Timing-dependence of insulin-receptor mitogenic versus metabolic signalling: a plausible model based on coincidence of hormone and effector binding

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Mitogenic signalling through the insulin receptor is enhanced compared with metabolic signalling for insulin analogues having slower dissociation kinetics than insulin itself. A plausible explanation in molecular terms of this timing-dependent specificity is lacking. We show here that if signalling is transmitted through a single effector, binding coincidentally with hormone to the insulin receptor and whose association and dissociation kinetics are slow relative to the hormone dissociation rate, the resulting biological effect is predicted to be dependent on hormone-binding kinetics. However, known primary effector molecules associating with the insulin receptor bind and interact rapidly with the receptor, contrary to the assumptions of the

single-effector model. A model with two effectors which must bind coincidentally with hormone for signalling to occur also gives the required dependence of signalling on hormone-binding kinetics, provided that at least one of the effectors has slow binding kinetics relative to hormone binding. In this case, the other effector can have rapid kinetics, which is consistent with the properties of the major known substrates of the insulin receptor, such as the insulin receptor substrate (IRS) molecules.

Key words: binding, feedback loops, insulin analogues, kinase, regulatory networks.

INTRODUCTION

Comparison of the biological effectiveness of different insulin analogues shows that their signalling potency depends on their equilibrium binding affinity for the insulin receptor. For example, stimulation of insulin receptor tyrosine kinase [1] or glucose metabolism [2] by various insulin analogues is correlated with the equilibrium affinity of the analogues for the insulin receptor over a wide range of affinities. However, this correlation with affinity does not proceed in parallel for mitogenic versus metabolic signalling. Rather, mitogenic signalling appears to be more sensitive to the kinetic properties of insulin or analogue binding. Quantitative analyses of the relationship between mitogenic potency and binding parameters show that those analogues with low dissociation rates (i.e. long average residence times on the receptor) have a high mitogenic signalling capability which is out of proportion to their equilibrium affinity [3–8]. One such analogue, with the mutation His^{B10} → Asp, has been shown to induce mammary tumours in laboratory rats on long-term treatment [9]. These experiments indicate that the level of mitogenic stimulation is better correlated with an analogue's kinetic dissociation rate than with its equilibrium affinity; that is, the characteristic residence time of the hormone on the receptor is a determinant of the specificity of receptor signalling.

A similar dependence of signalling specificity on the timing of hormone activation is seen in PC12 pheochromocytoma cells. In these cells, nerve growth factor (NGF) induces sustained activation of the mitogen-activated protein kinase (MAPK) signalling pathway, leading to translocation of MAPK to the nucleus and subsequent cell differentiation, whereas epidermal growth factor (EGF) induces only transient MAPK activation,

with no nuclear translocation of MAPK, and induces a cell proliferative response [10,11]. Similarly, insulin induces only a transient activation of MAPK [12]. All of these hormones seem to act, at least in part, through a common pathway, as indicated by the fact that overexpression of EGF [13] or insulin [12] receptors in these cells results in the hormone-induced sustained activation and nuclear translocation of MAPK, with subsequent neurite outgrowth. Thus the cell differentiation or proliferation response seems to be determined by the sustained or transient activation respectively of an event, presumably in the MAPK pathway, shared by NGF, EGF and insulin signalling [14].

In the PC12 system, the shared timing-dependent event is evidently downstream of the initial interactions with the receptors themselves, since NGF, EGF and insulin act through different receptors. In contrast, because the insulin analogues described above presumably all act through the insulin receptor [15], the timing-dependence of the different metabolic versus mitogenic signalling patterns can be traced back to the receptor itself, where specificity is directly dependent on the receptor-binding kinetics of the different analogues.

The increased mitogenic potency of long-acting insulin analogues cannot result directly from a high level of receptor occupancy, since even high concentrations of insulin are not carcinogenic. Indeed, at appropriate concentrations the average number of occupied and activated receptors in the presence of a faster-dissociating insulin analogue can be equal to the number of receptors occupied by a slower-dissociating analogue. The difference lies not in the average number of receptors activated, but in the time for which each receptor is activated (Figure 1). This implies an effect that must take place at the level of individual receptors, presumably through the induction of dif-

Abbreviations used: IRS, insulin receptor substrate; NGF, nerve growth factor; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase.

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Figure 1 Comparison of ligand occupancy at a single receptor for ligands with equal equilibrium affinities but fast or slow binding kinetics

There are fewer binding and dissociation events per unit time for ligands with slow kinetics, but the average time a given receptor is occupied is the same for ligands with slow and fast kinetics.

ferent modes of interaction of the receptor with downstream effector molecules, which is dependent on the timing properties of ligand binding.

