Cryptic receptors for insulin-like growth factor II in the plasma membrane of rat adipocytes – a possible link to cellular insulin resistance

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Abstract

To further elucidate the mechanisms for short-term regulation of the receptor for insulin-like growth factor II (IGF-II), we investigated effects of insulin, cAMP and phosphatase inhibitors on cell surface ^{125}I-IGF-II binding in rat adipocytes. Preincubation with the serine/threonine phosphatase inhibitor okadaic acid (OA, 1 μM) or the non-hydrolysable cAMP analogue N^6-mbcAMP (4 mM) markedly impaired insulin-stimulated ^{125}I-IGF-II binding. Furthermore, addition of OA enhanced the inhibitory effect exerted by N^6-mbcAMP. N^6-mbcAMP also induced an insensitivity to insulin which was normalized by concomitant addition of the tyrosine phosphatase inhibitor vanadate (0.5 mM). In contrast, vanadate did not affect the impairment in maximal insulin-stimulated ^{125}I-IGF-II binding produced by either OA or N^6-mbcAMP. Phospholipase C (PLC), which cleaves phospholipids at the cell surface, markedly enhanced cell surface ^{125}I-IGF-II binding in a concentration-dependent manner. Scatchard analysis demonstrated that the effect of PLC was due to an increased number of binding sites suggesting that 'cryptic' IGF-II receptors are associated with the plasma membrane (PM). PLC (5 U/ml) also reversed the N^6-mbcAMP-induced decrease of ^{125}I-IGF-II binding at a low insulin concentration (10 μU/ml). Taken together, these data indicate that cAMP, similar to its effects on the glucose transporter GLUT 4 and the insulin receptor, may increase the proportion of functionally cryptic IGF-II receptors in the PM through mechanisms involving serine phosphorylation, possibly of a docking or coupling protein. Tyrosine phosphorylation appears to exert an opposite effect promoting the full cell surface expression of receptors.

Keywords: IGF-II receptor; Phospholipase C; cyclic AMP; Phosphatase inhibitor; Insulin sensitivity; Plasma membrane; (Rat adipocyte)

1. Introduction

In basal non-stimulated rat adipocytes, the major proportion of the receptors for insulin-like growth factor II (IGF-II)/mannose-6-phosphate is located in an intracellular compartment [1]. Upon insulin stimulation, IGF-II receptors are translocated from the intracellular low density microsomal fraction to the plasma membrane (PM)

[1–3]. This stimulatory effect of insulin is similar to its action on glucose transporter translocation [4,5]. It is rapid and concentration-dependent, resulting in an enhancement of the number of IGF-II receptors at the cell surface up to 10- to 20-fold [3,6]. This effect of insulin is blunted in insulin-resistant cells, e.g., following cAMP treatment [6]. The mechanisms involved may include impaired insulin binding as well as signaling resulting in a reduced translocation of IGF-II receptors [6–9].

Detailed work on IGF-II receptor translocation has indicated that the marked effect of insulin in the intact cell is only partially recovered when binding to membranes is assessed following cell fractionation [1]. Moreover, a previous study from our laboratory demonstrated that, in insulin-resistant, cAMP-treated cells, the reduction in insulin-stimulated IGF-II binding to the cell surface, was not so marked when receptor translocation was assessed by

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Abbreviations: ADA, adenosine deaminase; BSA, bovine serum albumin; IGF-II, insulin-like growth factor II; MHC-I, major histocompatibility complex class I antigen; N^6-mbcAMP, N^6-monobutyryl cyclic AMP; OA, okadaic acid; PIA, N^6-(R-phenylisopropyl) adenosine; PLC, phospholipase C; PM, plasma membrane.

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IGF-II binding to solubilized PMs [6]. These observations indicate that not only the translocation of IGF-II receptors to the PM is impaired, but also an additional step, e.g., receptor activation or insertion appears to be altered. In the present study we therefore directly examined whether evidence for 'cryptic' IGF-II receptors within the PM in rat adipocytes could be obtained by using phospholipase C (PLC) under stimulated and non-stimulated conditions. The regulatory effects of serine and tyrosine phosphorylation events were also explored in experiments with the serine and tyrosine phosphatase inhibitors okadaic acid (OA) and vanadate, respectively.

