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HIRUDIN, A PROBE TO ANALYZE THE GROWTH-PROMOTING ACTIVITY OF THROMBIN IN FIBROBLASTS ; REEVALUATION OF THE TEMPORAL ACTION OF COMPETENCE FACTORS

E. Van Obberghen-Schilling, R. Pérez-Rodriguez, and J. Pouysségur
Centre de Biochimie, CNRS, Parc Valrose, 06034 Nice, FRANCE

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Two classes of mitogens, competence and progression factors, function synergistically to reinitiate DNA synthesis of quiescent cells in culture. Competence factors, such as Platelet-Derived Growth Factor and Fibroblast Growth Factor, deliver their mitogenic signal after only brief exposure. In contrast, progression factors, including insulin and the Insulin-like Growth Factors, are required throughout the prereplicative phase. We now report that thrombin behaves as a competence factor in Chinese hamster fibroblasts. In particular, thrombin displays a persistent effect on DNA synthesis after transient exposure (3 hours). However, use of [^{125}I]thrombin reveals that despite removal of thrombin from culture medium following the brief exposure, a significant amount of thrombin remains associated with cells. If cell-associated thrombin is totally removed (after a 3 hour incubation) with a specific thrombin inhibitor, hirudin, subsequent mitogenic action is totally abolished. Therefore, we propose that "competence" factors must occupy their receptors for the entire G1 period (more than 8 hours) of G0-arrested cells to trigger the mitogenic response.

INTRODUCTION

It has been suggested that normal cell growth is modulated by two different classes of mitogenic factors present in serum which function synergistically to promote cell division (1-3). One class is referred to as competence factors (4): Platelet-Derived Growth Factor (PDGF)(5,6), Fibroblast Growth Factor (FGF)(7) ..., and the second, progression factors (4) : somatomedin C, Multiplication Stimulating Activity (MSA), insulin, ... These factors differ drastically in their mode of action ; whereas a brief exposure of quiescent cells to a competence factor (PDGF, FGF) is sufficient to trigger DNA synthesis, progression factors are continuously required to allow "competent" cells to progress through G_1 and into the S phase

ABBREVIATIONS
PDGF, Platelet-Derived Growth Factor ; FGF, Fibroblast Growth Factor ; MSA, Multiplication-Stimulating Activity ; TCA, trichloracetic acid ; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid ; BSA, bovine serum albumin.

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of the cell cycle. We now report the dual control of Chinese hamster fibroblast cell growth by thrombin and insulin. The very close similarity of biochemical and biological effects elicited by thrombin and PDGF : stimulation of ionic fluxes(8), increased phosphorylation of a common set of proteins (8), synergy with progression factors (insulin, MSA), and persistent effect on DNA synthesis after a brief exposure (3 hours), strongly suggests that thrombin belongs to the defined class of competence factors. However, the peculiar mode of action of "competence" factors raises the question whether or not these factors are removed from the cells after the brief exposure. So far a clear answer to this question has not been provided.

In this study we demonstrate that if cell-associated thrombin is totally removed (after a 3 hour-pulse) with hirudin, a specific thrombin inhibitor (9), subsequent mitogenic action is abolished. Therefore, we propose a reevaluation of the temporal mode of action of the so-called "competence" factors (PDGF, FGF, thrombin,...).

MATERIALS AND METHODS

Materials and cell culture. Highly purified human thrombin (> 90% electrophoretically pure, minimum 3,000 N.I.H. units/mg) and crystalline bovine insulin (23.6 I.U./mg) were purchased from Sigma, bovine thrombin (1316-1516 units/mg) from Miles Laboratories, and hirudin from Diagnostica Stago. PDGF was prepared in this laboratory to the purification step of CM-Sephadex (5,6). Bovine pituitary FGF (7) was a gift of Dr. Y. Courtois and MSA of Dr. M. Rechler. [methyl-^{3}H]thymidine and carrier-free [^{125}I]NaI were obtained from CEA France and Amersham, respectively. The cell line derived from Chinese hamster fibroblasts, CCl39 (American Type Culture Collection) was cultivated as previously described (10).

DNA synthesis reinitiation. Reinitiation of DNA synthesis, as measured by [^{3}H]thymidine incorporation or autoradiographic determination of labeled nuclei, was assayed as detailed previously (10). In brief, cells were arrested in G0/G1 after reaching confluency by replacing the growth medium with a serum-free 1:1 mixture of Dulbecco's Modified Eagle Medium (H21) and Ham's medium (F12), referred to as H21/F12. After 30 hours of serum deprivation, cells were stimulated to reinitiate DNA synthesis by replacing the spent H21/F12 with fresh H21/F12 containing [^{3}H]thymidine and the mitogen(s) to be tested for 24 hours. Labeled cells were rinsed and fixed with 5% TCA then either solubilized and radioactivity of lysates counted by liquid scintillation, or processed for autoradiography.

