MOLECULAR PHARMACOLOGY, 23:282–288

Turnover *in Vivo* of *Alpha*$_1$-Adrenergic Receptors in Rat Submaxillary Glands

FRITZ SLADECZEK[1,2] AND JOËL BOCKAERT[2]

Laboratoire de Physiologie Cellulaire, Collège de France, 75231 Paris Cedex, France

Received July 21, 1982; Accepted October 12, 1982

SUMMARY

In submaxillary glands, vas deferens, and cerebral cortex, [^{3}H]prazosin labeled one homogeneous population of *alpha*$_1$-adrenergic receptors having a K_D of 0.1 nM. Intravenous injections of phenoxybenzamine blocked these receptors in a dose-dependent manner without changing the affinity of the remaining sites for [^{3}H]prazosin. The phenoxybenzamine efficiency was highest in submaxillary glands: 1 mg/kg completely blocked the *alpha*$_1$-adrenergic receptors but did not affect *alpha*$_2$-adrenergic, *beta*-adrenergic, and muscarinic receptors in this organ. After this blockade, the *alpha*-adrenergic receptors reappeared in the glands following a monoexponential time course. Analysis of this time course allows the determination of the rate constant for receptor degradation (k = 0.02 hr^{-1}) and the rate of receptor production (r = 1.86 fmoles/mg of protein per hour). The half-life of the receptor was 33 hr. The reappearing receptors corresponded to newly synthetized receptors since their reappearance was blocked by i.p. injections of cycloheximide. Blockade of *alpha*$_1$-adrenergic receptors with phenoxybenzamine (2 mg/kg) did not affect receptor reappearance. In contrast, higher doses (4–20 mg/kg) decreased the velocity of receptor reappearance.

INTRODUCTION

The density of hormonal or neurotransmitter receptors has been shown to be regulated by at least two main factors. The first factor is the intensity of receptor stimulation; therefore, when a large fraction of receptors is occupied for a long period of time by its own agonist, the number of receptors decreases (down-regulation) whereas in the absence of stimulating agents the number of receptors increases (up-regulation). The second factor is the regulation by various hormones not specific for the receptors studied. Two of the most interesting examples are thyroid hormones (1–3) and steroids (4–6). It is likely that these factors regulate the normal "turnover" of receptors, modifying either their synthesis or their degradation rates. Indeed, the down-regulation of insulin receptors seems to affect specifically their degradation rate (7, 8). For these reasons it is important to study the metabolism of receptors. A great number of studies have been done on acetylcholine, nicotinic (9), and insulin receptor (7, 10) turnover. In contrast, there are only few reports concerning *alpha*- or *beta*-adrenergic receptor turnover (11–14).

We reported recently that in a nonfusing muscle cell line the *alpha*$_1$-adrenergic receptor has a half-life of 23 hr, corresponding to a rate of receptor synthesis of 3.2 fmoles/mg of protein per hour and to a degradation rate of 0.03 hr^{-1} (14). Since the *in vitro* cultures involve transformed cells and reproduce neither the *in vivo* hormonal environment nor neuronal receptor stimulation, it was of importance to compare these results with an *in vivo* study of *alpha*$_1$-adrenergic receptor turnover.

EXPERIMENTAL PROCEDURES

Male Wistar rats (180–220 g; 7–9 weeks old) were used throughout.

Preparation of particulate fractions. Rats were killed by a blow on the head, and the glands were removed and separated from sublingual glands. The submaxillary glands were homogenized in 5 ml of ice-cold buffer (50 mM Tris-HCl, pH 7.5) by four 10-sec bursts at full speed with a Polytron PT-10. After dilution of the homogenate with 15 ml of buffer, it was centrifuged for 15 min at 30,000 × g at 4°. The pellet was homogenized in 10 ml of buffer with a Teflon-glass homogenizer and, after filtration through a double layer of silk screen (150-μm pore diameter) and dilution by one-half, recentrifuged for 15 min at 30,000 × g at 4°. The final pellet was resuspended in 10 ml of 50 mM Tris-HCl (pH 7.5).

Particulate fractions of vas deferens were prepared in

This work was supported by grants from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale and by the Centre CNRS-INSERM de Pharmacologie Endocrinologie de Montpellier, France.

[1] Recipient of a fellowship from the Délégation Générale à la Recherche Scientifique et Technique.

