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PERTUSSIS TOXIN INHIBITS THE ACTION OF INSULIN-LIKE GROWTH FACTOR-I

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Effect of pertussis toxin treatment on the actions of insulin-like growth factor-I (IGF-I) was examined in Balb/c 3T3 cells. In competent cells primed with epidermal growth factor, IGF-I stimulated both calcium influx and DNA synthesis. When these primed competent cells were treated with various concentrations of pertussis toxin for 2 hrs, IGF-I-induced calcium influx and DNA synthesis were inhibited in a similar dose dependent manner. The inhibitory action of pertussis toxin well coincided with the toxin-induced ADP-ribosylation of a 41 K-Da protein. These results suggest a possible involvement of a pertussis toxin-sensitive GTP-binding protein in the action of IGF-I. © 1987 Academic Press, Inc.

In density arrested Balb/c 3T3 cells, DNA synthesis is initiated by an addition of platelet-derived growth factor (PDGF) (1). PDGF renders quiescent cells to become 'competent' and competent cells are capable of progressing into S phase in response to growth factors in plasma (1). Stiles,

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Abbreviations: IGF, insulin-like growth factor; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; DME medium, Dulbecco's modified Eagle's medium containing 2.5 mM $NaHCO_3$ and 20 mM Hepes/NaOH (pH 7.4); CS, calf serum; PPP, platelet poor plasma, TCA, trichloroacetic acid: PBS, phosphate-buffered saline.

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Pledger and coleagues demonstrated that the major growth factor in plasma is dependent on pituitary (2). They showed that insulin-like growth factor-I (IGF-I) restores the ability of hypophysectomised rat plasma to stimulate DNA synthesis in PDGF-pretreated competent cells. Thus, IGF-I, in concert with an unknown factor in hypophysectomised plasma, promotes cell cycle progression in competent Balb/c 3T3 cells (3). Subsequent study has shown that epidermal growth factor (EGF) may be the unknown factor in hypophysectomised rat plasma (4). Thus, a combination of EGF and IGF-I reproduces the stimulatory effect of plasma on DNA synthesis in PDGF-treated competent cells. Despite its potent action on cell proliferation, the machanism of action of IGF-I is poorly understood. Like EGF and insulin, IGF-I receptor possesses a tyrosine specific protein kinase activity (5). It is, however, not clear whether this tyrosine specific protein kinase plays a key role in the promotion of cell proliferation.

Recently, Hildebrandt et al. (6) reported that pertussis toxin inhibits serum-induced stimulation of DNA synthesis in Swiss 3T3 cells. They suggest that a pertussis toxin-sensitive GTP-binding protein may be involved in the action of growth factors in serum. Since IGF-I is one of major growth factors in serum, we examined whether pertussis toxin inhibits a growth promoting action of IGF-I. Results obtained in the present study indicate that pertussis toxin blocks IGF-I-mediated calcium influx and DNA synthesis and suggest an involvement of pertussis toxin-sensitive GTP-binding protein in the action of IGF-I.

Materials and Methods

Cell Culture

Balb/c 3T3 cells (clone A31) were cultured in Dulbecco's modified Eagle's medium containing 2.5 mM $NaHCO_3$ and 20 mM Hepes/NaOH (pH 7.4) medium (DME medium) supplemented with 10% calf serum (CS). Quiescent cells were obtained as described previously (7). In short, cells were cultured at a split ratio of 1 : 5 and incubated in DME medium containing 10 % CS without renewing medium. Cells were further incubated for 24 hrs in DME medium containing 5% platelet poor plasma (PPP) to comfirm quiescency. To obtain primed competent cells, quiescent cells were incubated for 3 hrs in DME medium containing 20 U/ml PDGF. Cells were washed twice with DME medium and were incubated with DME medium containing 10 nM EGF for 20 min. Cells were then washed with DME medium for three times.

DNA synthesis

DNA synthesis was assessed by measuring [^{3}H]-thymidine incorporation into TCA-precipitable materials. Primed competent cells were obtained in a 24-well plate as described above. After an appropriate pretreatment, cells were incubated in DME medium containing 1 nM IGF-I and 1 uCi/ml [^{3}H]thymidine for 24 hrs. Radioactivity in TCA-precipitable materials was measured as described by McNiel et al. (8).

