Kinetic Relationships between Insulin Receptor Binding and Effects on Glucose Transport in Isolated Rat Adipocytes†

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ABSTRACT: The role of insulin receptor occupancy in the stimulation of glucose transport has been studied in isolated rat adipocytes. At 37 °C, under steady-state conditions, the time needed to fill the fraction of receptors (<1%) required for an initial measurable effect varied with insulin concentration from <10 s at 100 ng/mL to 90 s at 0.5 ng/mL. However, at all insulin concentrations there was an initial lag period before any activation was seen. The length of the initial lag was inversely related to the insulin concentration, lasting 2 min at 0.5 ng/mL and only 30–40 s at 5–500 ng/mL (maximal levels). A similar discrepancy was noted between dissociation of prebound insulin and the loss of insulin's effects on transport. At an insulin concentration of 0.3 ng/mL, half of the insulin effect was lost within 12 min; the $t_{1/2}$ of dissociation was 8 min. When the insulin concentration was increased to 10 ng/mL, the $t_{1/2}$ of dissociation increased only to 10 min while the $t_{1/2}$ of deactivation was now 60 min. In conclusion, (1) kinetic studies reveal a time-requiring step between insulin binding and early effects on glucose transport, (2) a low level of insulin binding (<1% occupancy) is all that is necessary to initiate the insulin stimulus–response sequence, and (3) the rate of deactivation is closely related to the steady-state level of insulin binding, and with increasing insulin concentrations this rate slows and diverges from the rate of dissociation of insulin from receptors.

The initial step in insulin action involves binding of the hormone to specific receptors on the cell surface (Roth, 1973). In general, insulin target cells possess more receptors than are necessary to generate a maximal biologic response to the hormone, indicating that spare receptors are present (Kono & Barham, 1971). Thus, depending on the tissue studied and biologic action measured, only a small percentage of the total cellular receptors need to be occupied in order for insulin to exert a maximal effect (Kono & Barham, 1971; Davidson & Frank, 1980; Fehlmann & Freychet, 1981; Meuli & Froesch, 1977). The concept and quantitation of spare receptors has evolved from studies which have measured both binding and biological responses under steady-state conditions (Kono & Barham, 1971). Therefore, relatively little information is available concerning the relationship between levels of receptor occupancy and the rate of onset of insulin action (Gliemann et al., 1975; Crofford, 1975; Häring et al., 1978). Similarly, the quantitative relationships between the number of insulin receptors occupied and deactivation of insulin's biologic effects are also largely unknown. In an earlier report we found that deactivation of insulin-stimulated glucose transport occurred at a much slower rate than dissociation of insulin from its receptors when a submaximal insulin concentration of 1 ng/mL was used (Ciaraldi & Olefsky, 1980). To extend these observations, and to better quantitate the relationship between fractional receptor occupancy and loss of insulin action, we have now studied the relationship between insulin occupancy of receptors and control of glucose transport activity at a variety of insulin concentrations.

Experimental Procedures

Materials. Porcine monocomponent insulin was a gift from Dr. Ronald Chance of the Eli Lilly Co. (Indianapolis, IN), and A^{14}-mono[^{125}I]iodoinsulin was supplied by Dr. Bruce Frank of Eli Lilly Co. 3-*O*-Methyl-D-[U-^{14}C]glucose, L-[1-^{14}C]glucose, and Na^{125}I were purchased from New England Nuclear (Boston, MA), collagenase was from Worthington Biochemical Corp. (Freehold, NJ), bovine serum albumin (fraction V) was from Armour Pharmaceutical Co. (Phoenix, AZ), phloretin was from Biochemical Labs (Redondo Beach, CA), silicon oil for binding studies was from Arthur H. Thomas Co. (Philadelphia, PA), and silicone oil for transport studies was from Union Carbide (New York, NY). All other chemicals were reagent grade and from standard sources.

Preparation of Isolated Adipocytes. All studies were performed on tissue removed from male Sprague-Dawley rats (180–220 g) which were given free access to lab chow. Animals were stunned by a blow to the head and killed by cervical dislocation, and the epididymal fat pads were removed. Isolated fat cells were prepared by a modification of the method of Rodbell (1964) using a modified Krebs–Ringer bicarbonate buffer containing collagenase (2 mg/mL), bovine serum albumin (BSA) (40 mg/mL), and glucose (3 mM).

Adipocyte counts were performed according to a modification (Olefsky, 1978) of method III of Hirsch & Gallian (1968). Counting was done by using a Coulter Model ZB counter with a 400-μm aperture, and all data were normalized to a cell number of 2×10^5 cells.