2. Materials and methods

2.1. Materials

3-[^{125}I]-Tyr-insulin-like growth factor II (IGF-II; spec. act. 263 μCi/μg) was from Amersham (Amersham, Buckinghamshire, UK). Human recombinant IGF-II, phopholipase C (type XIV: from C. perfringens), N^6-monobutyryl cyclic AMP (N^6-mbcAMP), bovine serum albumin (BSA; fraction V) and sodium orthovanadate (Na$_3$VO$_4$) were from Sigma Chemical Co. (St. Louis, MO, USA). Adenosine deaminase (ADA) and N^6-(R-phenyliso-propyl) adenosine (PIA) were from Boehringer-Mannheim (Mannheim, Germany). Human monocomponent insulin was a gift from Novo Nordisk (Copenhagen, Denmark). Okadaic acid was from LC Laboratories (Boston, MA, USA). Medium 199 was obtained from Gibco BRL, Life Technologies (Paisley, UK). A synthetic peptide fragment from the major histocompatibility complex class I (MHC-I) molecule (D^{K}69-85) was kindly donated by Dr. L. Olsson (Receptron, San Francisco, CA, USA).

2.2. Isolation of adipocytes

Male Sprague-Dawley rats, weighing 150–200 g and fed ad libitum, were stunned and decapitated. The epididymal fat pads were instantly excised and minced. Fat cells were isolated in medium 199 containing 0.6 mg/ml collagenase and 4% BSA at 37°C for 1 h in a shaking water bath ($\sim$ 120 rpm) as previously described [10].

2.3. ^{125}I-IGF-II binding to intact cells

After extensive washing, the cells (lipocrit 5–10%) were preincubated with 1 U/ml ADA and 0.1 μM PIA (a non-degradable adenosine analogue, to achieve a defined adenosine effect) and other indicated agents at 37°C for 20 min in a shaking water bath ($\sim$ 120 rpm). 2 mM KCN was then added for another 5 min in order to deplete the cells of ATP and stop receptor internalization and recycling [1,11]. Subsequently, cell surface binding of ^{125}I-IGF-II (0.2 ng/ml) was carried out at 24°C for 30 min or 60 min

in the absence or presence of PLC, which cleaves phospholipids at the cell surface [12]. After the incubation, cells and medium were separated by centrifugation through dinonyl phtalate and ^{125}I-IGF-II binding to cells was measured. Non-specific binding, defined as binding in the presence of 200 ng/ml unlabeled IGF-II, was subtracted.

2.4. ^{125}I-IGF-II binding to PMs

Isolated PMs were prepared as described by Weber et al. [13]. To obtain similar binding conditions as those in intact cells, binding of ^{125}I-IGF-II (0.2 ng/ml) was carried out at 24°C for 30 min in medium 199 containing 4% BSA, protease inhibitors (leupeptin 2 mg/l, pepstatin 1 mg/l and phenyl methylsulphonylfluoride 25 mg/l) and different concentrations of PLC. Then separation of radioactivity bound to the PM from free radioactivity was achieved by centrifugation through an oil mixture of dioctyl phthalate and dibutyl phthalate (2:3) at 12 000 $\times$ g for 20 min and ^{125}I-IGF-II binding to the PM (the pellet) was then measured.

^{125}I-IGF-II binding to receptors from solubilized PM was also performed. Solubilized PM was prepared as previously described [6]. Briefly, PM samples were solubilized in medium 199 containing 0.5% Triton X-100 for 60 min at 4°C in the presence of the protease inhibitors. The receptors were precipitated with polyethyleneglycol together with IgG. After vigorous vortexing, the samples were centrifuged at 11 000 $\times$ g for 5 min and the pellets were washed. After resuspending the pellets in medium, binding of ^{125}I-IGF-II in the presence or absence of 200 ng/ml unlabeled IGF-II (non-specific binding) was performed at 24°C for 30 min. The receptors were again precipitated and the pellets extensively washed three times.

