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Regulation of Insulin Receptors in Frog Erythrocytes by Insulin and Concanavalin A

Evidence for Discrete Classes of Insulin Binding Sites

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SUMMARY

In order to better understand the complex nature of insulin-receptor interactions we have investigated insulin-promoted receptor down-regulation in frog and turkey erythrocytes. We have quantitatively analyzed the data for equilibrium insulin binding to frog erythrocytes before and after preincubation with insulin (down-regulation) or concanavalin A. Preincubation of frog erythrocytes with insulin for 4 hr at 30° induced a 50% decrease in the ability of these cells to bind ^{125}I-labeled insulin. Under identical experimental conditions, turkey erythrocytes exhibited no insulin-mediated insulin receptor down-regulation. The extent of down-regulation of the insulin receptor in frog erythrocytes was dependent upon the concentration of insulin (10^{-10}–10^{-6} M) and the time of exposure to hormone. However, down-regulation of the receptor was only slightly temperature-dependent and was not blocked at 4° in these cells. When frog erythrocytes were incubated with ^{125}I-labeled insulin and the lysosomotropic amines chloroquine or methylamine, a 2-fold enhancement of cell-associated radioactivity was observed as compared with controls which received no chloroquine or methylamine. These findings suggest that the insulin-receptor complex in frog erythrocytes is internalized and degraded during the down-regulation process. By contrast, turkey eyrthrocytes which do not down-regulate their receptors also appear not to internalize insulin. Detailed insulin binding isotherms for frog erythrocytes were subjected to nonlinear least-squares curve fitting. It was found that these curves could be adequately fit with a model for two classes of independent binding sites: Site I being of high affinity, low capacity (K_H = 580 pM; R_H = 425 sites/cell) and Site II being of low affinity and high capacity (K_L = 130 nM, R_L = 4300 sites/cell). Computer analysis of the insulin binding data obtained from frog erythrocytes preincubated with insulin or perturbed by concanavalin A was consistent with an effect on Site I only. Insulin-induced receptor regulation promoted a significant 50% reduction in the number of high-affinity sites, and concanavalin A pretreatment resulted in a 2-fold decrease in the affinity of Site I. No significant effect on either the affinity or number of Site II was detected after pretreatment of the frog erythrocytes with insulin or concanavalin A. Under all experimental conditions the two-site model provides a rational basis for explaining the binding data. The excellent ability of the two-site model to fit the experimental binding data and the sensitivity of only the high-affinity sites to physiological or biochemical perturbations suggest that there may be two independent classes of insulin binding sites in the frog erythrocyte.

INTRODUCTION

We initially became interested in studying the insulin receptor in frog and turkey erythrocytes because of an interesting observation concerning the regulation of the *beta*-adrenergic receptor in these two cell systems. Incubation of these cells with the *beta*-adrenergic agonist isoproterenol causes the *beta*-adrenergic receptor to become uncoupled from its effector enzyme, adenylate cyclase, in both types of erythrocyte membranes (1–4). However, incubation with isoproterenol also leads to a

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down-regulation of *beta*-adrenergic receptors in frog erythrocytes, as judged by a loss of [^{3}H]dihydroalprenolol binding sites (1, 2, 4). By contrast, no loss of antagonist binding due to preincubation with isoproterenol is observed with turkey erythrocytes (3). We wondered whether other hormone receptors might be regulated in a similar fashion in these two erythrocytes. The insulin receptor was selected for study because it has been shown to be present in the membranes of both of these erythrocytes (5) and also because its binding properties (5, 6) and down-regulation (7–9) have been well characterized in a variety of cell systems.

In the present work we have utilized nonlinear least-squares curve-fitting procedures to analyze insulin binding data obtained with frog erythrocytes. Binding studies were performed with native cells as well as with cells perturbed by prior incubations with insulin (down-regulation) or with concanavalin A. Our results are consistent with the notion that the complex binding isotherm of insulin in frog erythrocytes may be dissected into two distinct classes of receptors. We found that only the high-affinity, low-capacity sites were regulated by insulin and concanavalin A whereas the low-affinity, high-capacity sites were not regulated by either.

EXPERIMENTAL PROCEDURES

Materials

Porcine insulin was a gift from the Eli Lilly Company (Indianapolis, Ind.). Chloroquine, methylamine, and concanavalin A were purchased from the Sigma Chemical Company (St. Louis, Mo.). [^{125}I]NaI was purchased from New England Nuclear Corporation (Boston, Mass.). ^{125}I-Labeled insulin was prepared by a modification of the chloramine T method (10) to a specific activity of 150–200 μCi/mg. Following iodination, ^{125}I-labeled insulin was separated from unreacted radioactive iodine on a Sephadex G-25 column. Aggregated or damaged insulin was then removed by passage over a Sephadex G-75 column. ^{125}I-Labeled insulin was stored at 4° and was repurified over a G-75 column weekly. Insulin was freshly iodinated every 2–3 weeks.

