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Insight into the molecular basis for the kinetic differences between the two insulin receptor isoforms

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Abstract

More than 20 years after the description of the two insulin receptor (IR) isoforms, designed IR-A (lacking exon 11) and IR-B (with exon 11) nearly every functional aspect of the alternative splicing both *in vitro* and *in vivo* remains controversial. In particular, there is no consensus on the precise ligand binding properties of the isoforms. Increased affinity and dissociation kinetics have been reported for IR-A in comparison to IR-B, but the reverse results have also been reported. These are not trivial issues considering the reported possible increased mitogenic potency of IR-A, and the reported link between slower dissociation and increased mitogenesis.

We have re-examined the ligand binding properties of the two isoforms using a novel rigorous mathematical analysis based on the concept of a harmonic oscillator. We found that insulin has 1.5 fold higher apparent affinity towards IR-A and a 2-fold faster overall dissociation rate. Analysis based on the model showed increased association (3-fold) and dissociation (2-fold) rate constants for binding site 1 of IR in comparison to IR-B. We also provide a structural interpretation of these findings based on the structure of the IR ectodomain and the proximity of the sequence encoded by exon 11 to the C-terminal peptide that is a critical trans-component of site 1.

Introduction

The insulin receptor (IR) belongs to a subfamily of receptor tyrosine kinases that comprises the type I IGF receptor (IGF-IR) and the insulin receptor-related receptor (for review see [1-3]). They are covalent disulfide-linked dimers made of two extracellular α -subunits that contain the ligand binding domains and two transmembrane β -subunits that contain the intracellular tyrosine kinase. The cloning and sequencing of the IR cDNA in 1985 by two different groups revealed two transcripts that predicted receptor sequences differing by the absence [4] or presence [5] of a 12-amino acid segment (718-729 in Ebina et al.'s numbering) at the C-terminal end of the IR α -subunit. The structure of the IR gene [6] was shown to consist of 22 exons and 21 introns. The 718-729 sequence is encoded by exon 11, which suggested that the two isoforms were generated by alternative splicing of that exon. The isoform lacking the insert is nowadays denoted IR-A, and the other IR-B. The existence of alternative splicing was confirmed by polymerase chain reaction [7-9].

The relative abundance of the two splice variants is highly regulated in a tissue-specific manner. Thus, liver expresses only IR-B, while muscle and placenta express both isoforms, and lymphocytes only IR-A [7-9]. The same tissue-specific pattern was also seen in the rat [10], suggesting functional significance of this alternative splicing. However, in other insulin target tissues significant species differences have been reported. In particular, in

skeletal muscle isoform expression is approximately 95% IR-A in rat [11,12], but approximately 70% IR-B in human [7,13,14,15]. There are also species differences in isoform expression in adipose tissue, kidney and spleen [9,11,16,17]. The IR-A is predominantly expressed in prenatal life and has a much higher affinity for IGF-II than the IR-B, and the IR-A thus plays an important role in fetal development and may play a role in certain cancers (for review, see [1]). IGF-I also binds better to IR-A [18], but not as well as IGF-II [19]. The IR-A is considered more involved in “mitogenic” signalling, while IR-B is more involved in “metabolic” signalling [1,20]. The functional consequences of this splicing remain controversial. An altered distribution of isoforms in adipocytes or muscle of patients with type 2 diabetes was reported by some but not others (see [1] for review). There is also variability in the reported ligand binding properties of the two isoforms of the IR. The IR-A was initially suggested to have a 5-fold higher affinity than the IR-B [21], while subsequent work using a variety of cell transfection systems reported a ~ 2-fold difference [8,18,22,23] or no difference [24,25]. In contrast, Denley et al. [26] found IR-B to have a two-fold higher affinity than IR-A. The dissociation rate of IR-A (by dilution) was reported to be slower than that of IR-B by Mc Clain et al. [27], but to be faster by Gu et al. [22] and Yamaguchi et al. [18]. Internalization of the IR-A has been reported to be faster than for the IR-B (in CHO cells: [18,23]; in rat-1 fibroblasts: [28]; in Hela cells: [20] while others found no difference in rat-1 fibroblasts [18,27]. The insulin affinity for hybrids with IGF-IR was found to be isoform-dependent by Pandini G., et al. (2002) [29] but not by Slaaby et al. [30] and Benyoucef, S. et al. [19].

