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# A New Approach to Determine Rates of Receptor Appearance and Disappearance *in Vivo*

## Application to Agonist-Mediated Down-regulation of Rat Renal Cortical $\beta_1$- and $\beta_2$-Adrenergic Receptors

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SUMMARY

We have developed a method for the assessment of agonist-induced down-regulation of receptors *in vivo* in terms of rates of receptor appearance and disappearance. This method involves computer-assisted analysis of the kinetics of receptor loss during agonist infusion and of the recovery of receptor number upon the removal of the agonist. These kinetics are analyzed in terms of a steady state model that allows estimation of the rate constants for receptor appearance, $k_{ap}$, and receptor disappearance, $k_{dp}$. Several tests establish that the model can fit experimentally derived data very well. In testing this model, we examined the *in vivo* down-regulation and recovery of rat renal cortical membrane $\beta_1$- and $\beta_2$-adrenergic receptors in response to infusion of the agonist isoproterenol from subcutaneously implanted osmotic minipumps. During recovery from down-regulation, the $\beta_1$-receptors have a $t_{1/2}$ of 45 hr and a $k_{ap}$ of 1.6%/hr, and the $\beta_2$-adrenergic receptors a $t_{1/2}$ of 18 hr and a $k_{ap}$ of 3.9%/hr. During down-regulation, the $t_{1/2}$ for both receptors is 12 hr, while $k_{ap}$ for $\beta_1$-receptors and $\beta_2$-receptors are 3 and 2.3%/hr, respectively. To the extent that the kinetics of recovery from down-regulation reflect "basal" receptor metabolism, the data indicate that enhanced receptor clearance of both receptor subtypes from the plasma membrane contribute to down-regulation, but changes in rates of receptor appearance may occur as well. The use of this computer modeling technique for defining kinetics of changes in receptor number from one steady state level to another should provide a generally useful means to assess hormone and neurotransmitter receptor metabolism *in vivo*.

### INTRODUCTION

A number of recent studies have examined the kinetics of agonist-induced down-regulation of peptide hormone receptors *in vitro* (1-7). These studies have attempted to determine how the processes of receptor synthesis and degradation are altered upon exposure of cells to agonist so that the cell membrane expresses a lower steady state level of receptors. While this type of analysis has provided valuable information concerning the mechanism of agonist-induced down-regulation in cell culture systems, the question remains as to how accurately these data reflect the processes occurring *in vivo* when an organism is exposed to high circulating levels of agonist.

In this report, we describe a new approach to examine the mechanisms mediating down-regulation of receptors *in vivo*. We have analyzed the kinetics of the development of and recovery from agonist-mediated down-regulation in terms of a steady state model for receptor number. Using a computer-assisted, nonlinear curve-fitting paradigm, we have quantitated the rate constants for appearance and disappearance of rat renal cortical $\beta$-adrenergic receptors in the down-regulated state and in the recovery phase after the removal of agonist. We reasoned that the renal cortex would be a useful tissue in which to test our kinetic approach because rat renal cortical membranes possess a mixed population of about 70% $\beta_1$- and 30% $\beta_2$-adrenergic receptors (8, 9). Thus, we could assess in parallel the impact of infusion of the $\beta$-agonist isoproterenol on the two different subtypes of $\beta$-adrenergic receptors.

Our results indicate that the model we describe accurately fits experimentally generated data and allows es-

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timates of the rates of receptor appearance and disappearance *in vivo*. To the extent that the rate constants derived during the recovery of these receptors from down-regulation reflect basal rates of receptor turnover, the results imply that an increase in rate of receptor disappearance plays a role in the decreases in $\beta$-receptor number in the renal cortex in response to infused agonist.

## EXPERIMENTAL PROCEDURES

*Infusion of catecholamines.* In order to raise circulating levels of catecholamines *in vivo*, osmotic minipumps (Alzet) were implanted subcutaneously on the backs of male Sprague-Dawley rats. The rats used were all 8 weeks old and weighed approximately 300 g at the time of minipump implantation. The pumps, which are designed to infuse drug continuously for up to 14 days at a constant rate, were filled with catecholamine at various dose levels prepared in 1 mM HCl. Animals were anesthetized with ether for minipump implantation and the time under anesthesia was less than 5 min. Following pump implantation, all rats were kept on the same light/dark cycle (12 hr each) with free access to food and water. Rats were sacrificed by cardiac puncture under pentobarbital anesthesia (80 mg/kg, intraperitoneal) at various times after minipump implantation. The kidneys were rapidly removed, and the cortices were dissected and then frozen in liquid nitrogen. Frozen cortices were stored at −70° until the time of assay (generally <2 weeks, over which period binding was stable). For studies of receptor recovery from down-regulation, minipumps were implanted in rats and removed 3 days later (by which time down-regulation was maximal) under ether anesthesia. Rats were then sacrificed at various times after pump removal and kidneys were handled as described above.

Plasma catecholamine levels were assayed by the radioenzymatic method of Durrett and Ziegler (10).

*Preparation of renal cortical membranes.* Renal cortical membranes were prepared as previously described (8, 11). Briefly, renal cortices were thawed slowly and homogenized at 4° with a Potter-Elvehjem Teflon-glass tissue homogenizer in buffer containing 0.32 M sucrose, 5 mM Tris, and 1 mM $MgCl_2$ (pH 7.5). The crude homogenate was filtered through four layers of cheesecloth. The resulting filtrate was centrifuged at 500 × *g* for 5 min and the pellet (which contained primarily nuclei and unhomogenized material) was discarded. The supernatant was centrifuged at 30,000 × *g* for 20 min. The resulting pellet was resuspended to a protein concentration of approximately 0.5 mg/ml in an incubation buffer containing 50 mM Tris, 10 mM $MgCl_2$, and 0.9% NaCl (pH 7.5).