[2] Present address, Centre CNRS-INSERM de Pharmacologie-Endocrinologie, 34033 Montpellier Cedex, France.

0026-895X/83/020282-07$02.00/0

the same way. The only differences in the preparation of cerebral cortex membranes were that the first homogenization was done in 10 ml of ice-cold buffer with a Teflon-glass homogenizer and that the final pellet was resuspended in 25 ml of buffer instead of 10 ml.

Blockade of alpha-adrenergic receptors with POB.[3] POB was dissolved in 2 mM tartaric acid and, after dilution by one-half with 150 mM NaCl, injected into the tail vein. To follow the reappearance of the *alpha*$_1$-adrenergic receptors, rats were kept during varying times with free access to food and water before the determination of the concentration of *alpha*$_1$-adrenergic receptors. In each case, this concentration was the maximal binding of [^{3}H]prazosin as determined by Scatchard analysis.

Binding assays. [^{3}H]Prazosin, [^{3}H]clonidine, and [^{3}H]QNB binding assays were performed in a total volume of 1 ml in a solution containing 50 mM Tris-HCl (pH 7.5), various concentrations of radioligand, and 100 μl of the membrane preparation corresponding to 100–200 μg of protein. The samples were incubated for 60 min at 25°. Incubations were terminated by rapid filtration of the entire mixture through Whatman GF/B glass-fiber filters and by washing three times with 5 ml of ice-cold incubation buffer. The filters were dried and counted in 8 ml of scintillation fluid.

For the [^{3}H]DHA binding assays, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 40 μl of the membrane fraction were used. The total volume was 400 μl and the incubation time 30 min at 25°. Following incubation, the samples were diluted with 600 μl of ice-cold incubation buffer and then treated as described above. The figures show specific binding, defined as the binding which was inhibited by 10^{-5} M phentolamine for [^{3}H]prazosin and [^{3}H]clonidine, 10^{-5} M atropine for [^{3}H]QNB, and 10^{-5} M alprenolol for [^{3}H]DHA.

Protein concentrations were determined by the method of Lowry *et al.* (15), using bovine serum albumin as a standard.

Blockade of protein synthesis by cycloheximide. During the 2 weeks preceding the experiment as well as during the entire experimental period, rats of 100–200 g at the beginning of the experiment were fed milk, vegetables, and the normal dry rat foods. Forty-eight hours before decapitation each rat received an i.p. injection of cycloheximide, 3 mg/kg body weight, preceded or not by an i.v. injection of POB; 24 hr later they received a second i.p. injection of cycloheximide, 3 mg/kg.

Protein synthesis was assessed by the incorporation of [^{3}H]leucine into submaxillary gland protein. For this assessment, the rats received i.p. injections of 10 μCi of [^{3}H]leucine. One hour later they were killed by a blow on the head and the glands were removed as previously described. After homogenization at 0° in 10% trichloroacetic acid plus 5 mM (−)-leucine with a Polytron PT-10, the suspension was centrifuged at 3000 × *g* for 5 min. The trichloroacetic acid-precipitable material was washed three times with the same medium at 0° and the final pellet was dissolved in 5 ml of 30% KOH at 80° during 1 hr. One milliliter of this solution was counted in scintillation fluid.

Materials. [^{3}H]Prazosin (28 Ci/mmole) and [^{3}H]QNB (32 Ci/mmole) were purchased from the Radiochemical Centre (Amersham, England). [^{3}H]Clonidine hydrochloride (23.8 Ci/mmole), [^{3}H]DHA (40.6 Ci/mmole), and L-[4,5-N-^{3}H]leucine (5.0 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.); (−)-epinephrine bitartrate, (−)-norepinephrine bitartrate, (−)-phenylephrine hydrochloride, (−)-isoproterenol bitartrate, yohimbine hydrochloride, (−)-alprenolol-D-tartrate, and cycloheximide were from Sigma Chemical Company (St. Louis, Mo.); phenoxybenzamine from Smith Kline & French (Philadelphia, Pa.), (−)-leucine from Calbiochem (San Diego, Calif.), clonidine from Boehringer Ingelheim (Ridgefield, Conn.), methoxamine hydrochloride from Burroughs Wellcome Company (Research Triangle Park, N.C.), (+)-norepinephrine bitartrate from Sterling-Winthrop Research Institute (New York, N. Y.), and prazosin hydrochloride from Pfizer Laboratories (New York, N. Y.). Phentolamine was a gift from Ciba-Geigy Corporation (New York, N. Y.).