Glucose Transport Studies. (A) Dose–Response Studies. Cells were diluted in a Krebs–Ringer phosphate buffer (KRP) containing 128 mM NaCl, 5.2 mM KCl, 1.2 mM $CaCl_2$, 1.29 mM $MgSO_4$, 1.29 mM KH_2PO_4, and 4% BSA (pH 7.4) to a cell concentration of 10^6 cells/mL. Tubes were capped, gassed with 95% O_2/5% CO_2, and incubated with or without insulin for 30 min in a 37 °C shaking bath. The cell suspension was used directly in the transport assay.

(B) Activation Studies. Cells were diluted to the same concentration and in the same buffer as for the dose–response studies. Procedures were the same as reported earlier (Ciaraldi & Olefsky, 1979) with basal transport measured at $t = 0$ followed by the addition of insulin in a small volume. Values were compared to the maximally stimulated transport rate seen in separate aliquots of cells preincubated with a maximally effective insulin concentration for 30 min. In these studies a 4% BSA buffer was used instead of a 1% BSA buffer

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0006-2960/82/0421-3475$01.25/0

(Ciaraldi & Olefsky, 1979), but both buffers gave identical results.

(*C*) *Deactivation Studies.* The cells from 2 g of tissue were diluted to 33 mL with KRP–1% BSA–1 mM glucose, and 3 mL was removed to a separate vessel to be used for determination of basal transport. Insulin was added to the larger volume of cells, and both were gassed and preincubated for 30–60 min at 37 °C. The excess, unbound hormone was removed by gentle centrifugation of the cell suspension and the buffer aspirated off. Dissociation and deactivation were initiated by adding a fresh 35-mL sample of insulin-free buffer (warmed to 37 °C) to the cells and transferring to large polypropylene flasks with gentle shaking in a 37 °C bath. Sampling was accomplished by removing 5-mL aliquots and centrifuging at 1000*g* for 10 s. Most of the buffer was removed to leave 0.5 mL of a concentrated cell suspension which was used in the transport assay. The cells which were not exposed to insulin were also concentrated and assayed for basal transport activity.

(*D*) *Measurement of 3-O-Methylglucose Transport.* Glucose transport was assessed by measuring initial rates of 3-*O*-methylglucose uptake according to a modification of the method of Whitesell & Gliemann (1979) as previously described (Ciaraldi & Olefsky, 1979). The final substrate concentration in the assay was 15.5 μM. The reaction was stopped after 4 s by the addition of 11 mL of a chilled solution of 0.3 mM phloretin in 0.9% NaCl–0.7% EtOH. Transport is stopped immediately upon exposure to phloretin. With this procedure the half-time of 3-*O*-methylglucose transport is 5 s or greater (Siegel & Olefsky, 1980), and the 4-s value lies on the linear portion of the time course and therefore is an accurate measure of the initial rate of transport. Two milliliters of silicone oil (viscosity = 125 cSt, specific gravity = 0.99) was layered over the mixture, and the cells were centrifuged at 2000*g* for 10 s and then collected for determination of radioactivity. The portion of cellular uptake due to trapping of the label in the extracellular water space was measured by performing parallel reactions with L-[^{14}C]glucose as substrate. All values of transport were corrected for the L-glucose value and normalized to 2×10^5 cells.

(*E*) *Insulin Binding Studies.* These were performed together with the dose–response studies of insulin action. Cells were taken up in the same KRP–BSA buffer at pH 7.4 for direct comparison of binding and action. Reactions were terminated as previously described (Olefsky et al., 1974). All values were corrected for nonspecific binding which was 5–10% of the total counts bound, and the results were normalized to 2×10^5 cells/mL.

(*F*) *Association Studies.* Cells were diluted in a KRP–1% BSA buffer (pH 7.6) at a cell concentration of (1.5–2.5) $\times$ 10^5 cells/mL. Reactions were started by adding cells (warmed to 37 °C) to the insulin. Duplicate 100-μL aliquots were removed at the designated times, placed in microfuge tubes containing 100 μL of silicone oil, and centrifuged in a Beckman microfuge for 30 s (Ciaraldi & Olefsky, 1979). The reaction was considered terminated when centrifugation was begun. A parallel time course of nonspecific binding was included in each study.