2.5. Statistics

Statistical significance of differences was tested with Student's two-tailed t-test for paired data. Results are means $\pm$ S.E.M. unless otherwise indicated.

3. Results

3.1. Effects of N^6-mbcAMP, vanadate and OA on ^{125}I-IGF-II binding

Preincubation with OA (1 μM) alone increased cell surface ^{125}I-IGF-II binding ($n = 4$, $P < 0.001$, Fig. 1). N^6-mbcAMP (4 mM) alone or together with OA (1 μM) had no effect on basal ^{125}I-IGF-II binding. However, both OA and N^6-mbcAMP decreased the maximal insulin-stimulated (1000 μU/ml) ^{125}I-IGF-II binding ($n = 4$, $P < 0.01$ and $P < 0.001$, respectively). Moreover, OA and N^6-mbcAMP when added together additionally decreased the maximal stimulation by insulin (1000 μU/ml) of
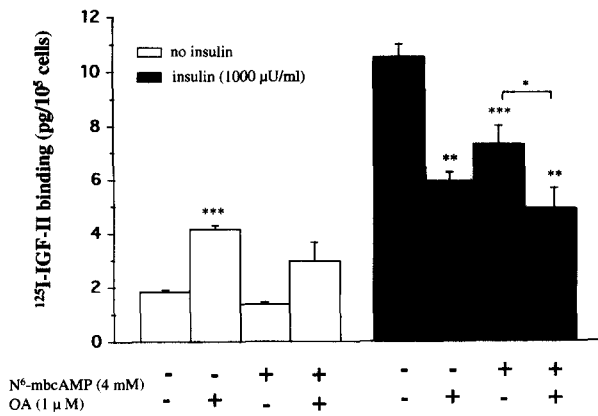
Fig. 1. Effects of insulin, N^6-mbcAMP and okadaic acid (OA) on cell surface ^{125}I-IGF-II binding. Rat adipocytes were preincubated at 37°C for 20 min with or without insulin (1000 μU/ml), OA (1 μM) and N^6-mbcAMP (4 mM) as indicated. Then, cells were treated with 2 mM KCN for 5 min at 37°C, and were transferred to 24°C and ^{125}I-IGF-II (0.2 ng/ml) was added. After 1 h, cell-associated radioactivity was determined. Data are means ± S.E.M. of 3–6 separate experiments. (* * $P <$ 0.01, * * * $P < 0.001$, compared to no addition or insulin alone).

^{125}I-IGF-II binding compared with that of N^6-mbcAMP plus insulin ($n = 4$, $P < 0.05$), but this was, however, not significantly different from the inhibition exerted by OA alone (Fig. 1).

N^6-mbcAMP (4 mM) shifted the dose–response curve for insulin-stimulated ^{125}I-IGF-II binding to the right, whereas vanadate (0.5 mM) added together with N^6-mbcAMP clearly restored insulin sensitivity to normal (Fig. 2). EC_{50} for insulin was 14 ± 3, 32 ± 4 ($P < 0.05$) and 14 ± 2 μU/ml for control cells and cells treated with N^6-mbcAMP or N^6-mbcAMP plus vanadate, respectively
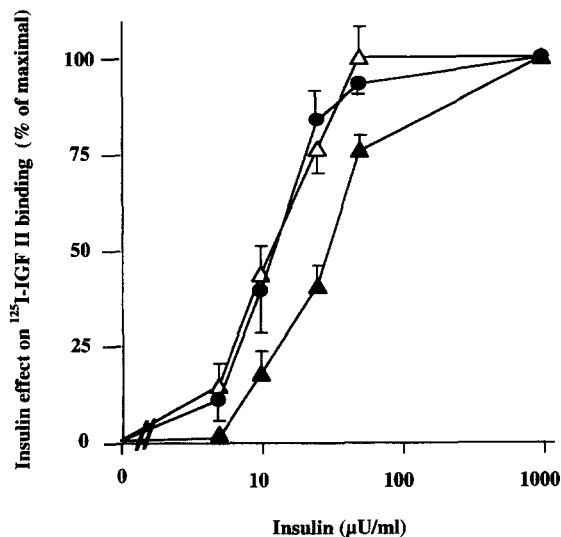

