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A Biochemical Study of Receptor Internalization during β-Adrenergic Receptor Desensitization in Frog Erythrocytes

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SUMMARY

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We have previously reported that in frog erythrocytes, a decrease in the number of β-adrenergic receptor recognition sites during subsensitivity of these receptors is associated with a translocation to the cytosol of β-adrenergic receptor recognition sites located on the red cell membrane. These results suggest that the recognition sites of β-adrenergic receptors located in the plasma membrane internalize following a persistent receptor stimulation. Experiments using cells incubated with (±)-[^{3}H]hydroxybenzylisoproterenol (HBI), a potent β-agonist, suggest that the agonist is bound to the internalized recognition sites of β-adrenergic receptors. The increase in the number of receptors found in cytosol and the decrease of those found in the particulate fraction were inhibited by lowering the preincubation temperature. The binding capacity of these internalized sites was blocked by Pronase but was resistant to phospholipase treatment. Dinitrophenol and cordycepin inhibited the agonist-elicited internalization of β-adrenergic receptors, suggesting an ATP-requiring metabolic process (protein phosphorylation?) may participate in causing this event. Preincubation of cells with doses of concanavalin A which induce erythrocyte agglutination was found to inhibit the internalization of the receptors induced by the agonist. Pretreatment with methylamine in millimolar ranges also reduced the extent of β-receptor internalization. These results strongly suggest that the internalization of β-adrenergic receptor recognition sites may be triggered by receptor clustering mediated by transglutaminase. The catalytic subunits of adenylate cyclase were not found to be internalized under conditions in which we found maximal internalization of β-adrenergic receptor recognition sites. Although guanyl-5′-yl imidodiphosphate (Gpp(NH)p) and GTP affected the binding of (−)-isoproterenol to β-adrenergic receptors in the particulate fraction, these guanine nucleotides failed to modify agonist binding to recognition sites of soluble internalized β-receptors.

INTRODUCTION

In a cell, the magnitude of the response elicited by a given agonist is proportional to the number of specific recognition sites bound to the membrane (1). In isolated frog erythrocytes during β-adrenergic receptor subsensitivity induced by the agonist, the number of β-receptor recognition sites bound to the surface membrane of the cell decreases while the affinity of these sites remains unchanged (2, 3). Such a loss of specific binding capacity is not due to a persistent receptor occupancy by the agonist (4). A certain binding capacity for β-adrenergic receptor ligands is also present in the cytosol of erythrocytes; the number of β-adrenergic receptor recognition sites present in this compartment of frog erythrocyte increases during receptor desensitization induced by (−)-isoproterenol (5, 6). The time constant of this increase is the mirror image of the decrease of the number of β-adrenergic receptor recognition sites bound to the erythrocyte membranes (5). The potency of various β-adrenergic agonists in changing the binding capacities of erythrocyte cytosol and membrane is also similar (5). These results suggest that the membrane-bound recognition sites of β-adrenergic receptors are internalized following their persistent stimulation, an event analogous to what has been described for many polypeptide hormones in various tissues (7–11).

MATERIALS AND METHODS

Chemicals. All the following chemicals were obtained from New England Nuclear: *levo*-[propyl-1,2,3,-^{3}H]dihydroalprenolol hydrochloride (47.4 Ci/mmol); *p*-[7-^{3}H]hydroxybenzylisoproterenol, (13.2 Ci/mmol); [2,8-^{3}H]aden-

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osine 5′-triphosphate, tetrasodium (32.5 Ci/mmol); and unlabeled (±)-hydroxybenzylisoproterenol. L-Isoproterenol-D-bitartrate, L-alprenolol-D-tartrate, 2-4-dinitrophenol, methylamine, phospholipase A_2 (from bee venom, 1470 units/mg protein), concanavalin A (from jack beans), and cordycepin (from *Cordyceps militaris*) are products of Sigma Chemical Company. Phosphoenolpyruvate, pyruvate kinase (from rabbit muscle, 10 mg/ml), Gpp(NH)p, Pronase (6 units/mg), ribonuclease (from bovine pancreas, 40 units/mg), and deoxyribonuclease I (from bovine pancreas, 2000 units/mg) were purchased from Boehringer-Mannheim Biochemicals. L- and D-propranolol hydrochloride were supplied by Ayerst Laboratory Inc. Phentolamine (Regitine mesylate) was a gift of Ciba-Geigy Corporation.

