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EVIDENCE THAT TYPE II INSULIN-LIKE GROWTH FACTOR RECEPTOR IS COUPLED TO CALCIUM GATING SYSTEM

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In competent Balb/c 3T3 cells primed with epidermal growth factor (primed competent cells), insulin-like growth factor-II (IGF-II) stimulated calcium influx in a concentration dependent manner with the ED_{50} of 450 pM. When receptor-bound [^{125}I]IGF-II was cross-linked by use of disuccinimidyl suberate, a 240 K-Da protein was radiolabeled. Excess amount of unlabeled IGF-II inhibited the affinity-labeling of the 240 K-Da protein. To further examine whether IGF-II stimulates calcium influx by acting on the type II IGF receptor, we employed polyclonal antibody raised against rat type II IGF receptor, R-II-PAB1. This antibody immunoprecipitated the type II IGF receptor and inhibited IGF-II binding in Balb/c 3T3 cell membrane without affecting IGF-I binding. In primed competent cells, R-II-PAB1 elicited an agonistic action in stimulating [^{3}H]thymidine incorporation. Under the same condition, R-II-PAB1 elicited a marked stimulation of calcium influx. These results suggest that, in Balb/c 3T3 cells, 1) relatively low concentrations of IGF-II act mainly on the type II IGF receptor; 2) the type II IGF receptor is coupled to a calcium gating system; and 3) binding of a ligand to the type II IGF receptor leads to the stimulation of DNA synthesis.

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Abbreviation: IGF, insulin-like growth factor; EGF, epidermal growth factor; DME, Dulbecco's modified Eagle's medium containing 20 mM Hepes/NaOH (pH 7.4); Hepes, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid; PDGF, platelet-derived growth factor; EDTA, ethylenediamine tetraenoic acid; SDS, sodium dodecyl sulfate; DSS, disuccinimidyl suberate.

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Insulin-like growth factors (IGFs) are potent mitogens in mammalian cells (1). At least two types of receptors for IGFs are identified (2, 3). The type I receptor resembles the insulin receptor in its structure. It is a heterotetramer of $(\alpha\beta)_2$ form and tyrosine-specific protein kinase activity localizes in its β-subunit. Unlike the type I receptor, the type II receptor is a single polypeptide with molecular weight of 250 K-Da and does not possess tyrosine specific kinase activity (4). The type II receptor has relatively high affinity for IGF-II and IGF-I binds to this receptor with lower affinity. However, the type II receptor has little or no affinity for insulin. Despite the fact that IGF-II is a potent mitogen, recent studies have cast doubt on the functional role of type II IGF receptor in stimulating cell growth. For instance, in H-35 hepatoma cells, IGF-II promotes cell proliferation by acting on the insulin but not type II IGF receptor (5, 6). Likewise, in human fibroblasts, IGF-II exerts its action by acting on the type I receptor (7). Thus, the function of type II IGF receptor remains to be elucidated.

We have recently shown that IGF-II stimulates calcium influx and DNA synthesis in competent Balb/c 3T3 cells primed with epidermal growth factor (EGF) (primed competent cells) (8, 9). The action of IGF-II is reproduced by high concentration of IGF-I but not by insulin, suggesting that IGF-II acts mainly on the type II receptor. Hence, Balb/c 3T3 cells may provide a good system to study the function of the type II IGF receptor. The present study was conducted to examine whether binding of a ligand to the type II IGF receptor leads to the stimulation of calcium gating and DNA synthesis in Balb/c 3T3 cells. For this purpose, we employed an antibody raised against rat type II IGF receptor, R-II-PAB1 (10). The results indicate that the type II IGF receptor is coupled to a calcium gating system in Balb/c 3T3 cells.