*Radioligand-binding assays.* [$^{125}$I]Iodocyanopindolol was prepared and $\beta$-adrenergic receptors and the subclasses were identified as previously described (8). Briefly, membranes were incubated with radioligand for 60 min at 37° in a shaking water bath (120 cycles/min). The reaction was terminated with the addition of 10 ml of the incubation buffer (50 mM Tris, 10 mM $MgCl_2$, 0.9% NaCl, pH 7.5) at 37°. Bound and free radioligands were then separated by rapid (<10 sec) filtration and washing over glass fiber filters (Whatman GF/C) on a Millipore filtration manifold. The radioactivity retained on the filters was counted in a gamma counter at 86% efficiency. Nonspecific binding was defined as the amount of [$^{125}$I]ICYP[2] binding measured in the presence of 1 $\mu$M (−)propranolol.

Competition for the binding of [$^{125}$I]ICYP to renal cortical $\beta$-adrenergic receptors was analyzed by a computer program that performs iterative, nonlinear regression (12). This program fits the binding data to equations based on the law of mass action for one or more classes of binding sites and, using analysis of variance, determines whether the fit for a two-site model is statistically better than that for a one-site model (or a three-site model). The output also includes affinities of the competing agent for one or more sites, as well as the relative properties of each site. Using subtype-selective unlabeled adrenergic antagonists competing with [$^{125}$I]ICYP, which binds equally to $\beta_1$- and $\beta_2$-receptors, this analysis allows one to determine the relative numbers of $\beta_1$- and $\beta_2$-receptors present in renal cortical membranes (8). The subtype-selective agent used was practolol, which binds with higher affinity to $\beta_1$- than to $\beta_2$-receptors. Previous studies indicate that analyses of $\beta$-adrenergic receptor subtypes with practolol yields estimates of $\beta_1$- and $\beta_2$-receptor populations that are identical to those obtained by zinterol, a $\beta_2$-receptor-selective compound (8, 9). For renal cortical membranes, the data modeled best to two sites; fitting the data to a three-site model did not improve the goodness of fit. The affinity of practolol was not altered by the addition of 100 $\mu$M Gpp(NH)p, a nonhydrolyzable analogue of GTP, indicating that this ligand is an antagonist in the renal cortex.

In order to determine the number of $\beta_1$- and $\beta_2$-receptors present in a membrane preparation, the relative percentage of each subtype (determined by practolol competition for [$^{125}$I]ICYP binding) was multiplied by the $B_{max}$ value derived from Scatchard analysis of a simultaneously run saturation isotherm or from analysis of specific binding at a single, near-saturating concentration of [$^{125}$I]ICYP. When a single concentration of radioligand was used to estimate $B_{max}$, the following equation was applied:

$$\frac{B}{B_{max}} = \frac{[L]}{[L] + K_D}$$

where $B/B_{max}$ is the proportion of $\beta$-receptors occupied, $[L]$ is the concentration of [$^{125}$I]ICYP, and $K_D$ is the dissociation constant of [$^{125}$I]ICYP. Several litters of rats were used to derive kinetic data, and for each experimental group, several saturation isotherms were performed on renal cortices from both control rats and those receiving isoproterenol infusion. The $K_D$ for [$^{125}$I]ICYP binding to renal cortical membranes was about 30 pM and this value did not change with isoproterenol treatment of up to 5 days. When a single concentration of [$^{125}$I]ICYP was used to estimate $B_{max}$, we used concentrations of [$^{125}$I]ICYP (300–400 pM), which would occupy in excess of 90% of the renal cortical $\beta$-receptors. Values for $B/B_{max}$ were corrected for the percentage of sites occupied in order to estimate $B_{max}$. Because values for the $K_D$ of [$^{125}$I]ICYP ranged only from 20–40 pM in membrane preparations from different animals, the greatest possible error in the estimate of $B_{max}$ in these experiments was less than 5%. In all experiments using renal cortices from rats receiving catecholamine infusions, binding assays were carried out in the presence of 50 $\mu$M Gpp(NH)p to prevent retention of agonist on tissue adrenergic receptors (13).

*Kinetic analysis of receptor appearance and disappearance.* For analysis of the kinetics of $\beta$-receptor down-regulation in response to isoproterenol and the recovery of these receptors upon the removal of agonist, we used a computer-assisted paradigm to model the data to a steady state rate equation (described in Results). The data were fit to this equation using the Marquardt analysis (14), a computer program which performs nonlinear regression using the method of least squares. Modeling the data provided estimates of $k_{ap}$, the rate constant for reappearance, and $k_{dp}$, the rate constant for receptor disappearance, as well as standard errors of the regression coefficients. Statistical analysis of differences in $k_{ap}$ and $k_{dp}$ between experimental groups was performed by calculating the $Z$ statistic:

$$Z = \frac{x_1 - x_2}{\sqrt{s.e._1 + s.e._2}}$$

where $x_1$ and $x_2$ represent the two values being compared and $s.e._1$ and $s.e._2$ represent their respective standard errors. $p$ values were then obtained from a table of the $Z$ distribution.

## RESULTS

*The model.* The kinetics of down-regulation of cell surface receptors can be described by a simple steady

[2] Abbreviations used are: ICYP, iodocyanopindolol; $B_{max}$, the maximal number of receptors estimated from $x$-intercept on Scatchard analysis; $K_D$, equilibrium dissociation constant; Gpp(NH)p, guanylylimidodiphosphate.

state model. This model is based on the assumption that at steady state the number of receptors present, $R_{SS}$, is equal to the ratio of the rate constants for receptor appearance, $k_{ap}$, and disappearance $k_{dp}$: $R_{SS} = k_{ap}/k_{dp}$. Thus, if the steady state level of receptors is reduced (as is the case with down-regulation), one or both of the rate constants must be altered. The following equation can be used to describe the time-dependent change in receptor number from one steady state level to another.

$$R_t = \frac{k_{ap}}{k_{dp}} (1 - e^{-k_{dp}t}) + R_0 e^{-k_{dp}t} \quad (1)$$

where $R_t$ is equal to the receptor concentration at time $t$, and $R_0$ is the number of receptors present at time 0.