(*G*) *Dissociation Studies.* Conditions were the same as for the deactivation studies. The 30-mL preincubation contained [^{125}I]iodoinsulin (0.3 ng/mL) and unlabeled insulin at the desired concentration. Triplicate 200-μL aliquots were obtained before centrifugation to remove unbound insulin; these provide the $t = 0$ values. Dissociation was initiated by dilution in fresh insulin-free buffer, and at appropriate times triplicate aliquots of the dilute cell suspension were removed for determination of total binding.

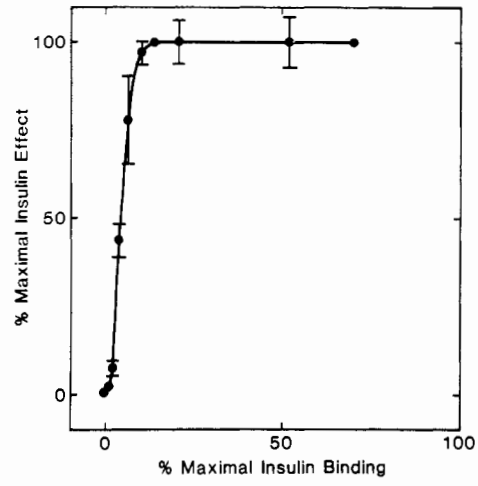

FIGURE 1: Comparison of insulin binding and stimulation of 3-*O*-methylglucose transport at the steady state. Binding and initial rates of 3-*O*-methylglucose transport were measured in the same buffer after 30 min of incubation at 37 °C. Transport results are expressed as a percent of the increase in the rate of glucose transport (taken as 100%) in the presence of a maximally effective insulin concentration. Basal transport was 0.14 ± 0.03 pmol $(2 \times 10^5 \text{ cells})^{-1}$ 4 s^{-1} while the maximally stimulated rate was 1.25 ± 0.11. The binding results are expressed as a percent of R_0 [total number of receptors available = 295 pg/$(2 \times 10^5$ cells)] as estimated from Scatchard analysis. Each point is the mean ± SEM of three to nine experiments.

Results

Relationship of Insulin Binding and Steady-State Effect. Exposure of adipocytes to insulin for 30 min at 37 °C is sufficient to permit binding to reach steady state (Ciaraldi & Olefsky, 1979) and for all hormone concentrations to express their full effects on glucose transport. Under these steady-state conditions it is possible to compare the fractional receptor occupancy achieved at different insulin concentrations with the proportion of maximal glucose transport stimulation elicited. As can be seen in Figure 1, significant transport stimulation occurs at very low levels of binding (1%), and 50% of the maximum response is seen when only 4–5% of the receptors are filled. Full transport activation is seen at approximately 10% receptor occupancy; thus, the remaining 90% of receptors can be regarded as spare. Therefore, at steady-state fractional receptor occupancies >10%, receptor binding is no longer limiting, and the capacity of the glucose transport system determines the final response.

Kinetics of Insulin Binding to Receptors and Glucose Transport Activation. The rate at which insulin binds to receptors in isolated adipocytes is a function of the insulin concentration. Figure 2 compares rates of binding at 37 °C and shows that the fractional receptor occupancy necessary for full stimulation, 10% [~20 pg/$(2 \times 10^5$ cells)], is reached very rapidly (<10 s) at 100 ng/mL and slower for 10 (90 s) and 5 ng/mL (5 min). The lower insulin concentrations studied had even slower time courses and never reached the spare receptor threshold. Figure 3 displays the time course of insulin stimulation of 3-*O*-methylglucose transport at 37 °C. Results are presented as a percent of the maximal steady-state effect seen with 100 ng/mL, which represents a 9–10-fold increase over basal. The curve for 100 ng/mL is the same as reported previously (Ciaraldi & Olefsky, 1979), consisting of an initial lag time of ~40 s and then a gradual rise to the full insulin effect within 10–15 min. The length of the lag period is nearly constant at insulin concentrations

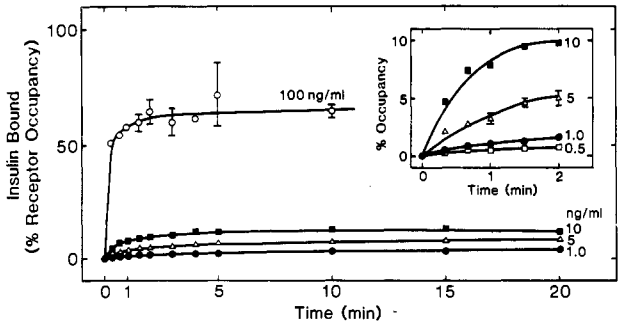

FIGURE 2: Time course of specific insulin binding at 37 °C for various insulin concentrations. A tracer concentration of [^{125}I]iodoinsulin (0.2 ng/mL) was added to cells with varying amounts of unlabeled insulin, and aliquots were removed at the indicated times for measurement of insulin binding. Maximum receptor number is determined from Scatchard analysis. (Inset) Expanded scale of first 2 min of reaction. Each value is the mean ± SEM of two experiments, each done in duplicate.