RESULTS

Irreversible blockade of alpha$_1$*-adrenergic receptors in different organs by POB.* In order to choose the organ in which the *alpha*$_1$-adrenergic receptors are most potently blocked by POB, we measured the effect of various concentrations of this compound on the receptor number of submaxillary glands, vas deferens, and cerebral cortex. In these three organs [^{3}H]prazosin labeled one homogeneous population of sites having similar dissociation constants ($K_D \simeq 0.1$ mM). The number of sites was about 100 fmoles/mg of protein in submaxillary glands and vas deferens, and slightly higher in brain cerebral cortex (Fig. 1). In all organs, i.v. injection of POB reduced the number of [^{3}H]prazosin binding sites without affecting the apparent K_D. Thirty minutes after POB injection the highest blockade was found in submaxillary glands: 0.8 mg/kg reduced by 95% the number of binding sites. At this dose, the reductions were 67% and 80% in vas deferens and cerebral cortex, respectively. Similar blockade was observed 60 min after POB injection.

Therefore we decided to study the reappearance of *alpha*$_1$-adrenergic receptors in submaxillary glands to avoid the use of higher doses of POB, which can, as we show later, influence the rate of *alpha*$_1$-receptor reappearance.

Specificity of [^{3}H]prazosin binding sites in submaxillary glands. The specificity of [^{3}H]prazosin binding in submaxillary glands was typically *alpha*-adrenergic (Fig. 2). The order of specificity was clonidine > (−)-epinephrine > (−)-norepinephrine > (−)-phenylephrine > methoxamine > (+)-norepinephrine > (−)-isoproterenol. The binding was stereospecific, since (−)-norepinephrine was 56 times more potent than (+)-norepinephrine. The antagonist specificity clearly indicated the *alpha*$_1$ specificity of the receptors labeled with [^{3}H]prazosin. Prazosin, an *alpha*$_1$-specific antagonist, was 2600 times more potent than yohimbine, an *alpha*$_2$-specific antagonist (Fig. 2).

[3] The abbreviations used are: POB, phenoxybenzamine; QNB, quinuclidinyl benzilate; DHA, dihydroalprenolol.

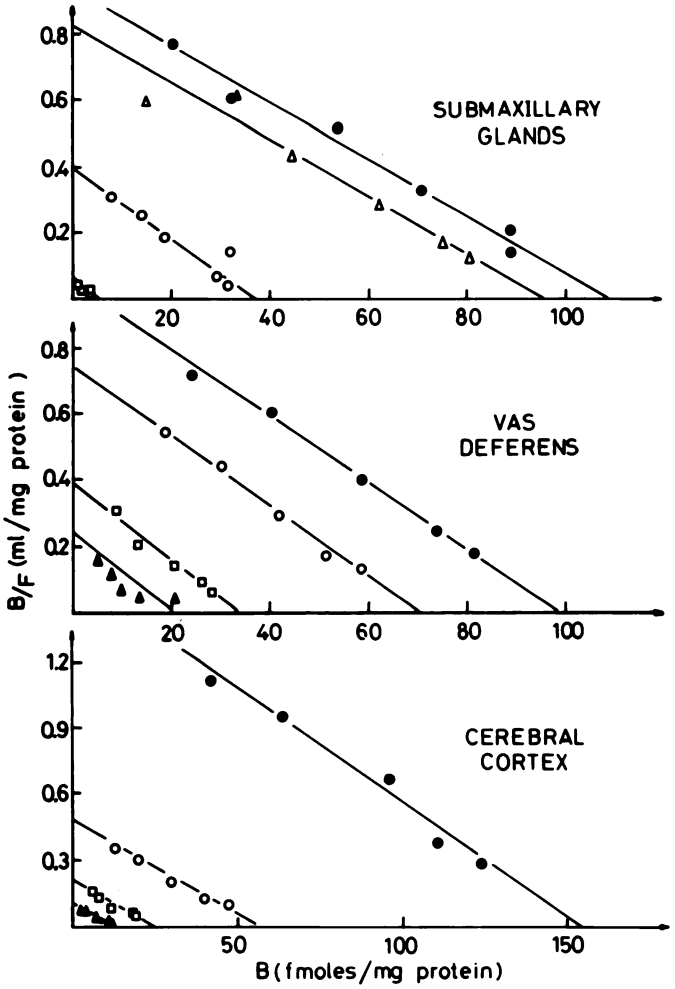

FIG. 1. *Blockade of alpha$_1$-adrenergic receptors in different organs following i.v. injections of POB*

Different doses of POB [△, 0.20 mg/kg; ○, 0.5 mg/kg; □, 0.8 mg/kg; ▲, 1 mg/kg; or vehicle (●) (1 mM tartaric acid in 0.5 ml of 0.9% NaCl)] were injected into rats. The rats were killed 30 min later, particulate fractions were prepared, and [^{3}H]prazosin binding was measured as described under Experimental Procedures.

