INSULIN INTERACTIONS WITH ITS RECEPTORS: EXPERIMENTAL EVIDENCE FOR NEGATIVE COOPERATIVITY

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SUMMARY. A simple method is reported to detect cooperative interactions in the binding of polypeptide hormones to their membrane receptors. The dissociation of radioiodinated hormone from the receptor is studied under two conditions: first, by diluting the hormone-receptor complex sufficiently to prevent rebinding of the dissociated tracer; second, by dilution to the same extent in a medium containing an excess of unlabeled hormone. If the sites are independent, the dissociation rates must be the same in both cases. If the presence of unlabeled hormone increases the dissociation rate of the tracer, negatively cooperative interactions must occur. Insulin receptors on cultured lymphocytes and liver plasma membranes show negative cooperative interactions. Growth hormone receptor sites lack these interactions.

The binding of polypeptide hormones to their specific receptors has been shown by direct methods with radioactively labeled hormones to be rapid, saturable and reversible, and precise measurements have been made under a wide variety of experimental conditions both with whole cells and fractions derived from the cells (1-3). To characterize these systems we and others have made observations under steady state conditions and have analyzed the data according to Scatchard (4) by plotting the bound/free ratio of the labeled hormone (B/F) as a function of the concentration of hormone that is bound to the receptors (B). Both linear and curvilinear plots have been observed (3); human growth hormone binding to its receptor sites on cultured lymphocytes (Fig. 1A) (5) is an example of the former, while insulin binding to the same cells (Fig. 1B) is an example of the latter (6). If certain assumptions are satisfied (e.g. that the steady state in fact represents a thermodynamic equilibrium, that the labeled and unlabeled hormones behave

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identically, that the interaction of hormone (H) with receptor (R) is a simple reversible bimolecular reaction, $H + R \rightleftarrows HR$, and that the receptor sites are acting independently) (4,7,8), then the slope of the line reflects the equilibrium constant for the interaction, K, and the intercept corresponds to the total concentration of receptor sites, $[R^\circ]$. Curvilinear data can be resolved into two or more linear components which correspond to sites of two or more orders; from the slope and intercept of each linear component, the approximate K and $[R^\circ]$ for each order of sites can be derived (9).

One of us (P.D.M.), in examining the insulin binding data, proposed that the nonlinear B/F versus B curve may not in fact indicate the presence of discrete orders of sites as had been suggested (10-16). Rather, the nonlinearity could result from cooperative interactions between the insulin binding sites. The affinity of any given site would then depend on the state (occupied or unoccupied) of the other sites. Most equilibrium or kinetic experiments cannot distinguish between these two models (17,18). The use of a labeled ligand, however, allowed us to design a simple method to detect site-site interactions.

METHODS AND RESULTS. Cultured human lymphocytes (IM-9) at high concentration in a single batch were reacted with radioiodinated hormone of high specific activity under conditions such that only a minority of receptor sites were occupied by the bound tracer (5,6). The cells were rinsed free of residual medium, resuspended in an identical volume, immediately divided into aliquots and diluted 100-fold in fresh medium; half were in hormone-free medium ("dilution only"), while half were in medium to which had been added an excess of unlabeled hormone ("dilution + unlabeled hormone"). Dissociation of the tracer from the receptor was monitored in both sets. In both cases the dilution factor by itself was sufficient to prevent rebinding of any dissociated labeled hormone (see below). Then, if the sites were independent, the presence of unlabeled hormone, which at the concentration used rapidly fills the empty sites, should not affect the dissociation rate of the labeled hormone. Such was the case for growth hormone (Fig. 1C).