In the present work, we have carefully re-examined the ligand binding kinetics of the two IR isoforms expressed in CHO cells using a recently developed structure-based mathematical model [31] of the insulin binding mechanism.

Insulin has been shown to contain two binding surfaces which are involved in binding to IR: the classical binding surface and a recently mapped binding site named the novel binding surface. The classical binding surface overlaps with the surface involved in dimer formation, and the novel – with the surface involved in hexamerization of insulin [32,33]. Insulin binding to the IR exhibits negative cooperativity, manifested by curvilinear Scatchard plots and acceleration of the dissociation rate of prebound ¹²⁵I-insulin in an infinite dilution in the presence of unlabeled ligand (for review, see [32]). Binding models have been proposed that imply bivalent crosslinking by site 1 and site 2 of insulin to two distinct sites on each IR a-subunit [32,34]. To explain negative cooperativity, De Meyts [32] proposed that sites 1 and 2 of the IR should have an antiparallel symmetry that allows alternative crosslinking to each pair of sites 1 and 2 of the IR. This model is supported by the recent crystallographic structure of the IR extracellular domain [35]. The extracellular domain of IR consists of two leucine-rich (L1 and L2) domains separated by a cysteine-rich (CR) domain, and followed by three fibronectin type III (Fn1-3) domains. The combination of photoaffinity crosslinking [36-39] and alanine scanning mutagenesis [40,41] studies suggest that the classical binding surface of insulin binds to the IR residues located in the central β -sheet of the L1 module and to a carboxy terminal peptide (704-719) (located next to the alternatively spliced 12 amino-acid sequence), which combine in trans to form “site 1”, whereas the novel binding surface binds to the less well characterized “site 2” of IR, which most likely corresponds to residues from the C-terminal portion of L2 as well as the loops at the junction of the Fn1 and Fn2 modules [42-45].

Kiselyov’s structure-based mathematical model of the IR kinetics [31] allows for the first time to describe accurately all the kinetic properties of IR using only five parameters: association rate constants a_1 , a_2 and dissociation rate constants d_1 , d_2 for site 1 and site 2, respectively, and a crosslinking constant k_{cr} . This model is based on an assumption that the IR conformational change upon ligand binding is analogous to a spontaneous oscillation of a

one-dimensional harmonic oscillator in thermal equilibrium with the surroundings. A highly simplified scheme of the harmonic oscillator model is shown in Fig. 1, in which an analogy from mechanics is used for depiction of the IR oscillator: the IR monomer is depicted as two rigid balls (representing sites 1 and 2) connected by a rigid concave rod. The force acting to restore the inactive symmetrical conformation is represented by a spring, which is assumed to be relaxed when the IR dimer is in the symmetrical (inactive conformation). The harmonic oscillator model confirms the intuitive predictions of the symmetrical bivalent model suggested by De Meyts [32] and allows robust determination of all of the kinetic parameters.

Since the carboxy terminal peptide (704-719) contributes to site 1 of IR, it is not unexpected that the two IR isoforms have different kinetic properties, since the 12-amino-acid long sequence (718-729 in Ebina et al.'s numbering) encoded for by exon 11 is located right next to it. We show that the association rate constant, a_1 , is approximately three-fold larger for the IR-A isoform than IR-B, while the dissociation rate constant d_1 is 2-fold increased, resulting in a 1.5 increase in the apparent high affinity. We explain this in terms of the recent structural information regarding the insulin-IR binding mechanism.

Materials and methods

Chemicals and reagents

^{125}I -labelled and non-labelled human insulin were provided by Novo Nordisk A/S, Denmark.