The dependence of signalling specificity on the coincident activation of multiple signal inputs is well known in the nervous system [16], in the stimulation of T-cells [17,18], and in G-protein signalling systems, where the molecular bases for such phenomena are perhaps best understood [16,19–23]. The behaviour of such systems can be described in terms of ‘coincidence detectors’ [16], which respond to specific combinations of simultaneously occurring inputs to activate a particular signalling pathway, and it has been shown that this can lead to a dependence of specificity on the timing properties of the interactions [21,24,25]. In insulin receptor signalling, hormone binding and consequent receptor activation are followed by the formation of different molecular complexes which trigger different biological signalling pathways. For example, ternary complexes of the insulin receptor, insulin receptor substrate-1 (IRS-1) and phosphatidylinositol 3-kinase can form [26] to stimulate a pathway associated with insulin metabolic signalling [27], while complexes of Grb2 with IRS-1 or Shc stimulate p21^{ras} [28,29], activating the MAPK pathway. The molecules responding to the formation of these complexes are therefore analogues to the coincidence detectors described above, and we suggest that the specificity of signalling through these alternative pathways may depend on the kinetic properties of the formation of the complexes, as well as those of the upstream events such as hormone binding.

In this paper, we consider a model in which coincident binding of one or more effectors to the ligand-bound insulin receptor and/or to its partners (e.g. IRS-1, IRS-2 or downstream elements) is necessary for signalling. We discuss how signalling can then depend on the kinetic properties of this coincident binding, and show that signalling can ‘prefer’ alternative pathways dependent on the hormone dissociation rate or, equivalently, on the characteristic residence time of the hormone on the receptor, as is seen for the metabolic versus mitogenic signalling of different insulin analogues.

DEVELOPMENT OF A COINCIDENCE-DEPENDENT MODEL OF MITOGENIC SIGNALLING BY THE INSULIN RECEPTOR

General considerations

We consider only models satisfying the following general assumptions. (1) The binding of hormone and effectors can be described classically, with constant on and off rates. (2) Only effects directly related to the binding of hormone and effectors

Figure 2 Comparison of receptor-population occupancy for ligands with equal equilibrium affinities but fast or slow binding kinetics

Open and filled circles represent unoccupied and occupied receptors respectively. For slow kinetics, the same receptors are occupied for longer times, whereas for fast kinetics different receptors are occupied over time. The fraction of receptors occupied at any instant is the same in both cases. Experiments indicate that metabolic effects are similar in the two cases, whereas mitogenesis is enhanced for slow-dissociating insulin analogues.

are important. We ignore the effects of molecular synthesis, degradation, desensitization, feedback, or receptor internalization, aggregation, etc. (In fact, it is known that receptor internalization, for example, affects the residence time of a hormone on the receptor [30]. For simplicity, in developing the model we will only refer to the classic association and dissociation binding parameters of the hormone, with the understanding that, in quantitative considerations, these will have to be replaced with effective binding parameters which take phenomena such as internalization into account.) (3) The effectors bind preferentially to hormone-occupied receptors. If hormone dissociates, this leads to the rapid dissociation and/or de-activation of effector(s). (4) Coincident binding of hormone and effectors is required for signalling; i.e. signalling occurs only from receptors occupied by both hormone and effector(s).

In its simplest form, the binding of a ligand to its receptor can be characterized by three parameters: the equilibrium association constant K_+ , the kinetic association constant k_+ and the kinetic dissociation constant k_- . These parameters are related, as the value of the equilibrium association constant in ordinary single-site binding will be equal to the ratio k_+/k_- . Because of this relationship, it is possible for different hormone analogues to have widely different kinetic binding parameters but similar equilibrium association constants. In order to focus attention on the kinetics of hormone binding, we restrict our attention to systems with fixed equilibrium parameters, e.g. a set of analogues with equal equilibrium affinities but different kinetic binding rates, and consider only the effects of varying kinetic rate parameters within this constraint.