Methods

Cell preparation. Frog and turkey erythrocytes were prepared as previously described (3, 4). Before experimental use, frog cells were washed three times in amphibian saline (100 mM NaCl, 10 mM Tris-HCl, pH 7.4), and turkey erythrocytes were washed twice in avian saline (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). One milliliter of packed frog cells is equivalent to 1×10^9 cells, and 1 ml of turkey erythrocytes equals 3×10^9 cells.[4]

Conditions for down-regulation experiments. Frog and turkey erythrocytes were resuspended with a 5 to 10-fold dilution in Buffer A [85 mM Tris, 25 mM NaCl, 10 mM dextrose, and bovine serum albumin (1 mg/ml)]. Buffer A was adjusted to pH 7.2 for frog erythrocytes and pH 7.8 for turkey erythrocytes. These are the respective pH optima for insulin binding in these two cell types (5), and buffers were adjusted accordingly for all experimental protocols. Unless otherwise indicated, down regulation of the insulin receptor was measured by incubating erythrocytes in the presence and absence of 1 μM porcine insulin for 4 hr at 30° in a shaking water bath. Following this incubation, free and cell-bound insulin were removed by a modification of the washing procedures used by Gavin *et al.* (7). Cells were washed twice with 40 ml of warm (25–30°) Buffer A in 50-ml plastic Sorvall tubes and were sedimented by centrifuging at $300 \times g$ for 5 min. Erythrocytes were then incubated for 30–40 min at 30° in a shaking water bath and subsequently washed once with Buffer A. Cells were incubated for another 30–40 min at 30° with 40 ml of Buffer A and then washed twice with Buffer A. The time required for the washing procedure is approximately 2 hr. Erythrocytes were then assayed for ^{125}I-labeled insulin binding.

^{125}I-Labeled insulin binding. The ^{125}I-labeled insulin binding assay employed is a modification of the method reported by Muggeo *et al.* (5). Binding incubations were performed in plastic tubes (17 × 100 mm) in a total volume of 0.5 ml of Buffer A. The tracer ^{125}I-labeled insulin was added at a concentration in the range of 300–900 pM. For competitive binding experiments, unlabeled insulin was added at concentrations from 0 to 58.4 μM. Frog erythrocytes ($1–2 \times 10^8$ cells/ml) were incubated in the assay mixture for 3 hr at 20°, and turkey erythrocytes ($5–7 \times 10^8$ cells/ml) were incubated for 2 hr at 15°. These conditions allow ^{125}I-labeled insulin binding to attain equilibrium (5). Duplicate 200-μl aliquots from each tube were then layered above 175 μl of 6% Ficoll in Buffer A (4°) in 400-μl Microfuge tubes and sedimented by centrifugation at $10,000 \times g$ for 30 sec in a Beckman microfuge. Supernatants were aspirated, the pellets were washed with 250 μl of Buffer A (4°), and the cells were centrifuged at $10,000 \times g$ for 10 sec. The supernatants were aspirated, and the bottoms of the Microfuge tubes were cut above the pellet and counted in a Beckman Gamma counter. Nonspecific binding is defined as radioactivity associated with the cell pellet when 10 μM unlabeled insulin is added to the binding assay. Nonspecific binding was subtracted from the total ^{125}I-labeled insulin binding to yield specific binding. ^{125}I-Labeled insulin binding was 85% specific for both frog and turkey erythrocytes.

Computerized data analysis. Competition binding data from each experiment was expressed as total concentration of ^{125}I-labeled insulin bound (including nonspecific binding) versus total concentration of unlabeled insulin added. The data from individual competition binding experiments obtained under the same experimental conditions (i.e., same lot of iodinated insulin, same concentration of radiolabeled hormone) were closely comparable to each other and were averaged. The competition binding data from the individual experiments were meaned and analyzed by weighted nonlinear least-squares curve fitting according to the method of Marquardt and Levenberg, as described by Magar (11). Experimental data points were weighted according to the reciprocal of their predicted variance (12). It was assumed throughout the computer modeling procedure that the binding properties of insulin were not significantly altered by the iodination process, so that native and labeled insulin could be considered as distinct ligands but with the same binding parameters (10, 13, 14).

[4] L. J. Pike, personal communication.

Competition curves were analyzed by using a model for the binding of the radioligand and the native hormone to one or several classes of independent binding sites according to mass action law, as previously described (12, 15). Briefly stated, a generalized model for multiple ligands binding to several classes of sites was used. A term for the nonspecific binding of the ligands was included. The model was repetitively used for fitting the ligand binding data, assuming each time a larger number of classes of binding sites. A model for two classes of sites was retained only when its use resulted in a statistically significant improvement of the fit over a one-site model. Testing for the statistical significance between fits of the data was performed by comparing the residual variance of the data fits according to the "extra sum of squares principle," using a partial F-test as previously described (12). Significance is defined as $p < 0.05$.