Preparation of supernatant and particulate fractions from bullfrog erythrocytes. Erythrocytes were isolated from the blood of bullfrogs (*Rana catesbeiana*) using a procedure described previously (5). The final preparation of cells was resuspended in a medium containing 20 mM potassium phosphate, pH 7.4, 85 mM NaCl, 2 mM KCl, 8 mM theophylline, and 1 mM $CaCl_2$ (medium A).[1] In our standard procedure, erythrocytes (20% hematocrit) in 2 ml medium A were incubated in a water bath at 30°C for 2 h in the absence or presence of (−)-isoproterenol (5 μM). Cells were then washed in 5 ml of medium A by centrifugation at 700*g* for 2 min at 4°C. The cell pellets were further washed an additional eight times. The final cell pellet was either lysed immediately or frozen and then lysed in 1.5 ml of buffer containing 25 mM Tris–HCl, pH 7.4, and 1 mM $MgCl_2$ (medium B). The lysate was centrifuged at 30,000*g* for 15 min and the supernatant was further clarified by centrifugation at the same speed. The final supernatant is termed cytosol and used as the source of soluble β-adrenergic receptors. The initial 30,000*g* pellet of erythrocyte lysate was homogenized in 5 ml of medium B followed by centrifugation at 30,000*g*. The pellet was resuspended by grinding in 1.0 ml of medium B and was used as source of erythrocyte plasma membrane.

Binding of [^{3}H]dihydroalprenolol [DHA] to the cytosol and membrane of bullfrog erythrocytes. Binding of [^{3}H]DHA to both fractions was carried out as described previously (5) with little modification. In brief, the binding of [^{3}H]DHA to the cytosol was performed in a total volume of 450 μl of medium B containing 15–20 mg of the cytosol protein and 20 nM (−)-[^{3}H]DHA, (the K_d for this binding is about 8 nM). After incubation at 30°C for 10 min, the binding mixture was loaded onto a Sephadex G-50 (superfine) column and immediately eluted with medium B. The fraction eluted in the void volume of the column was collected, deproteinized with acid, and then counted for ^{3}H radioactivity. The binding of (−)-[^{3}H]DHA to the membrane fraction was carried out in a reaction mixture of 150 μl of medium B containing 0.5–1.0 mg of membrane particulate protein and 10 nM (−)-[^{3}H]DHA (the K_d for this binding is about 2 nM). After incubation at 30°C for 10 min, the reaction was terminated by addition of ice-cold medium B and the mixtures were filtered immediately under reduced pressure through a Whatman GF/B filter. The filter was further washed with an appropriate volume of medium B and then counted for radioactivity. Nonspecific bindings of (−)-[^{3}H]DHA to the supernatant and particulate fractions were determined in the presence of 5 μM unlabeled (−)-alprenolol and subtracted from the total binding obtained in its absence. The specific activities of [^{3}H]-DHA bound to the cytosol and particulate fractions were about 25 and 30 cpm/fmol, respectively.

Assays of adenylate cyclase activity in the cytosol and membrane fractions of bullfrog erythrocytes. Adenylate cyclase activity was measured in a reaction mixture as described by Salomon *et al.* (12) except that [γ-^{32}P]ATP was replaced by [2,8-^{3}H]ATP and the cAMP added was unlabeled. The amounts of cytosol and membrane protein were 10–15 mg and 400–600 μg, respectively. After incubation, 200 μl of H_2O was added to the reaction mixture which was then heated immediately at 98°C for 2 min. The denatured proteins were removed by centrifugation and the supernatant was loaded onto a Dowex 50 AG × 4 column which was then eluted as described previously (12). The fraction containing most of the [^{3}H]cAMP formed was treated with $ZnSO_4$/$Ba(OH)_2$ as described by Krishna *et al.* (13) to remove the contaminating nucleotides before it was counted for radioactivity. The recovery of cAMP using this procedure was about 60–65%. Values obtained at zero time incubation were considered as the blank and subtracted.

Protein determination. Protein was determined by the method of Lowry *et al.* (14) using bovine serum albumin as the standard.

RESULTS

Internalization of β-adrenergic receptors during receptor desensitization and resensitization. The experiment in Table 1 confirmed our previous observation that

TABLE 1

Effects of various adrenergic agonists and antagonists on the [^{3}H]DHA binding in the erythrocyte cytosol and membrane derived from cells preincubated with (−)-isoproterenol

Erythrocytes (25% hematocrit) were preincubated at 30°C for 10 min in 1.6 ml of medium A in the absence or presence of the indicated adrenergic agent (10 μM). Medium A or (−)-isoproterenol (25 μM), in 0.4 ml, was added and the mixture was further incubated for 2 h at 30°C. The cytosol and membrane fractions were then prepared from the cells and the binding of [^{3}H]DHA to these fractions was measured as described under Materials and Methods. Values are mean ± SEM of four determinations.