Fig. 2. Effects of N^6-mbcAMP and vanadate on the dose–response curve for insulin-stimulated ^{125}I-IGF-II binding. Cells were treated as described in Fig. 1, but with the indicated concentrations of insulin in the presence of 4 mM N^6-mbcAMP (▲), 0.5 mM vanadate plus N^6-mbcAMP (Δ) or in the absence of these agents (•). Data are means ± S.E.M. of 5 separate experiments.
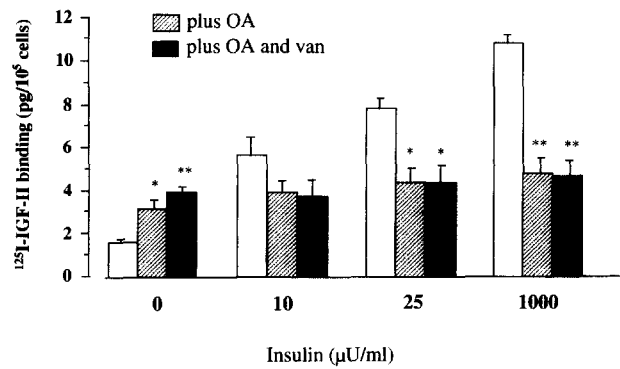

Fig. 3. Effects of okadaic acid (OA) and vanadate (van) on ^{125}I-IGF-II binding. Cells were treated as described in Fig. 1, but with different concentrations of insulin, 1 μM OA and 0.5 mM vanadate as indicated. Data are means ± S.E.M. of 4 separate experiments (* $P < 0.05$, * * $P <$ 0.01, compared to addition of insulin alone at the corresponding concentration).

($n = 5$). In contrast, vanadate did not significantly affect the blunted maximal insulin response following N^6-mbcAMP treatment (data not shown). Vanadate alone increased ^{125}I-IGF-II binding; 1.6 ± 0.2, 3.9 ± 0.3 and 5.6 ± 0.1 pg/10^5 cells for control, vanadate 0.5 mM ($P < 0.01$) and 4 mM ($P < 0.001$), respectively ($n = 4$). As shown in Fig. 3, vanadate did not, however, improve the impaired response to insulin following OA. Insulin sensitivity, measured as EC_{50} for insulin-stimulated ^{125}I-IGF-II binding, was not clearly affected by OA, but this was difficult to evaluate due to the blunted dose–response curve (Fig. 3).

Addition of the MHC-I peptide (30 μM) during the preincubation period clearly increased ^{125}I-IGF-II binding both in the presence or absence of insulin. This effect was also markedly reduced by concomitant or subsequent addition of N^6-mbcAMP (data not shown).

3.2. Effects of PLC on ^{125}I-IGF-II binding to intact cells, plasma membrane and solubilized receptors

As seen in Fig. 4, PLC in a concentration-dependent manner markedly enhanced ^{125}I-IGF-II binding to energy-depleted cells and EC_{50} for this effect was 2.8 ± 0.2 U/ml ($n = 4$). The maximal effect of PLC (25 U/ml) on ^{125}I-IGF-II binding was ~ 4-fold compared to control cells. However, at higher concentration of PLC (50 U/ml), ^{125}I-IGF-II binding declined to the control level. Scatchard analysis demonstrated that the effect of PLC (5 U/ml) on ^{125}I-IGF-II binding was due to an increase in the number of binding sites by 2.5-fold ($n = 3$, $P < 0.05$) without any clear change in receptor affinity (Fig. 4, inset).