Specific blockade of alpha$_1$-adrenergic receptors in submaxillary glands. Since POB has been shown to block with different potencies *alpha$_1$*-adrenergic, *alpha$_2$*-adrenergic, muscarinic (16), histamine H_1 (17), serotonin (18), dopamine (19), and opiate (20) receptors, it was important to verify that, at the dose used, *alpha$_1$*-adrenergic receptors were specifically blocked. As shown in Fig. 3, 30 min after i.v. injection of POB (1 mg/kg), *beta*-adrenergic (labeled with [^{3}H]DHA), muscarinic (labeled with [^{3}H]QNB), and *alpha$_2$*-adrenergic receptors (labeled with [^{3}H]clonidine) were unaffected by this treatment.

Reappearance of alpha$_1$-adrenergic receptors in submaxillary glands after irreversible blockade with POB. Rats received injections of POB (1 mg/kg) and were killed after different periods of time. The number of *alpha$_1$*-receptors and their dissociation constants were determined by Scatchard analysis. In all experiments the receptors which reappeared had the same affinity as the control ($K_D \simeq 0.1$ mM). When the receptor blockade was performed with 2-fold higher concentration of POB (2 mg/kg) and tested 48 hr later, the receptor reappearance was similar (data not shown).

Determination of synthesis and degradation rates of alpha$_1$-adrenergic receptors in submaxillary glands. We have previously shown (14) that the repopulation kinetics after irreversible blockade of a receptor can be described by the following equation

$$[R_t] = \frac{r}{k}(1 - e^{-kt}) \qquad (1)$$

where $[R_t]$ = receptor concentration at a given time, r = receptor production rate, and k = rate constant for degradation. When $t \rightarrow \infty$, $[R_t]$ approaches r/k, which is therefore equal to $[R_{ss}]$, the concentration of receptor at steady state. The $[R_{ss}]$ value in submaxillary glands was 87 ± 4 fmoles/mg of protein (n = 19) and did not vary significantly from the beginning to the end of the experiment (10 days).

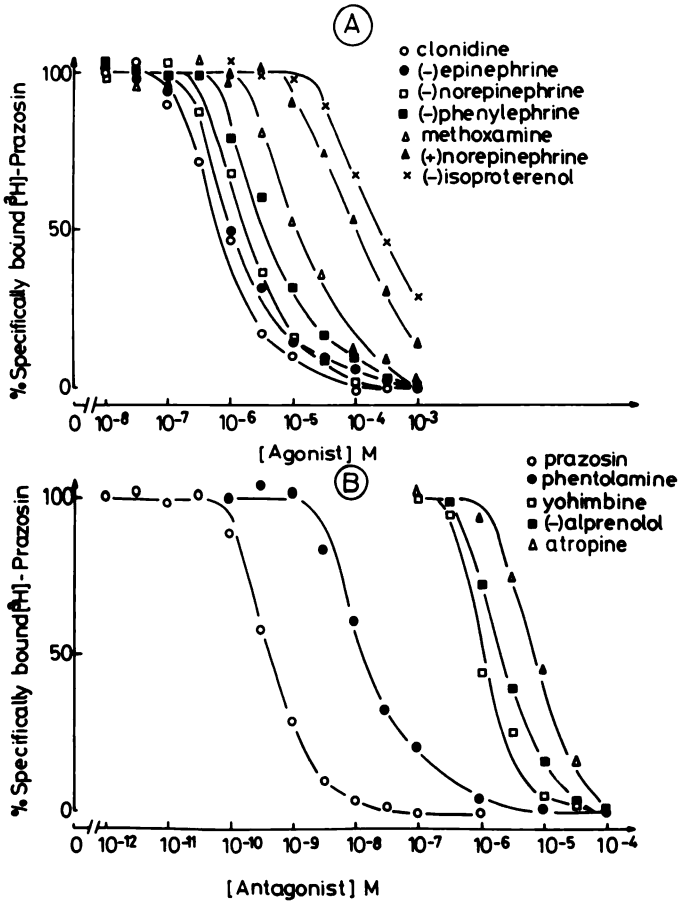

FIG. 2. *Comparison of relative potencies of various drugs competing with [^{3}H]prazosin binding sites*