Computer modeling was also used to compare experimental binding curves for control and pretreated cells by analyzing them simultaneously. In a first step, the curves were allowed to retain their individual binding parameters: affinity constants K_H and K_L and corresponding binding capacities R_H and R_L for the higher- and the lower-affinity classes of sites. Then in the following steps, curves from the control and pretreated cells were analyzed constraining one of the binding parameters to the same value for both curves. A statistically significant worsening of the fit of the data ($p < 0.05$) following application of the equality constraint indicated that the two corresponding parameters were different. Otherwise, the corresponding parameters were considered to have the same value.

All computations were performed with the use of interactive programs written in PL/1 for a PDP 11/45.

RESULTS

Insulin receptor down-regulation. Both frog and turkey erythrocytes possess cell surface receptors for insulin which are apparently similar to the insulin receptors present in mammalian tissues (5). We examined hormone-dependent receptor regulation in both types of erythrocytes. Specific ^{125}I-labeled insulin binding to whole cells was measured after a 4-hr incubation at 30° with 10^{-6} M insulin followed by a 2-hr washing procedure. The extensive washing procedure was employed to remove unlabeled insulin present in the medium or bound to cells, so as not to interfere with the subsequent ^{125}I-labeled insulin binding assay. The washing procedure reduced the concentration of insulin > 3000-fold. Moreover, we could demonstrate that the washing procedure was effective in dissociating the vast majority of specifically bound insulin. After 1 nM ^{125}I-labeled insulin established equilibrium with the cells (a concentration which binds primarily to high-affinity sites), more than 90% of the insulin specifically bound to frog and turkey erythrocytes could be removed by our washing procedure (data not shown). Thus, any decrease in ^{125}I-labeled insulin to the erythrocytes could not be explained by unlabeled insulin persistently bound to the receptor after the preincubation. In frog erythrocytes, pre-exposure to 10^{-6} M insulin caused a significant ($p = 0.001$) 50% reduction in the ability of cells to bind ^{125}I-labeled insulin. An identical insulin treatment with turkey erythrocytes produced no significant ($p > 0.2$) loss of insulin binding compared with controls which received no insulin during the preincubation.

Insulin receptor down-regulation in frog erythrocytes was dependent upon the concentration of insulin and the time of exposure. Figure 1 shows the time course and concentration dependence of this effect. Down-regulation occurred rapidly with 10^{-6} M insulin, as a significant decrease in ^{125}I-labeled insulin binding was observed after a 30-min exposure to insulin. On the other hand, very little effect was observed in the first 2 hr of exposure to 10^{-10} M or 10^{-8} M insulin. At 4 hr, a 45–50% decrease in ^{125}I-labeled insulin binding was achieved with both 10^{-8} and 10^{-6} M insulin. A considerable effect (25% decrease in binding) was observed with 10^{-10} M insulin. This observation is of interest since these low concentrations are within the physiological concentration range (16).

Figure 2 demonstrated the relative insensitivity of insulin receptor down-regulation in frog erythrocytes to temperature. Although down-regulation was somewhat enhanced at 30°, it was largely unaffected by temperatures ranging from 4° to 30°. The same extent of down-regulation was observed with 10^{-8} or 10^{-6} M insulin at each temperature. Interestingly, insulin receptor down-regulation was still quite pronounced when insulin was preincubated with cells at 4°. With turkey erythrocytes, no appreciable loss of ^{125}I-labeled insulin binding due to preincubation with insulin could be observed at temperatures between 4° and 37° (data not shown).

Down-regulation of hormone receptors may be mediated by receptor endocytosis. Evidence indicates that, once inside the cell, the hormone-receptor complex is degraded in lysosomes (17). Methylamine and chloroquine are agents which inhibit lysosomal degradation, presumably by raising the pH of lysosomes (18). By using these lysosomotropic agents to block intracellular deg-

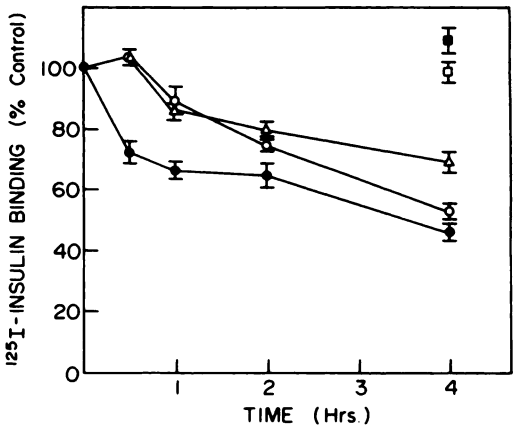

FIG. 1. *Concentration and time dependence of insulin receptor down-regulation in frog and turkey erythrocytes*