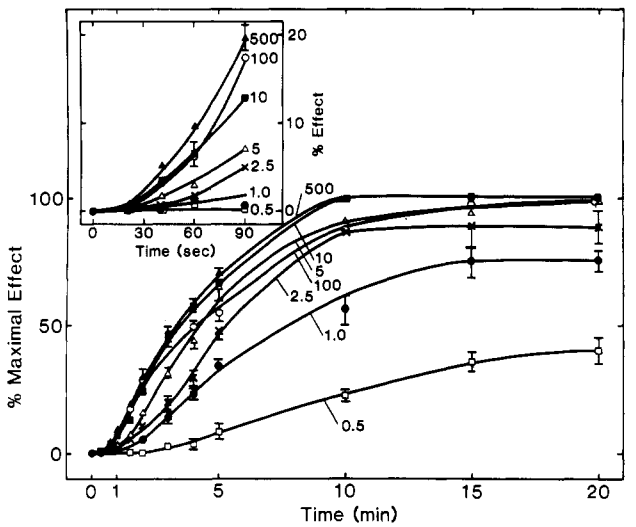

FIGURE 3: Time course of insulin action to stimulate initial rates of 3-*O*-methylglucose transport. Basal transport was measured and then insulin added in a small volume to cells. Aliquots were removed at the indicated times for rapid (4 s) assay of 3-*O*-methylglucose transport. Results are expressed as a percent of the maximal insulin effect, which was measured in cells exposed to 100 ng/mL insulin for 30 min, minus the basal value. Each value is the mean ± SEM of three to six experiments. (Inset) Expanded scale of first 90 s of reaction.

which produce steady-state levels of receptor occupancy that equal or exceed the spare receptor threshold (>10%). When the insulin concentration is lowered below this level, the duration of the lag phase increases to 1 min at 2.5 ng/mL, 2 min at 1 ng/mL, and 3–4 min at 0.5 ng/mL (Figure 3, inset). Thus, the greater the rate of binding, the faster the activation (shorter lag), provided concentrations less than those needed to achieve 10% receptor occupancy at steady state are used. Once binding exceeds the spare receptor threshold, regardless of the insulin concentration used (e.g., by 5 min at 5 ng/mL), then the rates of activation from that point on are similar and reach full stimulation at the same time. Therefore, as far as the rate of transport activation is concerned, a greater proportion of the cell's receptors are spare than the fraction needed for full stimulation at steady state, and at submaximal levels (2.5, 1, and 0.5 ng/mL), the initial lag phases are longer and the rates of activation are slower.

Kinetics of Insulin Dissociation and Deactivation of Glucose Transport. Next we evaluated the kinetics of insulin dissociation from receptors and deactivation of the stimulated glucose transport system. Adipocytes were incubated with various concentrations of insulin until steady-state binding

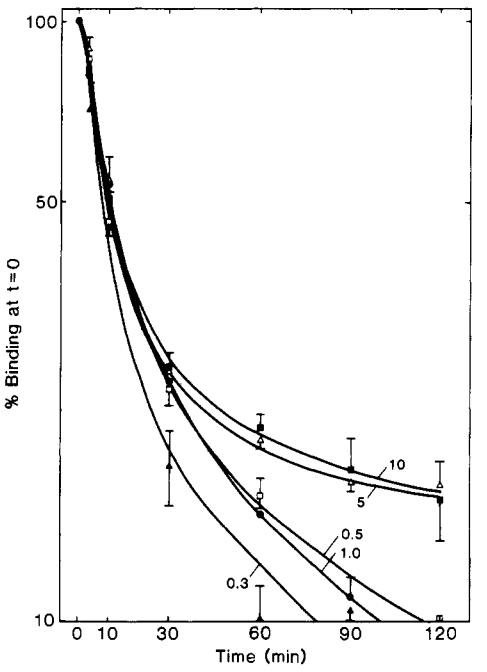

FIGURE 4: Time courses of dissociation of [^{125}I]iodoinsulin binding at 37 °C at various insulin concentrations. Dissociation was initiated after a single wash in the manner described under Experimental Procedures for studying deactivation. Each value is the mean ± SEM of two experiments, each done in triplicate.