The value of K_+ determines the fraction of receptors within the receptor population that will be occupied at any given instant of time or, equivalently, determines the fraction of time, averaged over many binding events, that any given receptor will be occupied by ligand. A given value of these fractions (i.e. a given value of K_+) can be achieved if, for example, both the kinetic on and off rates are high, or if they are both low, such that their ratio is constant. Under the constraint of constant equilibrium affinity, fast and slow kinetics can be distinguished qualitatively both at the single-receptor and at the receptor-population level. Figure 1 shows the effect at a single receptor. With slow kinetics, there are fewer association and dissociation events in a given time interval than for fast kinetics, while the average fraction of time the

Figure 3 Signalling through a single effector binding to a receptor coincidentally with hormone

Scheme 1 Transition diagram for the one-effector signalling model

The four possible states are unoccupied receptor (R_{00}), and receptor occupied by hormone alone (R_{h0}), effector alone (R_{0e}) or hormone plus effector (R_{he}). Possible transitions are indicated by arrows, along with their associated rate constants.

receptor is occupied is the same in both cases. At the receptor-population level (Figure 2), although the average number of receptors occupied at any instant is the same if the equilibrium affinities are the same, analogues with fast kinetics occupy more different receptors over a period of time compared with analogues with slow kinetics. It is these kinds of differences that must account for the experimental observation that slow kinetics leads to enhanced mitogenic signalling through the insulin receptor.

Single-effector coincident-binding model

Consider a signalling system where the signal is transmitted when a single effector binds to the receptor coincident with hormone occupancy, as shown in Figure 3. Signalling is assumed to occur only through the doubly-occupied receptor; all other receptor states transmit no signal.

The kinetic behaviour of this system is most easily visualized using a state-transition diagram, as shown in Scheme 1. In this representation, all transitions are described by first-order rate constants, and the transition rates depend on the concentrations of free hormone and effector (not shown in the Scheme). R_{00} , R_{h0} , R_{0e} and R_{he} are respectively the concentrations of unoccupied receptor, receptor occupied by hormone only, receptor occupied by effector only, and receptor occupied by both hormone and effector. In Scheme 1 we have made the special assumption that the binding of hormone to the effector-occupied receptor is the same as its binding to the unoccupied receptor, so that the hormone/receptor binding constants remain fixed at k_+ and k_- . To represent the preference of effector binding for the hormone-occupied receptor, we make k'_{e+} small and k'_{e-} large; that is, the effector associates slowly with and dissociates rapidly from the receptor in the absence of bound hormone.

Scheme 2 Simplified transition diagram for the one-effector signalling model

The transition at rate k_- from R_{he} to R_{00} occurs when hormone dissociates, followed immediately by dissociation of effector.

Requirement for non-equilibrium binding reactions

We first note that, if the system of Scheme 1 operates in isolation and at equilibrium, the relative occupancies of the four possible states will depend only on the equilibrium binding constants for the various transitions; that is, there will be no dependence on the kinetic binding constants independent of the equilibrium constants. This means that the set of reactions shown in Scheme 1 operating at equilibrium does not give the dependence on kinetic parameters that we want. Furthermore, application of microscopic reversibility conditions shows that the equilibrium binding parameters are not independent; the value of any one of them is fixed by specification of the values of the others (e.g. see [31]). It is easy to show, for example, that if the affinity for effector binding to the unoccupied receptor is low (i.e. k'_{e+}/k'_{e-} is small), then requiring that hormone binding is independent of effector binding implies that the affinity for effector binding to the hormone-occupied receptor (i.e. k_{e+}/k_{e-}) must also be low.

It is well established, however, that such signalling systems do not operate in isolation. Specifically, in the case of the insulin and related receptors, binding and/or activation of downstream signalling effectors depends on phosphorylation of the receptor subsequent to ligand binding, mediated by kinase and phosphatase enzymic reactions [32]. Because of this, the rate constants k_{e+} , k_{e-} , k'_{e+} and k'_{e-} in Scheme 1 in fact represent reactions which are a combination of effector binding and receptor phosphorylation/dephosphorylation; for example, the binding of effector to hormone-occupied receptor proceeds via an intermediate phosphorylation of the receptor. However, if the phosphorylation and dephosphorylation reactions are rapid compared with the effector binding rates (see Discussion), then the parameters in the Scheme will be a good approximation of the true effector binding rate constants. Such a system operates far from equilibrium, but can nevertheless reach a steady state [31], and differs from the equilibrium situation in that the rate parameters in Scheme 1 can now be considered to be independent of each other (see Appendix 1).

With the assumption that k'_{e+} is small and k'_{e-} is large, i.e. the effector associates slowly with and dissociates rapidly from the unoccupied/unphosphorylated receptor, a further simplification is possible. Because of the low value of k'_{e+} , there is no appreciable transition directly along the pathway from the unoccupied receptor (R_{00}) to the receptor occupied by effector alone (R_{0e}) and hence to its fully activated state (R_{he}). The hormone dissociation transition from R_{he} to R_{0e} occurs with rate k_- , and since R_{0e} is immediately converted into R_{00} because of the rapid effector dissociation at rate k'_{e-} , the state R_{0e} can be ignored in the reaction scheme, and the system takes the simplified form shown in Scheme 2.