Experiments with PLC were also performed with isolated PM as described in Section 2. PLC at the same concentrations as those used in intact cells, however, did not increase ^{125}I-IGF-II binding to PM nor was ^{125}I-IGF-II binding to receptors from solubilized PM affected by the
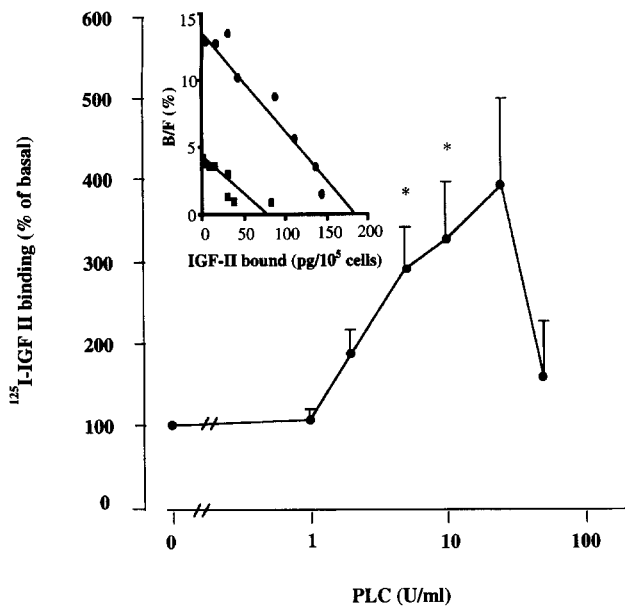
Fig. 4. Effects of PLC on cell surface ^{125}I-IGF-II binding. Cells were pretreated with 2 mM KCN at 37°C for 5 min. Binding of ^{125}I-IGF-II (0.2 ng/ml) was subseqently performed in the presence of different concentrations of PLC at 24°C for 30 min as described in Section 2. Data are means ± S.E.M. of 4 separate experiments. (* $P < 0.05$, compared to binding in cells without PLC treatment). The inset in Fig. 4 shows a Scatchard plot. Cells were pretreated with 2 mM KCN at 37°C for 5 min and then incubated with 0.2 ng/ml ^{125}I-IGF-II at 24°C for 30 min together with different concentrations of unlabelled IGF-II in the presence (●) or absence (■) of 5 U/ml PLC. The results are from one representative experiment repeated three times.

addition of PLC at different concentrations (data not shown).
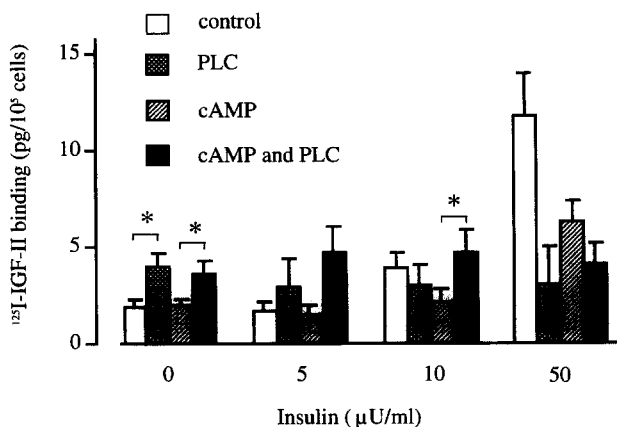
In cells pretreated with insulin and N^6-mbcAMP, ^{125}I-

Fig. 5. Effects of PLC on ^{125}I-IGF-II binding to insulin-stimulated cells. Cells were preincubated with or without 4 mM N^6-mbcAMP (cAMP) at 37°C for 5 min, then with the indicated concentrations of insulin for another 15 min. Following 2 mM KCN treatment for 5 min, binding of ^{125}I-IGF-II (0.2 ng/ml) was performed at 24°C for 30 min in the absence or presence of 5 U/ml PLC. Cell-associated radioactivity was subseqently determined. Data are means ± S.E.M. of 3–8 separate experiments (* $P < 0.05$, compared to corresponding group without PLC treatment).

