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Importance of receptor occupancy, concentration differences, and ligand exchange in the insulin-like growth factor I receptor system

(hormone–receptor interactions/binding complexity/$\alpha_2\beta_2$ heterotetramers/anticooperativity)

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ABSTRACT **We have investigated by use of placental membranes the mechanisms through which insulin-like growth factor I (IGF-I) comes to be associated with its $\alpha_2\beta_2$ receptor heterotetramer. Our results suggest that (*i*) at low ligand concentrations, the formation and disruption of IGF-I–receptor complexes are consistant with ligand binding *de novo* to empty receptors but not with equilibria involving ligand dissociation; (*ii*) at higher ligand concentrations, rapid exchange arising from the formation and collapse of bis-liganded receptors leads to a transiently perturbed receptor state; (*iii*) these nonclassical IGF-I receptor interactions depend on close communication between the $\alpha\beta$ halves of the $\alpha_2\beta_2$ holo-IGF-I receptor; and (*iv*) related processes based on ligand exchange have the potential for serving as biological sensors of changes in ligand concentration, while ordinary binding processes serve as sensors of ligand concentrations themselves. A model is presented in which one or two molecules of ligand can be bound to an $\alpha_2\beta_2$ IGF-I receptor heterotetramer, new ligand becomes associated with receptor by exchanging for a previously bound molecule of IGF-I, and fluctuating changes in free-ligand concentration might lead to enhanced IGF-I function.**

Complexity in the interactions of peptide hormones with their plasma membrane receptors is common and manifest by (*i*) ligand binding kinetics that can be modeled only in terms of two or more exponential processes, (*ii*) incomplete or multicomponent processes for ligand dissociation from receptor, (*iii*) curvilinear reciprocal plots reflecting receptor saturation at steady state, or (*iv*) multiple inflections during steady-state binding competition studies. In many cases, it is not clear whether this complexity arises from multiple (perhaps sequential) states of ligand–receptor interactions or from separate and noninteracting populations of ligand binding sites. Complexity in insulin-like growth factor I (IGF-I) interactions with the $\alpha_2\beta_2$ heterotetrameric receptor for IGF-I has been attributed to at least four different causes. Related studies have suggested that apparent ligand binding heterogeneity in the IGF-I receptor system arises from molecular differences in the α and β subunits of the $\alpha_2\beta_2$ receptor heterotetramer (including those attributable to both the protein and carbohydrate moieties of the receptor) (1–5), heterotetramer formation between $\alpha\beta$ dimers of the IGF-I receptor and those of the homologous insulin receptor (6–9), high- and low-affinity receptor populations that can be differentiated by their selective binding to various lectins or by other means (10, 11), and the existence of multiple binding sites specific for IGF-I on a single IGF-I receptor molecule (9, 12). Overall, experiments have identified heterogenous effects of various monoclonal antibodies on the inhibition of IGF-I binding to receptor (12–15), the indifference of IGF-II interactions with the IGF-I receptor to various inhibitory antibodies (12, 15), and the importance of dimer–dimer interactions in determining the affinity of IGF-I interactions with the IGF-I receptor (10, 11).

To gain insights into the causes of binding complexity in the IGF-I receptor system [one that serves as model for the related $\alpha_2\beta_2$ insulin receptor (16) and perhaps for other receptor systems as well (17, 18)], we reexamined how IGF-I complexes with the IGF-I receptor come to be formed. Our results suggest that under circumstances that might apply to the IGF-I receptor system *in vivo* (that is, under conditions of preexisting receptor occupancy and changing ligand concentrations), IGF-I exchange for a previously bound IGF-I molecule (rather than *de novo* IGF-I binding to unoccupied receptors) may represent the most important route.