This equation has two implicit assumptions: 1) receptor appearance occurs at a constant rate, and 2) the rate of receptor disappearance is proportional to the concentration of receptors present. These assumptions have been verified in cell culture systems for several classes of membrane receptors (1, 2, 15, 16). Receptor appearance was thus assumed to be a zero-order process and the rate constant $k_{ap}$ is expressed as femtomoles/mg/hr or %/hr. Receptor disappearance was assumed to be a first order process and the rate constant $k_{dp}$ is expressed in units of $hr^{-1}$.

Five tests of the ability of the model depicted by Eq. 1

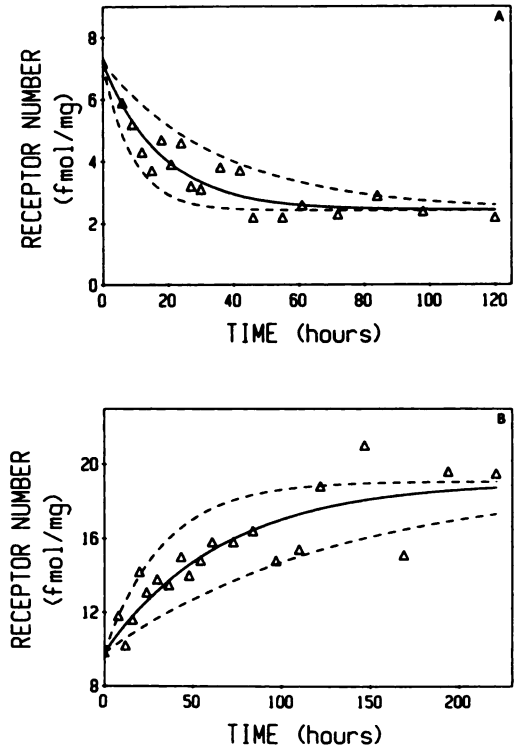


FIG. 1. *A test of $k_{ap}$ and $k_{dp}$ derived from computer modeling*

The data points shown are those obtained for the change in renal cortical membrane $\beta_2$-adrenergic receptors following isoproterenol infusion of 80 μg/kg/hr (A) or $\beta_1$-adrenergic receptors following infusion (50 μg/kg/hr) and subsequent removal of isoproterenol (B) and the computer-derived best fit line (——) to Eq. 1. The values of $k_{ap}$ and $k_{dp}$ derived from Marquardt analysis (A: $k_{ap} = 0.136$, $k_{dp} = 0.0558$; B: $k_{ap} = 0.249$, $k_{dp} = 0.0106$) were then doubled (- - - bottom) or halved (- - - top) so that the ratio of the two rates remained the same.

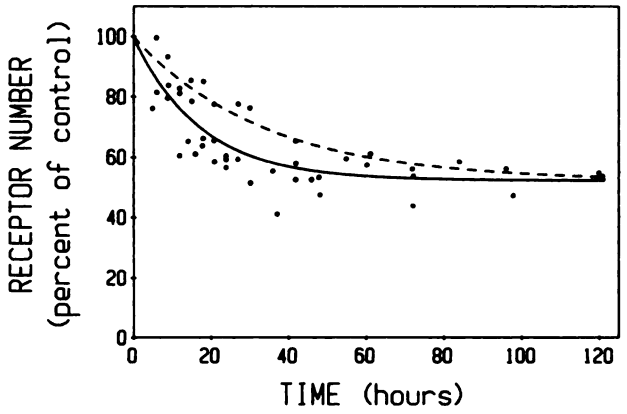


FIG. 2. *A test of whether selective increases in $k_{dp}$ fit the kinetics of $\beta_1$-adrenergic receptor down-regulation*

Data shown are those for the down-regulation of renal cortical $\beta_1$-adrenergic receptors (the same data as shown in Fig. 4). ——, fit of the data derived by Marquardt analysis: $k_{ap} = 3.02\%/hr$; $k_{dp} = 0.0580\ hr^{-1}$. - - -, theoretical curve generated if $k_{ap}$ is assumed to be equal to that determined for the recovery of $\beta_1$-receptors from down-regulation (see Table 4): 1.55%/hr. In order to arrive at the same steady state level of receptors, $k_{dp}$ must equal $0.0298\ hr^{-1}$ ($R_{SS} = k_{ap}/k_{dp}$). The data are not fit by the dashed line generated using these values for $k_{ap}$ and $k_{dp}$.

to fit experimentally derived data were conducted in order to determine the utility of this analysis for studying changes in receptor levels *in vivo*. Figs. 1 and 2 demonstrate that the computer-derived line fits the data reasonably well. In Fig. 1 (A and B), values for $k_{ap}$ and $k_{dp}$ were increased or decreased by a factor of 2 and the lines thus obtained provided a much worse fit of the data for both the development and the recovery phases of down-regulation than that derived from estimates provided by the Marquardt analysis. This indicates that the fit represents a unique solution for both values and not just for their ratio. In addition, when initial estimates of either $k_{ap}$ or $k_{dp}$ or both were altered 10-fold, identical estimates for the rate constants were obtained, indicating that a true minimum value of squared deviations had been reached.

As a third test of the accuracy of this model and as shown in Fig. 2, we compared the line generated by Marquardt analysis to a line generated by assuming that only a change in $k_{dp}$ was responsible for the difference in receptor number. The fit based on this assumption was much worse than that in which both parameters changed. This indicates that the method is sensitive to simultaneous changes in both parameters.