Measurement of Calcium Influx Rate

Unidirectional calcium influx rate was determined by measuring an initial uptake of radiocalcium as described previously (7). In short, primed competent cells grown in a 24-well plate were incubated for ten min in medium with or without IGF-I. Then cells were incubated for 15, 30 or 75 sec in medium containing 5 uCi/ml [^{45}Ca] $CaCl_2$ in the presence or absence of IGF-I and calcium uptake was measured. Calcium influx rate was calculated by using a linear regression line of calcium uptake.

ADP-ribosylation

Quiescent cells were washed with calcium-free PBS and were scraped off by using a rubber policeman. Cells were centrifuged at 300 x g for 5 min and the supernatant was discarded. Cells were resuspended in a cold hypotonic solution containing 25 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol and 50 units/ml aprotinin. Cells were then homogenized with a teflon-glass homogenizer and the homogenate was then centrifuged at 10000 x g for 3 min. The supernatant was aspirated and the pellet was sonicated for 20 sec in 100 mM potassium phosphate buffer (pH 7.5) containing 5 mM $MgCl_2$ and 50 units/ml aprotinin. The sonicated membrane suspensions (100-200 ug of protein) were then incubated with 10 uM [α-^{32}P]NAD and 20 ug/ml pertussis toxin at 30° C for 20 min in a 100 ul solution of 100 mM potassium phosphate buffer (pH 7.5) containing 2.5 mM $MgCl_2$ and 1 mM ATP (9). Proteins were then separated by SDS-polyacrylamide gel eletrophoresis (SDS-PAGE) according to the method by Laemmili (10).

For measurement of ADP-ribosylation in intact cells, primed competent Balb/c 3T3 cells were treated for indicated time with DME medium containing 0.1 ng/ml pertussis toxin. Cells were then washed, detached and homogenenized.[32] The sonicated membrane suspension was treated with [^{32}P]-NAD and pertussis toxin as described above.

Materials

Synthetic IGF-I was provided by Fujisawa Pharmatheutical Co. (Osaka, Japan). Pertussis toxin was obtained from Funakoshi (Kyoto, Japan). EGF was purchased from Collaborative Research (Lexington, MA,) Partially purified PDGF was prepared from out-dated human platelet rich plasma employing CM-Sephadex chromatography and Blue Sepharose chromatography (11). Platelet poor plasma (PPP) was prepared using human platelet poor plasma by the method of Pledger et al. (1).

Results and Discussion

In PDGF-pretreated 'competent' Balb/c 3T3 cells, DNA synthesis is initiated after 12 hr-treatment with PPP (1). Leof et al. (4) showed that a combination of EGF and IGF-I fully reproduces the progression activity of PPP. They also showed that EGF is required in an early half of 12 hrs while IGF-I should exist continuously throughout 12 hrs (4). We extended their observations in that only 20 min treatment with EGF is required prior to IGF-I (Figure I). When PDGF-pretreated competent cells were incubated with 1 nM IGF-I alone for 24 hrs, DNA synthesis was barely stimulated. However, when competent cells were treated with 10 nM EGF for 20 min before the addition of IGF-I, subsequent incubation with 1 nM IGF-I for 24 hrs resulted in a six-fold increase in [^{3}H]thymidine incorporation. Without IGF-I, a 20 min treatment with EGF did not affect [^{3}H]thymidine incorporation in subsequent 24 hrs. Thus, the action of IGF-I on DNA synthesis required priming action of EGF. It should be noted that, despite a brief treatment, the priming