Cell culture

CHO cells overexpressing the A- or B-isoform of the human IR (kindly provided by Bo Falck Hansen, Novo Nordisk A/S, Denmark) were cultured in D-MEM (1000g/l glucose: Na-pyruvate; Glutamix-1; pyridoxal) with 10% FBS, 1% Penicillin-Streptomycin, 1% MEM-NEAA and 0.45 mg/ml G418 (Gibco, Invitrogen, USA) at 37°C in a humidified atmosphere of 5% (v/v) CO_2 .

Preparation of cells for binding experiments

The cells were washed in 10 ml PBS without CaCl_2 and MgCl_2 (Gibco, Invitrogen, USA) and 10 ml of 100 μM EDTA (Sigma, USA) in PBS was added to the cells. 25 ml of HBB (Hepes Binding Buffer) (100 mM NaCl (Merck, Germany), 5 mM KCl (Merck, Germany), 1.2 mM MgSO_4 (Sigma, USA), 1 mM EDTA (Sigma, USA), 10 mM Glucose (Sigma, USA), 15 mM NaOAc (Merck, Germany), 100 mM Hepes (Sigma, USA), 1% BSA (Amresco, USA), pH=7.6 adjusted with NaOH (Sigma, USA)) was added and the cell suspension was centrifuged at 1000 rpm for 5 minutes in a Heraeus, Sorvall[®] Labofuge 400e (AXEB Laboratory Products, Denmark), and the supernatant was discarded. The cells were re-suspended in an appropriate volume of HBB, and the cell concentration was measured using a NucleoCounter (Chemometec, Denmark). The cells were centrifuged at 1000 rpm for 5 minutes, and the supernatant was discarded. The cell pellet was re-suspended in an appropriate volume of HBB to get the right cell concentration according to the assay the cells were used for.

Receptor binding assays

All experiments were performed three times in duplicate unless otherwise stated.

Homologous competition assay

The cell suspension was incubated for 4 hours at 15°C with increasing concentrations (1.67×10^{-11} M to 1.67×10^{-5} M) of unlabelled insulin, 50 μ l (20,000 cpm/50 μ l) of 125 I-labelled insulin, and HBB to a total volume of 0.5 ml. Duplicate aliquots were centrifuged after incubation and the bound activity counted. Two additional aliquots were not centrifuged but counted as total.

Dose-response assay for accelerated dissociation

125 I-labelled human insulin was added to 900 μ l of the cell suspension ($\sim 15 \times 10^6$ cells/ml) to a final concentration of 75,000 cpm/ml and incubated for 2 hours at 15°C. After pre-incubation the cells were centrifuged for 5 minutes at $1000 \times g$ at 4°C. The cell pellet was re-suspended in the original volume of HBB and 25 μ l of the cell suspension was transferred into duplicates of 15 tubes containing 500 μ l of an increasing concentration of cold ligand (1.67×10^{-11} M to 1.67×10^{-5} M) starting with the tubes with the lowest concentration. The 30 reaction tubes were incubated at 15°C for 0, 7.5, 15, 30, 60 or 120 minutes. Sextuples of 25 μ l of the cell suspension were counted as total (no centrifugation). After the dissociation the 30 reaction tubes were centrifuged in a 5810 R centrifuge (Eppendorf, Germany) for 5 minutes at 4000 rpm at 4°C and the radioactive ligand bound was counted in a gamma counter for 30 minutes.

Curve-fitting by a mathematical model

The binding experiments were modelled using a mathematical model developed in our department [31]. The model is based on the hypothesis that the receptor dimer with antiparallel disposition of the two half-receptors can be assimilated to a one-dimensional harmonic oscillator. Based on the assumptions of the model, the possible combinations of insulin and IR result in a set of 35 intermediaries and thus 35 differential equations that are fitted to the experimental data using the program Mathematica. The model describes the binding of insulin to IR by using only five binding parameters: a_1 and a_2 , the association rate constant to IR site 1 and 2 respectively; d_1 and d_2 , the dissociation rate constant to IR site 1 and 2 respectively and k_{cr} ; the receptor crosslinking constant (= the reciprocal of the time it takes for the oscillator to close). The goodness of fit between the experimental (X_i) and simulated (Y_i) data points can be evaluated with a score function equal to $1/N \sum (X_i - Y_i)^2 / (SD_i)^2$, where N is the number of data points and SD_i is the standard deviation of the experimental data point using a genetic algorithm.