conditions were reached. Following that, the cells were washed into insulin-free buffer, and Figure 4 displays the resulting dissociation curves at 37 °C. In these experiments it should be noted that the time at which 50% of the initial amount of insulin bound has dissociated is denoted as $t_{1/2}$. Dissociation is very rapid at all insulin concentrations, and the $t_{1/2}$ varies only slightly over the insulin concentration range 0.3–10.0 ng/mL. The $t_{1/2}$ of dissociation was the same at 0.5–10 ng/mL (10–12 min) and 8 min at 0.3 ng/mL.

Deactivation of insulin-stimulated glucose transport was measured under the same conditions as insulin dissociation from receptors. The results are presented in Figure 5, which reveals a concentration-dependent slowing of the rate of deactivation. At the lowest concentration tested (0.3 ng/mL), the half-time for deactivation is 12 min, a value in close agreement with the $t_{1/2}$ of dissociation (Figure 4). As the insulin concentration is increased, the rate of dissociation remains nearly constant, while the rate of transport deactivation slows; i.e., the two processes diverge. The time at which half of the insulin effect is lost increases from 25 min at 0.5 ng/mL to 60 min at 5, 10, and 20 ng/mL. This suggests that the number of receptors occupied during dissociation does not determine the rate of deactivation of the glucose transport system. Rather, the rate appears to be related to the level of steady-state receptor occupancy achieved during the activation process. This latter relationship can be most readily seen in Figure 6, which plots the fractional receptor occupancy reached at steady-state concentrations from 0.3 to 20 ng/mL, as a function of the half-time of deactivation of these same insulin levels. When steady-state occupancy achieved during the activation phase exceeds the spare receptor threshold (10%), then the $t_{1/2}$ of deactivation is constant, but below these levels the rate of deactivation is dependent upon the steady-state level of binding.

Discussion

Insulin's cellular action to stimulate glucose transport represents a complex multistep series of events. The first step

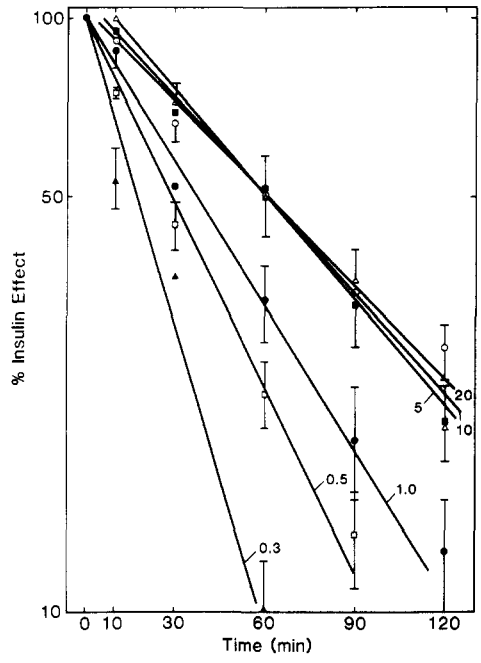

FIGURE 5: Time course of deactivation of insulin-stimulated glucose transport at 37 °C. Results are expressed as a percent of the insulin effect obtained at $t = 0$ at each concentration. Thus, the $t = 0$ value is 100% of the difference in glucose transport between cells incubated at steady state with the desired insulin concentration and a separately determined basal value. Each curve is labeled with the insulin concentration present during the preincubation. Each point is the mean ± SEM of three to six experiments.

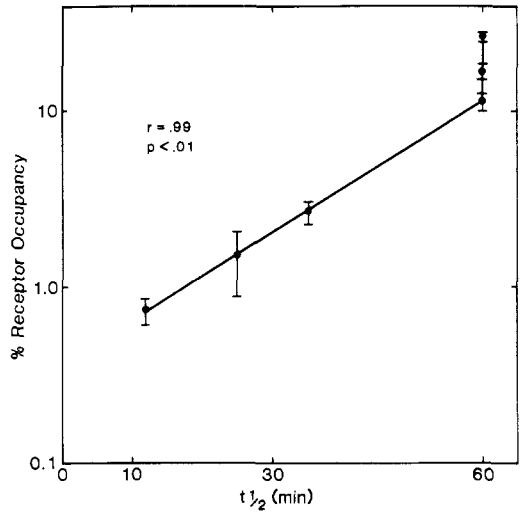

FIGURE 6: Relationship between steady-state fractional receptor occupancy and half-time of deactivation of glucose transport. Values are taken from Figures 1 and 5 where steady-state binding was determined after 30 min at 37 °C.