Frog erythrocytes were incubated in Buffer A containing no insulin or 10^{-10} M (△), 10^{-8} M (○), or 10^{-6} M (●) insulin for times ranging from 0.5 to 4 hr at 30°. Turkey erythrocytes were incubated in Buffer A with either 10^{-8} M (□) or 10^{-6} M (■) insulin for 4 hr at 30°. Cells were then extensively washed, and ^{125}I-labeled insulin was assayed. Results show the mean ± standard error of the mean of duplicate determinations from a representative experiment and are expressed as the percentage of a control which received no insulin during the preincubation. This experiment was replicated once with comparable results.

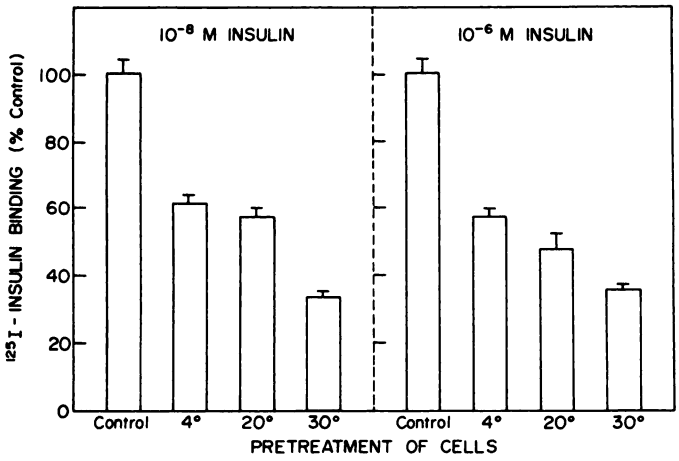

FIG. 2. *Temperature dependence of insulin receptor down-regulation in frog erythrocytes*

Frog erythrocytes were incubated in Buffer A containing 0, 10^{-8}, or 10^{-6} M insulin at 4°, 20°, and 30° in a shaking water bath for 4 hr. After extensive washing, cells were assayed for ^{125}I-labeled insulin binding. Results shown represent the mean ± standard error of the mean of duplicate determinations in a representative experiment and are expressed as the percentage of a control which was not preincubated with insulin. This experiment was replicated once with comparable results.

radation, it has been possible in some systems to document an accumulation of intact ^{125}I-labeled insulin within cells (19).

In order to explore whether the insulin receptor is internalized in frog or turkey erythrocytes, the effect of methylamine and chloroquine upon cell-associated ^{125}I-labeled insulin, not susceptible to dissociation with unlabeled insulin, was examined (Fig. 3). For these experiments, frog and turkey erythrocytes were incubated in the presence and absence of 15 mM methylamine or 75 μM chloroquine for 15 min. ^{125}I-Labeled insulin (1 nM) was then added and incubated with erythrocytes for between 1 and 6 hr at 30°. Methylamine and chloroquine did not affect ^{125}I-labeled insulin binding to either frog or turkey erythrocytes (data not shown). After this incubation, ^{125}I-labeled insulin bound at the cell surface was removed by a 2.5- to 3 hr-washing procedure in the presence of 10^{-6} M insulin. The addition of unlabeled insulin markedly enhances the dissociation of ^{125}I-labeled insulin bound to the surface of either frog or turkey erythrocytes (5). The cell-associated radioactivity remaining after this washing procedure is considered nonsusceptible to dissociation with unlabeled insulin and therefore presumably represents internalized insulin.

Figure 3 shows that cell-associated radioactivity in frog erythrocytes increases with the time of ^{125}I-labeled insulin incubation reaching a maximum at 4 hr. The time course of cell-associated radioactivity nonsusceptible to dissociation by insulin is similar to the time course measured for the down-regulation of the receptors (see Fig. 1). Cell-associated radioactivity does not appreciably increase with the time of ^{125}I-labeled insulin incubation in turkey erythrocytes. Both 15 mM methylamine and 75 μM chloroquine were able to increase by 2-fold the cell-associated radioactivity in frog erythrocytes. The maximal amount of cell-associated radioactivity in the presence of lysosomotropic amines is equivalent to 5% of the total ^{125}I-labeled insulin bound before the dissociation with unlabeled insulin. In contrast, methylamine-treated turkey erythrocytes exhibited the same low amount of cell-associated radioactivity as the untreated controls. The finding that methylamine and chloroquine stimulate cell-associated, nondissociable radioactivity in frog but not turkey erythrocytes is consistent with the observation that the insulin receptor is down-regulated only in frog erythrocytes.