MATERIALS AND METHODS

Membranes were isolated from normal-term human placenta by published procedures (19) and were stored at −80°C. In this system the affinities of the insulin receptor for IGF-I and of the IGF-I receptor for insulin have been shown (20) to be <1% of those that apply to the homologous ligand/receptor pairs. Accordingly, results reported here can be assigned specifically to interactions of IGF-I with the IGF-I receptor rather than with the insulin receptor or with other receptor forms that exhibit high affinity for insulin (see refs. 1–12). ^{125}I-labeled IGF-I (^{125}I-IGF-I; purified by HPLC) was purchased from Amersham. Unlabeled IGF-I was from GroPep (Adelaide, Australia), and monoclonal antibody αIR-3 was from Oncogene Science. Placental membranes were washed in 50 mM Tris buffer (pH 8) containing 2.5 mg of bovine serum albumin per ml (binding buffer) and were used at a final density of 0.2–0.4 mg of membrane protein per ml. Membrane incubations were performed in plastic Microfuge tubes at 22°C for selected periods usually at 0.25-ml total volume. Radiolabeled tracer was usually used at a concentration of 120,000 cpm/ml with unlabeled hormone sometimes being added. Incubations were terminated by the addition of ice-cold binding buffer (1 ml) and by subsequent centrifugation for 2 min at 4°C in a Microfuge; the supernatant fluid was removed by aspiration, and the membrane pellet was assayed for radioactivity.

For incubations involving ligand dissociation, membranes were preincubated with radiolabeled tracer with or without added unlabeled IGF-I for 30 min at 22°C, followed by dilution with ice-cold buffer, centrifugation, and resuspension to the original volume in buffer. Additions of unlabeled hormone were made by use of small volumes to limit membrane dilution. For experiments involving conversion of membrane-associated $\alpha_2\beta_2$ IGF-I receptor heterotetramers to $\alpha\beta$ heterodimers, placental membranes were incubated with 4 mM dithiothreitol in 75 mM Tris buffer (pH 8.5) containing 1 mM EDTA for 15 min at 22°C (11) prior to

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Abbreviation: IGF-I, insulin-like growth factor I.

centrifugation, washing with binding buffer, and resuspension in binding buffer for further use. Details of the experiments are provided in the figure legends. Experiments were performed on multiple occasions, with typical results being shown. When appropriate, data were modeled by computer-based, nonlinear methods by use of the program KalidaGraph.

RESULTS AND DISCUSSION

Initial experiments assessed the time courses of ^{125}I-IGF-I binding to and dissociation from placental receptors. The data of Fig. 1*A* suggest that radiolabeled IGF-I associates with placental membranes by an apparently simple binding process in which doubling the amount of hormone tracer doubles the amount of receptor-bound ligand. Nevertheless, Fig. 1*B* identifies that (*i*) the dissociation of previously bound hormone from receptor is barely detectable when membranes are reincubated in buffer alone, (*ii*) dissociation is induced by the addition of unlabeled ligand, (*iii*) the rate of ligand dissociation is enhanced in proportion to ligand concentration, and (*iv*) the extent of ligand-induced hormone dissociation reaches a concentration-dependent limit in each case. The observed rate constants for the fast components of ligand dissociation were modeled to be 0, 0.08, 0.16, and 4.4 nM·min^{-1} for dissociation induced by buffer or by 10, 30, or 750 nM IGF-I, respectively. Although the values of these rate constants vary considerably, the intrinsic rate constants corresponding to the three nonzero rates ($k_{intrinsic} = k_{observed}$/hormone concentration) are very similar (0.0064 ± 0.0014 min^{-1}, see Table 1), a result suggesting that the applicable mechanism of ligand dissociation is the same in all cases. Membranes incubated at 37°C exhibited the same near-zero rate of ligand dissociation into buffer (and the same enhancement of ligand dissociation due to added IGF-I) as that shown in Fig. 1*B* for membranes incubated at 22°C (data not shown).