[^{125}I]thrombin binding. Human thrombin was iodinated using chloramine-T in the presence of benzamidine to protect the active site. [^{125}I]thrombin, prepared with a specific activity of 10 Ci/g, retained > 90% of its enzymatic activity. Binding experiments were performed on quiescent monolayers in 24-well (16-mm) plates. Prior to assay, cell cultures were washed twice with binding buffer (Dulbecco's Modified Eagle Medium containing 5 mM glucose, 25 mM Hepes, 5 mg/ml BSA, pH 7.6). Incubation with [^{125}I]thrombin (.01 U/ml) was carried out in a volume of 0.25 ml at 15°C ; afterwards, cells were washed 5 times at 0°C (twice with binding buffer and 3 times with phosphate-buffered saline), solubilized with 0.5 ml of 0.1 N NaOH and the lysates assayed in a gamma spectrometer at 80% efficiency.

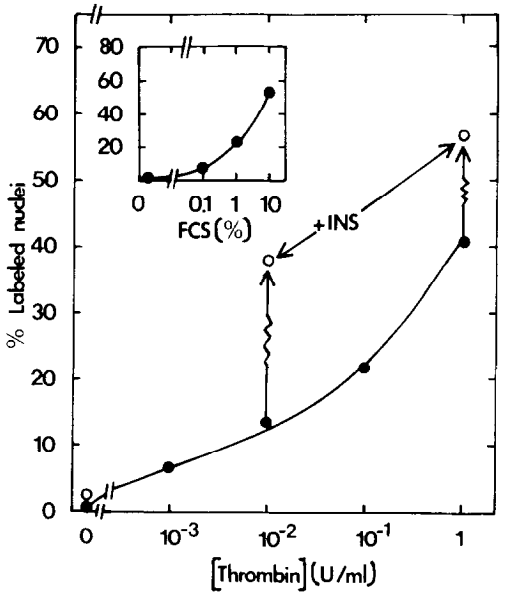

Figure 1: Synergistic Effect of Thrombin and Insulin on DNA Synthesis. Stimulation of DNA synthesis reinitiation in growth-arrested cells (30 hours of serum deprivation) was evaluated by determining the percentage of labeled nuclei following a 24 hour incubation with 5 μCi/ml [^{3}H]thymidine and varying concentrations of bovine thrombin added alone (●), or in the presence of insulin at 10 μg/ml (○). The inset shows the response of cells to fetal calf serum (FCS).

RESULTS

Highly purified human thrombin (E.C. 3.4.4.13) potently stimulates the reinitiation of DNA synthesis in quiescent cultures of Chinese hamster fibroblasts. Thirty hours of serum deprivation arrests these cells in G_0/G_1, following stimulation they enter the S phase after a lag of 8-10 hours (11). Reinitiation of DNA synthesis was measured by determining the fraction of labeled nuclei in cell populations exposed to increasing concentrations of thrombin and [^{3}H]thymidine for 24 hours (Fig.1). Maximal response to thrombin was observed with 1 U/ml (10 nM). Insulin at 10 μg/ml potentiated the thrombin-induced response, whereas insulin alone was not mitogenic (2% labeled nuclei). Thrombin plus insulin stimulated DNA replication as effectively as 10% fetal calf serum (FCS) (inset, Fig.1). Note that extremely elevated concentrations of insulin were required to enhance the mitogenic response, suggesting that insulin exerts this effect via a growth factor receptor (12). Accordingly, the Insulin-like Growth Factor, MSA (13), induced DNA synthesis synergistically in the presence of thrombin, as did insulin, but at a 10-fold lower concentration (unpublished results). Similarly to insulin, MSA alone was unable to stimulate

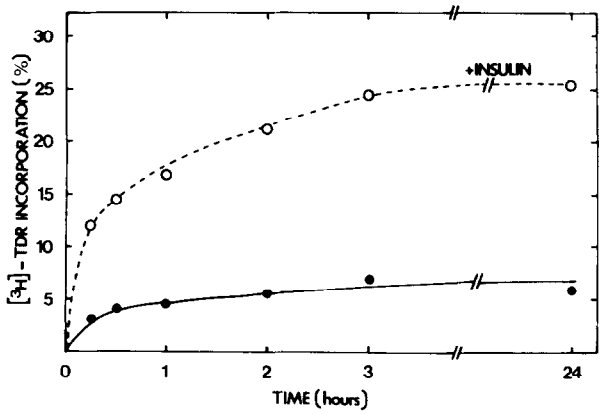

Figure 2: Effect of Transient Exposure to Thrombin on DNA Synthesis. Cells were arrested in G0/G1 as described in "Methods". Quiescent cells were incubated for the indicated period of time with human thrombin at a concentration of .01 U/ml. Cultures receiving thrombin for 0-3 hours were rinsed 5 times with serum-free medium immediately after the pulse and subsequently incubated for 24 hours in H21/F12 containing [^{3}H]thymidine (1 μCi/ml) in the absence (●) or in the presence (○) of insulin (10 μg/ml). Control cultures exposed to thrombin for 24 hours received [^{3}H]thymidine throughout the same 24-hour incubation period. DNA synthesis was determined by measuring the radioactivity incorporated into acid-precipitable material (10). Results are expressed as percent maximal stimulation of [^{3}H]thymidine incorporation relative to 10% FCS.