The [^{3}H]Prazosin concentrations were 0.26 nM and 0.18 nM in A and B, respectively. The K_D values for the different agonists (nanomolar) were as follows: clonidine, 175; (−)-epinephrine, 278; (−)-norepinephrine, 494; (−)-phenylephrine, 1,106; methoxamine, 3,120; (+)-norepinephrine, 27,800; and (−)-isoproterenol, 69,800. The K_D values for the different antagonists (nanomolar) were as follows: prazosin, 0.16; phentolamine, 4.8; yohimbine, 420; (−)-alprenolol, 800; and atropine, 2,530. Particulate fractions were prepared as described under Experimental Procedures.

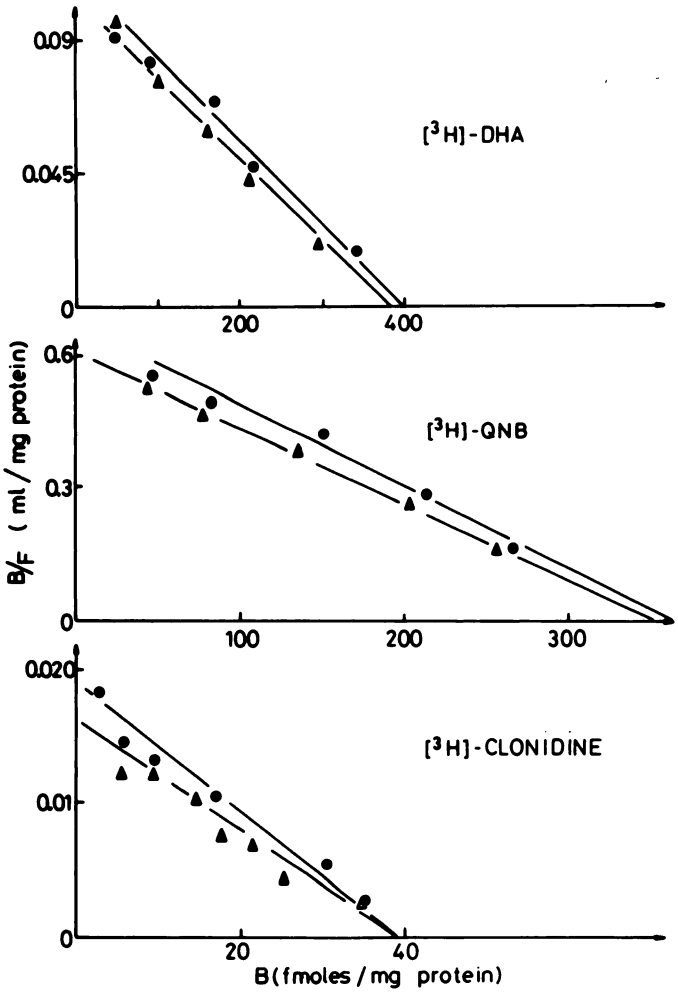

FIG. 3. *Absence of effect of POB (1 mg/kg) on beta-adrenergic, alpha$_2$-adrenergic, and muscarinic receptors in submaxillary glands*
Rats were killed 30 min after an i.v. injection of POB (1 mg/kg) (▲) or vehicle (●).

The logarithmic transformation of Eq. 1 gives

$$\log \frac{[R_{ss}]}{[R_{ss}] - [R_t]} = kt$$

The entire time course of reappearance was studied and is described in Fig. 4A. The logarithmic transformation of this time course gave a straight line (Fig. 4B), indicating that Eq. 1 correctly described the repopulation kinetics as a monoexponential process. From these data it is possible to calculate the rate constant for receptor degradation (k = 0.02 hr^{-1}) and the receptor production rate (r = 1.86 fmoles/mg of protein per hour). The half-life of the receptor was 33 hr.

