In Vitro Desensitization of Beta Adrenergic Receptors in Human Neutrophils

ATTENUATION BY CORTICOSTEROIDS

ALBERT O. DAVIES and ROBERT J. LEFKOWITZ, *Howard Hughes Medical Institute, Departments of Medicine (Divisions of Pulmonary Medicine and Cardiology) and Biochemistry, Duke University Medical Center, Durham, North Carolina 27710*

A B S T R A C T   The receptor alterations involved in catecholamine-induced desensitization of adenylate cyclase in human neutrophils have been investigated as has the ability of hydrocortisone to modify such alterations. Incubation of human neutrophils with isoproterenol for 3 h in vitro resulted in an 86% reduction in the ability of isoproterenol to stimulate cyclic AMP accumulation in the cells. Two types of receptor alterations were documented. There was a 40% reduction in the number of beta adrenergic receptors (42 vs. 25 fmol/mg protein, $P < 0.005$) present after desensitization as assessed by [^{3}H]dihydroalprenolol ([^{3}H]DHA) binding. In addition the receptors appeared to be relatively uncoupled from adenylate cyclase. This uncoupling was assessed by examining the ability of the agonist isoproterenol to stabilize a high-affinity form of the receptor, detected by computer modelling of competition curves for [^{3}H]DHA binding. Desensitized receptors were characterized by rightward-shifted agonist competition curves. When hydrocortisone was added to the desensitizing incubations (combined treatment) there was a statistically significant attenuation in the desensitization process as assessed by the ability of isoproterenol to increase cyclic AMP levels in the cells. Although combined treatment did not prevent the decline in receptor number, it did attenuate the uncoupling of the receptors. Combined treatment resulted in competition curves intermediate between the control and the rightward-shifted desensitization curves. Prednisolone was similar to hydrocortisone in attenuating isoproterenol-induced uncoupling. Thus, steroids appeared to attenuate agonist-induced desensitization of the beta adrenergic receptor-adenylate cyclase system by dampening the ability of agonists to uncouple receptors without modifying their ability to promote down-regulation of beta adrenergic receptors.

INTRODUCTION

Beta adrenergic receptors undergo regulation in a variety of situations (1). Catecholamine exposure from endogenous or exogenous sources may lead to diminished physiologic responsiveness to subsequent catecholamine challenge in lung (2) and other tissues. Steroid hormones appear to increase beta adrenergic responsiveness as evidenced by enhanced inotropic responses in heart muscle (3), enhanced vascular response (4, 5), and enhanced hepatic glucose production (6) upon catecholamine stimulation. Dog lungs undergo desensitization of the bronchodilatory response to isoproterenol upon prolonged isoproterenol exposure. Methylprednisolone administration restores isoproterenol responsiveness in the dog lung (7). The relationship between these opposing regulatory influences is of interest, since both catecholamines and steroid hormones are secreted in acute and chronic states of stress. Further, both catecholamines and steroid hormones may be simultaneously administered in the management of critical illness.

The beta adrenergic receptor-adenylate cyclase system appears to be composed of at least three components: the receptor (R),[1] the nucleotide regulatory site

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[1] *Abbreviations used in this paper*: C, catalytic moiety of adenylate cyclase; cAMP, cyclic AMP; [^{125}I]CYP, [^{125}I]iodocyanopindolol; [^{3}H]DHA, [^{3}H]dihydroalprenolol; K_H, dissociation constant for high-affinity state; K_L, dissociation constant for low-affinity state; N, nucleotide regulatory site; R, receptor.

(N), and the catalytic moiety of adenylate cyclase (C) (8). Curve modelling methods applied to competition binding data have demonstrated that a high-affinity complex is formed between the agonist and the beta adrenergic receptor (9, 10). Studies in frog erythrocyte membranes have demonstrated that the high-affinity complex appears to be composed of the hormone (H), receptor (R), and N-site as a high-affinity ternary complex, HRN (11, 12). This N-site normally assists in the "coupling" of receptor occupation to enzyme activation through the action of guanine nucleotides. Guanine nucleotides such as guanosine triphosphate act through the N-site once the HRN complex is formed to convert the high-affinity state of the receptor into a low-affinity state, resulting in release of bound hormone and activation of the catalytic moiety of adenylate cyclase. Thus, the formation of the high-affinity state and the subsequent actions of the N-site are essential to normal beta adrenergic receptor function (8).

The overall process of isoproterenol-induced desensitization (diminished receptor action) may be composed of both down-regulation (diminished receptor amount) and uncoupling. Studies in frog erythrocytes demonstrate that upon chronic exposure to isoproterenol there is both a loss of beta adrenergic receptor sites as well as a functional uncoupling, apparent as a loss of the stability of the high-affinity state of the receptor. This destabilization of the high-affinity state is apparent as a decreased ratio of the dissociation constants of the agonist for the two forms of the receptor, i.e., a decreased K_L/K_H ratio. Conversely, steroid exposure results in enhanced stabilization of the high-affinity state of the receptor, reflected in an increased K_L/K_H ratio (13). The purpose of the present work was twofold. First, we wished to assess whether changes in receptor number or coupling or both were present in a human model of desensitization and second, we wished to assess whether any changes occurring in desensitization might be altered by exposure to hydrocortisone.