In addition, a different program was used to analyze the data. This program also performs nonlinear regression by the method of least squares, but uses an alternative paradigm (program kindly provided by Dr. Michael Bolger, University of Southern California). Using this program, the same values were obtained for $k_{ap}$ and $k_{dp}$ and for the standard errors of the regression coefficients as were obtained using the Marquardt analysis. Finally, as an overall estimate of the "appropriateness" of the model for fitting the experimentally derived data, no consistent deviation was observed when the residuals of the computer fit (the values for receptor number determined experimentally minus the computer-derived

values) were plotted versus their corresponding time values. That is, deviation of the data around the "best fit line" was random.

Thus, the model presented in Eq. 1 appears to describe adequately the kinetics of receptor loss during agonist-induced down-regulation and the recovery of receptor number following agonist removal. We then used this paradigm to examine the kinetics of agonist-induced changes in $\beta_1$- and $\beta_2$-adrenergic receptors in the rat renal cortex.

*Down-regulation of β-adrenergic receptors by isoproterenol infusion.* Isoproterenol was infused into rats via subcutaneously implanted osmotic minipumps at three different doses: 50, 80, and 110 μg/kg/hr. The levels of circulating isoproterenol achieved by these three doses are shown in Table 1, as is the decrease in total β-receptor number on renal cortical membranes. All three doses achieved similar levels of β-receptor down-regulation. The $\beta_2$-receptors were down-regulated to a slightly greater extent than were $\beta_1$-receptors; in five experiments, the mean values for percentage of receptors remaining after 72 hr were 52.1 ± 2.5 and 39.4 ± 3.5 for $\beta_1$- and $\beta_2$-receptors, respectively. This difference was, however, not statistically significant at the 5% level. Also shown in Table 1 are the levels of plasma isoproterenol remaining 8 hr after the removal of the osmotic minipumps. By this time, circulating isoproterenol levels had returned to normal (close to zero) and, thus, analysis of data during this phase was used to assess receptor recovery from down-regulation.[3] As previous data indicate that depletion of tissue catecholamines with 6-hydroxydopamine fails to alter renal β-receptor number or the proportion of $\beta_1$- and $\beta_2$-receptors (8), changes in expression of renal β-adrenergic receptors in our studies likely result from the observed changes in plasma concentrations of isoproterenol.

Fig. 3 shows the down-regulation and recovery of total renal cortical β-receptors ($\beta_1 + \beta_2$) in response to infusion of the three doses of isoproterenol. At all three dose levels, β-receptor number was maximally decreased within 50 hr and recovered to control levels by 120 hr after cessation of agonist infusion. The changes in receptor number did not appear to differ at these three infusion rates of isoproterenol. In addition, no changes were observed in the numbers of $\alpha_1$- and $\alpha_2$-adrenergic receptors on renal cortical membranes with isoproterenol infusion (data not shown). Control rats, in which pumps contained only vehicle (1 mM HCl), showed no altera-

TABLE 1

*Plasma isoproterenol levels and down-regulation of β-adrenergic receptors in response to three different doses of infused isoproterenol*

Isoproterenol was infused into rats via osmotic minipumps at the doses shown. Plasma isoproterenol levels (in picograms/ml) from two to six rats are shown for control rats (no infusion), rats into which isoproterenol was infused for 72 hr, and rats 8 hr after removal of pumps which had been infusing drug for 72 hr. Controls are presented for each dose level because different groups of rats were used. Positive values for isoproterenol in the control situation reflect some cross-reaction with plasma dopamine in the radioenzymatic assay used for these determinations. Also shown is the maximum percentage of receptors lost at the three doses of isoproterenol.

| | Plasma isoproterenol level at dose | | |
|---|---|---|---|
| | 50 μg/kg/hr | 80 μg/kg/hr | 110 μg/kg/hr |
| | *pg/ml* | | |
| Control (no infusion) | 6 | 29 | 69 |
| 72-hr down-regulation | 1044 | 1130 | 3218 |
| 8-hr recovery | 0 | 2 | 4 |
| % down-regulation of total β ($\beta_1 + \beta_2$)-adrenergic receptors | 48 | 48 | 54 |

[3] Recent observations indicate that the $t_{1/2}$ of plasma isoproterenol is <4 min, thus implying that plasma isoproterenol would decrease >95% in <20 min (M. G. Ziegler, unpublished observations).

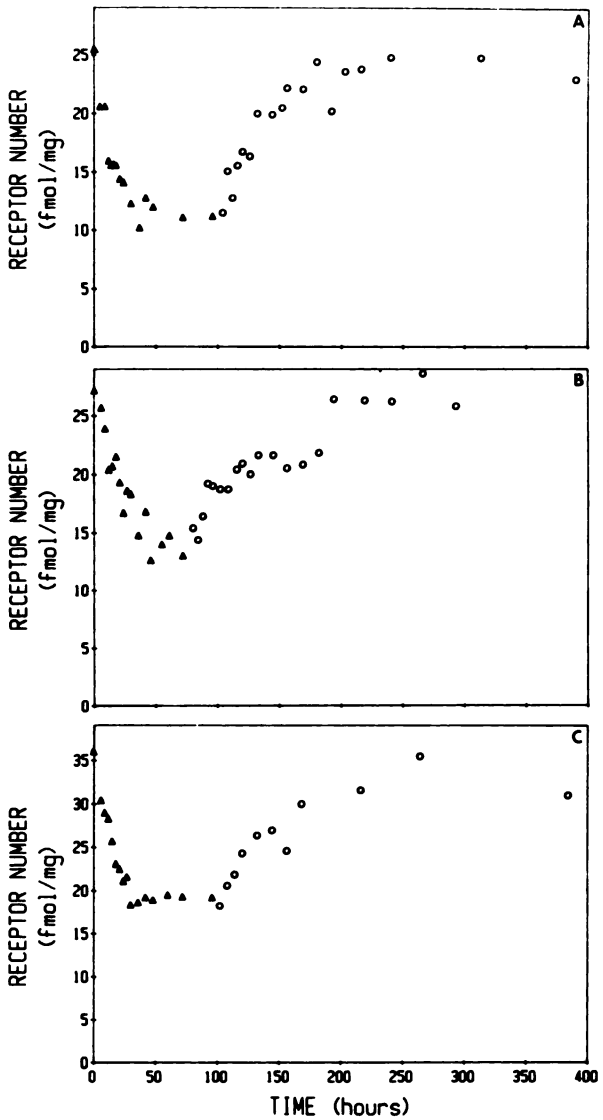


FIG. 3. *Kinetics of down-regulation of renal cortical membrane β-adrenergic receptors by infusion of isoproterenol*