The kinetics of the two occupied receptor states for the reduced system are described by eqns. (1), where H and E are the concentrations of free hormone and effector respectively.

$$\begin{aligned}\frac{dR_{h0}}{dt} &= k_+ H R_{00} - k_- R_{h0} - k_{e+} E R_{h0} + k_{e-} R_{he} \\ \frac{dR_{he}}{dt} &= k_{e+} E R_{h0} - k_{e-} R_{he}\end{aligned}\quad (1)$$

The parameters k_- and k_{e-} are (approximately; see above) the true dissociation rate constants of hormone and effector. The term $-k_- R_{he}$ appears in the second equation because of the assumption that the effector dissociates immediately upon dissociation of hormone. To simplify the calculations, we will assume that hormone and effector are in excess, so that their free concentrations remain effectively constant. This assumption does not change the essential properties of the model.

It can be shown through simulation that, if we consider only various values of k_- at constant K_+ (e.g. a set of constant-affinity analogues) and constant values of the other parameters, both R_{h0} and R_{he} initially increase more rapidly for high k_- than for low k_- . However, at later times the situation reverses for R_{he} , which now increases more rapidly for low k_- . Because we are interested in mitogenic signalling, which occurs on a time scale generally considerably longer than that of the binding of hormone (see the Discussion section), it is these later times that are significant. We therefore compute the steady-state solutions, by setting the derivatives to zero in eqns. (1) and solving the resulting algebraic equations. The predicted signalling rate is proportional to the total fraction of receptors occupied by both hormone and effector (i.e. the fraction of receptors in the R_{he} state):

$$\text{Signal} \propto \frac{R_{he}}{R_{00} + R_{h0} + R_{he}} = \frac{K_+ H}{1 + K_+ H} \cdot \frac{k_{e+} E}{k_{e+} E + k_{e-} + k_-} \quad (2)$$

where $K_+ = k_+/k_-$ is the equilibrium affinity constant for hormone binding. It can be seen that, at constant K_+ , an inverse dependence on k_- (i.e. a positive dependence on hormone residence time) occurs if both $k_{e+} E$ and k_{e-} are comparable with or smaller than k_- . This means that the binding kinetics of the effector must be slow compared with the dissociation kinetics of the hormone; in other words, the effector binds slowly and also remains on the receptor for a long time, perhaps until the hormone itself dissociates. The slow association means that there is a relatively long average time delay between the association of hormone and the association of effector, and that the inherent residence time of the effector on the receptor is comparable with or longer than that of the hormone itself. (The dissociation rate k_{e-} , or rather its inverse, represents the residence time of the effector on the receptor in the continuing presence of bound hormone. By the assumptions of our model, the effector will dissociate immediately upon hormone dissociation, regardless of the value of k_{e-} .)

These requirements make it difficult for the single-effector model described here to account for the observed signalling phenomena in the case of the insulin receptor. Even with the acceleration of insulin dissociation rates by receptor internalization [30] or negative co-operativity [33] (not considered in this work), typical hormone residence times are at least several minutes, whereas the first observed downstream events in insulin signalling typically take place in significantly shorter times after insulin binding. For example, tyrosine phosphorylation of IRS-1, which is perhaps the most important signalling molecule associating directly with the insulin receptor, occurs rapidly in cultured cells [34] and in intact animals [35], so that the kinetic properties of activation of these primary effectors do not seem to

Figure 4 Signalling requiring the coincident binding of two effectors to the receptor along with hormone

satisfy the requirement for slow effector kinetics compared with hormone dissociation. Therefore any reasonable model must allow for rapid kinetics in the early stages of activation of the signalling pathway, and cannot depend on the existence of long time delays in the activation of primary effectors associating with the insulin receptor. In other words, the single-effector coincidence model, although inherently giving a timing-dependence, does not satisfactorily account for the data, and we must consider more complex models.

Two-effector coincident-binding model

Next we investigate the signalling behaviour predicted if a signal is generated only when a second effector binds coincidentally to the receptor along with a first effector and hormone. The model describing this behaviour is shown in Figure 4, where it is assumed that the two effectors bind independently to the hormone-occupied receptor. Signalling does not occur when only one of the effectors binds the receptor, but begins when the two effectors are bound to the receptor coincidentally with hormone.