in this sequence involves binding of the hormone to its cell surface receptor (Roth, 1973). Following this, the insulin receptor complex is coupled, through some signal mechanism, to the glucose transport effector system (Czech, 1977). It is well established that target cells possess more receptors than are necessary in order to generate a maximal biologic insulin effect (Kono & Barham, 1971). For insulin-stimulated adipocyte glucose transport, approximately 90% of the receptors are spare; i.e., occupancy of 10% of the total available receptor complement will lead to the same maximal glucose transport stimulation (Kono & Barham, 1971; Figure 1).

Recent information demonstrates that the biologic effect of insulin to stimulate glucose transport does not occur with the same time course as binding to the receptor (Ciaraldi & Olefsky, 1979; Häring et al., 1978). For example, at 37 °C, exposure of adipocytes to a high concentration of insulin (100 ng/mL) results in a fractional receptor occupancy which exceeds the spare receptor threshold (fractional receptor occupancy of 10%) within 10 s (Figure 2). However, there is an absolute initial time lag of about 40 s before any stimulation of glucose transport can be observed, which is then followed by a gradual increase in transport activity until full effects are reached by 10–15 min (Ciaraldi & Olefsky, 1979; Häring et al., 1978, 1982). This lag period represents the time required for the mechanisms which couple occupied insulin receptors to the glucose transport system to proceed and is a time-, temperature-, and energy-dependent process (Ciaraldi & Olefsky, 1979; Siegel & Olefsky, 1980; Häring et al., 1981).

These findings were elucidated in our initial studies of this activation process and used only a single high (100 ng/mL), maximally effective insulin concentration. When a wider range of insulin concentrations (Figures 2 and 3) was used, additional features of this activation mechanism can be appreciated. As can be seen in Figure 3, the absolute time lag for the onset of insulin action is the same (about 40 s) at all maximally effective insulin concentrations, despite the fact that the amount of insulin bound over this time interval varies greatly over the insulin concentration range 5–500 ng/mL. Of particular note is the fact that at the lowest maximally effective insulin concentration employed (i.e., 5 ng/mL), the spare receptor threshold (10% fractional receptor occupancy) is not reached for 5 min. At maximally effective insulin concentrations, not only is the initial lag period the same but also the rate of activation and the time period until full activation is achieved are quite comparable. In contrast, at submaximally effective insulin levels, the length of the lag time is inversely related to the insulin concentration used, as is the rate of activation and the time elapsed until the full steady-state effect is achieved. Thus, it would appear that the rate of glucose transport stimulation is proportional to initial receptor occupancy until maximally effective insulin concentrations are used (in these experiments 5 ng/mL and greater). This is true despite the fact that the number of receptors occupied during the first few minutes of the activation phase is much less than the number of receptors which need to be occupied to elicit a maximal biologic effect under steady-state conditions. These findings demonstrate that when one considers the rate of onset of insulin action, cells possess a much higher proportion of spare receptors than when one only considers the final steady-state effect. This would have the physiologic result of allowing the cell to respond at a maximal rate to extremely small changes in circulating insulin levels, which has the advantage of shortening the functional time delay between insulin secretion and final hormonal effect at the target tissue.

We have previously reported that at a submaximal insulin concentration of 1 ng/mL, the $t_{1/2}$ of insulin dissociation from its receptors was 10 min while the $t_{1/2}$ for glucose transport deactivation was 40 min (Ciaraldi & Olefsky, 1980). This showed that the rate of decay of this biologic effect was independent of residual receptor occupancy, in the sense that although dissociation and deactivation are temporarily related they are not directly coupled. Conceptually similar concepts can be drawn from the work of Crofford (1975) and Gliemann et al. (1975). Furthermore, Häring et al. (1982) have carried out experiments similar to our own with essentially identical results, showing that the rate of adipocyte glucose transport deactivation is considerably slower than the rate of insulin dissociation from receptors.