Isoproterenol was infused at 110 (panel A), 80 (panel B), or 50 μg/kg/hr (panel C) into rats and renal cortices were prepared at the indicated time points as described in Experimental Procedures. Each point shown represents the mean of determinations of $B_{max}$ using [$^{125}$I] ICYP from two animals sacrificed at each time point. The triangles represent determinations during the isoproterenol infusion and circles are determinations after removal of the osmotic minipump.

tions in renal cortical β-adrenergic receptor number for up to 14 days after implantation compared to rats receiving no infusions.

*Computer modeling of the kinetics of $\beta_1$-adrenergic receptor down-regulation.* Measurements of $\beta_1$-adrenergic receptor number in membranes prepared from renal cortices of rats receiving isoproterenol infusions yielded data which were fit to Eq. 1. Table 2 shows the values for $k_{ap}$ and $k_{dp}$ generated by computer modeling of kinetic data from down-regulation and recovery of $\beta_1$-receptors in three separate experiments. The most striking difference between the down-regulated and recovery states was the higher value for $k_{dp}$ obtained for $\beta_1$-receptors during down-regulation, which ranged from 2-fold greater in experiment 2 to nearly 6-fold in experiment 1, with a mean in the three experiments of 3.5-fold. The $k_{dp}$ for $\beta_1$-receptors was also higher in each of the three experiments by a mean value of 2-fold.

The values for $k_{ap}$ and $k_{dp}$ estimated from recovery data were very consistent, yielding mean values of 0.282 fmol/mg/hr and 0.0143 $hr^{-1}$, respectively. These correspond to an appearance rate of 1.4%/hr, assuming a control value of 19.9 ± 0.9 fmol/mg of $\beta_1$-receptors ($n$ = 21) for all experiments, and a $t_{1/2}$ in the membrane of 48 hr for renal cortical membrane $\beta_1$-adrenergic receptors.

*Computer modeling of the kinetics of $\beta_2$-adrenergic receptor down-regulation.* Table 3 presents the estimates for $k_{ap}$ and $k_{dp}$ of $\beta_2$-adrenergic receptors generated by modeling the data from three experiments to Eq. 1. A comparison between the rate constants for receptor disappearance in the recovery and down-regulated states illustrates that, as with the $\beta_1$-adrenergic receptors, $k_{dp}$ was greater in the down-regulated state in each of the three experiments. The difference, however, was smaller for $\beta_2$-receptors: a mean difference of 1.8-fold. In contrast with results for $\beta_1$-receptors, the $k_{ap}$ for $\beta_2$-receptors was lower in the down-regulated state by a mean value of 50%. The number of $\beta_2$-receptors from renal cortices of 19 rats was 6.8 ± 0.3 fmol/mg. The mean value for $k_{ap}$ from the three kinetic experiments yielded an appearance rate of 4.4%/hr during the recovery phase. The mean $k_{dp}$, 0.0402 $hr^{-1}$, yielded a $t_{1/2}$ of 17.2 hr for rat renal cortical $\beta_2$-adrenergic receptors during this phase.

*Computer modeling of combined kinetic data for down-regulation of $\beta_1$- and $\beta_2$-receptors.* Because the three infusion rates of isoproterenol used to induce β-receptor down-regulation appeared to be equally effective [all three doses down-regulated $\beta_1$- and $\beta_2$-adrenergic receptors to the same extent with similar kinetics (Tables 1–3)], we combined the data from all three doses and applied the modeling paradigm to generate overall estimates for the kinetic parameters. In order to do this, values for receptor number were expressed as a percentage of the control values for β-receptor number determined for each experiment. The results of modeling to the combined data are shown in Figs. 4 and 5 and in Table 4. As was the case when experiments were analyzed separately, down-regulation of $\beta_1$-receptors was complete by 48 hr (Fig. 4) and recovery from down-regulation was complete by 150 hr after the removal of the osmotic minipumps (Fig. 5). The analysis of combined data indicated that the rate constant for $\beta_1$-receptor disappearance in the down-regulated state was nearly 4-fold higher than that obtained during recovery (Table 4). The rate constant for receptor appearance in the down-regulated state, was greater by approximately 2-fold. The $t_{1/2}$ of $\beta_1$-adrenergic receptors during recovery was 45 hr, in close agreement with the value of 48 hr obtained from the mean $k_{dp}$ of the three experiments shown in Table 2. Similarly, the rate of receptor appearance during the recovery phase was 1.55% $hr^{-1}$ (or 0.3 fmol/mg/hr) compared to the value of 1.4% $hr^{-1}$ obtained from the mean of individual experiments. In the down-regulated state, the rate of receptor appearance was 3.02% $hr^{-1}$ (or 0.6 fmol/mg/hr) while the $t_{1/2}$ of $\beta_1$-adrenergic receptors was 12 hr.