Computer modeling of insulin competition binding data. In an attempt to better understand the mechanism of insulin-induced loss of ^{125}I-labeled insulin binding to frog erythrocytes, competition binding curves of ^{125}I-labeled insulin versus unlabeled insulin (Figs. 4A and 5A) were analyzed by nonlinear least squares curve-fitting procedures (see Experimental Procedures). The shallow competition binding curve for insulin to frog erythrocytes is consistent with curvilinear Scatchard plots for insulin binding previously reported using these cells (5). The curvilinear nature of the Scatchard plot is particularly acute for insulin binding to frog erythrocytes (see Figs. 4B and 5B). Historically, the complex nature of insulin equilibrium binding data has been interpreted either as

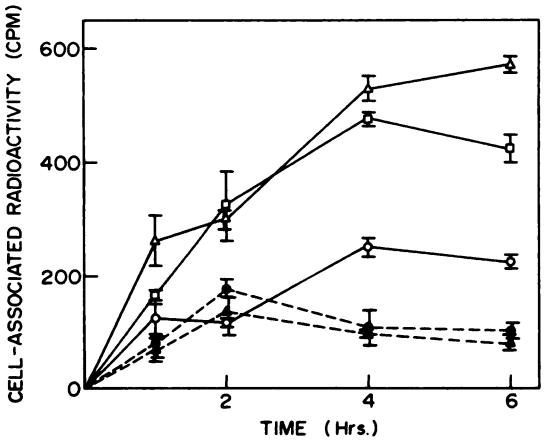

FIG. 3. *Effect of methylamine and chloroquine on cell-associated radioactivity, nonsusceptible to dissociation with unlabeled insulin, remaining after incubation of frog and turkey erythrocytes with ^{125}I-labeled insulin*

Frog erythrocytes (3×10^8 cells/ml) were incubated in the absence of (○) or presence of 15 mM methylamine (△) or 75 μM chloroquine (□) for 15 min at 30°. Turkey erythrocytes (5×10^8 cells/ml) were incubated in the absence (●) or presence of 15 mM methylamine (▲) for 15 min at 30°. ^{125}I-labeled insulin (1 nM) was added to both erythrocytes and incubated at 30° for times ranging from 1 to 6 hr. Nonspecific binding was determined by adding 10 μM insulin. Cells were then washed twice with 40 ml of Buffer A (25°) and incubated for 45 min at 25° with 15 ml of Buffer A containing 1 μM insulin. Cells were washed once with 40 ml of Buffer A and incubated again for 45 min at 25° with 15 ml of Buffer A containing 1 μM insulin. After washing once more with Buffer A, cells were resuspended in 1 ml of Buffer A. The washing procedure required approximately 2.5 hr. Three 200-μl aliquots from each tube were layered above 6% Ficoll in Buffer A, centrifuged, washed, and counted as described for ^{125}I-insulin binding under Experimental Procedures. The remaining cell-associated radioactivity, corrected for counts remaining from nonspecific ^{125}I-labeled insulin binding, is expressed in counts per minute. Data shown represent the mean ± standard error of the mean of duplicate determinations in a representative experiment. This experiment was replicated twice with comparable results.

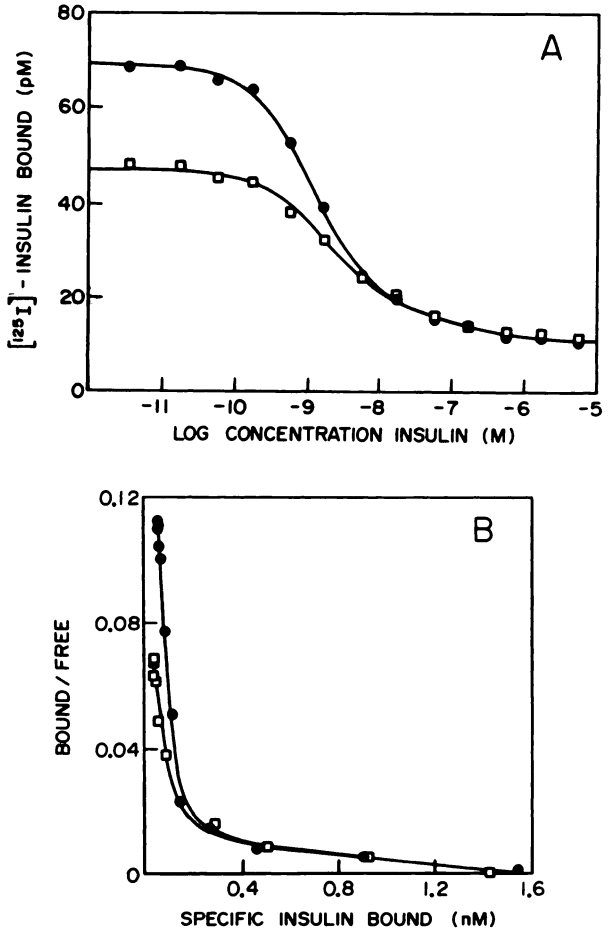

FIG. 4. *Computer modeling of ^{125}I-labeled insulin competition binding curves in control and insulin down-regulated frog erythrocytes*