Preincubation condition		[^{3}H]DHA bound (fmol/ml cell)	
First	Second	Cytosol	Membranes
Buffer	Buffer	103 ± 10	5039 ± 193
Buffer	(−)-Isoproterenol	456 ± 18	3552 ± 210
(−)-Propranolol	(−)-Isoproterenol	121 ± 9	5001 ± 285
(+)-Propranolol	(−)-Isoproterenol	467 ± 21	3577 ± 172
Phentolamine	(−)-Isoproterenol	433 ± 18	3790 ± 169
Buffer	(−)-Propranolol	96 ± 11	4894 ± 226

[1] Abbreviations used: medium A, 20 mM potassium phosphate, pH 7.4, 85 mM NaCl, 2 mM KCl, 8 mM theophylline, and 1 mM $CaCl_2$; medium B, 25 mM Tris–HCl, pH 7.4, and 1 mM $MgCl_2$; DHA, dihydroalprenolol; HBI, hydroxybenzylisoproterenol.

preincubation of frog erythrocytes with (−)-isoproterenol results in the redistribution of the recognition sites for β-adrenergic receptors from the membrane to the cytosol (5); this redistribution was termed internalization (5). This internalization was agonist specific and was prevented by the presence of (−)-propranolol, a specific blocker of β-adrenergic receptors. Neither (+)-propranolol, the inactive stereoisomer, nor phentolamine, an α-adrenergic blocker, was active in blocking the redistribution of β-adrenergic receptor recognition sites elicited by (−)-isoproterenol.

As we have reported previously (5), the number of β-adrenergic agonist recognition sites recovered in the cytosol fraction could account for 25–30% of the number of sites lost from the membrane preparation. Repeated exposure of erythrocytes to (−)-isoproterenol at 4 and 8 h after the initial receptor stimulation failed to induce an additional increase in the number of β-adrenergic receptor recognition sites present in the cytosol or a further decrease in the number of membrane-bound recognition sites (data not shown).

In the experiment described in Fig. 1 frog erythrocytes were incubated with labeled (±)-hydroxybenzylisoproterenol, ((±)-[^{3}H]HBI), a potent agonist for β-adrenergic receptors. After removing the unbound agonist by repeated washings, the label bound to high-molecular-weight material present in the cytosol was determined by gel filtration. An aliquot of the radioactivity was recovered in the void volume of a Sephadex G-50 column. This aliquot of radioactivity was greatly reduced when the cells were incubated in the presence of an excess of unlabeled (±)-HBI or (−)-isoproterenol; however, the stereoisomer, (+)-isoproterenol, failed to cause such a reduction. When the erythrocytes were incubated with [^{3}H]DHA, little or no radioactivity was found to be associated with the material appearing in the void volume.

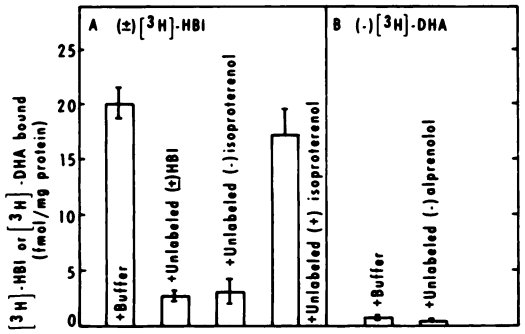

FIG. 1. *The content of ^{3}H bound to macromolecules in the cytosol derived from erythrocytes incubated with (±)-[^{3}H]HBI or (−)-[^{3}H]-DHA*

Cells (40% hematocrit) were incubated at 30°C for 1 h in 1 ml of medium A containing 10^{-4} M catechol and 5×10^{-7} M of (±)-[^{3}H]HBI (A) or (−)-[^{3}H]DHA (B). When used, the concentration of unlabeled ligand was 10^{-4} M. After incubation, cells were washed eight times in 5 ml of medium A by centrifugation at 700*g*. The washed cells were then lysed in 2 ml of medium B and the lysate was centrifuged at 30,000*g* for 20 min. An aliquot of 350 μl of the supernatant was loaded onto a Sephadex G-50 superfine column and the ^{3}H bound to material eluated in the void volume was determined as described under Materials and Methods. Values are mean ± SEM for three independent determinations.

In an attempt to study the time constant of the redistribution of β-adrenergic receptor recognition sites elicited by isoproterenol, we incubated erythrocytes for 2 h with (−)-isoproterenol and after an extensive washing the erythrocytes were reincubated in a (−)-isoproterenol-free medium. The number of β-receptor recognition sites in the erythrocytes' membrane and cytosol was measured at 14, 18, 22, 38, and 45 h after the second incubation (Fig. 2). The cytosol content of β-adrenergic receptor recognition sites declined with a half-life of about 40 h. At this time, the number of membrane-bound β-adrenergic receptors was restored to about 80% of the value found in membrane preparations from erythrocytes that were not incubated with (−)-isoproterenol.