To test more fully the importance of ligand concentration on ligand dissociation from the IGF-I receptor, membranes previously incubated with radiolabeled IGF-I were washed and then incubated sequentially in solutions containing increasing concentrations of unlabeled hormone. Fig. 1*C* shows that (*i*) the partial dissociation of radiolabeled hormone achieved by incubating membranes at low or intermediate concentrations of unlabeled hormone is enhanced by subsequently incubating the membranes at higher hormone concentrations, and (*ii*) time-independent plateaus are achieved in each case. These findings suggest that ligand-induced dissociation from the IGF-I receptor depends on changes in IGF-I concentration and on the perturbations of preexisting steady states. The control experiment of Fig. 1*D* identifies that unlabeled IGF-I remaining after one step of ligand dissociation is in fact capable of stimulating dissociation further but is somehow without effect once an apparent steady state has been reached. Notwithstanding the expected character of the curves shown in Fig. 1*A*, these results imply that simple equilibrium binding is not applicable to the IGF-I receptor system.

Although results related to those of Fig. 1*B* are sometimes interpreted in terms of anticooperativity (21, 22), the addition of ligand to a negatively homotropic system can usually be expected to enhance the rate of ligand dissociation without intervening plateaus. Ligand exchange involving (*i*) the for-

Table 1. Rate constants applicable to IGF-I–receptor interactions

Experimental format	Reaction direction	Apparent intrinsic rate constant, min^{-1}
Dissociation (Fig. 1*B*)	Reverse	0.0064 ± 0.0014
Association (Fig. 2*A*)	Forward	
	Fast	0.14 ± 0.031
	Slow	0.012 ± 0.0025
Ongoing flux (Fig. 2*C*)	Forward	0.0052
	Reverse	0.0054

Observed rate constants derived by mathematical modeling were corrected for the concentration of ligand to arrive at the apparent intrinsic rate constants reported. The direction of reaction is defined as forward when radiolabeled ligand–receptor complexes are being formed and as reverse when these complexes are being disrupted.