In an apparent conflicting report, Laursen et al. (1981) have shown rapid deactivation of the adipocyte glucose transport system with rates comparable to that of insulin dissociation from receptors. However, the data reported in the current study would appear to reconcile the apparent discrepancy with the report by Laursen et al. These latter workers used only a single low submaximal insulin concentration (0.4 ng/mL) in their experiments. The data in Figures 4 and 5 demonstrate that the rate of insulin dissociation from its receptors is very similar at all submaximally effective insulin concentrations, whereas the rate of transport deactivation is inversely related to the insulin concentration used. Thus, at 0.3 ng/mL, transport deactivates at approximately the same rate as insulin dissociates from its receptors ($t_{1/2}$ = 12 min), similar to the results of Laursen et al. However, at higher, but still submaximal insulin concentrations, insulin dissociation rates remain nearly constant ($t_{1/2}$ = 10–12 min) whereas deactivation rates progressively slow as one raises the insulin concentration throughout the submaximal insulin concentration range. Since Laursen et al. used only a single low (0.4 ng/mL) insulin concentration, they were unable to observe this relationship. Thus, while deactivation can be initiated by a fall in receptor occupancy, the rate of deactivation of the insulin-stimulated glucose transport system is independent of the rate of insulin dissociation from its receptors. It is also likely that the absolute amount of insulin bound during deactivation does not determine the rate of this process, since the rates of deactivation after exposure to 5, 10, and 20 ng/mL were the same, despite the fact that the amount of insulin bound differs greatly over this insulin concentration range.

It should be noted that Karnieli et al. (1981) reported a rapid deactivation ($t_{1/2}$ = 9 min) of transport even at a maximal insulin concentration of 4 ng/mL. However, Karnieli et al. employed a large excess of insulin antibody to reverse insulin's effects compared to the methods of Crofford (1975), Gliemann et al. (1975), Laursen et al. (1981), Häring et al. (1982), and the current report, which all used washing and dilution. There may be some unique aspect of this technique which accounts for the difference.

Further evidence for the independence of dissociation and deactivation also exists. Kono et al. have shown that deactivation of transport does not proceed unless intracellular ATP is available, whereas insulin dissociation from receptors is unaffected by energy depeletion (Kono et al., 1981; Vega et al., 1980). Additionally, deactivation is greatly slowed when glucose is omitted from the buffer but can be restored to normal rates by adding back fructose or pyruvate (Ciaraldi & Olefsky, 1979, 1981). These results suggest that some aspect of ongoing intracellular metabolism is necessary to allow deactivation of transport to proceed (Kono et al., 1981; Ciaraldi & Olefsky, 1981). Laursen et al. (1981) and Karnieli et al. (1981) found that omission of glucose from the buffer did not alter the rate of deactivation. However, differences in the preparation and treatment of cells may result in differences in the energy charge of the cells, which would control the substrate needs for later metabolism. This could easily be the case in the work of Laursen et al. (1981) where cells are exposed to glucose until the start of deactivation, making it quite possible that the cells had sufficient substrate stores during the deactivation time of the study.

In contrast to the lack of correlation with the rate of insulin dissociation, the rate of deactivation correlates well with the fractional receptor occupancy achieved during the steady-state phase of activation (Figure 6). These results are consistent with the formulation that insulin stimulates glucose transport by generating a chemical signal which then activates transport, possibly mediating the translocation of glucose transport proteins from an intracellular location to the plasma membrane (Karnieli et al., 1981; Suzuki & Kono, 1980). With this line of reasoning it would be the level of this chemical signal, rather than the degree of receptor occupancy, which is responsible for the magnitude of transport stimulation, and the level of signal would be proportionate to binding until the spare receptor level is reached. Beyond that point additional binding would no longer cause generation of more signal. A decrease in receptor occupancy would then be sufficient to initiate the process of deactivation, but the rate of deactivation would be dependent on the rate of decay or degradation of this chemical mediator. Thus, as insulin levels are increased through the range of submaximal insulin concentrations, more of this signal is generated, necessitating a greater time for its decay or degradation, leading to progressively slower rates of transport deactivation. Only at very low insulin concentrations would the rates of insulin dissociation from receptors and deactivation of transport appear to be tightly coupled, and this would be due to the coincidental circumstance of two distinct processes (i.e., dissociation and deactivation) occurring at similar rates. However, it must be stressed that these two processes are indeed causally linked since dissociation must begin before deactivation can proceed.

Acknowledgments

We thank Dr. Hans Häring for permission to cite work in press and Elizabeth J. Martinez for her expert secretarial assistance.

