cells further incubated for 30 min. Thereafter the medium was aspirated, the cultures were washed twice with the low Na^+ medium and received 1 ml of this medium, containing 10 α-thrombin-inactivating units/ml of hirudin. At varying times, the initial rate of amiloride-sensitive $^{22}Na^+$ uptake was assayed as follows: the cultures were washed twice with a K^+-free medium pH 7.4 (containing 2 mM Na^+ as above), and then incubated in 0.8 ml of the same medium, containing 1 mM ouabain and 2 μCi/ml of $^{22}Na^+$, with or without 50 μM diethylamiloride. After 3 min, $^{22}Na^+$ uptake was terminated by five washes with ice-cold 0.1 M $MgCl_2$. Cells were extracted with 1 ml of 5% TCA at 4°C, and radioactivity was measured in a γ-counter.

S6 phosphorylation

To determine the phosphorylated state of ribosomal protein S6, cell monolayers in 12-well multidishes were pulse-labeled by addition of 500 μCi/ml ^{32}P for 10 min and acid-soluble proteins were extracted according to Glover (1982). Subsequently, cultures were rinsed twice with ice-cold PBS and scraped from the dish in 0.2 ml H_2SO_4 (0.2 M) at 0°C. Wells were rinsed once with the same volume of acid. Extraction was carried out for 1 h on ice in 1.5 ml Eppendorf tubes after which time the precipitated material was pelleted by centrifugation (10 000 *g*; 10 min). The supernatant was supplemented with 10 μg bovine serum albumin and precipitated in the presence of 20% w/v TCA (1 h; 0°C). Following centrifugation (10 000 *g*; 10 min), the pellet was rinsed one time with 0.2% HCl in acetone, once with acetone then dried and solubilized in 40 μl lysis buffer (SDS 2%, Tris-HCl 10 mM, pH 6.8). Samples were heated at 95°C for 5 min in the presence of 0.1 M dithiothreitol, 10% glycerol and 0.01% bromophenol blue. SDS-PAGE was carried out in slab gels of 11% polyacrylamide according to Laemmli (1970). Mol. wt. standards shown are: carbonic anhydrase (mol. wt. 30 000) and ovalbumin (mol. wt. 45 000) (Bio-Rad).

Preparation of RNA and Northern hybridization analysis

Cells grown to confluence in 15-cm culture dishes were arrested by 24 h of serum deprivation. For each experimental point, cells from one dish ($\sim 2 \times 10^7$) were rinsed twice with PBS at room temperature before lysis with: 3 ml 5 M guanidinium isothiocyanate (Bethesda Research Laboratories), 50 mM Tris 10 mM EDTA, pH 7.6, 8% β-mercaptoethanol. Total cellular RNA was recovered from lysates by precipitation at 4°C (24–72 h) with 4 M LiCl, phenol-chloroform then chloroform extraction and ethanol precipitation. The yield was ~250 μg total RNA per dish. Poly(A)$^+$ RNA was isolated by affinity chromatography according to Bantle *et al.* (1976), denatured and separated on 1.2% formaldehyde-agarose gels. Transfer to nitrocellulose filters was performed according to Thomas (1980), followed by pre-hybridization for 4 h (68°C) in 5 × SSC, 5 × Denhart's solution, 100 μg/ml denatured salmon sperm DNA, 0.1% SDS. Filters were hybridized with $\sim 2 \times 10^6$ c.p.m. of the ^{32}P-labeled nick-translated probe indicated in the figure legend, washed at 60°C in 0.1 × SSC, 0.1 SDS and exposed to Kodak XAR film at −70°C. The plasmid DNA probes used in this study were: pSV c-*myc*-1 which carries a rearranged form of the mouse c-*myc* gene (Land *et al.*, 1983), and pRGAPDH 13 (Fort *et al.*, 1985) containing rat glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) cDNA.

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