Characteristics of the redistribution of β-adrenergic receptor recognition sites elicited by (−)-isoproterenol. The internalization of β-adrenergic receptor recognition sites elicited by the incubation of erythrocytes with (−)-isoproterenol is a temperature-dependent process (Fig. 3). Little or no increase in the number of soluble recognition sites was found when the preincubation with (−)-isoproterenol was performed at 0°C. The increase in the number of cytosol recognition sites and the decrease of the sites bound to erythrocyte membranes were significant when these cells were incubated at 14°C. Both changes were greater when the incubation temperature was raised to 30°C. Since the binding of (−)-isoproterenol to its recognition sites is facilitated at low temperature (15, 16), our results suggest that the redistribution of the

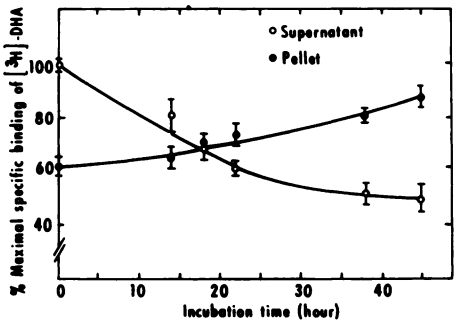

FIG. 2. *Time course of the changes in the [^{3}H]DHA binding sites in the supernatant and pellet fractions derived from cells preincubated with isoproterenol followed by incubation in an isoproterenol-free medium*

Cells (20% hematocrit) were preincubated in 2 ml of medium A for 2 h at 30°C in the presence of 5 μM (−)-isoproterenol and then washed in 5 ml of medium A by centrifugation at 700*g* for 2 min. The washing was repeated six additional times and then resuspended in 2 ml of medium A containing 100 units/ml and 100 μg/ml of penicillin and streptomycin, respectively. The cell suspensions were further incubated at 30°C in the dark for the time periods indicated in the figure. After incubations cells were washed two times by centrifugation in 5 ml medium A and then lysed in 1.5 ml of medium B. Supernatant and pellet fractions were prepared from the lysates and the binding of [^{3}H]DHA to these fractions was measured as described under Materials and Methods. ○, Binding of [^{3}H]DHA to the supernatant fraction. ●, Binding of [^{3}H]DHA to the pellet fraction. Values are mean ± SEM for four experiments. The maximal (100%) binding to the supernatant was 2.04 fmol/mg protein. The maximal (100%) specific binding to the pellets was 193 fmol/mg protein which was obtained using pellet fractions derived from cells treated in parallel but were preincubated without (−)-isoproterenol. This value did not change significantly throughout the subsequent incubation periods.

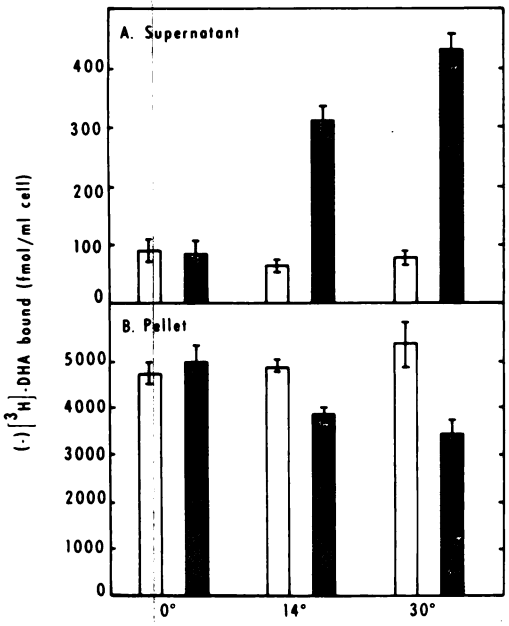

FIG. 3. *Effects of the preincubation temperature on the number of β-adrenergic receptors in the supernatant and pellet fractions derived from control and isoproterenol-treated cells*

Cells (20% hematocrit) were preincubated in 2 ml of medium A for 1 h at the temperature indicated, in the absence or presence of 5 μM (−)-isoproterenol followed by extensive washing to remove the free agonist. The supernatant and pellet fractions were then prepared and the binding of [^{3}H]DHA to these fractions was determined. Details of these experimental conditions are described under Materials and Methods. □, Results obtained with samples derived from untreated cells. ■, Results obtained with samples derived from isoproterenol-treated cells. Values are mean ± SEM for four determinations.

recognition sites triggered by (−)-isoproterenol depends on some metabolic event that is temperature sensitive. The data in Table 2 show that incubation of red blood cells with 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, lowered the contents of soluble β-adrenergic receptor recognition sites in normal and isoproterenol-treated erythrocytes. Such an inhibition was dose dependent; at 1 mM the inhibition was reduced by 60 and 80% in normal and treated cells, respectively. In erythrocytes treated with cordycepin (17, 18) the number of soluble β-receptor recognition sites was reduced (Table 2); this inhibitor of ATP-dependent protein phosphorylation caused a 50% inhibition at a 1 mM concentration, Scatchard analysis revealed that the effects of dinitrophenol and cordycepin were due to a decrease in the number of soluble β-adrenergic receptors with no change in the binding affinity for [^{3}H]DHA (data not shown).