As for the single-effector model, we derive a transition diagram, as shown in Scheme 3. In this, as in Scheme 2, the system operates not at equilibrium but at steady state, due to hormone-dependent receptor phosphorylation reactions. The kinetic equations for the bound states of the receptor are as follows:

$$\begin{aligned}\frac{dR_{h0}}{dt} &= k_+ H R_{00} - k_- R_{h0} - k_{e+}^1 E_1 R_{h0} + k_{e-}^1 R_{h1} - k_{e+}^2 E_2 R_{h0} \\ &\quad + k_{e-}^2 R_{h2} \\ \frac{dR_{h1}}{dt} &= k_{e+}^1 E_1 R_{h0} - k_{e-}^1 R_{h1} - k_{e+}^2 E_2 R_{h1} + k_{e-}^2 R_{h12} - k_- R_{h1} \\ \frac{dR_{h2}}{dt} &= k_{e+}^2 E_2 R_{h0} - k_{e-}^2 R_{h2} - k_{e+}^1 E_1 R_{h2} + k_{e-}^1 R_{h12} - k_- R_{h2} \\ \frac{dR_{h12}}{dt} &= k_{e+}^1 E_1 R_{h1} - k_{e-}^1 R_{h12} + k_{e+}^2 E_2 R_{h2} - k_{e-}^2 R_{h12} - k_- R_{h12}\end{aligned}\quad (3)$$

where R_{00} is the concentration of free receptor, and R_{h0} , R_{h1} , R_{h2} and R_{h12} are respectively the concentrations of receptor occupied by hormone alone, hormone plus effector 1, hormone plus effector 2 and hormone plus both effectors. The parameters k_{e+}^1 , k_{e-}^1 and k_{e+}^2 , k_{e-}^2 are the association and dissociation rate constants for binding of the first and second effector respectively to the hormone-occupied receptor. These equations can be solved for

Scheme 3 Transition diagram for the two-effector binding model

Effectors bind independently, and signalling occurs when both effectors plus hormone are bound to the receptor. Dissociation of hormone causes immediate dissociation of both effectors.

Figure 5 Signalling against hormone residence time for the two-effector coincidence model

Solid line, both effectors have rapid kinetics compared with hormone binding; dotted line, one effector has rapid kinetics and one has slow kinetics; dashed line, both effectors have slow kinetics. Both signal and hormone residence time are normalized to those of native insulin.

the fraction of signalling receptors (i.e. the fraction of receptors in state R_{h12}) at steady state to give:

$$\begin{aligned} \text{Signal} &\propto \frac{R_{h12}}{R_{00} + R_{h0} + R_{h1} + R_{h2} + R_{h12}} \\ &= \frac{K_+ H}{1 + K_+ H} \\ &\quad \cdot \frac{k_{e+}^1 E_1 k_{e+}^2 E_2 (k_{e+}^1 E_1 + k_{e-}^1 + k_{e+}^2 E_2 + k_{e-}^2 + 2k_-)}{(k_{e+}^1 E_1 + k_{e-}^1 + k_-) (k_{e+}^2 E_2 + k_{e-}^2 + k_-)} \quad (4) \\ &\quad (k_{e+}^1 E_1 + k_{e-}^1 + k_{e+}^2 E_2 + k_{e-}^2 + k_-) \end{aligned}$$

Examination of this expression shows that an inverse dependence on k_- occurs if at least one of the effectors has association and dissociation kinetics which are comparable with or slower than the hormone dissociation rate k_- . If both effectors

have slow kinetics, then the dependence on k_- is proportional to $1/k_-^2$. Figure 5 shows these dependencies as a function of T_{res} , the characteristic hormone residence time on the receptor, which we take to be equal to $1/k_-$. When both effectors have fast kinetics the signal develops rapidly, but has little or no dependence on T_{res} . When one of the effectors has slow kinetics, the signalling develops more slowly and is dependent on T_{res} , and when both of them have slow kinetics the dependence is weak at low T_{res} but increases at higher T_{res} . At very high T_{res} all three curves flatten and become independent of T_{res} (not shown in Figure 5). Therefore, in the case of both effectors having slow kinetics, the curve has a sigmoid shape, which is a classical sign of switching behaviour with respect to the variable T_{res} [36]. It should be noted that the assumption that the effectors bind independently to the receptor is not crucial; similar behaviour is seen (calculations not shown) if the second effector binds only when the first is already bound, e.g. by binding directly to the first effector and not to the receptor itself.