Frog erythrocytes were incubated in the presence (□) and absence (●) of 10^{-6} M insulin for 4 hr at 30° followed by a 2-hr washing procedure. Binding assays were performed as described under Experimental Procedures. The concentration of ^{125}I-labeled insulin was 600 pM. The experimental points represent the mean of duplicate determinations from four independent experiments. A, The line through the points represents the "best fit" from the computer modeling procedure according to a two-site model. B, Transformation of the same data and fit into Scatchard plot coordinates.

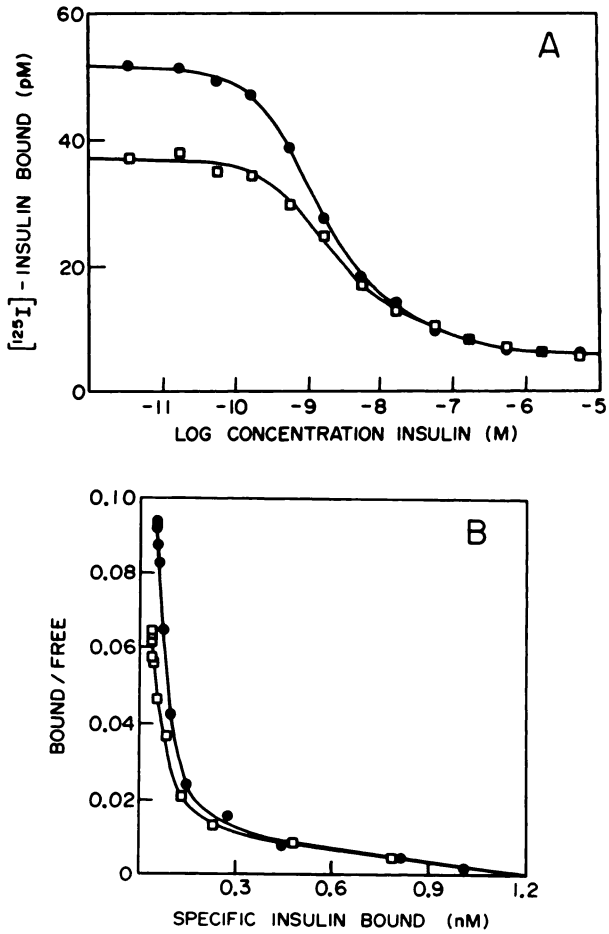

FIG. 5. *Computer modeling of ^{125}I-labeled insulin competition curves for unlabeled insulin with Con A-treated and untreated frog erythrocytes*

Frog erythrocytes (4×10^8 cells/ml) were incubated in the presence (□) and absence (●) of Con A (10 μg/ml) for 1 hr at 30°. Binding assays were performed as described under Experimental Procedures. The concentration of ^{125}I-labeled insulin was 550 pM. The data points represent the mean of duplicate determinations from three independent experiments. The line through the data points represents the "best fit" from the computer modeling procedures according to a model for two sites. A, Untransformed data. B, Transformation into Scatchard plot coordinates.

indicative of independent classes of insulin binding sites (20, 21) or as negatively cooperative interactions among insulin receptors (22, 23). Utilizing computer-aided analysis, it can be seen in Figs. 4 and 5 that a two-site model can accommodate all of the experimentally obtained binding data in frog erythrocytes. One site (Site I) was of high affinity and low binding capacity (K_H = 578 pM, R_H = 425 sites/cell) and the other (Site II) was of low affinity and high binding capacity (K_L = 132 nM; R_L = 4300 sites/cell).

Competitive binding curves from control cells and cells down-regulated with insulin were analyzed by these procedures, and the various parameters of Site I and Site II binding were compared. Figure 4 and Table 1 show the analysis of the averaged results from four independent experiments. The insulin receptors on the frog erythrocytes were down-regulated by incubation in the presence of 10^{-6} M insulin for 4 hr at 30°. Our analysis revealed that down-regulation with insulin perturbed only the high-affinity, low-capacity receptors (Site I). Erythrocytes incubated with insulin showed a statistically significant 50% loss ($p < 0.01$) in the number of Site I receptors with no significant alteration in receptor affinity. Both the concentration and the affinity of Site II receptors were statistically unaltered by insulin preincubation.

In order to investigate further whether the high- and low-affinity receptor populations could be functionally distinguished, we examined the effects of another agent known to perturb insulin binding. Con A[5] is a plant lectin which has been observed to mimic a variety of insulin-like responses, such as inhibition of lipolysis and stimulation of glucose transport in adipocytes (24). Furthermore, Con A inhibits the binding of ^{125}I-labeled insulin to whole cells and solubilized insulin receptors apparently

[5] The abbreviation used is: Con A, concanavalin A.