With insulin and its receptors a different result was obtained. A 100-fold dilution in the presence of unlabeled insulin (1.7×10^{-7} M) produced a much more rapid dissociation of the bound labeled insulin than did dilution alone; the half-time for dissociation of the bound hormone differed by 10-fold (Fig. 1D). This suggests that insulin-filled sites lower the affinity of other sites for this hormone. The following two experiments show that none of the difference in dissociation that we observed could be attributed to rebinding of labeled hormone from the bulk solution. First, dilution alone was compared with dilution plus unlabeled hormone over a wide range of

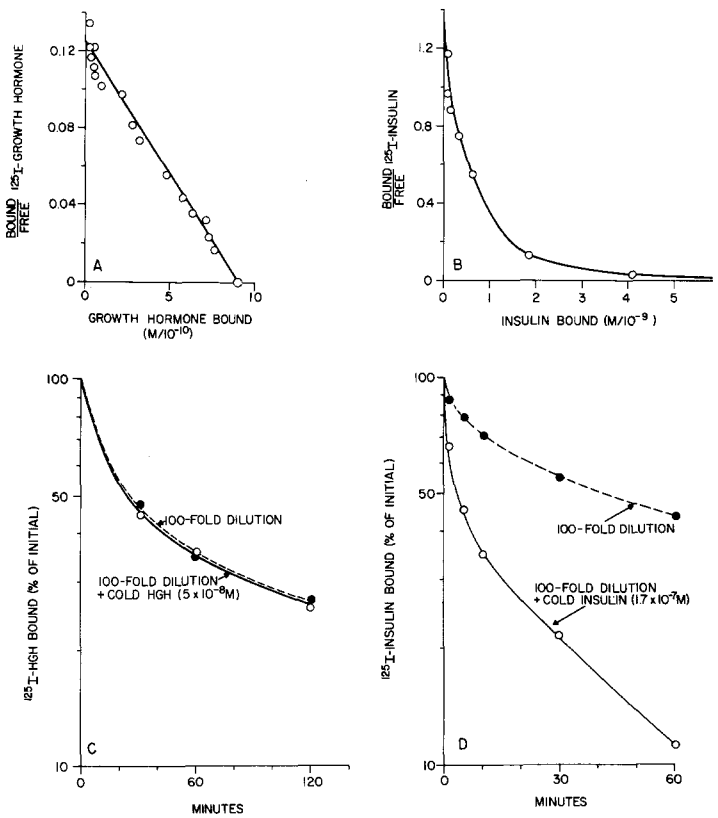

FIG. 1A and 1B. Binding of human growth hormone (hGH) or porcine insulin to human lymphocytes. [^{125}I]hGH (2×10^{-11} M) at 30° or [^{125}I]porcine insulin (7×10^{-12} M) at 15° was incubated with 1.7×10^{6} cultured lymphocytes (cell line IM-9) in the absence and presence of unlabeled hormone; at steady state, the cells were sedimented and radioactivity in cell pellet counted (5,6). The bound/free of labeled hormone is plotted as a function of hormone bound to the cells; "nonspecific" binding, which constituted less than 5% of the total binding, has been subtracted (5,6). FIG. 1C and D. Dissociation of [^{125}I]hGH or [^{125}I]insulin from lymphocytes. [^{125}I]hGH (10^{-10}M) was incubated for 90 min at 30° and [^{125}I]insulin (5×10^{-11} M) for 30 min at 15° with 2.5×10^{7} cells/ml, after which the cells were sedimented at 4°, the supernatant was replaced by an equal aliquot of chilled fresh medium, the cells were resuspended and aliquots (0.1 ml) were transferred to a series of tubes that contained 10 ml of medium in the presence and absence of unlabeled hormone (5×10^{-8} M, 30° for hGH; 1.7×10^{-7} M, 15°, insulin). At intervals, two tubes of each set were centrifuged, and the radioactivity in the cell pellet was counted. The radioactivity on the cells expressed as a percentage of the radioactivity present at t = 0, is plotted as a function of the time elapsed after the dilution of the system. Each point is a mean of duplicates; duplicates differed by less than 5%. The radioactivity at t = 0 was measured at the completion of the incubation, both before and after the first sedimentation step; both results were the same, indicating that trapped radioactivity and dissociation during the analyses were insignificant. At present we have no explanation why the dissociation of [^{125}I]hGH was not single order.