Results

In order to compare the binding properties of the two IR isoforms, we tested first if there is a difference in the receptors' affinity for insulin. For this purpose, binding of human insulin tracer (at equilibrium) to cells over-expressing either the IR-A or IR-B in the presence of various concentrations of unlabeled insulin was analysed. As shown in Fig. 2, insulin competed more efficiently when IR-A was used. Fitting of the binding data to a two-site sequential model produced the following values for the apparent high affinity (= crosslinked sites 1 and 2) dissociation constant (KD): 236 ± 86 pM and 349 ± 128 pM for the IR-A and IR-B, respectively. From this we conclude that the IR-A has a 1.5-fold higher affinity in comparison with the IR-B. The KD values for the second site (= uncrosslinked site 1) were 1.75 ± 5.86 nM for IR-A and 2.62 ± 8.80 nM for IR-B, which are in agreement with previously reported data for site 1 affinity [24,31].

According to the harmonic oscillator model [31], the rate of the IR dissociation at maximum acceleration is proportional to the dissociation coefficient for site 1, and can be estimated with high accuracy as $0.5 d_1$. Thus, measurement of the maximum dissociation rate

allows to easily compare the d_1 values for the two receptor isoforms. In order to estimate the maximum dissociation of IR, we tested the rate of the insulin tracer dissociation at various concentrations of the unlabelled insulin and established that the maximum acceleration was achieved at 100 nM concentration for both receptor isoforms. As can be seen from Fig. 3, the rate of the IR dissociation at maximum acceleration is higher for the IR-A, and fitting of the dissociation data to a mono exponential decay shows that the maximum dissociation rate for the IR-A is approximately two fold higher than that for the IR-B. This indicates that the d_1 value, or dissociation coefficient for site 1, is two fold higher for the IR-A than the IR-B.

The K_D value for the apparent high affinity binding site of IR can be calculated from the a_1 , a_2 (association coefficients for site 1 and 2, respectively), and d_1 , d_2 (dissociation coefficients for site 1 and 2, respectively) and k_{cr} (crosslinking constant) according to an approximate formula [31]:

$$K_D = d_1/a_1 \times d_2/k_{cr}$$

We have shown above that the d_1 value for the IR-A is two fold higher than for the IR-B. At the same time, the K_D value for the IR-A is 1.5 fold lower. From the above formula, it is obvious that in order for K_D to become 1.5 fold lower (assuming d_1 is 2 fold higher), either

- a_1 has to increase 3 fold or
- d_2 has to decrease 3 fold or
- k_{cr} has to increase 3 fold.

Since the crosslinking constant describes the oscillatory properties of the whole IR molecule, it is unlikely that the presence of exon 11, which represents only a very small fraction of the IR total mass, can substantially change the value of the crosslinking constant. Thus, it is likely that either a_1 is increased 3 fold or d_2 decreased 3 fold in order for the B isoform to have 1.5 fold lower affinity.

To test if either the a_1 or d_2 parameters are affected, we observe that the rate of the IR dissociation (in the absence of unlabelled ligand) is given by $d_1 \times d_2/k_{cr}$ [31]. This means that if the d_2 values are the same for the two isoforms, then the rate of the IR dissociation is expected to be 2 fold faster for the IR-A since we have already established that d_1 is 2 fold higher for the IR-A. As appears from Fig. 3, the rate of the IR dissociation for the IR-A is indeed faster than for the IR-B, and fitting of the mono exponential decay to the dissociation data shows that the difference in the dissociation rate is approximately 2 fold.