Experimental Procedures

Cell Culture

Balb/c 3T3 cells (clone A31) were grown in Dulbecco's modified Eagle's medium containing 20 mM Hepes/NaOH (pH 7.4) (DME) under humidified conditions at 37°C. Quiescent cells were prepared as described previously (8). To obtain IGF-responsive primed competent cells, quiescent cells were incubated for 3 hrs in DME containing 20 U/ml platelet-derived growth factor (PDGF). Cells were then washed two times with DME and incubated for 20 min with DME containing 10 nM EGF, after which time cells were washed twice with DME.

Binding of [^{125}I] IGF-I and II

IGF-I and II were labeled with [^{125}I] by chrolamine T method to the specific acitivity of 880 and 600 μ Ci/nmol, respectively. Binding study was done as described by Lee et al. (11). Aliquots containing 20 ug membrane protein were incubated with 200 pM iodinated IGF-I or II, in the presence and absence of unlabeled IGF at 4 C for 15 hrs. The membrane was washed with several volumes of ice-cold buffer and bound radioactivity was measured. Nonspecific binding was determined in the presence of 1 μM unlabeled IGF.

Affinity Cross-linking and Immunoprecipitation of IGF Receptor

IGF receptors in Balb/c 3T3 cells were cross-linked to [^{125}I]IGF-II by using DSS (12). Cells were detached and suspended in the binding buffer. An aliquot of cell suspension containing 5 x 10^5 cells in 0.5 ml was incubated for 2 hrs at 10 °C in a siliconized glass tube with 1 nM [^{125}I]IGF-II in the presence or absence of unlabelled IGF-II. Cells were then washed twice with ice-cold binding buffer and were resuspended in 0.5 ml of binding buffer containing 250 uM DSS. After 15 min incubation at 4 C, the reaction was stopped by diluting with ice-cold buffer containing 250 mM sucrose, 10 mM Tris/HCl (pH 7.5) and 1 mM EDTA. Cells were centrifuged and resuspended in 10 mM Tris/HCl (pH 7.0) buffer containing 1% Triton X-100, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. After 60 min incubation at 4°C, the detergent insoluble material was removed by centrifugation at 30000 x g for 20 min. For immunoprecipitation, cells were washed once with phosphate-buffered saline and homogenized by Dounce homogenizer in solution S containing 5 mM NaCl, 5 mM Tris/HCl (pH 7.5), 5 mM $MgCl_2$ and 2 mM dithiothreitol. The homogenate was centrifuged at 400 x g for 10 min and the supernatant was centrifuged at 10000 x g for 60 min. The pellet was solubulized by incubating at 4°C for 40 min in soluation S containing 1 % Triton X-100. The solution was centrifuged at 100000 x g for 60 min. The solubilized membrane was incubated for 4 hrs in solution containing 20 mM Tris/HCl (pH 7.5), 60 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS and 50 ug/ml anti-type II receptor antibody on ice after which time 10 volumes of 50 % Protein A-Sepharose CL4B (Pharmacia, Uppsila, Sweden) were added. After extensive washes with buffer S, samples were diluted with four volume of sample buffer containing 25 mM Tris/HCl (pH 6.8) 10% glycerol, 1% SDS and 0.1% -mercaptoethanol and boiled. Proteins were separated by polyacrylamide gel electrophresis according to the method by Laemmli (13). Autoradiography was performed by using Kodak XO-Mat film.

Measurement of Calcium Influx

Primed competent cells were obtained in a 24-well dish by incubating quiescent cells sequentially with PDGF and EGF. Unidirectional calcium influx rate was determined by measuring initial uptake of [^{45}Ca] as described previously (9).

Measurement of DNA Synthesis

DNA synthesis was assessed by measuring [^{3}H]thymidine incorporation into trichloroacetate-precipitable materials. Primed competent cells were incubated for 24 hrs in DME containing 1 μCi/ml [^{3}H]thymidine and either IGF-II or antibody. [^{3}H]Thymidine incorporation was measured as described by McNiel et al. (14).