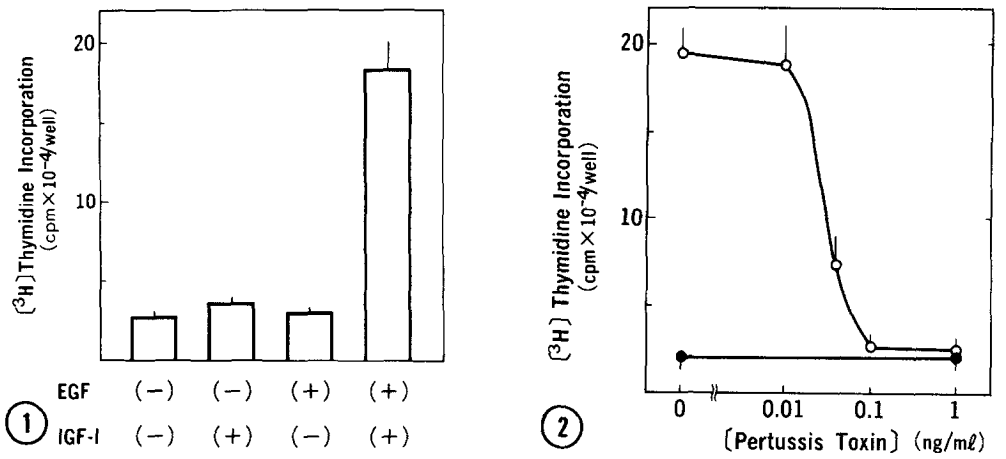

Figure 1. Effect of EGF pretreatment on IGF-I-stimulated DNA synthesis in competent Balb/c 3T3 cells.

PDGF-pretreated competent cells were incubated for 20 min with or without 10 nM EGF. Cells were washed with DME medium for three times and then incubated for 24 hrs in DME medium containing [^{3}H]thymidine in the presence or absence of IGF-I. Values are the mean ± S.E. for four determinations.

Figure 2. Dose response curve for pertussis toxin action on IGF-I-mediated DNA synthesis.

Primed competent cells were incubated with DME medium containing various doses of pertussis toxin or saline for 2 hrs. Cells were then washed two times with DME medium and were then incubated for 24 hrs in DME medium containing [^{3}H]thymidine in the presence (○) or absence (●) of 1 nM IGF-I. Values are the mean ± S. E. for three determinations.

action of EGF remained effective for at least 2 hrs even if EGF is removed (see Figure 2).

To determine the effect of pertussis toxin treatment on IGF-I action, we incubated competent Balb/c 3T3 cells primed with EGF ('primed competent' cells) in DME medium containing various doses of pertussis toxin for 2 hrs. After the removal of pertussis toxin, cells were incubated with 1 nM IGF-I for 24 hrs. As shown in Figure 2, pertussis toxin inhibited IGF-I-mediated DNA synthesis in a dose dependent manner. Effect of pertussis toxin was

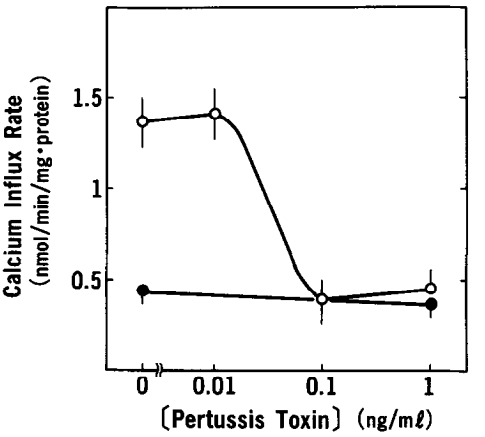

Figure 3. Dose response curve for pertussis toxin action on IGF-I-induced calcium influx

Primed competent cells were incubated for 2 hrs in DME medium containing various doses of pertussis toxin or saline. Cells were then washed two times with DME medium. Calcium influx rate was measured 10 min after the addition of 1 nM IGF-I as described in Methods. Values are the mean ± S.E. for three determinations.

observed at a concentration as low as 0.05 ng/ml. At 0.1 ng/ml, pertussis toxin inhibited IGF-I-mediated DNA synthesis almost completely and higher doses of pertussis toxin did not further reduce the action of IGF-I.