Thus we have demonstrated that the IR-A binds to insulin with 1.5 fold higher affinity when compared to the IR-B, and our kinetic analysis indicates that this difference is caused solely by the perturbation of the site 1 rate constants, namely: the presence of exon 11 leads to a 2 fold decrease of d_1 , and a 3 fold decrease of a_1 .

In order to verify the above conclusions we decided to estimate the a_1 , a_2 , d_1 , d_2 and k_{cr} parameters for the two IR isoforms by curve fitting the harmonic oscillator mathematical model to the dose response of the accelerated dissociation for the two IR isoforms. As can be seen from Fig. 4, the dose response for the IR-A is characterised by faster dissociation in comparison with the IR-B. The data are also represented in Fig. 5 in which the dissociation is plotted as a function of time instead of concentration. Since fitting results might be affected by the initial choice of the parameters values, we performed 10 independent parameter estimations for each IR isoform using a genetic algorithm (as described in Kiselyov et al. [31]) with randomly selected values of the initial parameters. The average values of the parameters and their standard deviations are shown in Table 1. As can be seen in Table 1, some parameters have large standard deviations, which imply that a relatively large range of parameter values allow to make a good fit of the model to the experimental data. Thus, when

comparing the parameter values between the two IR isoforms, the uncertainty of the parameter estimation (represented by the standard deviation) should be taken into account. To characterise the significance of the difference between the parameter values for the A and B receptor isoforms, we calculated a significance factor, defined as the difference between the parameter values divided by the sum of the parameter standard deviations. As can be seen from Table 1, the a_1 parameter values for the two isoforms differ from each other by almost 4 standard deviations (column named “Significance”), the d_1 – by approximately 1.5 standard deviations, and all the other parameters – by much less than 1 standard deviation. From this we can conclude that only the parameters of site 1 (a_1 and d_1) differ substantially between the two isoforms, namely: the presence of exon 11 leads to a 1.6-fold decrease of d_1 , and a 2.3-fold decrease of a_1 , which is in good agreement with our previous kinetic analysis.

Discussion

We have shown here that the IR-A has a slightly higher affinity (1.5-fold) than IR-B. This agrees well with some but not all previous findings [8;18;22]. The faster association rate constant a_1 that we determined agrees with the only study that looked at association rate, which was found to be faster for IR-A [18]. As for the dissociation rate, our observation of a faster off rate by dilution alone agrees with the data of Gu et al. [22] and Yamaguchi et al. [18] but not McClain et al. [27]. Only one study previously examined the dissociation rate in the presence of unlabeled insulin and like here found the IR-A to dissociate faster [22]. Discrepancies between our study and previous reports could be due to differences in assay conditions such as the nature of receptor preparation, buffer, pH and temperature. It would be interesting to extrapolate these studies to include a more physiologically relevant temperature. However, internalization of IR at 37°C is significantly increased in comparison to 15°C, which makes data interpretation difficult unless you have a more complicated model, which can take into account the higher degree of internalization.

The further analysis of the receptor kinetics demonstrated that the presence of exon 11 in the IR-B affects only the receptor site 1 by decreasing the a_1 and d_1 rate constants approximately 3- and 2-fold, respectively. This can be explained in the light of the insulin receptor ectodomain structure [46]. In the sequence of the IR-B, exon 11 (718-729 in Ebina et al.'s numbering) is located next to the CT peptide (residues 704-719). The CT peptide together with the L1 module form the receptor's site 1, and as can be seen from the crystal structure of the IR ectodomain (IR-A), the exon 11 residues are expected to be exposed on the surface of IR in the vicinity of site 1. Docking of insulin into IR by means of molecular dynamics simulations shows that insulin can get access to site 1 only from the outside of the receptor, and from the inside the access is expected to be blocked by the residues connecting the two subunits [47]. This means that the exon 11 residues are expected to obstruct binding of insulin to site 1 and thus decrease the rate of association to this site when compared to the IR isoform lacking the exon. By the same reasoning, once insulin is bound to site 1, the residues of exon 11 are expected to obstruct the dissociation of insulin and therefore decrease its dissociation rate from site 1. Although it does not seem possible to predict quantitatively how the association and dissociation rate constants of site 1 are expected to be affected by the presence of exon 11, the proposed explanation appears to be in excellent qualitative agreement with the presented experimental data.