Similar data were obtained for the kinetics of the change in $\beta_2$-adrenergic receptor number during down-

TABLE 2

*Rate constants for $\beta_1$-adrenergic receptor appearance and disappearance in control (recovery) and down-regulated states*

Values presented are computer-derived estimates for the rate constants of $\beta_1$-receptor appearance, $k_{ap}$, and disappearance, $k_{dp}$, for modeling of three experiments of the types described in Experimental Procedures. All values shown are ± standard errors of the regression coefficients. Although three different dose levels of isoproterenol were used to treat the three separate groups of rats, there was no consistent difference in the kinetics of down-regulation and recovery of $\beta_1$-receptors. Values obtained by modeling the recovery of $\beta_1$-receptors from down-regulation are assumed to represent control rate constants for receptor turnover as described in the text.

| Dose of isoproterenol | $n$ | | $k_{ap}$ | $k_{dp}$ |
|---|---|---|---|---|
| *μg/kg/hr* | | | *fmol/mg/hr* | *$hr^{-1}$* |
| 1. 50 | 32 | Down-regulation | 0.855 ± 0.147 | 0.0596 ± 0.0082 |
| | 22 | Recovery | 0.249 ± 0.059 | 0.0106 ± 0.0030 |
| 2. 80 | 36 | Down-regulation | 0.303 ± 0.090 | 0.0308 ± 0.0066 |
| | 40 | Recovery | 0.288 ± 0.064 | 0.0151 ± 0.0041 |
| 3. 110 | 28 | Down-regulation | 0.599 ± 0.156 | 0.0676 ± 0.0121 |
| | 36 | Recovery | 0.308 ± 0.077 | 0.0173 ± 0.0052 |

TABLE 3

*Rate constants for $\beta_2$-adrenergic receptor appearance and disappearance in control (recovery) and down-regulated states*

Values presented are computer-derived estimates for the rate constants for $\beta_2$-receptor appearance, $k_{ap}$, and disappearance, $k_{dp}$, for modeling of three experiments of the types described in Experimental Procedures. All values shown are ± standard errors of the regression coefficient. Although three different dose levels of isoproterenol were used to treat the three separate groups of rats, there was no consistent difference in the kinetics of down-regulation and recovery of $\beta_1$-receptors. Values obtained by modeling the recovery of $\beta_2$-receptors from down-regulation are assumed to represent control rate constants for receptor turnover as described in the text.

| Dose of isoproterenol | $n$ | | $k_{ap}$ | $k_{dp}$ |
|---|---|---|---|---|
| *μg/kg/hr* | | | *fmol/mg/hr* | *$hr^{-1}$* |
| 1. 50 | 32 | Down-regulation | 0.229 ± 0.062 | 0.0665 ± 0.0133 |
| | 22 | Recovery | 0.436 ± 0.059 | 0.0471 ± 0.0072 |
| 2. 80 | 36 | Down-regulation | 0.136 ± 0.030 | 0.0558 ± 0.0081 |
| | 40 | Recovery | 0.244 ± 0.068 | 0.0388 ± 0.0121 |
| 3. 110 | 28 | Down-regulation | 0.101 ± 0.052 | 0.0562 ± 0.0168 |
| | 36 | Recovery | 0.230 ± 0.044 | 0.0346 ± 0.0078 |

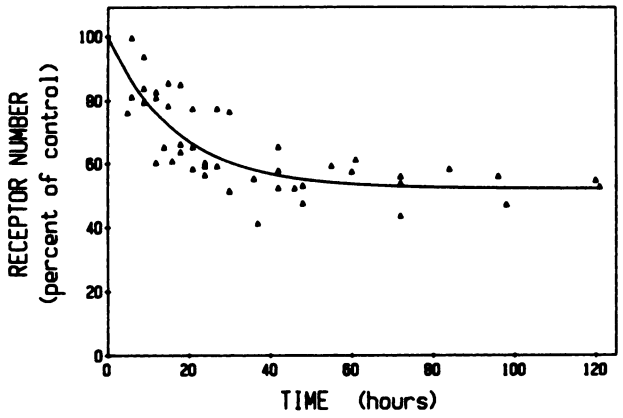


FIG. 4. *Computer modeling of the kinetics of down-regulation of $\beta_1$-adrenergic receptors by isoproterenol*

Results from the three sets of experiments shown in Table 2 were combined by expressing $\beta_1$-adrenergic receptor numbers from each experiment as a percentage of the control value determined for that particular set of rats. The control values for the three groups of rats were: group 1, 27.7 fmol/mg; group 2, 19.9 fmol/mg; group 3, 20.4 fmol/mg. The levels of down-regulation achieved in the three groups were: group 1, 47%; group 2, 46%; group 3, 55%. Each point represents the percentage of control $\beta_1$-receptors remaining at that particular time after minipump implantation and is the mean value obtained from duplicate rats. ——, computer-derived fit of the model to Eq. 1 as described in the text. Values for $k_{ap}$ and $k_{dp}$ derived from this analysis are shown in Table 4.

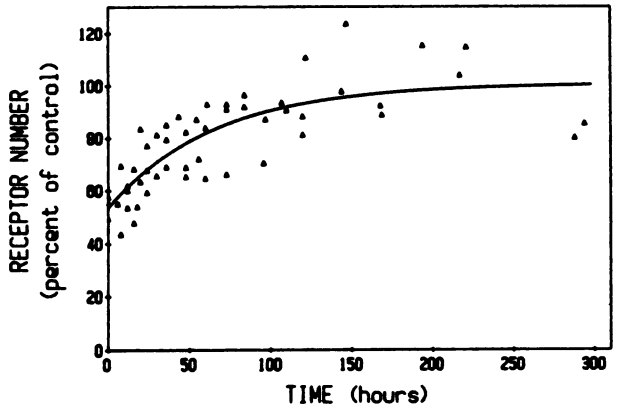


FIG. 5. *Computer modeling of the recovery of $\beta_1$-adrenergic receptors from down-regulation by isoproterenol*

Results from the three sets of experiments shown in Table 2 were combined by expressing $\beta_1$-adrenergic receptor numbers from each experiment as a percentage of the control value determined for that particular set of rats. The control values for the three groups of rats were: group 1, 27.7 fmol/mg; group 2, 17.0 fmol/mg; group 3, 18.6 fmol/mg. The levels of down-regulation achieved in the three groups were: group 1, 47%; group 2, 42%; group 3, 51%. Each point represents the percentage of control $\beta_1$-receptors present at that particular time and is the mean value obtained from duplicate rats. ——, computer-derived fit of the model to Eq. 1 is described in the text. Values for $k_{ap}$ and $k_{dp}$ derived from this analysis are shown in Table 4. The $\beta_1$-receptors of the three groups recovered to 85% (group 1), 111% (group 2), and 96% (group 3) of the control values.