In this two-effector model, the first effector to bind may have fast kinetics (i.e. $k_{e+}^1 E_1$ and k_{e-}^1 may be larger than k_-), but if the second effector has slow kinetics, an inverse dependence of signalling on k_- is predicted, i.e. signalling increases with T_{res} . This means, for example, that the first effector has kinetics analogous with those seen for IRS-1, whereas the second, as yet unidentified, effector would be responsible for the activation of the k_- -dependent signalling pathway, in our example leading to mitogenic signalling. This model can therefore predict the increase in signalling as T_{res} increases (e.g. for analogues with equal equilibrium affinities but different kinetics), while still allowing binding of the first effector to the receptor to have rapid kinetics, as is seen experimentally.

DISCUSSION

We have presented models with simple properties that illustrate kinetics-dependent signalling behaviour. These models are based on the premise that, upon hormone binding, downstream effectors must bind coincidentally to trigger activation of a specific pathway, which in our example is mitogenic signalling through the insulin receptor. Our analysis shows that a dependence of signalling on hormone dissociation kinetics can occur if signalling is transmitted through a single effector which binds to the

occupied receptor with association and dissociation rates that are lower than the dissociation rate of the hormone binding itself. However, because the best known molecules (e.g. IRS-1 and IRS-2) interacting directly with the insulin receptor seem to have rapid kinetics in relation to the dissociation rate of insulin, a second effector was introduced into the model, such that the first effector has rapid kinetics while the second effector has slow kinetics and is primarily responsible for the timing-dependence of signalling specificity. In this way, the role of the first effector with rapid kinetics can be assigned to IRS-1 or other primary insulin-receptor substrates.

In fact, it is not necessary that the slow kinetic component be at a second effector; it could be farther down the signalling chain. In other words, the kinetic dependence would still be observed if transmission of the signal is through an initial series of effector molecules with fast kinetics, followed by one or more with slow kinetics which represent the steps with the longest lag times in the signalling pathway. This possibility emphasizes the important point that the coincidence detector need not reside at a single molecule, but may be distributed among two or more molecular loci [16]. Indeed, in our example the entire signalling chain can be thought of as a coincidence detector for the association and activation of its multiple molecular components, and the work here shows that the behaviour of such a system is sensitive to the kinetics of the slowest reaction in the chain.

The basis of our model is that effectors bind to the receptor and to each other in a complex which is the active element in receptor signalling. In general terms, however, the rate parameters can be assigned, not specifically to binding and dissociation, but to activation and de-activation of the effectors. That is, the conclusions of the model are not necessarily invalidated, for example, if an effector somewhere in the signalling pathway disengages its predecessor before interacting with subsequent elements in the pathway. The essential requirement is that the activation/de-activation reactions are reasonably tightly coupled to each other and to hormone binding. For example, if the dephosphorylation of the receptor lags behind the dissociation of hormone, as discussed above, the timing-dependence of signalling should be retained so long as this lag is not large compared with the average hormone residence time on the receptor. We have shown that this concept can be expressed quantitatively in a model, based on logical analysis, which considers the signalling pathway as a sequence of effector activations with specific time delays of activation and de-activation [37]. In such a model, activation of a pathway depends on whether the hormone residence time is greater or less than a critical time which depends on the participating effectors' activation and de-activation times and which is analogous to the duration of coincident binding of the hormone, receptor and effector in the model presented here.

In the context of our model, the mitogenic signalling pathway should include an activation step whose kinetics are slow in relation to the residence time of insulin on the receptor, whereas the metabolic pathway should contain only relatively rapid components. Metabolic signalling does seem to be rapid, since glucose transport in fat cells begins less than 1 min after insulin binding and reaches an apparent plateau within about 4 min [38]. Evidence for slow components in the mitogenic pathway is limited; however, based on the fact that a signalling switch to neuronal differentiation in PC-12 cells stimulated by NGF is associated with protracted MAPK activation and translocation of MAPK to the nucleus [10,11], one can speculate that similar slow nuclear translocation events are associated with mitogenic signalling in insulin-stimulated cells.