TABLE 1

Computer analysis of insulin competition curves from control frog erythrocytes and cells down-regulated with 10^{-6} M insulin

The following binding parameters were obtained from the competition curves presented in Fig. 4 utilizing a model for two independent classes of sites as described under Experimental Procedures. Data are from four experiments.

Sites and binding parameter	Insulin concentration during preincubation		
	None	1 μM	
High affinity			
R_H (sites/cell)	425 ± 40[a]	230 ± 40	($p < 0.01$)
K_H (nM)	0.58 ± 0.09	0.60 ± 0.19	NS[b]
Low affinity			
R_L (sites/cell)	4300 ± 1800	4250 ± 150	NS
K_L (nM)	130 ± 70	71 ± 25	NS

[a] Values represent means ± standard error of the parameter estimate.

[b] NS, Not significantly different, i.e., no significant worsening of the fit when these two binding parameters are constrained to a common value.

by binding directly to the receptor, which is a glycoprotein (25, 26). Con A has also been employed previously in the study of insulin binding to human erythrocytes to discriminate between independent classes of insulin binding sites (27). When incubated with frog erythrocytes, Con A (10 μg/ml) also produced a 50% reduction in ^{125}I-labeled insulin binding. At this concentration of Con A, no agglutination of the red blood cells occurred.

Figure 5 shows the average of three insulin competition binding curves from frog erythrocytes preincubated in the presence and absence of Con A (10 μg/ml) for 1 hr at 30°. The analysis using the two-site model (Table 2) revealed that the loss of ^{125}I-labeled insulin binding after Con A incubation was again due to an alteration in the Site I receptors. Con A incubation produced a significant change ($p < 0.015$) in the affinity of Site I, raising the K_H from 516 to 1106 pM. No alteration was observed in the number of high-affinity receptor sites. Also, no difference was detected in the affinity K_L or binding capacity R_L of Site II between Con A-treated and untreated cells. Thus, as with insulin-induced receptor down-regulation, Con A appeared to affect specifically the high affinity insulin binding component. Site II binding was not significantly affected by either insulin or Con A incubation. The two-site model thus provides a mechanistic explanation for the regulatory effects of insulin and Con A on the frog erythrocyte insulin receptor.

TABLE 2

Computer analysis of insulin competition curves from Con A-treated and untreated frog erythrocytes

The following binding parameters were obtained from competition curves presented in Fig. 5 utilizing a model for two independent classes of sites as described under Experimental Procedures. Data are from three experiments.

Sites and binding parameter	Con A treatment		
	None	10 μg/ml	
High affinity			
R_H (sites/cell)	300 ± 30[a]	310 ± 40	NS[b]
K_H (nM)	0.52 ± 0.09	1.11 ± 0.18	($p < 0.01$)
Low affinity			
R_L (sites/cell)	3700 ± 780	4800 ± 1200	NS
K_L (nM)	79 ± 24	130 ± 45	NS

[a] Values represent means ± standard error of the parameter estimate.

[b] NS, Not significantly different, i.e., no significant worsening of fit when these two binding parameters are constrained to a common value.

DISCUSSION

In these studies we have investigated insulin-mediated receptor regulation in frog erythrocytes, and our results provide evidence for two distinct classes of insulin binding sites on these cells. The characteristics of the insulin-induced loss of ^{125}I-labeled insulin binding to frog erythrocytes appear similar to insulin receptor down-regulation which has been documented, *in vitro*, for several types of mammalian cells (7–9). Our results suggest that insulin-promoted down-regulation in frog erythrocytes is a function of high-affinity receptor occupancy by the hormone and therefore represents a true and physiologically relevant form of receptor regulation. Although the molecular mechanism is not completely understood, there is reason to believe that down-regulation is mediated by hormone-induced receptor endocytosis (28). Using fluorescent and ferritin-labeled derivatives of insulin as well as other peptide hormones, it has been possible to demonstrate the clustering and subsequent internalization of hormone-receptor complexes (29). After internalization, insulin is generally reported to be found associated with lysosomes, ultimately resulting in the degradation of the hormone (17, 19). Our results are consistent with the notion of internalization, since the lysosomotropic agents cholorquine and methylamine promote an accumulation of a cell-associated radioactivity in frog, but not turkey, erythrocytes after incubation with ^{125}I-labeled insulin.