regulation and recovery (Table 4). $\beta_2$-Receptors were maximally down-regulated by 60 hr and recovered completely within 100 hr. These receptors were thus lost more slowly and recovered more quickly than did $\beta_1$-receptors. The differences in $k_{ap}$ (lower) and $k_{dp}$ (higher) between the down-regulated and recovery states were significant ($p < 0.05$ in both cases) and the results obtained modeling to the combined data were similar to those obtained when the experiments were analyzed separately (Tables 3 and 4). The $t_{1/2}$ of the $\beta_2$-receptor during recovery was 17.8 hr, virtually identical to the value of 17.2 hr obtained from the mean $k_{dp}$ of the three individual experiments. The $t_{1/2}$ was 12 hr in the down-regulated state. The $k_{ap}$ of 3.9% $hr^{-1}$ during recovery was similar to the mean value of 4.4% $hr^{-1}$ calculated from the three individual experiments.

In comparing the values of $k_{ap}$ and $k_{dp}$ for $\beta_1$- versus $\beta_2$-adrenergic receptors, the rate constant for disappearance of the $\beta_2$-receptor (0.039 $hr^{-1}$) was greater than that of the $\beta_1$-receptor (0.015 $hr^{-1}$, $p < 0.05$) during recovery and both values increased to ~0.06 $hr^{-1}$ in the presence of agonist. The rate constant for appearance of $\beta_2$-receptors (3.90% $hr^{-1}$) was also significantly greater than that of the $\beta_1$-receptor (1.55% $hr^{-1}$, $p < 0.005$) and the rate constant for receptor appearance of the two subtypes changed in opposite directions in the down-regulated state. During exposure to agonist, the two $\beta$-receptor subtypes yielded similar rate constants for both receptor appearance and disappearance ($p > 0.05$, Table 4).

## DISCUSSION

We have presented a kinetic model that accurately describes both the loss of receptors in response to elevated levels of circulating agonist and the recovery of receptor number upon the removal of the agonist. Use of this computer-modeling technique allows the description of changes in receptor number in terms of rates of receptor appearance and disappearance and represents a first step toward a more detailed understanding of the kinetics and mechanisms of receptor turnover *in vivo*. Furthermore, the use of this technique *in vitro*, as well as *in vivo*, should facilitate an understanding of how well results obtained in cell culture reflect the processes that occur in the intact animal. In addition, this method should be widely applicable to different receptor systems, thereby allowing comparisons of the turnover rates of a number of different receptor types in different organs as well as assessment of the role that changes in rates of receptor appearance and disappearance play in changes in receptor number that are evoked by various stimuli.

While a number of tests illustrate that the model accurately describes experimentally derived data and is sensitive to changes in $k_{ap}$ and $k_{dp}$, the method itself and the interpretation of data derived from it are subject to certain limitations. Of primary concern is the fact that the kinetic model described by Eq. 1 is probably an oversimplification. The events that occur *in vivo* leading to an alteration in the steady state number of receptors in response to agonists are undoubtedly complex. A more sophisticated model has recently been described to account for down-regulation of insulin receptors (3). However, this model is not applicable to our study because it requires a knowledge of the concentration of ligand at the receptor; such knowledge is not available in an *in vivo* system. In addition, the rate constants $k_{ap}$ and $k_{dp}$ likely include multiple processes. The rate constant of

TABLE 4

*Rate constants for appearance and disappearance of $\beta_1$- and $\beta_2$-adrenergic receptors in control (recovery) and down-regulated states*

Values for the rate constants for receptor apppearance, $k_{ap}$, and disappearance, $k_{dp}$, were derived from computer fitting of the combined data shown in Tables 2 and 3 and are presented ± standard errors of the regression coefficients. These estimates represent values from the analysis of the combined data from experiments conducted at three dose levels of infusions of isoproterenol.

| | $\beta_1$ | | $\beta_2$ | |
|---|---|---|---|---|
| | $k_{ap}$ | $k_{dp}$ | $k_{ap}$ | $k_{dp}$ |
| | %/hr | $hr^{-1}$ | %/hr | $hr^{-1}$ |
| Down-regulation | 3.02 ± 0.47[a] | 0.0580 ± 0.0087[b] | 2.29 ± 0.39[a] | 0.0600 ± 0.0069[a] |
| Recovery | 1.55 ± 0.31[c] | 0.0154 ± 0.0037[c] | 3.90 ± 0.67 | 0.0390 ± 0.0076 |

[a] $p < 0.05$ down-regulation compared to recovery.
[b] $p < 0.005$ down-regulation compared to recovery.
[c] $p < 0.005$ $\beta_1$ compared to $\beta_2$.

appearance no doubt subsumes rate constants for both receptor synthesis and insertion into the plasma membrane; these events cannot be distinguished using our methodology. Similarly, $k_{dp}$ likely includes rate constants for receptor internalization and degradation. However, as our steady state model fits the observed data well, we conclude that Eq. 1 provides a simple means to describe the system and may be compatible with what transpires *in vivo*. Further studies will be needed to ascertain the contribution of the various events subsumed by $k_{ap}$ and $k_{dp}$.

A further limitation of this method is inherent in studies of receptors in tissues from intact animals: the results obtained probably reflect processes acting on receptors located on a variety of cell types. This is especially true for the renal cortex which is a very heterogeneous tissue and for which some evidence exists from cell culture and autoradiographic studies supporting the idea that the $\beta_1$- and $\beta_2$-adrenergic receptors are located on different renal cells (17, 18).[4] In this sense also, the model we present here is an oversimplification. Thus, the more rapid recovery of $\beta_2$-receptors after removal of agonist may reflect a general property of $\beta_2$-receptors or, alternatively, this result may be indicative of a faster rate of metabolism of receptors by the cells on which the $\beta_2$-receptors are located. Such questions can only be resolved by additional studies but the more rapid recovery of one receptor subtype *in vivo* is nonetheless a new and somewhat unexpected observation.