A critical component of our coincident-binding model is that

the effectors can bind to the receptor only if the receptor is occupied by hormone. If effectors can bind equally well to the unoccupied receptor, it can be shown that the dependence of signalling on hormone residence time disappears. This means that, in order to observe a kinetic dependence in signalling specificity, hormone binding must directly trigger some change in the receptor which allows effector or effectors to bind; for the insulin receptor this change is evidently receptor phosphorylation [32,39], possibly accompanied by conformational changes [40,41]. Conversely, effectors must dissociate rapidly from the active complex when the hormone dissociates. If it is receptor phosphorylation that enables effector binding to occur, this implies that the receptor must be rapidly dephosphorylated upon hormone dissociation. There is indirect evidence for this from studies with permeabilized adipocytes [42] and intact rat liver endosomes [43]. In both of these studies, [γ - 32 P]ATP incorporated into insulin receptors through insulin stimulation was 'chased' with an excess of unlabelled ATP, and loss of label was measured over time. The results showed that the labelled phosphate was lost within approx. 2 min, indicating that the receptor phosphorylation reaction is opposed by a rapid dephosphorylation reaction, presumably mediated by one or more phosphatases. The importance of phosphatase activity in insulin signalling is illustrated by the insulin-mimetic effects of the phosphatase inhibitor vanadate [44]. These results, taken together with the observation that the insulin receptor on cultured cells is rapidly tyrosine-phosphorylated (within 10–60 s) in response to insulin treatment [45], indicate that receptor tyrosine phosphorylation is tightly coupled to the binding of hormone. It is possible, however, that this coupling is somewhat looser than the above experiments indicate, as there is evidence to suggest that internalized receptors persist in a phosphorylated state for several minutes after insulin dissociation [46].

For the model presented here, the dissociation parameter k_- must take into account both the inherent binding affinity of insulin or analogue plus the modifying effects of other factors, such as receptor internalization. At 37 °C, at which temperature receptors are actively internalized into an acid endosomal environment [47], insulin leaves the receptor with a half-time of less than 10 min [30,48], but much longer half-times are observed at 15 °C or lower [33], when receptor internalization is minimal [49]. In our model, therefore, it is these relatively short hormone residence times that must be compared with the characteristic on and off times of the effectors in order to determine how sensitive the signalling is to hormone residence time. That is, the characteristic times of the rate-limiting step in the timing-dependent pathway need only be comparable with or longer than times of the order of 10 min in order to observe dependence of signalling on the hormone dissociation rate. It is unclear at present which events in the insulin signalling pathways might have such characteristics, but careful experiments measuring the kinetics of interaction of signalling molecules should reveal likely candidates.

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APPENDIX

Non-equilibrium behaviour of extended reaction system including enzymic reactions

For closed-loop reaction schemes such as that shown in Scheme 1, equilibrium analysis reveals that the binding parameters are constrained, such that one of them is completely dependent on the others. Furthermore, the distribution of the receptor population among the possible states at equilibrium depends only on the equilibrium parameters, and not on the kinetic parameters explicitly. We show here that, if effector binding depends on receptor phosphorylation, and the phosphorylation is mediated by kinase and phosphatase enzymic reactions operating far from equilibrium, the constraints among the parameters in Scheme 1 are relaxed and the steady-state behaviour can now depend explicitly on the hormone kinetic binding parameters. Similar discussions have been given by others (e.g. see [1]).

We have constructed a reaction system, shown in Scheme A1, with the following properties. (1) Hormone binding is not dependent on receptor phosphorylation or effector occupancy. (2) The effector binds strongly to the phosphorylated receptor, but weakly to the unphosphorylated receptor, independent of hormone occupancy. (3) The kinase and phosphatase reactions are strongly forward-directed under the assumed non-equilibrium conditions. (4) The hormone-occupied receptor is a good substrate for the kinase, but a poor substrate for the phosphatase; and the non-hormone-occupied receptor is a good substrate for the phosphatase, but a poor substrate for the kinase.

In Scheme A1, R_{00} , R_{h0} , R_{0e} and R_{he} are respectively the concentrations of receptor bound by neither hormone nor effector, by hormone alone, by effector alone and by both

hormone and effector. Phosphorylated receptor is represented by the superscript p. In the Scheme we use a shorthand notation for the rate parameters; for example, the symbol k represents both the forward (k_+) and reverse (k_-) kinetic constants for hormone binding to the receptor. Similarly, k_e and k'_e represent effector binding to phosphorylated and unphosphorylated receptor respectively. The reactions are taken to be such that k_+ , k_{e+} and k'_{e+} are the rates in the rightward and upward directions, and k_- , k_{e-} and k'_{e-} are the rates in the opposite directions.

The symbols p and $\bar{p}$ represent the kinetic reaction parameters for the kinase and phosphatase reactions respectively on the hormone-occupied receptor, and p' and $\bar{p}'$ are the corresponding rates for the non-hormone-occupied receptor, all in the absence of bound effector. The corresponding rate parameters subscripted with 'e' (e.g. p_e) specify the same reactions for the effector-occupied receptor. The enzymic cofactors A and B for the kinase reaction, and C and D for the phosphatase reaction, shown only in the lower right and upper left of the Scheme, are taken to be the same for all kinase and phosphatase reactions in the reaction scheme. For all phosphorylation reactions, the direction towards increased phosphorylation (e.g. from R_{h0} towards R_{hp}) will be represented by a plus sign, and the opposite direction by a minus sign. The arrows indicate the directions of the rapid, nearly irreversible, reactions under non-equilibrium conditions, and the broken lines show those phosphorylation reactions for which the receptor is a weak substrate. It is important to note that assumption (4) implies that both the forward and backward

Scheme A1 Complete reaction scheme including receptor kinase and phosphatase reactions

Notation is described in the text.

phosphorylation rate constants are small for the weak-substrate reactions, but says nothing about the ratio of these rate constants, i.e. the equilibrium constants (see below). Solid lines with no arrows represents the rate-limiting reactions for the system, and correspond to the reactions shown in Scheme 2.