Materials

[^{45}Ca]$CaCl_2$, [^{125}I]NaI and [^{3}H]thymidine were purchased from New England Nuclear (Boston, MA). DSS was obtained from Pierce Chemical (Rockford, IL). Recombinant IGF-I was supplied by Fujisawa Pharmaceutical (Osaka, Japan). Multiplication stimulating activity, the rat IGF-II, was kindly provided by Dr. Peter Nissley of National Institute of Health (Bethesda, MD). Partially purified PDGF was prepared as described by Hasegawa-Sasaki (15). EGF was obtained from Collabrative Reaserch (Lexington, MA). Platelet poor plasma was prepared as described by Pledger et al. (16). Polyclonal anti-rat type II IGF receptor antibody, R-II-PAB1, was prepared as described previously (10) and Ig-G fraction obtained by ammonium sulfate precipitation was used in the present study. Preimmune serum was treated in the same way.

Results

Effect of IGF-II on Calcium Influx

We have recently reported that IGF-II stimulates calcium influx in primed competent Balb/c 3T3 cells (9). Figure 1 depicts the dose response relationship for IGF-II-induced calcium influx. IGF-II action was detected at 100 pM and was saturated at 1 nM. The ED_{50} was 450 pM. By contrast, approximately 50 times more IGF-I was needed to stimulate calcium influx in this condition (9).

Affinity Cross-linking of IGF Receptors

In an attempt to determine the type of receptor to which IGF-II binds, we affinity-labeled cellular receptors for IGF-II by cross-linking intact cells with cell-bound [^{125}]IGF-II using the homobifunctional agent, disuccinimidyl suberate (DSS). Figure 2 demonstrates a typical autoradiogram from SDS polyacrylamide electrophoresis gel of Balb/c 3T3 cells cross-linked to [^{125}I]IGF-II. Cells were sequentially incubated in the presence of 1 nM [^{125}I]IGF-II and DSS. The cross-linked cells were solubilized by Triton X-100 and the detergent-solubilized

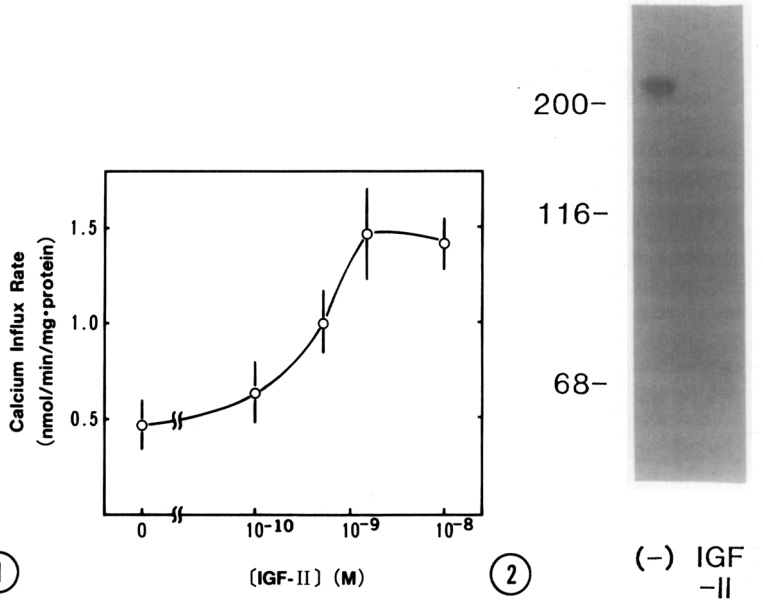

Fig. 1 Dose Response Relationship of IGF-II Action on Calcium Influx in Primed Competent Cells.
Primed competent cells were stimulated by varying concentrations of IGF-II. Calcium influx rate was determined as described in Methods. Values are the mean ± S. E. for four determinations.