Influence of protein synthesis on the alpha$_1$-receptor reappearance. Although we have previously shown (14) that after blockade of *alpha*$_1$-adrenergic receptors with POB in BC_3H_1 cells their reappearance was dependent on protein synthesis, it was important to verify this point in intact animals. However, it is well known that blockade of protein synthesis in intact animals is a difficult task. Cycloheximide doses (4 mg/kg) which completely blocked protein synthesis in submaxillary glands led to a 90% mortality rate within the following 48 hr. Therefore we decided to use a protocol leading to a partial blockade of protein synthesis. It consisted of injecting cycloheximide (3 mg/kg) at the beginning of the experiment followed by a second injection 24 hr later. All of the rats were still alive 48 hr after the beginning of treatment. The pattern of protein synthesis inhibition is given in Fig. 5. During the 48-hr period the average protein synthesis was about 30% of the control. We have also verified that POB (1 mg/kg) did not modify the protein synthesis.

As shown in Fig. 6 this inhibition of protein synthesis reduced by 61% the degree of *alpha*$_1$-receptor reappearance following POB treatment. In contrast, inhibition of protein synthesis alone did not significantly modify the concentration of *alpha*$_1$-adrenergic receptors as compared with control rats.

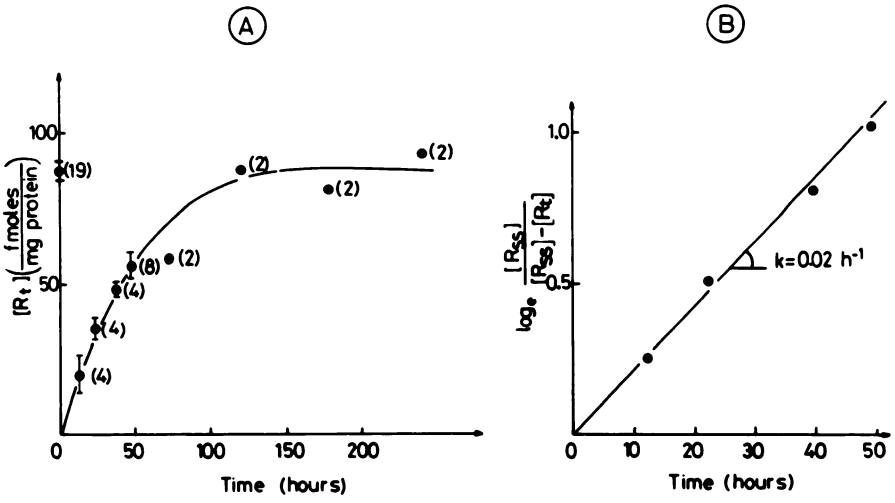

FIG. 4. *Time course of alpha$_1$-adrenergic receptor reappearance in submaxillary glands after blockade with POB*
A. Maximal [^{3}H]prazosin specific binding (R_t) was determined by Scatchard analysis at different times after i.v. injection of POB. The number of experiments is indicated in parentheses. Values are means ± standard error of the mean.
B. Semilogarithmic plot of the time course of *alpha*$_1$-adrenergic receptor reappearance. [R_{ss}] is the steady-state receptor concentration (87 ± 4 fmoles/mg of protein). The slope of this line is equal to the rate constant for degradation of the receptor (k).

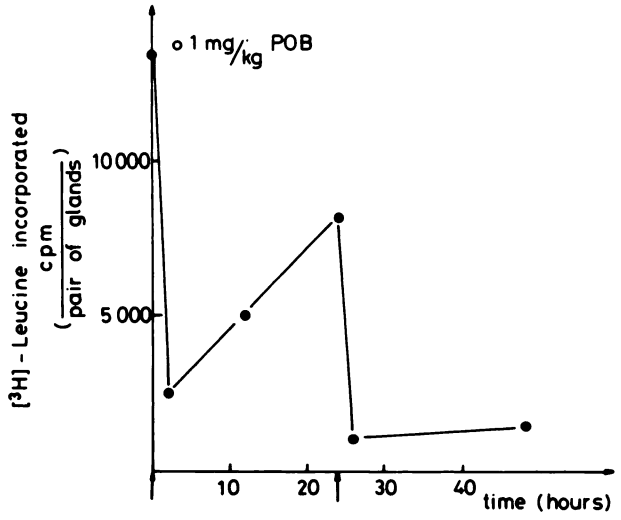

FIG. 5. *Effect of cycloheximide and POB on protein synthesis in submaxillary glands*

Rats were treated with cycloheximide (3 mg/kg) at 0 time and 24 hr later (*arrows*). Protein synthesis was assessed by incorporation of [^{3}H]leucine as described under Experimental Procedures (●). Protein synthesis was also assessed 1 hr following i.v. injection of POB (1 mg/kg) (○).