IGF-II binding was decreased compared to cells treated with insulin alone (Fig. 1). PLC (5 U/ml), however, increased ^{125}I-IGF-II binding to N^6-mbcAMP-treated cells to a similar magnitude as in control cells (Fig. 5). PLC also reversed the N^6-mbcAMP-induced decrease of ^{125}I-IGF-II binding at a low insulin concentration (10 μU/ml) ($n = 4$, $P < 0.05$). In the presence of higher concentrations of insulin, PLC did not increase ^{125}I-IGF-II binding and this was also true for cells treated with insulin plus N^6-mbcAMP (Fig. 5).

To examine whether PLC induces an insulin signal we also performed control experiments with immunoblots with phosphotyrosine antibodies on cell lysates. However, PLC treatment (1 or 5 U/ml) alone of cells did not alter tyrosine phosphorylation of insulin-regulated proteins including the insulin receptor β-subunit (data not shown).

4. Discussion

Previous studies [1,6] showed that the stimulating effect of insulin as well as the counteracting effect of cAMP on cell-surface IGF-II binding are not fully accounted for by receptor translocation. In experiments using cell fractionation, receptor distribution between the cell interior and the PM was not sufficient to explain the effects on IGF-II binding found in intact cells [1,6]. This suggests that additional mechanisms are involved in the regulation of cell surface IGF-II binding. In analogy with findings with respect to the glucose transporter GLUT 4 [14] and the insulin receptor [10,15,16], one possibility is the docking, fusion and/or insertion at the PM, whereby the IGF-II receptor is functionally expressed at the cell surface. This would tentatively be promoted by insulin and impaired by cAMP.

Both tyrosine and serine phosphorylation events are involved in the insulin signaling pathways [17–19]. In the present study both vanadate and okadaic acid, a tyrosine and a serine/threonine phosphatase inhibitor [18,20], respectively, exerted insulin-like effects on ^{125}I-IGF-II binding. Even though OA alone displayed an insulin-like action, it exerted an opposing effect when added together with insulin. This suggests that serine/threonine phosphorylation of key insulin-regulated proteins impairs insulin action. Since insulin responsiveness was attenuated by OA as well as N^6-mbcAMP, post-receptor steps appear to be involved. These may include insulin signaling proteins, such as the insulin receptor substrate-I (IRS-I) [20], but also the IGF-II receptor itself which may become serine phosphorylated [21,22].

The cAMP-analogue N^6-mbcAMP inhibited both insulin responsiveness and sensitivity as was also previously shown [6,23]. Cyclic AMP can induce serine phosphorylation of the insulin receptor leading to a decrease in tyrosine kinase activity and, possibly, also to an incomplete functional expression of PM-associated insulin receptors at

the cell surface [9,16,24]. This may account for the insensitivity to insulin produced by cAMP treatment. Moreover, one cannot exclude the possibility that cAMP via serine phosphorylation of the IGF-II receptor itself [22] impairs its full insertion in the PM. The finding of a more pronounced inhibitory effect of cAMP when OA was also present supports the concept of a serine-phosphorylation step impairing the functional availability of IGF-II receptors at the cell surface. Further support for a PM mechanism for the inhibitory action of cAMP on IGF-II receptors was obtained with experiments with the MHC-I peptide fragment which effectively inhibits receptor internalization [25]. Even in the presence of this peptide, when IGF-II receptors are accumulated and 'locked' in the PM, N^6-mbcAMP produced a clear impairment of IGF-II binding. Vanadate was capable of restoring insulin sensitivity in cAMP-treated, but not insulin responsiveness in cAMP- or OA-treated cells. Tyrosine phosphorylation of key proteins in the insulin signaling pathway is critical for insulin action [17]. Possibly, the effect of vanadate is partly exerted at the level of the insulin-receptor, since it was previously shown at our laboratory that vanadate can enhance cell-surface insulin binding capacity, even in the presence of cAMP [10]. Taken together, these data suggest that in addition to translocation between the interior and the surface of the cell, also mechanisms in the PM regulate the functional insertion of IGF-II receptors. Tyrosine and serine phosphorylation appear to have opposite effects in this regard. This is similar to what has recently been found concerning insulin receptor insertion in the PM (J.W. Eriksson et al., to be published). The PM-associated mechanisms vis-à-vis the IGF-II receptor may involve either the conformation of the receptor, its insertion in the PM or both.