Fig. 2 Affinity Cross-linking of Type II IGF Receptor
Balb/c 3T3 cells were incubated with 1nM [^{125}I]IGF-II in the absence and presence of 1 μM unlabeled IGF-II. Cells were washed and were incubated for 15 min with 0.25 mM DSS. Cells were then solubilized by 1 % Triton X-100 and the solubilized material was subjected to SDS polyacrylamide gel electrophoresis in the presence of 50 mM dithiothreitol followed by autoradiography.

material was subjected to electrophoresis followed by autoradiography. A 240 K-Da protein was specifically labeled with [^{125}I]IGF-II. Excess amount of unlabeled IGF-II almost completely inhibited the labeling of the 240 K-Da protein. Furthermore, [^{125}I]IGF-II, at least in this condition, did not label a 130 K-Da protein, α-subnit of the type I receptor.

Effect of Anti-type II Receptor Antibody

Anti-rat type II IGF receptor antibody, R-II-PABl, was previously shown to immunoprecipitate type II IGF receptor and to inhibit the binding of [^{125}I]IGF-II in rat 18, 54-SF cells (10). We examined whether R-II-PABl immunoprecipitates type II IGF receptors in Balb/c 3T3 cells. The IGF receptors of Balb/c 3T3

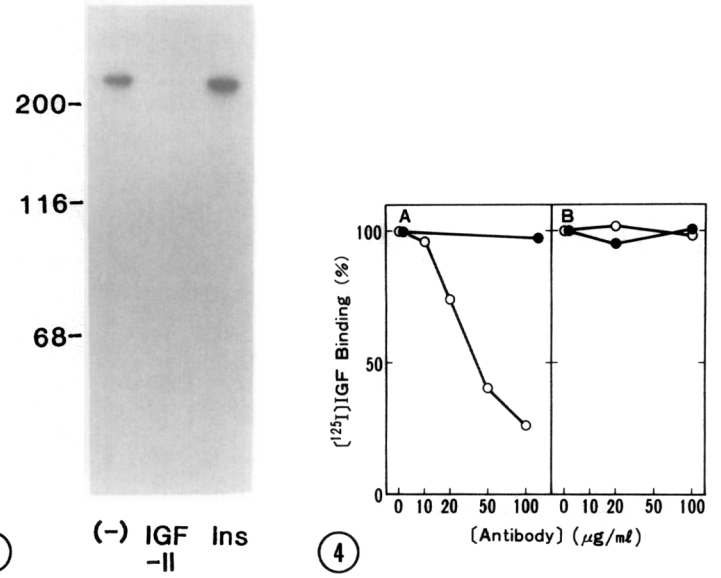

Fig. 3 Immunoprecipitation of Type II IGF Receptor
[^{125}I]IGF-II was cross-liked by use of DSS as described in Methods. Balb/c 3T3 cells were then solubilized by Triton X-100, and immunoprecipitated by anti-type II IGF receptor antibody in the presence and absence of 1 μM IGF-II or insulin, as indicated.

Fig 4. Effect of Anti-type II IGF Receptor Antibody on Binding of IGF-I and IGF-II
Binding of [^{125}I]IGF-II (A) and [^{125}I] IGF-I (B) were determined in the presence of various concentrations of anti-type II IGF receptor antibody (○) or preimmune serum (●). Values are the mean of three determinations.

membranes were cross-linked to [^{125}I]IGF-II by using DSS. The crude membrane fraction was solubilized and immunoprecipitated with R-II-PABl. As shown in Figure 3, a 240 K-Da protein was immunoprecipitated. When solubilized membrane was incubated with R-II-PABl in the presence of 1 μM IGF-II, the 240 K-Da protein was not immunoprecipitated. In contrast, 1 μM insulin did not affect the immunoprecipitation of the 240 K-Da protein. It should be noted that R-II-PABl did not immunoprecipitate the 130 KDa protein cross-linked to [^{125}I]IGF-I (data not shown). In the next set of experiments, we examined whether R-II-PABl inhibits [^{125}I]IGF-II binding in mouse Balb/c 3T3 cell membrane. Figure 4A demonstrates binding of [^{125}I]IGF-II in the presence and absence of R-II-PABl. The antibody inhibited binding of

[^{125}I]IGF-II in a concentration dependent manner. At 100 μg/ml, R-II-PABl inhibited [^{125}I]IGF-II binding by approximately 70%, which is as potent as 10 nM IGF-II (9). The potecy of R-II-PABl in displacing [^{125}I]-IGF-II in Balb/c 3T3 cells was nearly identical to that observed in rat 18, 54 SF cells (10). Ig-G prepared from preimmune serum did not block the binding of [^{125}]IGF-II. In contrast, R-II-PABl did not affect the binding of [^{125}I]IGF-I (Fig 4B).