Effect of high doses of POB on alpha$_1$-receptor reappearance. When rats were treated with POB doses which were 4–20 times higher than those giving complete *alpha$_1$*-receptor blockade, the receptor reappearance was reduced (Fig. 7). This reduction, apparent with doses of 4 mg/kg and higher, was not present at 2 mg/kg (data not shown). Note that, whatever the dose of POB used, the K_D for [^{3}H]prazosin was unaffected.

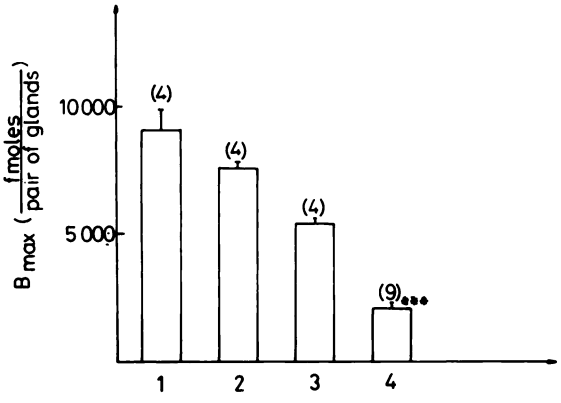

FIG. 6. *Blockade of alpha$_1$-adrenergic receptor reappearance by cycloheximide in submaxillary glands*

Maximal [^{3}H]prazosin specific binding was determined by Scatchard analysis 48 hr after the beginning of treatments, which were as follows: *1*, vehicle injection at 0 time; *2*, cycloheximide (3 mg/kg) injections at 0 time and 24 hr later; *3*, POB injection (1 mg/kg) at 0 time; *4*, POB (1 mg/kg) and cycloheximide (3 mg/kg) injections at 0 time followed by a second cycloheximide injection 24 hr later. The number of experiments is indicated in parentheses. Groups 3 and 4 were statistically different (but not Groups 1 and 2) when compared by Student's *t*-test. ***$p < 0.001$.

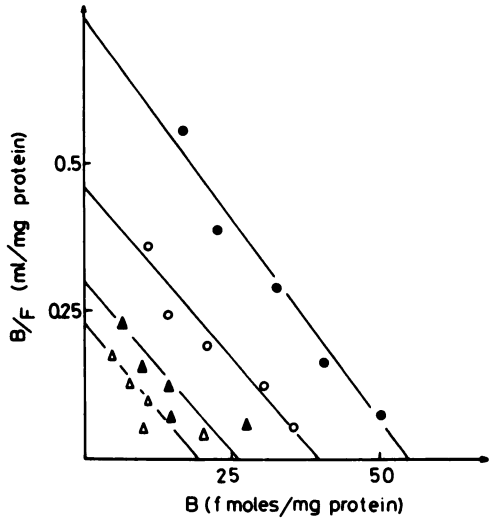

FIG. 7. *Effects of high doses of phenoxybenzamine on alpha$_1$-adrenergic receptor reappearance*

Rats received injections of POB, 1 mg/kg (●), 4 mg/kg (○), 10 mg/kg (▲), or 20 mg/kg (△); [^{3}H]prazosin binding was measured 48 hr later.

DISCUSSION

Two main methods have been used to study the turnover of receptors. The first consists of labeling newly synthetized receptors with either radioactive or heavy amino acids or sugars (21–23). The second method consists of irreversibly blocking receptors and measuring their reappearance (9, 11–14). The second method is more indirect but is also the only one which can be used *in vivo*. The main conceptual problem of this approach is the possibility that the blockade of the receptor might influence the rate of receptor synthesis or degradation. This pitfall was not observed for extrajunctional nicotinic receptors, as shown by studies using the two methods described above (9). However, such a demonstration remains to be done for other receptors, including *alpha$_1$*-adrenergic receptors. Furthermore, with the blocking method it is necessary to demonstrate that the product used is really irreversibly bound during the entire period of receptor reappearance. This is generally done by blocking protein synthesis with cycloheximide or other inhibitors (9). Such inhibitors are very difficult to use *in vivo*. However, we have found an injection protocol which reduces incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable proteins by 70% during a 48-hr period and also decreases by 61% the receptor reappearance (Fig. 6). This strongly suggests that receptors which reappear are newly synthetized. It was surprising that in submaxillary glands of control rats treated with cycloheximide alone, the number of *alpha$_1$*-adrenergic receptors did not decrease as compared with the control. This could be due either to the presence of precursor receptors in the cell which are incorporated even in the presence of cycloheximide, as seen for nicotinic receptors (24), or to the necessity for receptor degradation of a protein having a short half-life, as suggested by studies of insulin receptor metabolism (25).