The system shown in Scheme A1 comprises 16 reactions, as shown in eqns. (A1):

H and E are the concentrations of free hormone and effector respectively, and A – D are the concentrations of cofactors A – D . The equilibrium solution of these equations gives the thermodynamic constraints among the parameters resulting from the

closed loops in the reaction scheme. The equilibrium equations have the form:

$$\begin{aligned} R_{h0} &= K H R_{00} & B R_{00}^p &= P' A R_{00} \\ R_{he} &= K H R_{0e} & B R_{h0}^p &= P A R_{h0} \\ R_{h0}^p &= K H R_{00}^p & B R_{0e}^p &= P'_e A R_{0e} \\ R_{he}^p &= K H R_{0e}^p & B R_{he}^p &= P_e A R_{he} \\ R_{0e} &= K'_e E R_{00} & D R_{00}^p &= \tilde{P}' C R_{00} \\ R_{he} &= K'_e E R_{h0} & D R_{h0}^p &= \tilde{P} C R_{h0} \\ R_{0e}^p &= K_e E R_{00}^p & D R_{0e}^p &= P'_e C R_{0e} \\ R_{he}^p &= K_e E R_{h0}^p & D R_{he}^p &= \tilde{P}_e C R_{he} \end{aligned} \quad (A2)$$

where we use upper-case letters for the equilibrium constants; i.e. $K = k_+/k_-$, $P' = p'_+/p'_-$, $P = p_+/p_-$ etc. These equations give eight constraints among the equilibrium constants, which can be written as

$$\begin{aligned} P/P_e &= K'_e/K_e & P' &= P \\ P'/P'_e &= K'_e/K_e & P'_e &= P_e \\ \tilde{P}/\tilde{P}_e &= K'_e/K_e & \tilde{P}' &= \tilde{P} \\ \tilde{P}'/\tilde{P}'_e &= K'_e/K_e & \tilde{P}'_e &= \tilde{P}_e \end{aligned} \quad (A3)$$

The constraints on the left of eqns. (A3) show that, if the effector binds preferentially to the phosphorylated receptor ($K'_e \ll K_e$), as we have assumed, then the kinase reaction must be stronger and the phosphatase reaction weaker for the receptor with effector bound compared with these reactions for the receptor without effector bound. The constraints on the right state that the equilibrium constants for the kinase and phosphorylation reactions must also be independent of hormone binding. This is a direct consequence of our assumption that hormone binding is independent of receptor state, and, as for the constraints on the left of eqns. (A3), illustrates the symmetry of the dependence of

binding and phosphorylation on each other. The physical meaning of this is that, even though the individual rate constants for the weak-substrate phosphorylation reactions (listed in assumption 4 above) are small, the equilibrium constants for the reaction (i.e. the ratios of the rate constants) must be independent of hormone occupancy if the binding of hormone is itself independent of receptor phosphorylation.

Now, if the reaction in Scheme A1 is driven far from equilibrium by making A/B large and C/D small, receptor phosphorylation will be mediated primarily by the kinase, and receptor dephosphorylation by the phosphatase, as indicated by the arrows on the phosphorylation reactions in Scheme A1. The weak-substrate reactions can be ignored because of their low forward and backward rates, and if the primary enzymic reactions and the effector dissociation rate (k'_e) from the unphosphorylated

receptor are rapid compared with the rate-limiting hormone and effector binding steps represented by k and k_e , the entire reaction scheme can be approximated by the simple system shown in Scheme 2 and eqns. (1), involving only the three receptor states R_{00} , R_{h0} and R_{he}^p . Note that the requirement $P/P_e = \tilde{P}'/\tilde{P}'_e = K'_e/K_e \ll 1$, according to the equations on the left in eqns. (A3), must still hold, but since all kinase and phosphatase reactions under far-from-equilibrium conditions are assumed to be rapid in their respective forward directions, none of them will be rate-limiting, and the approximation of Scheme 2 is still valid.

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