The regulatory properties of insulin receptors in frog and turkey erythrocytes show remarkable similarities to those of *beta*-adrenergic receptors in these two cell types. Interestingly, both insulin and *beta*-adrenergic receptors are down-regulated in frog erythrocytes whereas no such regulation has been observed for either hormone receptor in turkey erythrocytes (3, 30). There are also many analogies in the regulation of insulin and *beta*-adrenergic receptors in frog erythrocytes. A high concentration of insulin or isoproterenol produces a maximal 50% down-regulation of their respective receptors. Furthermore, the time course for down-regulation is similar for the *beta*-adrenergic and insulin receptors (1, 4). Of particular note is the observation that insulin or *beta*-adrenergic receptor down-regulation in these cells is only slightly diminished and is not abolished at 4°.[6] This finding is in contrast to mammalian systems, in which low temperatures block down-regulation (7). This may relate to the fact that frogs are poikilotherms.

Since the binding characteristics of the insulin receptors in both frog and turkey erythrocytes are closely comparable (5) and the overt morphology of these cells is quite similar, it is not clear why one cell (frog) down-regulates its hormone receptors but the other (turkey)

[6] J. M. Stadel, D. Mullikin-Kilpatrick, and R. J. Lefkowitz, unpublished results.

does not. It is possible to speculate from our data using lysosomotropic amines that a correlation exists between the ability of these erythrocytes to internalize insulin (presumably as an insulin-receptor complex) and receptor down-regulation. Hormone-promoted receptor internalization has been implicated in down-regulation of insulin receptors in mammalian systems (28, 29, 31), and receptor internalization has also been suggested as the mechanism of *beta*-adrenergic receptor regulation in frog erythrocytes (32).

The binding of insulin to a variety of tissues, including frog and turkey erythrocytes, is characterized by curvilinear Scatchard plots (5, 6). This observation was originally interpreted as indicating heterogeneous populations of insulin receptors (20). On the basis of additional kinetic experiments demonstrating enhanced dissociation of cell bound ^{125}I-labeled insulin by unlabeled insulin, De Meyts and co-workers (22, 23) proposed a model of negatively cooperative interactions among insulin receptors to explain complex insulin binding properties. In the present work, our analysis of the equilibrium binding data in frog erythrocytes demonstrates an extremely good fit of the data by a model based on two discrete classes of insulin binding sites. This is true not only for insulin binding to control erythrocytes but also for erythrocytes preincubated with 10^{-6} M insulin or Con A (10 μg/ml). Our modeling indicates that only the high-affinity, low-capacity sites (Site I) were affected by these perturbations of the receptor. Although we cannot rule out small alterations, the analysis did not detect a significant change in either the affinity or number of Site II receptors following insulin or Con A pretreatment of the frog cells.

Our results do *not* exclude the possibility that a negative cooperativity model might also accommodate the binding data in frog erythrocytes. It should be pointed out that the notion of negatively cooperative site-site interactions does not imply a specific relationship between fractional receptor occupancy and receptor affinity for the hormone. The lack of a precise, yet general, mathematical formulation for a negative cooperativity model precludes a definitive comparison of the ability of a "negative cooperativity" and a "two-site" model to explain the equilibrium binding data. Complex relationships between receptor occupancy and affinity can be envisaged which would probably accommodate the data as well as the two-site model. Ultimately, however, the appropriateness of any model of hormone-receptor interactions should be judged by its ability to provide reasonable explanations for the experimental observations. In this respect, a simple model for two independent classes of sites can adequately explain our insulin binding data in the frog erythrocyte under control circumstances as well as under conditions of perterbation by insulin or Con A.

Our modeling approach thus provides a consistent conceptual framework for interpreting all of our binding data in the frog erythrocytes. Site I receptors most likely represent the physiologically important insulin binding component. The concentration of Site I receptors can be regulated by prolonged insulin exposure, and its affinity is modulated by concanavalin A. Furthermore, the K_H of Site I (~ 500 pM) is within the range of insulin concentrations that produce half-maximal biological effects in mammalian tissues (33, 34) and is in accordance with the physiological range of insulin concentration in the peripheral blood of mammals (16). The physiological significance of Site II receptors is at present unknown. A possible interpretation of low-affinity insulin binding was suggested by Wheeler *et al.* (35), who recently proposed a two-site model for the binding of insulin to cultured embryonic heart cells. They suggest that the low-affinity binding component of insulin (i.e., Site II) may represent insulin's weak cross-reactivity with the multiplication-stimulating activity receptor. Both competitive binding potency of insulin for ^{125}I-labeled multiplication-stimulating activity binding sites and the ED_{50} for insulin stimulation of amino acid transport in embryonic heart cells correlate with low-affinity insulin binding. Thus insulin binding to low-affinity sites in cells may be consistent with the growth-promoting effects that have been observed in cell culture in the presence of high concentrations of insulin (36).

As yet no unique model has been able to encompass all of the many observations that have been reported concerning insulin binding to its receptor. The present work suggests that in the frog erythrocyte there may be distinct classes of insulin binding sites with only the high-affinity ones subject to physiological down-regulation or biochemical regulation by Con A.

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