Concanavalin A, a plant lectin that binds to membrane glycoproteins, reduced the content of soluble β-adrenergic receptors in the normal and isoproterenol-treated cells in a dose-dependent manner (Table 3). Such a decrease was concomitant with an increase in the number of β-adrenergic recognition sites in membranes of erythrocytes treated with (−)-isoproterenol. At 200 μg/ml of concanavalin A, the number of membrane-bound β-receptors in isoproterenol-treated cells was virtually restored to the control value. Methylamine inhibits the clustering and subsequent internalization of insulin, epidermal growth factor, and α_2-macroglobulin bound to their specific surface receptors (19, 20). Methylamine in the range of millimolar concentration inhibited the redistribution of β-adrenergic recognition sites triggered by (−)-isoproterenol (Table 3). However, unlike the effect of concanavalin A, methylamine failed to completely antagonize the decrease of recognition sites for β-adrenergic receptors. It should be mentioned that neither concanavalin A nor methylamine added directly to the binding mixtures in concentrations up to 200 μg/ml and 4 mM, respectively, affects the binding of [^{3}H]DHA and (−)-isoproterenol to membrane and cytosol preparations of frog erythrocytes (data not shown).

In the experiment described in Table 4, we examined

TABLE 2

Effects of preincubation of cells with dinitrophenol and cordycepin on the contents of soluble β-adrenergic receptors in control and isoproterenol-treated cells

Erythrocytes (25% hematocrit) were preincubated at 30°C for 30 min in 1.6 ml of medium A in the absence or presence of dinitrophenol or cordycepin. Medium A or (−)-isoproterenol (25 μM), in 0.4 ml, was added to the mixtures which were further incubated at 30°C for 2 h. Supernatant fractions were prepared from the control and isoproterenol-treated cells and the binding of [^{3}H]DHA to these fractions was determined as described under Materials and Methods.

Preincubation condition	[^{3}H]DHA bound [fmol/mg protein (%)]	
	Control cells	Treated cells
Buffer	0.51 (100)	2.05 (100)
Dinitrophenol (mM)		
0.2	0.32 (63)	1.20 (59)
0.5	0.24 (47)	0.50 (24)
1.0	0.21 (41)	0.39 (19)
Cordycepin (mM)		
0.5 mM	0.38 (74)	1.53 (75)
1.0	0.25 (49)	1.18 (57)
2.0	0.24 (47)	1.04 (50)

TABLE 3

Effects of concanavalin A and methylamine on the number of [^{3}H]-DHA binding sites in the supernatant and pellet fractions derived from erythrocytes preincubated with isoproterenol

Cells (25% hematocrit) were preincubated at 30° for 1 h in 1.6 ml of medium A in the absence or presence of the indicated concentration of concanavalin A or methylamine. Medium A or (−)-isoproterenol (25 μM), in 0.4 ml, was added to the cell suspension which was further incubated at 30°C for 2 h. Supernatant and pellet fractions were derived from the cell preparations and the binding of [^{3}H]DHA to these fractions was determined. Details were described under Materials and Methods. Values are mean ± SEM of three determinations.

Preincubation condition		[^{3}H]DHA bound (fmol/mg protein)	
First	Second	Cytosol	Membranes
Buffer	Buffer	0.4 ± 0.03	167 ± 9
Buffer	Isoproterenol	2.54 ± 0.08	114 ± 5
Concanavalin A (μg/ml)			
20	Isoproterenol	2.62 ± 0.11	113 ± 4
100	Isoproterenol	1.43 ± 0.07	124 ± 5
200	Isoproterenol	0.86 ± 0.07	146 ± 7
Methylamine (mM)			
2 mM	Isoproterenol	1.88 ± 0.10	80 ± 5
5	Isoproterenol	0.86 ± 0.09	86 ± 6
10	Isoproterenol	0.92 ± 0.09	102 ± 9

TABLE 4

The activities of adenylate cyclase and [^{3}H]DHA binding in the supernatant and pellet fractions derived from erythrocytes preincubated with or without isoproterenol

Cells (20% hematocrit) were preincubated at 30°C for 2 h in 2.0 ml of medium A in the absence or presence of (−)-isoproterenol (5 μM). These cells were then washed, the supernatant and pellet fractions were prepared, and the activities of adenylate cyclase and the [^{3}H]-DHA binding in these fractions were measured. Details of these procedures are described under Materials and Methods. When used the concentrations of NaF, Gpp(NH)p, and (−)-isoproterenol were 10 mM, 100 μM, and 50 μM, respectively. Values are mean ± SEM of four such experiments.

	Cytosol		Membranes	
	Control cells	Treated cells	Control cells	Treated cells
Adenylate cyclase activity (pmol [^{3}H]-cAMP/mg protein/min)				
Basal	ND[a]	ND	9.8 ± 0.6	9.5 ± 1.1
+ NaF	ND	ND	171 ± 10	174 ± 12
+ Gpp(NH)p	ND	ND	130 ± 7	153 ± 10
+ (−)-Isoproterenol	ND	ND	272 ± 17	138 ± 6
[^{3}H]DHA binding (fmol bound/mg protein)	0.3 ± 0.04	2.1 ± 0.04	242 ± 11	169 ± 9

[a] Not detectable.