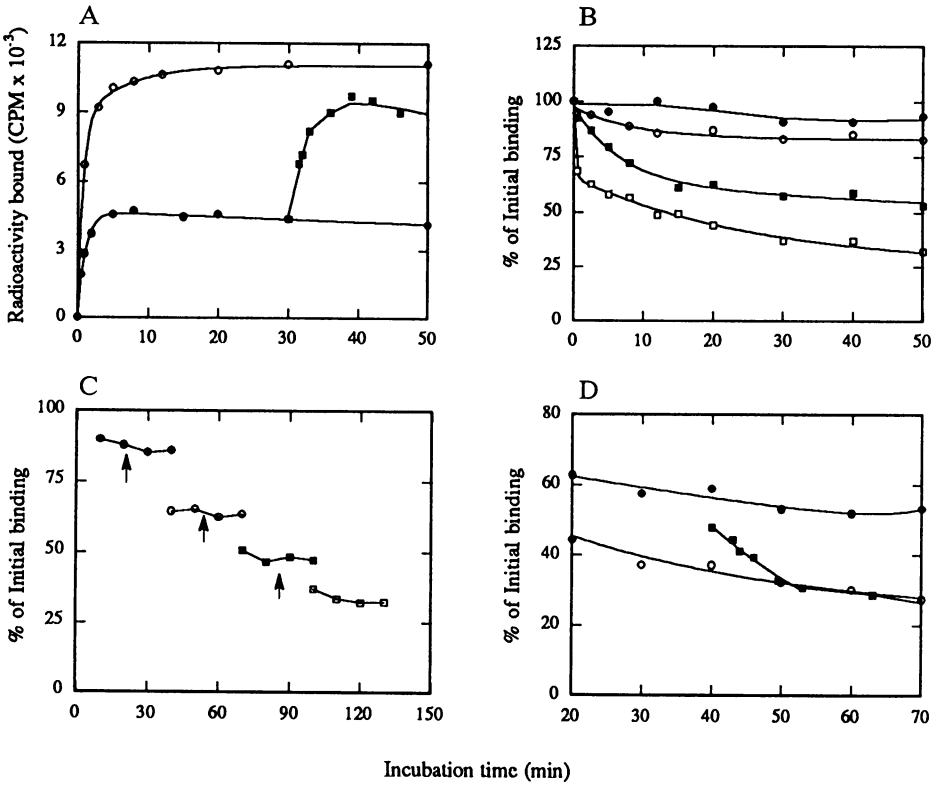

FIG. 1. Time courses of ^{125}I-IGF-I association with and dissociation from membrane receptors. (*A*) Ligand association using 30,000 (●) or 60,000 (○) cpm of ^{125}I-IGF, or 30,000 cpm followed by a second addition of the tracer to double the concentration after the initial steady state had been achieved (■). (*B*) Dissociation of previously bound ligand in buffer alone (●) or in solutions containing 10 (○), 30 (■), or 750 (□) nM unlabeled IGF-I. (*C*) Dissociation of previously bound ligand, beginning from buffer alone (●), due to the sequential addition (arrows) of 0.01 (○), 0.04 (■), and 1 (□) μM hormone, respectively; measurements were initiated 20 min after each addition. (*D*) Dissociation of previously bound ligand resulting from subsequent incubations of membrane suspensions with 33 (●) or 750 (○) nM IGF-I or from mixing equivalent membrane suspensions after dissociation had taken place during an initial 40-min period (■).

mation of a transient intermediate and (*ii*) the return of the corresponding perturbed state to a new equilibrium could represent a better model for the relevant process. Whereas homologous exchange processes involving radiolabeled and unlabeled ligand are often described by the expression $L + {}^*LR \rightleftharpoons LR + {}^*L$ (where L and ${}^*L$ = unlabeled and radiolabeled ligand, respectively, and R = the binding protein), the process is better presented by considering the expression $L + LR \rightleftharpoons LRL$ (where LRL = the bis-liganded protein), an expression that identifies relevant molecular participants rather than the experimental basis for detecting protein-bound ligand. Notably, the first expression implies (in the absence of isotope effects) that the result of exchange involves a zero sum in Gibbs standard free energy, while the second implies that the process of exchange involves both a potentially significant standard free energy and the participation of a transient intermediate. The intermediate relevant to IGF-I–receptor interactions is the bis-liganded receptor resulting from occupancy of both $\alpha\beta$ halves of the $\alpha_2\beta_2$ receptor heterotetramer. In this context, exchange can be considered to represent an extreme example of anticooperativity in which (*i*) the rate of dissociation of the first molecule of ligand bound to receptor is near zero in the absence of additional ligand, and (*ii*) the rate of binding of the second molecule of ligand is much less than its corresponding rate of dissociation from the bis-liganded complex. The bis-liganded receptor represents under this set of circumstances an unstable intermediate that contributes in only a minor way to equilibrium concentrations relevant to describing ligand–receptor interactions. Overall, the addition of ligand to an exchanging system is formally analogous to a concentration jump in rapid-reaction kinetics (23, 24), and is consistent with the concentration-dependent rates and limits of dissociation shown in Fig. 1 *B–D*.

Further experiments were designed to test the influence of preexisting receptor occupancy and of ligand concentration on the kinetics of IGF-I interactions with the IGF-I receptor. Fig. 2*A* shows that the rate and character of association of 20 nM IGF-I with placental membranes diverge from those expected for a single exponential function when membranes preincubated with unlabeled IGF-I are washed and reincubated with a mixture of labeled and unlabeled hormone. Each of these curves can be modeled only in terms of two exponential processes. The rapid phase of association (representing 80%, 58%, and 34% of the total for membranes preincubated in buffer alone or in 10 or 20 nM IGF-I, respectively) corresponds to one reflecting ligand binding to empty receptors. The slower phase of association (representing 20%, 42%, and 66% of the total for the three respective conditions described above) depends on the existence of previously