Thus our data provide a structural insight into the mechanism of the exon 11's regulation of the insulin receptor binding.

It is of interest that IR-A has a slightly faster dissociation rate while it is considered by some authors (20) to be more “mitogenic” than IR-B. Previous work has suggested that mitogenicity of insulin analogues correlates with a slower dissociation rate from the IR and an

increased residence time [48,49]. Another important factor in this case may be linked to internalization. Jensen et al. [50] have shown that an insulin mimetic peptide, S597, that is not internalized, is a much weaker mitogen than insulin and a poor activator of the MAPK/ERK cascade, suggesting that signalling from the endosome is critical for MAPK/ERK activation and mitogenesis [50,51]. While the data in the literature about the respective internalization rates of IR-A and IR-B are equivocal, we believe that the elegant recent studies of Giudice et al. [20] using fluorescent –tagged ligands and receptors demonstrate convincingly an enhanced internalization of IR-A compared to IR-B in transfected Hela cells, with a resulting enhanced activation of ERK1/2 by IR-A, while IR-B was more potent than IR-A in activating AKT.

Much remains to be done to establish the functional significance of the alternatively spliced forms of the insulin receptor and the hybrids thereof.

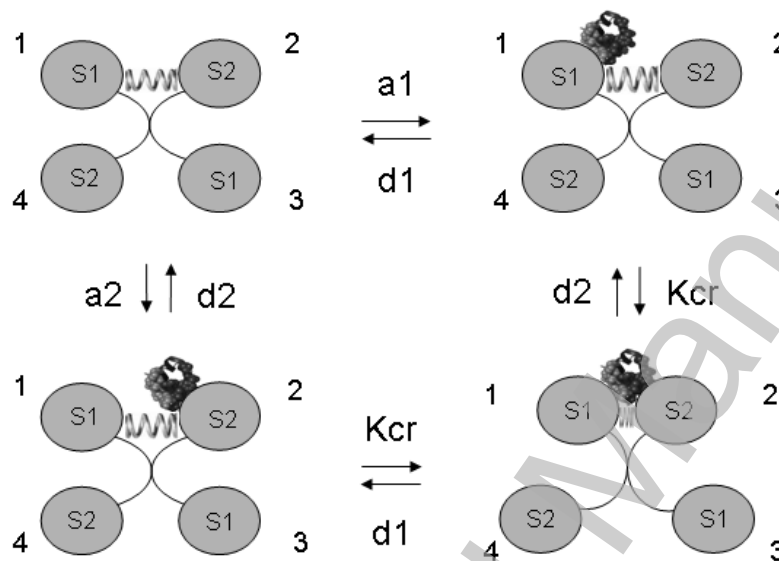


Figure 1: Simplified scheme of the IR harmonic oscillator model

a_1 and a_2 are the association rate constants for binding site 1 and 2, respectively ($M^{-1}s^{-1}$), and d_1 and d_2 are the dissociation rate constants for binding site 1 and 2, respectively (s^{-1}). K_{cr} is the crosslinking constant (s^{-1}). S1 and S2 are the binding sites 1 and 2 of the IR, respectively. The binding of insulin to IR is only shown for site 1 and 2 numbered 1 and 2, respectively. It is assumed that there are no differences between site 1 numbered 1 or 3, or between site 2 numbered 2 or 4. The insulin molecule is pig insulin (PDB accession number: 9INS). Graphics program: Pymol.