To determine the action of R-II-PABl, we examined the effect of the antibody on [^{3}H]thymidine incorporation. As reported previously (8), Balb/c 3T3 cells become responsive to IGF-II when quiescent cells are pretreated sequentially with PDGF and EGF. In these primed competent cells, R-II-PABl stimulated [^{3}H]thymidine incorporation in a concentration dependent manner (Figure 5A). At 100 μg/ml, the antibody caused ten-fold

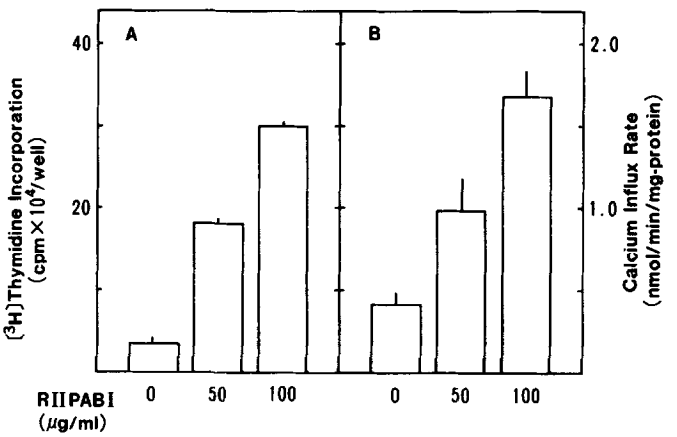

Fig 5. Effect of Anti-type II IGF Receptor Antibody on DNA Synthesis and Calcium Influx in Primed Competent Balb/c 3T3 Cells.

Cells were rendered primed competent by sequential treatment with PDGF and EGF as described in Methods. [^{3}H]Thymidine incorporation (A) and calcium influx rate (B) were measured in primed competent cells in the presence of various concentration of R-II-PABl. Values are the mean ± S.E. for four determinations. Note that [^{3}H]thymidine incorporation and calcium influx rate in the presence of 100 μg/ml IgG fraction of preimmune serum were 25341 ± 2398 cpm/well and 0.48 ± 0.15 nmol/min/mg protein, respectively.

stimulation of [^{3}H]thymidine incorporation. When 100 μg/ml R-II-PAB1 and 1 nM IGF-II were added simultaneously, there was no further stimulation of [^{3}H]thymidine incorporation (data not shown). The Ig G fraction of preimmune serum did not affect [^{3}H]thymidine incorporation in primed competent cells. Thus, the anti-type II receptor antibody R-II-PAB1 has an agonistic action in Balb/c 3T3 cells.

Employing this antibody, we examined whether activation of type II IGF receptor results in a stimulation of calcium influx. As demonstrated in Figure 5B, 50 μg/ml R-II-PAB1 caused approximately 2.5-fold increases in calcium influx rate. At 100 ug/ml, R-II-PAB1 induced approximately four-fold increase in calcium influx rate. The antibody-induced calcium influx resembled, in many aspects, that induced by IGF-II. First, 2.5-fold stimulation of calcium influx was observed in primed competent cells but not in either quiescent or competent cells. Second, antibody-induced calcium influx was sensitive to tetramethrin (data not shown), a compound which blocked IGF-II-mediated calcium influx (9). It is noteworthy that tetramethrin also blocked R-II-PAB1-induced [^{3}H]thymidine incorporation. Third, antibody-induced calcium influx was not affected by nitrendipine (data not shown), which does not inhibit IGF-II-induced calcium influx (9).