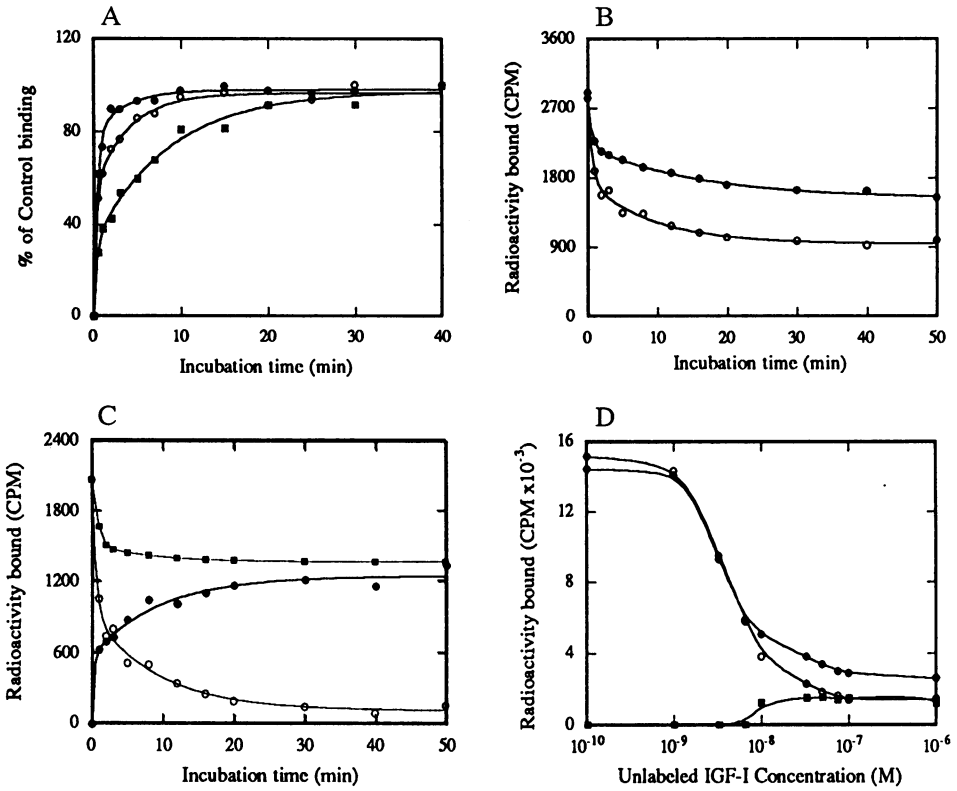

FIG. 2. IGF-I association with receptor and dissociation from receptor under conditions emphasizing prior and continuing occupancy of receptors by ligand. (*A*) Time courses of ligand association with receptors for membrane suspensions preincubated in buffer alone (●), preincubated with 10 nM IGF-I (○), or preincubated with 20 nM IGF-I (■), in each case prior to removing any unbound ligand and subsequent incubation with 20 nM IGF-I plus ^{125}I-IGF-I. Control binding is radioactivity becoming membrane-associated after 40 min of incubation (3700, 3300, and 1800 cpm for the three conditions, respectively). (*B*) Time courses of ligand dissociation from receptors for membrane suspensions preincubated with 20 nM IGF-I plus ^{125}I-IGF-I for 30 min prior to removing unbound ligand and subsequent incubation in buffer alone (●) or in buffer containing 20 nM unlabeled IGF-I (○). (*C*) Time courses of ligand interactions with receptors for membrane suspensions incubated for 30 min with 20 nM unlabeled IGF-I prior to centrifugation and subsequent incubation with 20 nM unlabeled IGF-I plus ^{125}I-IGF-I (●) or incubated for 30 min with 20 nM unlabeled IGF-I plus ^{125}I-IGF-I prior to centrifugation and incubation with 20 nM unlabeled IGF-I (○); data were modeled as the sum of two exponential functions. ■, Arithmetic sum of the curves reflecting ligand flux at 20 nM IGF-I. (*D*) Dependence of the formation of dissociable and dissociation-resistant IGF-I–receptor complexes on initial IGF-I concentration. Membranes were incubated with ^{125}I-IGF-I plus unlabeled IGF-I at the concentrations indicated for 30 min. One set of samples was processed to determine total bound ligand. The other set was centrifuged, the membranes were resuspended to their original volume with buffer, and the samples were incubated for 30 min prior to determining the amount of ligand that was resistant to dissociation. ●, Total receptor-bound radioactivity; ○, dissociation-resistant radioactivity; ■, dissociable radioactivity (determined by difference).