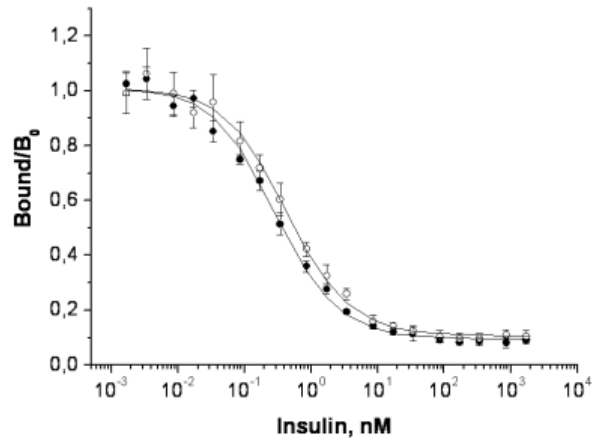


Figure 2: Homologous competition experiments

The experiments were performed with insulin as the ligand and CHO cells over-expressing either IR-A (●) or IR-B (○). The dots are the experimental data points and the lines correspond to the fitted curves using a sequential model. The apparent high affinity (=crosslinked sites 1 and 2) dissociation constant (KD) value for insulin when using IR-A was 236 ± 86 pM and 349 ± 128 pM when using IR-B. The KD values for the second site (corresponding to uncrosslinked site 1) were 1.75 ± 5.86 nM for IR-A and 2.62 ± 8.80 nM for IR-B.

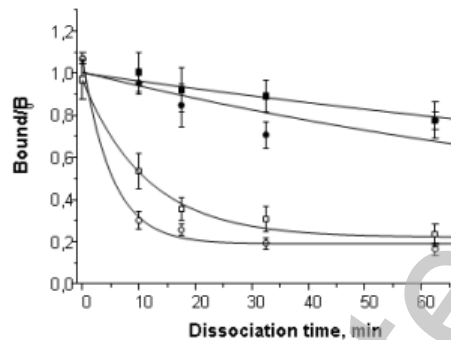


Figure 3: Dissociation of insulin from IR-A or IR-B at zero and 100 nM cold ligand

Prebound insulin was allowed to dissociate for various time periods (0; 7.5; 15; 30; 60 and 120 min) in dilution alone from IR-A (●) or IR-B (■), or in the presence of an excess (100 nM) of cold insulin from IR-A (○) or IR-B (□).

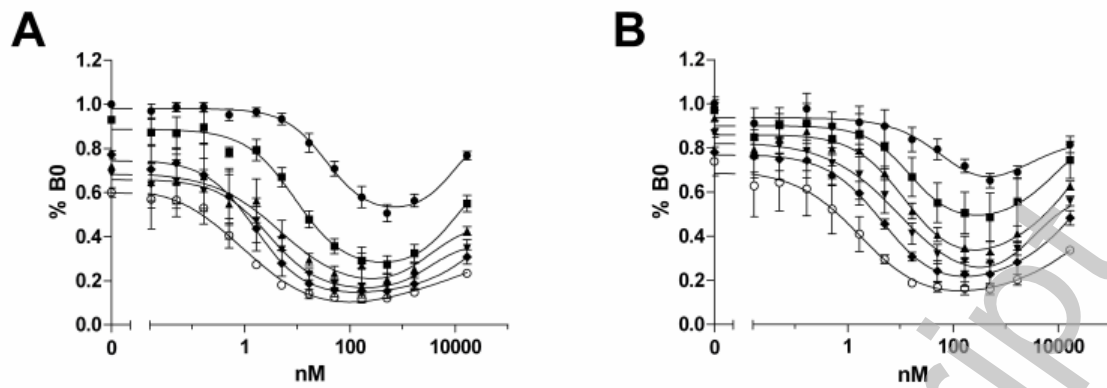


Figure 4: Dissociation kinetics of insulin or insulin analogues using IR-A and IR-B cells
 Cells expressing IR-A (panel A) or IR-B (panel B) were pre-incubated with ^{125}I -insulin for 2 hours, and then pre-bound ^{125}I -insulin was allowed to dissociate in dilution alone or dilution in the presence of increasing amounts of cold ligand for 0 minutes (●), 7.5 minutes (■), 15 minutes (▲), 30 minutes (▼), 60 minutes (◆) or 120 minutes (○). The data were plotted as bound ^{125}I -insulin divided by bound ^{125}I -insulin at zero concentration cold ligand and zero time, as a function of concentration of added unlabelled ligand. Each experiment was performed in duplicates and repeated three times on different days.