TABLE 5

Effects of various hydrolytic enzymes on the [^{3}H]DHA binding activity in the supernatant and pellet fractions derived from erythrocytes incubated with isoproterenol

Supernatant and pellet fractions were prepared from cells preincubated with (−)-isoproterenol and the binding of [^{3}H]DHA to these fractions was then determined as described under Materials and Methods, except that samples were preincubated at 30°C for 10 min in the absence or presence of the indicated concentrations of hydrolytic enzymes prior to the addition of [^{3}H]DHA.

Treatment (μg/ml)	[^{3}H]DHA bound [fmol/mg protein (%)]	
	Cytosol	Membranes
None	2.18 (100)	195 (100)
Pronase		
10	1.49 (68)	131 (67)
20	0.85 (39)	81 (41)
Phospholipase A_2		
10	2.16 (99)	71 (36)
20	2.16 (99)	59 (30)
DNase 40	2.13 (98)	186 (95)
RNase 40	2.03 (93)	197 (101)

whether adenylate cyclase is also redistributed following stimulation of frog erythrocytes with β-adrenergic receptor agonists. Neither the basal nor the NaF or Gpp(NH)p-sensitive adenylate cyclase activities were detected in the cytosol of cells preincubated in the absence or presence of (−)-isoproterenol. Consistent with data that had been reported previously (2), the adenylate cyclase activity in the membrane preparations derived from untreated and agonist-treated cells was similar. During the isoproterenol-induced internalization of β-adrenergic receptors, the activity of isoproterenol-sensitive adenylate cyclase was diminished in the membrane; however, no such activity could be detected in the cytosol (Table 4).

Properties of the internalized β-adrenergic receptor recognition sites. When the cytosol or membrane was treated with various hydrolytic enzymes (Table 5), the membrane capacity to bind [^{3}H]DHA was destroyed by Pronase as well as phospholipase A_2, confirming observations by others (21). However, the [^{3}H]DHA binding activity of the cytosol was sensitive to Pronase but resistant to phospholipase A_2 at the concentrations examined. Neither DNase nor RNase affected the [^{3}H]DHA binding in cytosol or membrane fractions.

The association and dissociation rates of [^{3}H]DHA to the cytosolic recognition sites are shown in Fig. 4. At 20 nM and 30°C, the ligand binding reached a plateau within 30 min (Fig. 4A). Figure 4B shows the dissociation rate of [^{3}H]DHA from the soluble receptors in the presence of an excess of unlabeled (−)-alprenolol after an incubation with [^{3}H]DHA at 30°C for 30 min. The $t_{1/2}$ of the dissociation was about 12 min. The results in Fig. 5 show that the binding of [^{3}H]DHA to the cytosol prepared from cells preincubated with or without (−)-isoproterenol is linear with respect to cytosol protein concentrations up to 20 mg/assay.

It has been reported that the guanine nucleotides (GTP, GDP, and Gpp(NH)p) regulate the binding of β-adrenergic agonists to their receptors (22, 23). We therefore examined whether the internalized β-adrenergic receptors are also regulated by GTP and Gpp(NH)p. The results in Fig. 6A show that the displacement curve of (−)-isoproterenol for the [^{3}H]DHA binding to the cytosol derived from cells preincubated with isoproterenol was unaffected by the presence of Gpp(NH)p (100 μM). Similar results were obtained when GTP was used to replace

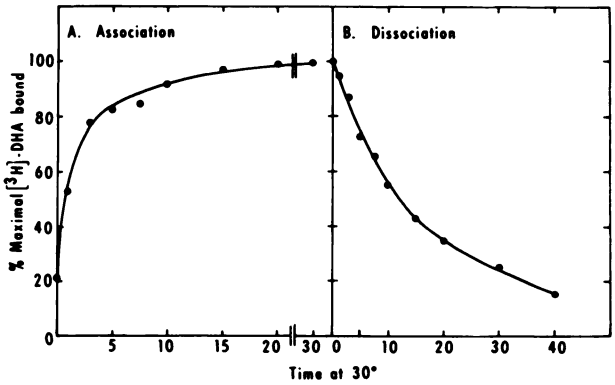

FIG. 4. *The rate of association and dissociation of [^{3}H]DHA binding for the cytosol derived from erythrocytes preincubated with (−)-isoproterenol*

The cytosol fraction was prepared from cells treated with (−)-isoproterenol and aliquots were used to assay for the binding of (−)-[^{3}H]DHA as described under Materials and Methods except that the incubation times were varied as indicated in (A). In (B), [^{3}H]DHA was prebound to the cytosol by incubation at 30°C for 30 min under the standard binding conditions as described under Materials and Methods. Unlabeled (−)-alprenolol in 50 μl was added to the binding mixture to give a final concentration of 5 μM. Zero time was that of the addition of unlabeled ligand. At various times thereafter, samples were assayed for the [^{3}H]DHA bound to the cytosol using a gel filtration column as described under Materials and Methods.