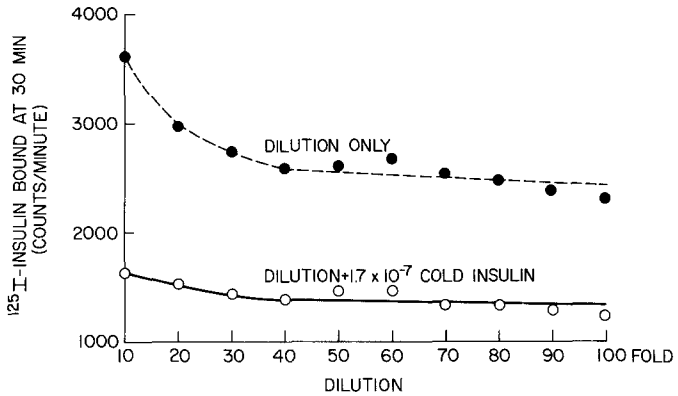

FIG. 2. Effect of dilution on dissociation of [^{125}I]insulin. Cells were incubated with [^{125}I]insulin at 15° and rinsed as described in legend to Fig. 1C and D. After the cells were resuspended in the fresh medium, 0.1 ml aliquots were transferred to series of tubes that contained 1 to 10 ml of medium, in the presence and absence of unlabeled insulin. After 30 min at 15°, the cells were sedimented; the radioactivity in the cell pellet is plotted as a function of the dilution factor during the dissociation.

dilutions; the difference between the two at 1:40 dilution was the same as at higher dilutions (Fig. 2). If this difference had been accounted for by rebinding of the dissociated tracer, it would have been greater at the lower dilutions. In a second experiment the two sets of cells (dilution only and dilution + unlabeled hormone) were subdivided again, and an additional equal aliquot of fresh cells that had never been exposed to hormone was added to one set of each; in both sets, dissociation was unaffected by addition of fresh cells. If dissociation had been followed by rebinding, the latter would have been magnified by the more than doubling of the concentration of unoccupied receptors.

Accelerated dissociation of [^{125}I]insulin was detected with as low as 2.5×10^{-10} M insulin (Fig. 3), well within the physiological range of hormone concentrations. The effect increased as a function of the insulin concentration up to 1.7×10^{-7} M. Further increase in the insulin concentration reduced the effect (Fig. 3); with insulin at 10^{-4} M (not shown), the dissociation of [^{125}I]insulin was the same as that observed in the absence of unlabeled insulin.

Only insulin and its analogues accelerated the dissociation of [^{125}I]-insulin; other polypeptide hormones, glucagon,[1] adrenocorticotropin,[2] thyrocalcitonin,[3] and growth hormone[2] were without effect. Prostaglandin[4]

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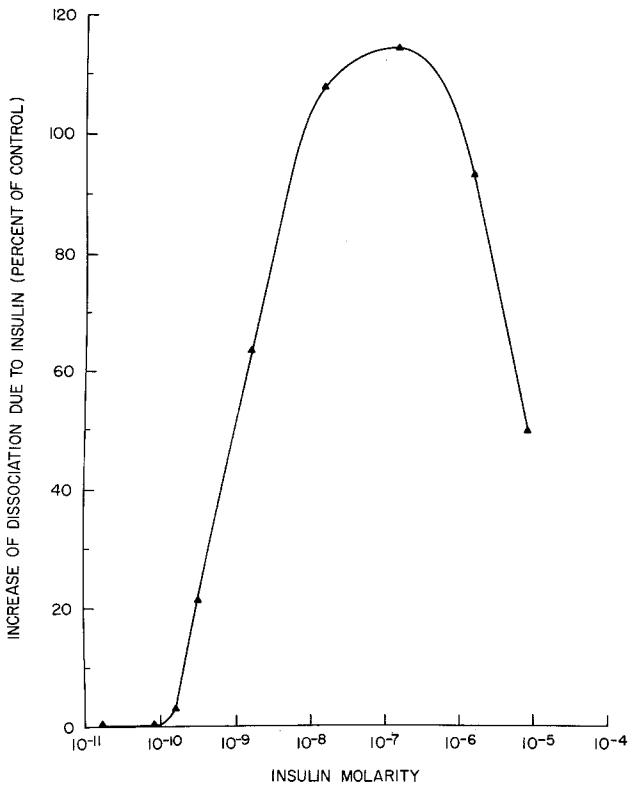