formed ligand–receptor complexes and presumably reflects ligand exchange at already occupied receptors. Applicable rate constants are presented in Table 1. Fig. 2*B* shows that hormone–receptor complexes formed during incubations involving high ligand concentrations behave differently from those formed during incubations with low ligand concentrations (see Fig. 1*B*). That is, significant dissociation occurs in buffer alone, notwithstanding that the extent of loss of radiolabeled IGF-I is further enhanced by subsequent incubation in the presence of the unlabeled hormone.

Results presented above imply that significant flux in receptor-bound ligand occurs at high IGF-I concentrations but not at low IGF-I concentrations and that the molecular characteristics of complexes formed under the two sets of circumstances must differ. The experiment of Fig. 2*C* shows that rates of ligand association with and dissociation from the receptor are in fact both high at high ligand concentrations. The half-time for ligand turnover is <1 min under these circumstances, notwithstanding that total ligand occupancy (as assessed by the near-zero slope of the curve reflecting the sum of the two processes) does not change. The similarity of the intrinsic rate constants applicable to IGF-I turnover at the receptor under conditions of constant ligand concentration and to IGF-I-induced ligand dissociation (see Table 1) suggests that both processes rely on the same mechanism involving the bis-liganded receptor intermediate.

Fig. 2*D* provides data assessing the concentration dependence for the formation of IGF-I–receptor complexes that are subject to simple dissociation. Notably, previously formed radiolabeled hormone complexes remain stable to subsequent dissociation into buffer when they were formed by IGF-I concentrations ≤ 4 nM (the concentration of ligand resulting in about 50% saturation of receptors), whereas they exhibit progressively higher degrees of dissociation into buffer as the concentration of ligand during the preincubation is increased. At very high IGF-I concentrations, about half of receptor-bound hormone is unstably bound and subject to dissociation. Taken together, the findings of Figs. 1 and 2 suggest that (*i*) receptor-bound IGF-I can exist in two different states, (*ii*) the formation of these two states depends critically on ligand concentration and receptor occupancy, (*iii*) fluxes of receptor-bound hormone are undetectable at low IGF-I concentrations but are high at high ligand concentrations, and (*iv*) both the ligand-dependent loss of previously bound hormone during dissociation experiments and the occupancy-dependent association of new hormone during binding experiments reflect transient perturbations of preexisting steady states.

Since the exchange model for IGF-I–receptor interactions requires that receptors behave in a bivalent fashion, further experiments were designed to test (through the preferential reduction of interdimer disulfides by use of 4 mM dithiothreitol; see refs. 6, 9–11) the importance of dimer–dimer interactions on ligand dissociation from the IGF-I receptor. Dithiothreitol treatment did not significantly alter the ability of placental membranes to bind ^{125}I-labeled IGF-I, consistent with the results of others obtained by use of solubilized receptors (11); however, the apparent dissociation constant of receptors for ligand was increased about 6-fold for dithiothreitol-treated membranes (data not shown). A comparison of Fig. 3 *A* and *B* reveals that (*i*) in contrast to results obtained by use of untreated membranes, ^{125}I-labeled IGF-I dissociation from dithiothreitol-treated membranes is significant even in buffer and is unaffected by the addition of unlabeled hormone; and (*ii*) whereas the monoclonal receptor antibody αIR-3 induces only partial dissociation of previously bound ligand from control membranes (as expected; see refs. 12–15), it induces complete dissociation of ligand from membrane receptors in which interdimer disulfides have been reduced. Accordingly, it seems that interdimer communication is required for ligand-induced ligand dissociation in the IGF-I receptor system and that the same communication gives rise to the apparent receptor heterogeneity, which is implied by the partial inhibition of ligand binding to the holoreceptor by antibody αIR-3.