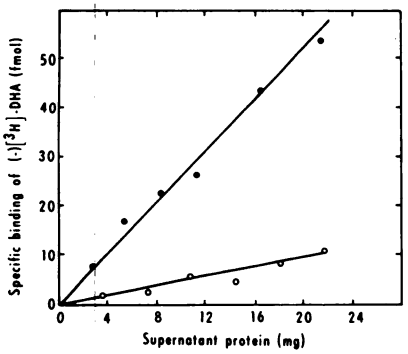

FIG. 5. *Relationship between the amounts of cytosol protein and the contents of [^{3}H]DHA bound to the cytosol*

The cytosol fractions derived from control and isoproterenol-treated cells were used for the assay of the binding of [^{3}H]DHA as described under Materials and Methods except that the amounts of cytosol proteins were varied as indicated. ○, Binding to cytosol derived from untreated cells. ●, Binding to cytosol derived from cells treated with isoproterenol.

Gpp(NH)p. In confirming previous reports (22, 23), we found a shift to the right of the agonist displacement curve for the [^{3}H]DHA binding to the membranes derived from the same cell preparation in the presence of guanine nucleotides (Fig. 6B).

DISCUSSION

In the present report we have extended our previous studies (5) and obtained further evidence that in frog erythrocytes the recognition sites of β-adrenergic receptors located on the membrane are redistributed following the erythrocyte incubation with β-adrenergic receptor agonists. This redistribution of the number of recognition sites present in membranes is termed internalization. Since this internalization is temporarily related to the decrease of the number of membrane-bound β-adrenergic receptors elicited by (−)-isoproterenol, one may surmise that internalization can be relevant to explain receptor subsensitivity. Both events are agonist specific and are prevented by the presence of β-adrenergic antagonists (Table 1). Experiments with erythrocytes incubated with [^{3}H]HBI (Fig. 1) indicate that the agonist bound to the β-receptor recognition sites reaches the cytosol.

The number of recognition sites that can be measured in the cytosol accounts for about 25% of the sites lost from the erythrocytes' membranes. Since we were unable to detect any β-receptor recognition sites in the incubation medium (data not shown), this percentage may represent a minimal value and reflect a dynamic equilibrium between internalization and destruction of internalized receptors. An underestimation of the number of internalized recognition sites could be caused by the occupancy of the sites by (−)-isoproterenol, or by the inactivation of some recognition sites within the membrane bilayers. The time course of the reduction of the number of β-adrenergic receptor recognition sites in cytosol which has increased during internalization is similar to that of the recovery of the membrane-bound recognition sites during resensitization (Fig. 2). These results might be explained by assuming that the internalized recognition sites, at least in part, may be rebound to the plasma membrane, thereby restoring some of the surface recognition sites lost during the desensitization. Other mechanisms may be operative during resensitization, as for instance, a new synthesis of receptor proteins (24).

We have also investigated the mechanisms whereby β-adrenergic receptor recognition sites are internalized during desensitization. The internalization is temperature and energy dependent and is inhibited by cordycepin, concanavalin A, and methylamine. It is of interest to note that the internalization of insulin and epidermal growth factor bound to their specific receptors, as visualized with fluorescence microscopy, involves an initial clustering of receptors around coated pits on the plasma membrane; this step is temperature sensitive and is prevented by methylamine presumably through an inhibition of the transglutaminase activity (7, 19, 20). Subsequently, the clustered receptors are invaginated into the cytoplasm and bound to subcellular organelles; this event appears to require metabolic energy (7). Thus the inhibition of internalization by methylamine suggests that transglutaminase activity and receptor clustering are involved in the internalization of β-adrenergic receptor recognition sites. However, it should be stressed that the internalized β-receptor recognition sites do not seem to be bound to subcellular structures because 80% of the [^{3}H]DHA binding activity remains in the supernatant following centrifugation at 100,000*g* up to 4 h. The 20% binding activity that is lost during high-speed centrifugation probably is bound to small fragments of plasma membrane or to other intracellular structures. In frog erythrocytes this loss is minimized because the number