DNA synthesis. An excellent correlation was found between thrombin-stimulated [^{3}H]thymidine incorporation and its corresponding effect on cell proliferation.

This synergistic action of thrombin and insulin seen in hamster fibroblasts closely resembles that observed in Balb/c-3T3 cells with the competence factor, PDGF, and the progression factor, somatomedin C. The functional similarity between thrombin and the class of competence factors (PDGF, FGF) is further reinforced by the fact that all these factors, as opposed to progression factors, elicit common early biochemical responses : stimulation of Na^+ influx and increased phosphorylation of a common set of proteins (8). Finally, as expected for a competence factor, we found that thrombin exerts its mitogenic effect after only transient exposure. Incubating cells with thrombin for various periods of time (Fig.2) revealed that the thrombin effect occured quite rapidly, a one hour pulse (.01 U/ml) was already sufficient to elicit 65% of the maximal mitogenic response in the presence of insulin. No difference was observed between the stimulation of cultures exposed for only 3 hours to thrombin and parallel cultures exposed to thrombin for 24 hours. This could be taken as evidence in favor of the idea that the information ne-

cessary for cells to proceed to synthesize DNA is relayed within the first 3 hours of thrombin incubation.

One of the most exciting aspects of the "competence" concept is that competence factors deliver their mitogenic signal to arrested cells after only a brief exposure. However, this fundamental nature of mitogen action holds only if competence factors (usually "sticky peptides") are totally removed from cells after the short period of incubation. Thusfar, complete removal has been assumed to occur during washing procedures. Considering the importance of the completeness of mitogen removal in the definition itself of "competence", we used hirudin to remove specifically bound thrombin from its receptors on our cells. Hirudin, an inhibitor of thrombin (9), is a protein purified from leech pharyngeal glands which rapidly forms an essentially non-dissociable complex with thrombin at the active site of enzyme (Km : 0.8×10^{-10}). It has been demonstrated in platelets that hirudin blocks the binding of thrombin to specific sites and causes rapid dissociation of thrombin bound to these sites, thereby inhibiting thrombin-induced platelet stimulation(14). Using [^{125}I]labeled thrombin we were able to directly monitor the binding of thrombin to hamster fibroblasts and the effect of hirudin on the dissociation of the thrombin-receptor complex. Association of [^{125}I]thrombin (.01 U/ml) reached a maximal steady state after 2-3 hours (Fig.3). Hirudin enhanced the dissociation of receptor-bound thrombin from cells in a time-dependent fashion with complete dissociation of specifically bound thrombin within 2 hours. On the contrary, slow and incomplete ($\simeq$35%) dissociation of [^{125}I]thrombin was observed in the absence of hirudin after the normal washing procedure (5 times with serum-free medium).

Given these results, we reexamined the temporal action of thrombin on DNA synthesis reinitiation. Interestingly, we found that if hirudin was added to cells after a 3-hour thrombin pulse and allowed to remain until the end of the thymidine incorporation period, the thrombin-induced response was virtually abolished as well as the synergistic effect with insulin (Fig.4A). Furthermore, hirudin blocked the mitogenic response even when added as late as 8 hours after thrombin (data not shown). These findings indicate that brief exposure of cells to thrombin is not sufficient to cause activation of DNA synthesis, rather a persistent presence of the factor (at least 8h) is required. It is important to note that hirudin had no deleterious effect on the stimulation of DNA synthesis induced by growth factors unrelated to thrombin such as FGF or PDGF (Fig.4B),