FIG. 3. Effect of insulin concentration on dissociation of [^{125}I]insulin bound to cells. The binding of [^{125}I]insulin to cells was the same as described in the legend to Fig. 1C and D; 0.1 ml of the rinsed resuspended cells were transferred to 10 ml of medium in the presence and absence of unlabeled insulin (10^{-11} to 10^{-5} M). After 30 min at 15°, the cells were sedimented and the radioactivity in the cell pellet was counted. The increase of dissociation due to unlabeled insulin =

$$\left(\frac{[^{125}\text{I}]\text{insulin dissociated with dilution + cold insulin}}{[^{125}\text{I}]\text{insulin dissociated with dilution only}} - 1 \right) \times 100$$

is plotted as a function of the concentration of unlabeled insulin.

PGE_1 (10^{-9} to 10^{-6} M), GTP (10^{-5} M), and theophylline (10^{-3} M) were likewise without effect.

Zinc-free insulin,[5] fish insulin,[6] guinea-pig insulin,[7] proinsulin[5] and nonsuppressible insulin-like activity-soluble (NSILA-S),[8] which vary 300-fold in their relative biological potency, bind to insulin receptors with an affinity proportional to their biological potencies in vitro (19). At high concentrations, they can inhibit completely the binding of labeled insulin to the receptors and then can occupy the receptors to the same extent as porcine

insulin. In our experiments all of these derivatives accelerated the dissociation of labeled insulin in direct proportion to their ability to inhibit the binding of porcine [^{125}I]insulin to the receptors (manuscript in preparation).

Two analogues, desalanine-desasparagine (DAA)-insulin^{5,9} and desoctapeptide (DOP)-insulin^{5,9}, had no effect on the dissociation of [^{125}I]insulin, although both are as active as guinea-pig insulin in bioassay and occupy the receptors to the same extent (19). This suggests that the regions of the insulin molecule needed for binding to the receptor and related to bioactivity are distinct from the region that produces the site-site interactions.

Other data (manuscript in preparation) suggest that dimers of insulin, which form spontaneously at high concentrations, also bind to receptors but do not accelerate the dissociation of the labeled hormone, and that dimer formation accounts for the reduction in the effect of insulin at concentrations greater than 10^{-7} M (Fig. 3).

DISCUSSION. Our results show that filling of empty sites by cold insulin increases the dissociation of labeled insulin from other sites. Our interpretation of the data is that filling sites produces site-site interactions which increase the dissociation rate constant of other sites, and that this process explains at least in part why the apparent affinity constant is lowered with increased occupancy of the receptor population. This is usually referred to as negative cooperativity (20).

An alternative explanation invokes the concept of an unstirred layer adjacent to the receptor, which is not in convective equilibrium with the bulk solution, and from which rebinding after dissociation could occur. Dilution by unlabeled hormone in the unstirred layer would increase the apparent dissociation rate of the tracer. This model is considered unlikely, since increased dissociation was not observed for the growth hormone-receptor system in the same cells, or with DAA-insulin and DOP-insulin, or with the highest concentrations of insulin in the dilution medium.

Similar studies with highly purified plasma membranes from rat liver (21) gave comparable results (manuscript in preparation), suggesting that these cooperative interactions are an intrinsic physico-chemical property of the membrane-bound receptor system, independent of the integrity of the cellular metabolism.

These findings have an important implication in the analysis of binding data. One of the important assumptions for the interpretation of Scatchard plots (B/F versus B) in terms of K and [R°] values for discrete subpopulations of sites was the absence of inter-site interactions. If these are present,

these apparent K and [R°] values do not have a precise physico-chemical meaning (18). The physical meaning of the fall in [R°] in insulin-resistant states in man and laboratory animals reported by this laboratory (22-24) will then require rethinking.

All receptor systems for which nonlinear Scatchard plots have been interpreted as indicating heterogeneity of binding sites are potential candidates for negative cooperativity: receptors for polypeptide hormones; glucagon (11); ACTH (10); oxytocin (12); TSH (13); receptors for other hormones; epinephrine (11,14); estrogens (15); nonhormonal systems; Ca^{2+} (11); phlorizin (16). Negative cooperativity has in fact been explicitly postulated for acetylcholine binding to its receptor (25).