Results presented here provide a framework for understanding IGF-I–receptor interactions by mechanisms involving both ligand binding and ligand exchange. Fig. 4*A* illustrates how IGF-I association with αβ receptor heterodimers can be described in terms of simple ligand binding and dissociation; the enhancement of ligand dissociation from the IGF-I receptor by the receptor antibody αIR-3 is rapid and complete under this circumstance, whereas it is not under circumstances where molecular communication between both dimers is retained in the holoreceptor. In contrast, Fig. 4*B* illustrates that IGF-I association with the $\alpha_2\beta_2$ receptor heterotetramer involves a branched pathway consisting of IGF-I binding to empty receptors (with the consequent formation of mono-liganded receptors, a process for which the reverse reaction is very slow), and subsequent IGF-I binding to mono-liganded receptors to form unstable $(IGF\text{-}I)_2\text{-}\alpha_2\beta_2$ ligand–receptor complexes. Dissociation of a single molecule of IGF-I from this bis-liganded complex to yield a mono-liganded receptor apparently represents a facile process that corresponds to the proposed mechanism for IGF-I exchange. The efficiency of the exchange reaction depends in fact on the ready formation and stability of IGF-I–$\alpha_2\beta_2$

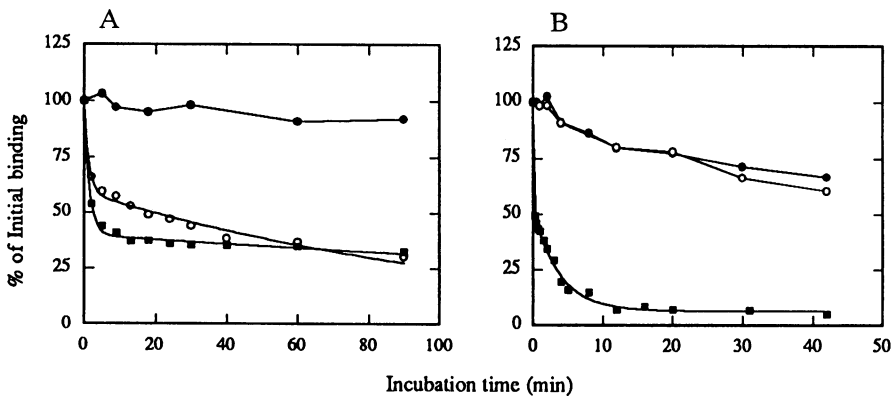

FIG. 3. Effects of converting $\alpha_2\beta_2$ receptor heterotetramers into αβ dimers on IGF-I dissociation from receptors. (*A*) Untreated membranes: dissociation of ^{125}I-IGF-I from $\alpha_2\beta_2$ heterotetramers because of subsequent incubations in buffer alone (●), 1 μM IGF-I (○), or 25 nM monoclonal receptor antibody αIR-3 (■). (*B*) Dithiothreitol-treated membranes: dissociation of ^{125}I-IGF-I from αβ heterodimers because of subsequent incubations in buffer alone (●), 1 μM IGF-I (○), or 25 nM receptor antibody αIR-3 (■). Control studies showed that the concentration of antibody chosen was maximally effective under both conditions.