Discussion

In Balb/c 3T3 cells, relatively low concentrations of IGF-II stimulate calcium influx while approximately 50 times more IGF-I is needed to reproduce the action of IGF-II. Furthermore, insulin is essentially without effect (9). These observations suggest that IGF-II acts on the type II IGF receptor. Consistent with this notion, a 240 K-Da protein is specifically radiolabeled when cellular IGF receptors are affinity cross-linked with 1 nM

[^{125}I]IGF-II by using DSS. Thus, it is reasonable to conclude that relatively low concentrations of IGF-II stimulate calcium influx by acting mainly on the type II IGF receptor.

Further support for the idea that type II IGF receptor is coupled to a calcium gating system comes from experiments using anti-type II IGF receptor antibody. As shown in Figure 3, the anti-rat type II receptor antibody R-II-RAB1 immunoprecipitates a 240 K-Da protein affinity-labeled with [^{125}I]IGF-II. That unlabeled IGF-II, but not insulin, inhibits the immunoprecipitation establishes the 240 K-Da protein as the type II IGF receptor. In addition, R-II-PAB1 inhibits the binding of [^{125}I]IGF-II in a dose-dependent manner. Thus, at least one epitope for R-II-PAB1 may be a site very close to the IGF binding site. Of particular importance is the fact that, instead of inhibiting the action of IGF-II, R-II-PAB1 by itself has an agonistic action in Balb/c 3T3 cells. This action of R-II-PAB1 is rather specific to Balb/c 3T3 cells since R-II-PAB1 has an inhibitory action in rat 18, 54-SF cells (Rosenfeld, R. G., Pham, H., James, P., Shah, R., Diaz, G. and Wyche, J., submitted for publication). Differences in species may account for this discrepancy. In any case, the present results support an idea that the type II IGF receptor is biologically active in stimulating cell proliferation. This notion is consistent with recent reports (17, 18) that IGF-II stimulates cell growth in K-562 cells by acting on the type II IGF receptor. Although R-II-PAB1 is a polyclonal antibody, a remote possibility that R-II-PAB1 is acting on the type I receptor is excluded by two lines of observations: first, R-II-PAB1 does not block [^{125}I]IGF-I binding and, second, R-II-PAB1 does not immunoprecipitate the type I receptor. R-II-PAB1 therefore provides a useful tool for evaluating the role of type II IGF receptor in Balb/c 3T3 cells.

Indeed, R-II-PABl stimulates calcium influx in primed competent cells. Furthermore, the antibody-induced calcium influx resembles in many aspects that caused by IGF-II. Hence, binding of a ligand to the type II IGF receptor leads to the activation of calcium gating system, namely calcium channel (9). These results support our proposal that IGF-II stimulates calcium influx in primed competent Balb/c 3T3 cells by acting on the type II IGF receptor (8). Accordingly, we have recently observed that IGF-II increases opening probability of a voltage-independent calcium-permeable cation channel in primed competent Balb/c 3T3 cell and that R-II-PABl stimulates calcium influx by opening this IGF sensitive cation channel (19) Taken together, these results provide evidence that the type II IGF receptor is coupled to a cation channel which augments the entry of calcium into the cell. These results together with those in our previous study (9) indicate that the type II IGF receptor is functionally linked to a cation channel by a pertussis toxin-sensitive mechanism.

The primary sequence of the type II IGF receptor has been recently determined by deducing from its complementary DNA. Morgan et al. (4) showed that the type II IGF receptor is identical with the cation-independent mannose-6-phosphate receptor (20, 21). They suggested that the type II IGF receptor functions as a mannose-6-phosphate receptor and may play a role in the targeting of lysosomal enzymes to the lysosomes. On the other hand, a recent study by Hari et al. (22) demonstrates that type II IGF receptor mediates an insulin-like response in human hepatoma cells. Furthermore, recent studies indicate that the type II IGF receptor mediates mitogenic action of IGF-II in K-562 cells (17, 18). These observations as well as the present results indicate that the type II IGF receptor is unique not only in its structure but also in its function.

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