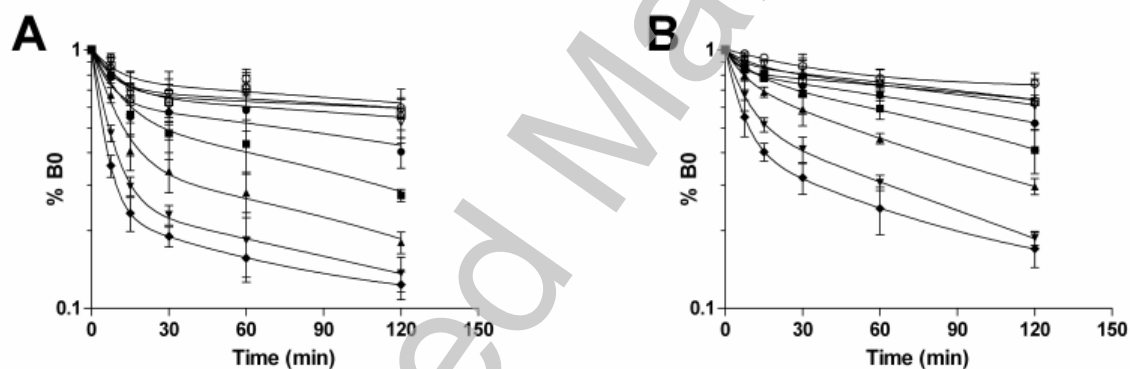


Figure 5: The dissociation kinetics as a function of time
 Cells expressing IR-A (panel A) or IR-B (panel B) were pre-incubated with ^{125}I -insulin for 2 hours, and then pre-bound ^{125}I -insulin was allowed to dissociate for various time points in the presence of 0 nM (○), 1.67×10^{-11} M (□), 5.1×10^{-11} M (△), 1.67×10^{-10} M (▽), 5.1×10^{-10} M (●), 1.67×10^{-9} M (■), 5.1×10^{-9} M (▲), 1.67×10^{-8} M (▼), or 5.1×10^{-8} M (◆). The data in this figure, are the same data presented in Fig.4, although here presented as the amount of bound ^{125}I -ligand normalised to the amount bound ligand at zero time and concentration as a function of time.

	IR-A ± SD	IR-B ± SD	Significance*	IR-A/IR-B ± SD
Kcr (s ⁻¹)	0.79 ± 0.23	0.70 ± 0.23	0.22	1.13 ± 0.50
a1 (M ⁻¹ s ⁻¹)	306 ± 23 x 10 ³	135 ± 21 x 10 ³	3.94	2.27 ± 0.39
d1 (s ⁻¹)	0.0057 ± 0.0006	0.0036 ± 0.0008	1.48	1.58 ± 0.39
a2 (M ⁻¹ s ⁻¹)	25 ± 18 x 10 ³	33 ± 23 x 10 ³	0.18	0.76 ± 0.76
d2 (s ⁻¹)	0.015 ± 0.006	0.012 ± 0.0005	0.58	1.25 ± 0.72

Table 1. The parameters obtained using the mathematical model

* The significance parameter describes how significant the difference between the IR-A and IR-B isoforms for a particular parameter is and is calculated as the difference between the two parameters divided by the sum of their standard deviations. In addition to the significance parameter, the IR-A/IR-B ratios as well as its standard deviations are given. A1, association rate constant; a2, association rate constant 2; d1, dissociation rate constant 1; d2, dissociation rate constant 2.

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Conflict of interests

Pierre De Meyts and Vladislav V. Kiselyov were employees of Novo Nordisk A/S. Pierre De Meyts and Louise Knudsen own Novo Nordisk stocks.

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