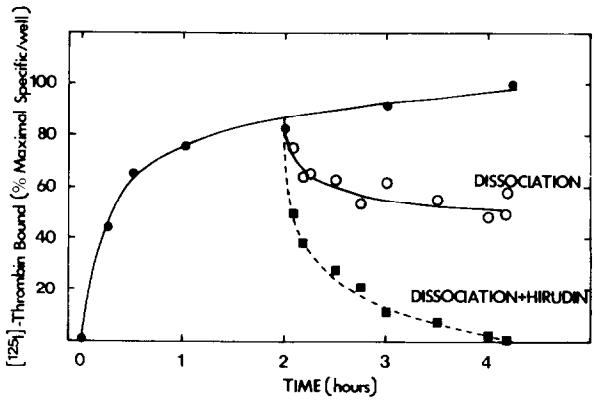

Figure 3 : Effect of Hirudin on Dissociation of [^{125}I]Thrombin. Cells were incubated with .01 U/ml of tracer at 15°C for the indicated periods of time. Following incubation, cultures were harvested as described in "Methods" and cell-associated radioactivity was determined. Percentage of maximal specific [^{125}I]thrombin bound per tissue culture well is plotted as a function of incubation time. Non-specific binding was determined in the presence of a large excess of thrombin (10 U/ml). After 2 hours of association parallel cultures were washed 5 times and the dissociation of [^{125}I]thrombin followed by measuring the residual specifically bound tracer in the absence (○) or presence (■) of a 100-fold excess of hirudin (1 thrombin-neutralizing unit/ml). Total [^{125}I]thrombin bound at equilibrium (4 hours) amounted to 11.9% of tracer added ; 80% of the total binding was specific.

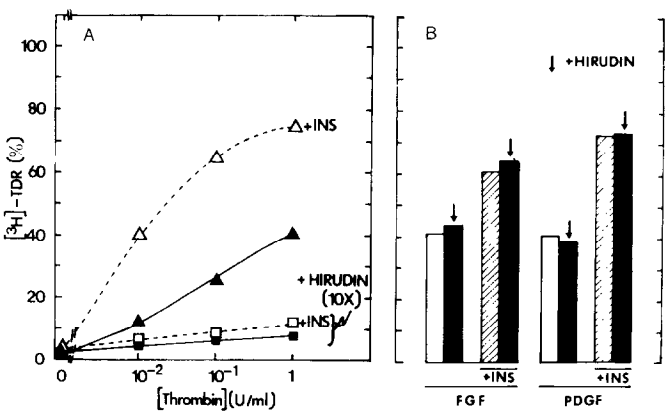

Figure 4 : Effect of Hirudin on Competence Factor-induced DNA Synthesis: Specific Inhibition of Thrombin Action.
A. Percentage of [^{3}H]thymidine incorporated during the 24-hour labeling period, relative to 10% FCS, is expressed as a function of the thrombin concentration present during a 3-hour thrombin preincubation. The data represent DNA synthesis of thrombin-treated cells labeled in serum-free medium plus : no addition (▲) ; insulin (10 µg/ml), (△) ; hirudin (10-fold excess), (■) ; or hirudin plus insulin, (□).

B. Fig.4B shows the extent of DNA synthesis stimulation, relative to 10% FCS, in cultures exposed to 100 ng/ml bovine pituitary FGF (left) or 240 µg/ml CM-Sephadex purified PDGF (right), alone (open bars) or in the presence of insulin at 10 µg/ml (striped bars). Solid bars represent stimulation when hirudin at 10 U/ml is present throughout the incubation in addition to the mitogen.

nor did preincubation of cells with hirudin alter subsequent responsiveness to thrombin.

DISCUSSION

The three factors, PDGF, FGF, and thrombin are all capable of reinitiating DNA synthesis of G_0/G_1-arrested CC139 cells, and in addition, share the following in common : a marked synergy with progression factors (insulin, MSA), activation of an amiloride sensitive Na^+ porter, and stimulation of the phosphorylation of a common set of polypeptides with apparent Mr 62,000, 33,000 and 27,000 (8). This similarity of action, which is not displayed by insulin or MSA, suggests that PDGF, FGF, and thrombin deliver identical signals. Clearly, the results of the present study demonstrate that persistent occupancy of the thrombin receptor is absolutely required for subsequent stimulation of DNA synthesis. In the light of these findings we propose that FGF and PDGF, likewise, mediate their mitogenic action through a continuous occupancy of their respective receptors. The recent elegant study of Smith and Stiles (15) does not rule out the possibility that a low concentration of PDGF persists after extensive washings of PDGF-treated cells.

On the basis of the present study we would like to propose that the so-called "competence" factors (PDGF, FGF, thrombin...) can exert their mitogenic action (either solely or in synergy with progression factors) only if they are maintained during the entire G_1 period of G_0-arrested cells. A similar conclusion was reached recently by Dicker and Rozengurt with regard to the mitogenic action of phorbol esters (16).

Our view of the temporal mode of action of competence factors which can reconcile contradictory reports, is that during the first 3-4 hours of reinitiation, a high concentration of these factors is required to bring about maximal activation of the cell population. Progression of "competent" cells through G_1 and into the S phase of the cell cycle requires, in addition to progression factors, continual presence of "competence" factors yet at a much lower concentration (not removed after washing). We are currently testing this hypothesis

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