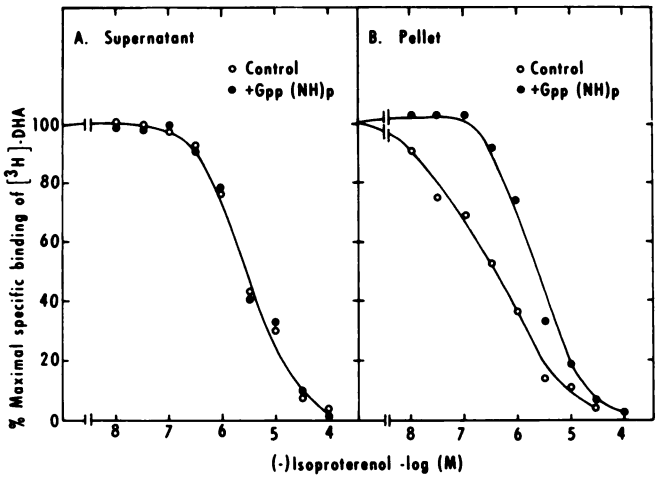

FIG. 6. *Effects of Gpp(NH)p on the binding of* (−)*-isoproterenol to the soluble and membrane-bound β-adrenergic receptors*

Supernatant and pellet fractions were derived from erythrocyte lysates of cells treated with isoproterenol as described under Materials and Methods. The binding of [^{3}H]DHA to the supernatant and pellet fractions was then performed in the absence or presence of increasing concentrations of (−)-isoproterenol. When used, the concentration of Gpp(NH)p was 100 μM. Details of the binding of [^{3}H]DHA to the cytosol and particulate fractions are described under Materials and Methods. The maximal (100%) [^{3}H]DHA binding to the soluble fraction was 2.55 and 2.43 fmol/mg protein in the absence and presence of Gpp(NH)p, respectively. The maximal [^{3}H]DHA binding to the pellet fraction was 139 and 151 fmol/mg protein in the absence and presence of Gpp(NH)p, respectively.

of intracellular organelles is smaller than in other cells.

Cordycepin has been shown to inhibit cyclic nucleotide-dependent and independent protein phosphorylation through competition with ATP, the PO_4^{-3} donor (17, 18). We have found that this drug at relatively high concentration (2 mM) diminished by about 50% the extent of the recognition site internalization during receptor subsensitivity elicited by (−)-isoproterenol (Table 2). Moreover, we have previously demonstrated that the incorporation of labeled PO_4^{-3} into membrane proteins of 60,000 and 38,000 daltons is selectively increased when maximal desensitization is operative (24). These results are in line with the hypothesis that the phosphorylation of membrane protein participates in causing the internalization of β-receptor recognition sites during receptor subsensitivity elicited by prolonged exposure to (−)-isoproterenol. Our results, however, leave open the possibility that other ATP-requiring processes also participate in causing receptor internalization. Recently, Hirata *et al.* (25) have demonstrated that stimulation of β-adrenergic receptor in turkey erythrocyte membranes results in an activation of phospholipid methylation and a concomitant increase of membrane fluidity. We have not yet studied the relationship between the increase in membrane fluidity and the internalization of β-receptor recognition sites in frog erythrocytes. The experiment using concanavalin suggests the participation of membrane glycoproteins in eliciting the internalization of β-adrenergic receptor recognition sites (Table 3). The specificity of this finding is supported by results that drugs affecting microtubular protein assembly (such as colchicine and vinblastine) or inhibiting microfilament function (such as cytochalasin B) failed to inhibit the internalization of receptor recognition sites during desensitization of β-adrenergic receptors (data not shown).

Not all the receptor proteins appear to translocate from the plasma membrane during β-adrenergic receptor subsensitivity in frog erythrocytes. The catalytic subunits of adenylate cyclase are not redistributed from the membrane into the cytosol (Table 4). In fact, we found that the cytosolic protein content of untreated or isoproterenol-treated erythrocytes is virtually identical. This finding has provided further credence for the idea that the translocation of the recognition sites during receptor subsensitivity is not due to an artifactual release of some small plasma membrane vesicles during cell lysis. Such an artifact can be excluded also by showing that phospholipase affects only the binding of [^{3}H]DHA to the membrane but not to the recognition sites present in cytosol (Table 5). Moreover, guanine nucleotides regulate the agonist binding to membrane but fail to affect the agonist binding to the cytosol (Fig. 6).

It should be stressed that the molecular mechanisms participating in the desensitization of β-adrenergic receptors are very complex and differ in various tissues. Although a decrease in receptor density is associated with the desensitization of β-receptors in other systems such as S49 mouse lymphoma cells (26) and human astrocytoma cells (27), the number of β-adrenergic receptors is unchanged while the synthesis of a protein inhibitor and the production of cyclic AMP is required to elicit the subsensitivity of β-receptors in C6–2B astrocytoma cells (28). In other tissues, subsensitivity appears related to a disruption of the coupling between β-adrenergic receptors and adenylate cyclase (29, 30). Hence internalization may not be a general mechanism for the subsensitivity of β-adrenergic receptors in every tissue. Indeed, our preliminary results show that during subsensitivity of C6 glioma cells induced by exposure to (−)-isoproterenol, the number of β-receptor recognition sites in the membrane or in cytosol fails to change. Further experiments are now in progress to determine whether β-adrenergic receptor internalization can be demonstrated in cells other than those of frog erythrocytes.

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