When a low dose of POB (1 mg/kg) was injected into rats, a complete blockade of *alpha*$_1$-adrenergic receptors was observed in submaxillary glands. Partial blockade occurred with lower doses without any change in the dissociation constant of [^{3}H]prazosin. In contrast, with an alkylating derivative of alprenolol recently used to study the turnover of *beta*-adrenergic receptors *in vivo* (13), which does not cross the blood-brain barrier, phenoxybenzamine blocked both peripheral and central *alpha*$_1$-adrenergic receptors (Fig. 1).

Following *alpha*$_1$-receptor blockade, the time course of receptor reappearance followed monoexponential kinetics in submaxillary glands. This suggests (a) that the reappearance is dependent only on the production rate of receptor, which is a constant, and on the degradation of receptors, which is proportional to the receptor concentration in the cell; and (b) that, besides synthesis and degradation, it does not seem to have additional phenomena affecting the receptor reappearance. Such a simple model has been shown to describe correctly acetylcholine and insulin receptor turnover (9, 23).

The half-life of *alpha*$_1$-adrenergic receptors in submaxillary glands was very close to that of *alpha*$_1$-adrenergic receptors in BC_3H_1 cells (14) (33 and 23 hr, respectively) and close to that found for extrasynaptic nicotinic receptors (9). The difference between the half-life *in vivo* and that *in vitro* was due both to a lower rate constant for degradation (0.02 and 0.03 hr^{-1}, respectively) and to a lower rate of receptor synthesis (1.86 and 3.2 fmoles/mg of protein per hour). These similarities in the turnover rate measured in intact animals and BC_3H_1 cells in culture indicate that these cells could be used as a good model with which to study the modulation of receptor metabolism under different hormonal or pharmacological situations.

Although a dose of POB 2-fold higher (2 mg/kg) than the dose used to block 100% of receptors (1 mg/kg) was without consequence on receptor reappearance (Fig. 4), a 10-fold higher POB dose (10 mg/kg) reduced the reappearance by 50% (Fig. 7). Furthermore, preliminary experiments indicate that, in this condition, the kinetics of reappearance does not follow a simple monoexponential process. In cell culture a 10-fold increase in the POB concentration needed to block completely the *alpha*$_1$-adrenergic receptors was without any significant effect (14). Thus far we can only speculate on the nature of this phenomenon in intact animals. At least two hypotheses can be proposed. It is possible that, when administered at high doses, POB is trapped in the cell membrane or adipose tissue and is slowly liberated, thereby blocking newly appearing receptors. Another possible explanation is that high doses of POB enhance the norepinephrine content of the synapse, leading to a down-regulation of the newly synthetized receptors. An increase in norepinephrine liberation induced by high doses of phenoxybenzamine could be the result of an observed blockade of *alpha*$_2$-adrenergic receptors (data not shown). For those reasons, one should be careful in interpreting results on receptor reappearance obtained with high doses of POB. Recently McKerman and Campbell (26) have followed the *alpha*$_1$-receptor reappearance in brain after injection of POB (8 mg/kg). They found that the receptor recovery occurred with a half-life of approximately 5–6 days. Considerable differences have also been reported in half-lives of *beta*-adrenergic receptors in rat heart, rat lung *in vivo* (12, 13), and human lung VA_2 cells *in vitro* (11). It is important to determine whether the difference in the half-life of adrenoreceptors between these different tissues is due to a fundamental difference of receptor metabolism or to a problem induced by the doses used to block these receptors.

ACKNOWLEDGMENTS

We thank Dr. Mathieu and Miss Euvrard, of Roussel-Uclaf, for a grant given to F. Sladeczek, and Mrs. du Parc for preparation of the manuscript.

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Send reprint requests to: Fritz Sladeczek, Centre CNRS-INSERM de Pharmacologie-Endocrinologie, B.P. 5055, 34033 Montpellier Cedex, France.