The present work further suggests the idea of a critical insertion step in the PM, since PLC, which cleaves phospholipids at the cell surface [12], markedly increased ^{125}I-IGF-II binding to rat fat cells. This was attributed to an increase in the number of binding sites without any change in receptor affinity suggesting uncovering of functionally cryptic receptors associated with the PM [12]. Additional experiments with IGF-II binding to solubilized PM argue against a direct effect of PLC on the affinity of the IGF-II receptor. Since the protocol used in the ^{125}I-IGF-II binding experiments included cellular ATP depletion with KCN, possible PLC effects on insulin signaling and receptor translocation are very unlikely as was also shown with the phosphotyrosine immunoblots. Another possible error would be that PLC-induced cell leakage leads to ^{125}I-IGF-II binding to non-specific cell structures. However, this is unlikely, since non-specific binding of ^{125}I-IGF-II in PLC-treated cells was not consistently changed compared to control cells (data not shown). Taken together, a plausible interpretation of the PLC effect would be that unexposed and/or incompletely inserted IGF-II receptors are associated with the PM. A similar effect of PLC has previously

been demonstrated with respect to 'cryptic' insulin receptors in the PM [12].

The failure to demonstrate a PLC effect directly in the PM preparations may be explained by the possibility that unexposed and/or incompletely inserted IGF-II receptors associated with the PM in intact cells become fully inserted during the preparation of PM. Our recent data on the insulin receptor suggest such a possibility (J.W. Eriksson et al., to be published).

An interesting finding is that PLC can reverse the impairment of IGF-II binding induced by N^6-mbcAMP in cells stimulated with a low insulin concentration (10 μU/ml; see Fig. 5). This observation suggests that insulin-stimulated insertion of the IGF-II receptors in the PM is impaired by cAMP and this could be one of the mechanisms for cAMP-induced cellular insulin insensitivity with respect to IGF-II binding. In contrast, PLC markedly reduced IGF-II binding in cells pretreated with high insulin concentrations both in the presence and absence of N^6-mbcAMP and the reasons for this are not clear. Possibly, high insulin concentrations lead to full IGF-II receptor insertion even in the presence of cAMP. In contrast, insulin binding is enhanced by PLC also when cells have been stimulated with high concentrations of insulin (J.W. Eriksson et al., to be published) and there may possibly be cryptic insulin but not IGF-II receptors after full insulin stimulation. Moreover, the IGF-II receptor is a monomeric, single-stranded peptide with a large extracellular domain [2,26], and it may be more fragile than the tetrameric insulin receptor and it might perhaps be released from the PM upon PLC treatment when it has already been fully inserted in response to a high insulin concentration.

Effects of cAMP, similar to those presently reported, have also been suggested with respect to PM fusion and cell-surface exposure of yet another insulin-regulated protein, the glucose transporter GLUT 4 [14,27]. Therefore, the impairment of exposure of insulin-regulated proteins resulting in a reduced availability at the cell surface may be a common perturbation in cAMP-induced insulin resistance. Moreover, specific tyrosine and serine phosphorylation events, e.g., of docking proteins or, to some extent, of the receptors and transporters per se, may directly regulate the fusion and insertion processes of all these insulin-responsive proteins - the insulin and IGF-II receptors and GLUT 4 - in the PM as also suggested by previous work [28–30].

In conclusion, this study suggests that IGF-II receptors, similar to insulin receptors and glucose transporters, can be associated with the PM, without being functionally available at the cell surface. The proportion of such cryptic receptors may increase in insulin-resistant states, e.g., elevated cAMP levels, probably through mechanisms involving serine phosphorylation. If this is a general phenomenon, as current data suggest, incomplete receptor insertion may be of importance for the impaired sensitivity and responsiveness to insulin in these states.

Acknowledgements

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