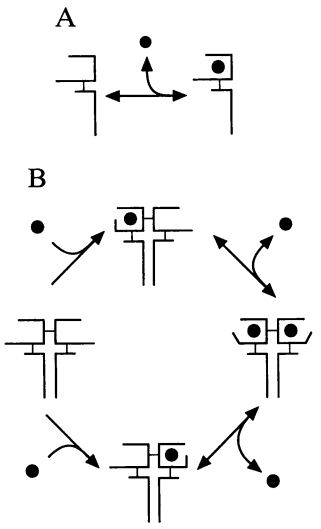

FIG. 4. Diagrammatic representation of ligand interactions with the IGF-I receptor. (*A*) Interactions of ligand with $\alpha\beta$ receptor halves; the scheme identifies the simple reversible binding of ligand to noncommunicating binding sites. (*B*) Interactions of ligand with $\alpha_2\beta_2$ holoreceptors; the scheme identifies the essentially irreversible binding of ligand to empty receptors (left open conformation) to produce mono-liganded receptors (upper and lower middle closed conformation) and the reversible binding of ligand to mono-liganded receptors to produce unstable bis-liganded receptors (right perturbed conformation). The scheme in *B* requires communication between each $\alpha\beta$ half of the $\alpha_2\beta_2$ receptor tetramer. Bis-liganded receptors represent the transient intermediate through which ligand comes to be receptor-associated by relaxation of the perturbed state toward a new equilibrium.

ligand–receptor complexes as much as it does on the relative instability of $(IGF\text{-}I)_2\text{–}\alpha_2\beta_2$ complexes. As noted earlier, exchange in this context represents an extreme example of anticooperativity. Whereas *de novo* ligand binding may represent a useful route for IGF-I association with receptor at low IGF-I concentrations, the exchange of previously bound ligand for new ligand increases as the level of preexisting receptor occupancy and the IGF-I concentration are raised.

Since IGF-I is seldom (or is never) absent from the cellular environment under biologically important circumstances, and since its dissociation from monoliganded receptors is slow, (*i*) a subset of total cell-surface IGF-I receptors is probably always occupied by ligand, (*ii*) evaluation of IGF-I–receptor interactions beginning from zero receptor occupancy is probably relevant only to laboratory situations, and (*iii*) ligand exchange at occupied receptors probably occurs at low rates during any defined steady state. The bis-liganded intermediate for the exchange of receptor-bound IGF-I should probably best be viewed as a perturbed state of the occupied receptor. While this receptor state always exists when IGF-I is already present, its formation is enhanced when IGF-I concentrations are increased above their previous levels as long as prior receptor occupancy (to produce the monoliganded receptor) is significant. That is, because the bis-liganded intermediate is unstable compared with the monoliganded receptor, an increase in IGF-I concentration will represent a ligand concentration jump leading to the transient formation of the unstable intermediate, followed by relaxation toward a new steady state (23, 24). Accordingly, an exchanging receptor is capable of sensing differences in ligand concentrations as well as absolute ligand concentrations themselves. It is important in this respect that the half-time for ligand exchange on the IGF-I receptor (<1 min at 20 nM hormone, see earlier) is very much less than that required for receptor-mediated ligand internalization and subsequent hormone processing (25).

For IGF-I, the binding of previously secreted ligand by any of the extracellular IGF-I binding proteins (26) could cause a local decrease in free ligand concentration (without an appreciable decrease in the level of mono-liganded receptors) and could initiate a transient rise in the level of the bis-liganded exchange intermediate due to ongoing IGF-I secretion. Similar considerations could apply to any system capable of interacting with two ligand molecules at the same time and could help in understanding (*i*) the extent to which both halves of other $\alpha_2\beta_2$ receptor heterotetramers are capable of simultaneously binding ligand and (*ii*) whether receptor autophosphorylation occurs predominantly in a cis or trans fashion with respect to receptor-bound ligand.

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