We have recently found that IGF-I causes a slow but sustained increase in calcium influx rate in primed competent Balb/c 3T3 cells (Kojima, I., Nishimoto, I. and Ogata, E., submitted for publication). We suggested that calcium influx may be a mitogenic message of IGF-I. In the next set of experiment, we tested whether pertussis toxin attenuates IGF-I-induced calcium influx in primed competent cells. As shown in Figure 3, treatment of primed competent cells with 0.1 ng/ml pertussis toxin almost completely inhibited subsequent IGF-I-induced calcium influx. By contrast, 0.01 ng/ml pertussis toxin had little effect. Dose

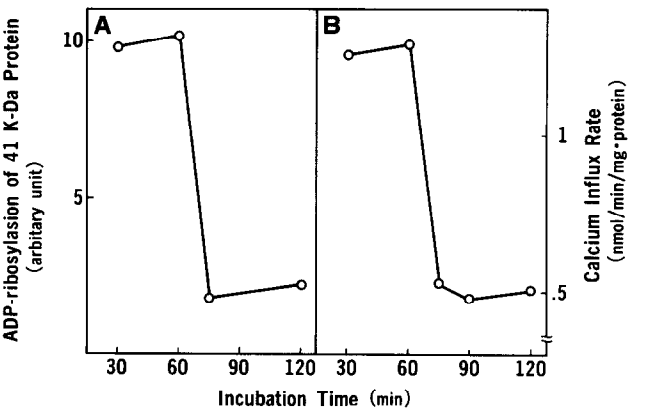

Figure 4. Time course of effect of pertussis toxin on ADP-ribosylation of the 41 K-Da protein and IGF-I-induced calcium influx

Primed competent cells were incubated in DME medium containing 10 ng/ml pertussis toxin for indicated time. Cells were then washed twice with DME medium and ADP-ribosylation of the 41 K-Da protein (A) and IGF-I-induced calcium influx (B) were measured. For measurement of ADP-ribosylation, cells were homogenized and the sonicated membrane was incubated in vitro with excess amount of pertussis toxin and [^{32}P]NAD. The radioactivity in the 41 K-Da protein was counted.

response relationship for pertussis toxin-induced inhibition of calcium influx well agrees with toxin-induced inhibition of [^{3}H]thymidine incorporation.

It is accepted that most, but not all, of the action of pertussis toxin is exerted through ADP-ribosylation of α-subunit of GTP binding proteins (12). We have recently shown that pertussis toxin ADP-ribosylates a 41 K-Da protein in Balb/c 3T3 cell membrane (7). When ADP-ribosylation is studied in intact cells, 0.01 ng/ml pertussis toxin does not significantly ADP-ribosylate the 41 K-Da protein while 0.1 ng/ml toxin ADP-ribosylates the 41 K-Da protein almost completely (7). Therefore, doses of pertussis toxin which causes ADP-ribosylation coincide well with those to inhibit IGF-I actions. Figure 4A demonstrates the time course of pertussis toxin-induced ADP-

ribosylation of the 41 K-Da protein in primed competent cells. It takes approximately 75 min for the toxin to ADP-ribosylate the 41 K-Da protein. In accordance with this, pertussis toxin inhibited IGF-I-induced calcium influx when cells were incubated with the toxin for longer than 75 min. Thus, there is a good correlation between the toxin-induced ADP-ribosylation and inhibition of IGF-I action. These results suggest that the 41 K-Da protein may be critical in the action of IGF-I.

To date, an involvement of a GTP-binding protein in the action of IGF-I has not been demonstrated. Tell et al., however, showed that IGF-I inhibits basal as well as agonist-stimulated adenylate cyclase activity in fat cells, liver cells, lymphocytes and chondrocytes (13). These observation suggest a possibility that G_i may be coupled to IGF-I receptor (12). Alternately, a GTP-binding protein different from G_i is coupled to IGF-I receptor and binding of IGF-I to the receptor leads to the liberation of $\beta\delta$-subunit which in turn inhibits adenylate cyclase. Our present results together with the observations by Tell et al. (13) suggest that a pertussis toxin-sensitive G protein, possibly G_i, is coupled to the type 1 IGF receptor. Furthermore, the fact that pertussis toxin inhibits both IGF-I-induced calcium influx and DNA synthesis supports a critical role of calcium influx in the mitogenic action of IGF-I.

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