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Binding of Simple Peptides, Hormones, and Neurotransmitters by Calmodulin†

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ABSTRACT: We have prepared a fluorescent conjugate of porcine calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride that is highly sensitive to both calcium binding and protein binding. We have used the fluorescence of this conjugate in addition to the intrinsic peptide fluorescence to show that adrenocorticotropic hormone (ACTH), β-endorphin, glucagon, and substance P undergo calcium-dependent binding by calmodulin, with competition for common binding sites. The dissociation constants determined in the presence of 0.85 mM $CaCl_2$ and 0.2 N KCl, pH 7.3 at 25 °C, range from 1.5 μM to 3.4 μM. The α-melanocyte-stimulating hormone, bombesin, and somatostatin also bind, with dissociation constants between 60 μM and 90 μM. Angiotensins I and III, bradykinin, neurotensin, physaelemin, substance P octapeptide, insulin, and Leu- and Met-enkephalin show little or no binding. Sequence comparisons show that the peptides that bind calmodulin well contain regions structurally similar to the recognition sequence for the cAMP-dependent protein kinase and to the sequences surrounding phosphorylated serine residues in several calmodulin binding proteins. This result suggests that modification of calmodulin binding sites in calmodulin-dependent proteins is one of the functions of protein kinase. Calcium has a dual role in peptide binding by calmodulin. The occupation of calcium binding sites having a $pK \sim 4$ results in a 2-fold increase in peptide binding affinity.

The intricate workings of the nervous system and endocrine system, evolved by animals for intercellular coordination, converge on two known major intracellular messengers. Cell surface receptors for hormones such as adrenocorticotropic hormone (ACTH), glucagon, and epinephrine transfer information across the cell membrane with cAMP[1] acting as the second messenger [cf. reviews by Greengard (1978) and by Krebs & Beavo (1979)]. The nervous system stimulates membrane depolarization and the release of *calcium* recognized by calmodulin and related intracellular proteins [cf. reviews by Kretsinger (1979), Cheung (1980), and Means (1981)]. The two systems are closely interconnected, as shown by the following examples. First, the enzymes catalyzing cAMP synthesis and degradation, adenylate cyclase and cyclic nucleotide phosphodiesterase, are both activated by the calcium–calmodulin complex (Cheung, 1971, 1981; Cheung et al., 1975; Brostrom et al., 1975; Kakiuchi et al., 1970). cAMP and calcium nearly always act together in mediating cellular response. Rasmussen (1980) has applied the term synarchic in reference to this dualism. Examples of synarchic control are found in phosphorylase kinase [cf. review by Fischer et al. (1975)] and in smooth muscle myosin light chain kinase (Conti & Adelstein, 1981), strictly calmodulin-dependent enzymes with activities stimulated and inhibited, respectively, after phosphorylation by the cAMP-dependent protein kinase. Finally, the nervous and endocrine systems seem to use some of the same peptide messengers. Peripheral hormones including somatostatin, angiotensin II, insulin, glucagon, and members of the gastrin/cholecystokinin group occur in the brain, where they may function as neurotransmitters [cf. reviews by Hokfelt et al. (1980), Synder & Innis (1979), and Snyder (1980)].

Weiss et al. (1980) found that ACTH and β-endorphin inhibit the purified cyclic nucleotide phosphodiesterase and showed that the peptides compete with the enzyme for calmodulin. We have pursued this observation with fluorescence measurements on the binding of 17 different peptides by calmodulin. When possible, we have used the intrinsic peptide fluorescence to detect interaction. We have also prepared and used a fluorescent conjugate of porcine calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride that is exceptionally responsive to both calcium and protein binding. Our goal of obtaining information on the protein binding specificity of calmodulin was fulfilled. The peptides that calmodulin binds well contain regions structurally homologous to the marker sequence for the cAMP-dependent protein kinase and to the sequences surrounding phosphorylated serine residues in several calmodulin binding proteins.

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received January 13, 1982.* Supported by reasearch grants from the Muscular Dystrophy Association and from the National Institutes of Health (AM 13912). Dedicated to Professor Alessandro Rossi-Fanelli of the Instituto di Chimica Biologica, Rome, Italy, in honor of his 75th birthday.

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; cAMP, adenosine 3′,5′-phosphate; CaM, calmodulin; TnI, troponin I; MLCK, smooth muscle myosin light chain kinase; α-MSH, α-melanocyte-stimulating hormone; SP, substance P; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; ρ, rotational relaxation time; τ, lifetime of the excited state; Q, quantum yield; p_0, limiting polarization; $\bar{A}$, anisotropy; K, dissociation constant; F_∞, fluorescence of totally bound ligand; F_0, fluorescence of unbound ligand; F, observed fluorescence; ϕ, fractional degree of saturation; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/82/0421-3480$01.25/0