Cooperative mechanisms, widespread in various areas of biochemistry (26-29), are likely to play a role at various steps of hormone action (30) (D. Rodbard, manuscript in preparation). Few data are available showing cooperative mechanisms in the binding of hormones to antibodies (31) or receptors (32). We now demonstrate negative cooperativity at the binding level by a direct method. Considerable kinetic and other data will be necessary to define the model more precisely.

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1. Lefkowitz, R. J., Pastan, I. and Roth, J. (1969) NIH Fogarty International Center Proceedings 4, 88
2. Goodfriend, T. and Lin, S-Y. (1969) Clin. Res. 17, 243
3. Roth, J. (1973) Metabolism 22, 1059
4. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660
5. Lesniak, M. A., Roth, J., Gorden, P. and Gavin, J. R. III (1973) Nature New Biol. 241, 20
6. Gavin, J. R. III, Gorden, P., Roth, J., Archer, J. and Buell, D. N. (1973) J. Biol. Chem. 248, 2202
7. Rodbard, D. (1973), in press
8. Kahn, C. R. (submitted for publication)
9. Klotz, I. M. and Hunston, D. L. (1971) Biochemistry 10, 3065
10. Lefkowitz, R. J., Roth, J. and Pastan, I. (1970) Science 170, 633
11. Marinetti, G. V., Shlatz, L. and Reilly, K. (1972) in Insulin Action (Fritz, I., ed), p. 207, Academic Press, New York
12. Bockaert, J., Imbert, M., Jard, S. and Morel, F. (1972) Mol. Pharmacol. 8, 230
13. Winand, R. and Kohn, L. (personal communication)
14. Lefkowitz, R. J. and Haber, E. (1971) Proc. Nat. Acad. Sci. U. S. A. 68, 1773
15. Ellis, D. J. and Ringold, H. J. (1971) in The Sex Steroids Molecular Mechanisms (McKerns, K. W., ed), p. 73, Appleton Century Crofts, New York
16. Glossman, H. and Neville, D. M. Jr. (1972) J. Biol. Chem. 247, 7779
17. Harper, E. T. (1973) J. Theor. Biol. 39, 91
18. Fletcher, J. E., Spector, A. A. and Ashbrook, J. D. (1970) Biochemistry 9, 4580

19. Freychet, P., Roth, J. and Neville, D. M. Jr. (1971) Proc. Nat. Acad. Sci. U. S. A. 68, 1833
20. Levitzki, A. and Koshland, D. E. Jr. (1969) Proc. Nat. Acad. Sci. U. S. A. 62, 1121
21. Neville, D. M. Jr. (1968) Biochim. Biophys. Acta 159, 540
22. Kahn, C. R., Neville, D. M. Jr. and Roth, J. (1973) J. Biol. Chem. 248, 244
23. Goldfine, I. D., Kahn, C. R., Neville, D. M. Jr., Roth, J., Garrison, M. M., and Bates, R. B. (1973) Biochem. Biophys. Res. Commun. 53, 852
24. Archer, J. A., Gorden, P., Gavin, J. R. III, Lesniak, M. A. and Roth, J. (1973) J. Clin. Endocrinol. Metab. 36, 627
25. Eldefrawi, M. E., Eldefrawi, A. T., Seifert, S. and O'Brien, R. D. (1972) Arch. Biochem. Biophys. 150, 210
26. Monod, J., Wyman, J. and Changeux, J. P. (1965) J. Mol. Biol. 12, 88
27. Koshland, D. E. Jr., Némethy, G. and Filmer, D. (1966) Biochemistry 5, 365
28. Whitehead, E. (1970) Progr. Biophys. Mol. Biol. 21, 323
29. Herzfeld, J. and Stanley, H. E. (1972) Biochem. Biophys. Res. Commun. 48, 307
30. Rodbell, M., Krans, H. M. J., Pohl, S. L. and Birnbaumer, L. (1971) J. Biol. Chem. 246, 1872
31. Weintraub, B. D., Rosen, S. W., McCammon, J. A. and Perlman, R. J. (1973) Endocrinology 92, 332
32. House, P. D. R. (1971) FEBS Letters 16, 339