The applicability of this method to measurements of receptor turnover *in vivo* was demonstrated by the kinetic modeling of down-regulation of renal cortical β-adrenergic receptors by the agonist isoproterenol and the recovery of these receptors following agonist removal. Three doses of isoproterenol were initially chosen in an attempt to generate a dose-response relationship between agonist concentration and down-regulation. However, all three doses turned out to be maximally effective in decreasing β-receptor number. Thus, we were able to combine the data obtained at the three different levels of agonist. The model fit the experimentally derived data very well, whether the data for each dose of agonist were analyzed separately or simultaneously. Significant differences were observed in the values for rate constants of receptor appearance and disappearance between the down-regulated and recovery states. Furthermore, quite different rate constants were derived for $\beta_1$- and $\beta_2$-receptors in the recovery phase, while the values for $k_{ap}$ and $k_{dp}$ during down-regulation were similar. These results are consistent with the idea that similar mechanisms may be involved in the down-regulation of both renal cortical β-receptor subtypes, while quite different processes may be at work during the recovery of receptor number upon the removal of agonist.

It is unclear whether the values of $k_{ap}$ and $k_{dp}$ obtained from the recovery of receptors from down-regulation represent the basal turnover rates of receptors. Virtually all previous work on metabolism of adrenergic receptors has involved the use of protein synthesis inhibitors and/or irreversible antagonists. In the latter experiments, receptors are blocked with an irreversible antagonist and the recovery of radioligand-binding sites is monitored over time. After irreversible blockade, receptor recovery results from synthesis of new receptors as well as their transport and insertion into the plasma membrane. By contrast, our approach measures receptors reappearing in the membrane following the rapid elimination of agonist. Although down-regulated receptors do not recognize ligands, they may not be completely degraded and may be sequestered in an intracellular pool in an altered state. If sequestered receptors recover from down-regulation more rapidly than if *de novo* synthesis of receptor molecules is required, values derived from the kinetics of receptor recovery may not provide accurate estimates of the basal rates of receptor appearance and disappearance. On the other hand, if under basal conditions receptor turnover were to involve rapid movement of receptors to and from a preformed sequestered pool, rates of receptor turnover determined during recovery from agonist-mediated down-regulation may be a more accurate reflection of basal receptor turnover than methods which require *de novo* synthesis. Moreover, one imagines that receptor turnover under "basal" conditions is in large part determined by circulating and neuronally released agonists. The model that we present is applicable to studies of receptor recovery after irreversible blockade and such studies should help to clarify this issue. Furthermore, this model can also be used in experiments using immunological approaches to study receptor turnover. Studies of this type will likely prove of importance

[4] D. Healy, P. Münzel, and P. A. Insel, manuscript submitted.

in defining mechanisms involved in the regulation of receptor number *in vivo*.

The values for the $t_{1/2}$ of the membrane bound $\beta_1$- and $\beta_2$-receptors in the renal cortex derived from the recovery of these receptors from down-regulation (45 and 18 hr, respectively) are similar to certain previous values reported for adrenergic receptors in cell culture studies using irreversible antagonists: 24 hr for $\alpha_1$-adrenergic receptors in BC3H-1 cell membranes (16, 19)[5] and about 30 hr for $\beta_2$-adrenergic receptors in cultured human lung ($VA_2$) cells and S49 lymphoma cells (20, 21), although other studies have noted considerably longer half-times for $\beta_1$- and $\beta_2$-receptors on cultured 132N1 astrocytoma and BC3H-1 muscle cells, respectively (19, 22).[5] In addition, Sladeczek and Bockaert (23) used recovery from blockade with phenoxybenzamine and reported a $t_{1/2}$ for rat submandibular gland $\alpha_1$-receptors *in vivo* of 33 hr. By contrast, studies involving the use of the irreversible antagonist bromoacetylalprenololmenthane to block $\beta$-adrenergic receptors in rat heart and lung *in vivo* (24, 25) indicated that receptor recovery is slow (10–30 days to full recovery versus ≤5 days in our studies). This slow recovery might result from the partitioning into fat and then slow release of a lipophilic blocker like bromoacetylalprenololmenthane. In the absence of control experiments to eliminate persistent receptor blockade, the results obtained with bromoacetylalprenololmenthane are somewhat difficut to interpret.[6] Thus, our results compare favorably with other data on adrenergic receptor turnover *in vitro* and *in vivo* (16, 19–23, 26), while the recovery rates estimated with the irreversible $\beta$-adrenergic blocker are much slower.

To the extent that the $k_{ap}$ and $k_{dp}$ values derived from the recovery of $\beta$-adrenergic receptor subtypes from down-regulation reflect basal rates of receptor turnover, the major change in $\beta$-receptor metabolism during down-regulation appears to be an enhanced rate of receptor disappearance, although the degree of this change is different for the two receptor subtypes. To the best of our knowledge, this is the first comparison of the relative rates of turnover of $\beta_1$- and $\beta_2$-adrenergic receptors in the same tissue *in vivo*. More importantly, we believe that the application of computer-assisted kinetic analysis provides a first step in defining events in target cells that are responsible for agonist-mediated down-regulation in an intact animal. The relative simplicity of the approach described here should make it applicable to studies of receptor metabolism in a variety of different systems both *in vivo* and *in vitro*.

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[5] R. J. Hughes and P. A. Insel, manuscript submitted.

[6] In recent studies, workers in our laboratory have found partitioning of this compound into certain cultured cells and long-lived blockade of $\beta$-receptors in both a competitive and noncompetitive manner.

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