METHODS

Subjects. Healthy volunteers aged 18 to 36 yr gave informed consent to participate in this study in a manner approved by the Duke University Medical Center Committee for Clinical Investigations. Phlebotomy was performed as previously stated (14).

Binding studies. The methods for preparing and assaying neutrophil membranes for beta adrenergic receptors were previously described (14). Tritiated dihydroalprenolol ([³H]DHA) was obtained from Amersham Corp. (Arlington Heights, IL, 86 Ci/mmol sp act). Neutrophils obtained from individual subjects were divided into aliquots, then incubated for 3 h in the presence of either medium alone (control), (−)-isoproterenol (10^{-4} M final concentration, isoproterenol treatment), or both (−)-isoproterenol (10^{-4} M final

concentration) and hydrocortisone sodium succinate (final concentration 133 µg/dl or 2.7 µM, the equivalent of 100 µg/dl hydrocortisone, isoproterenol + hydrocortisone treatment). The cells were supported by the specially designed medium, MEMP, as described (13). This medium contains minimal essential medium (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), Earle's salts, heparin, dextran, penicillin, and streptomycin maintained at pH ~7.4. The final incubation medium consists of ~80% autologous plasma. The added hydrocortisone binds to plasma proteins yielding a free hydrocortisone concentration of ~20 µg/dl (0.5 µM). This free concentration of hydrocortisone was chosen to simulate concentrations achievable in severe stress or major glucocorticoid therapy. A high initial isoproterenol concentration was chosen since it was anticipated that substantial losses would occur over the 3-h incubation from autooxidation and enzymatic degradation (from plasma and neutrophil enzymes).

Neutrophils from different individuals were separately exposed to similar conditions (e.g., isoproterenol treatment), then combined when the cells were to undergo polytron treatment. It was necessary to pool cells in order to accumulate enough receptors for detailed binding curves, especially after down-regulation. Each experiment, thus, consisted of neutrophils obtained from three to four subjects aliquoted and exposed to the incubation conditions noted. Saturation experiments were conducted with [³H]DHA concentrations between 0.5 and 3 nM with specific binding defined as that binding displaceable by 10^{-3} M (−)-isoproterenol. Specific binding was generally in the range of 70 to 80% of the total binding. Formation of the high-affinity state by the agonist isoproterenol was assessed by assays of the competition of (−)-isoproterenol (between 10^{-9} and 10^{-3} M final concentration) for [³H]DHA binding to beta adrenergic receptors. The [³H]DHA concentration in the competition assays was 1.5–2 nM.

During the course of this study the radioligand [¹²⁵I]iodocyanopindolol ([¹²⁵I]-CYP) became available. Binding parameters such as receptor density, EC_{50}, K_H, K_L, etc., determined from [¹²⁵I]CYP binding data are very similar to those determined from [³H]DHA binding data. The advantage of using [¹²⁵I]CYP lies in its very high affinity (K_d ~ 100 pM) and high specific radioactivity, allowing much smaller amounts of receptor to be used in binding studies. Assay conditions are similar to those for [³H]DHA (14), with the following exceptions: the incubation buffer was 75 mM Tris, 7.5 mM MgCl₂, pH 7.9 and incubation time was 40 min. Binding was stereoselective, rapid, and had appropriate rank-order selectivity. Specific binding was generally 85–95%. Competition assays were performed at 0.05 nM [¹²⁵I]CYP. The [¹²⁵I]CYP was synthesized from cyanopindolol in our laboratories.

[¹²⁵I]CYP was used in the prednisolone studies. The prednisolone studies were performed in a manner similar to those noted above for hydrocortisone. Prednisolone sodium succinate was substituted for hydrocortisone sodium succinate in the combined treatment groups. The total concentration of prednisolone sodium succinate was set at 20 µg/dl (equivalent to 75 µg/dl or 2 µM hydrocortisone) or 12 µg/dl (equivalent to 45 µg/dl or 1.2 µM hydrocortisone). Prednisolone was chosen as a more potent steroid to ensure that our observations with hydrocortisone were not simply due to its high concentration. Further, we could examine the general dose-response relationship of steroid effect.

Data from each individual competition assay was combined to form composite competition curves constructed from the mean radioligand binding values at each competing

isoproterenol concentration. The details of the analysis of these curves is described elsewhere (9, 13). Briefly, iterative curve modelling methods are used to derive each of the binding parameters. The EC_{50} (the concentration of isoproterenol reducing specifically bound radioligand by 50%) was derived by fitting the data with a four-parameter logistic equation (9). Dissociation constants for isoproterenol were derived by applying the law of mass action to the experimentally determined binding data. This procedure tests for the existence of one or more binding states of the receptor; the several fits were compared by F test to search for improvement in the goodness-of-fit by successively more complicated models. We accepted a two-state model only if it statistically significantly improved the fit over a one-state model. When two states of the receptor were observed their dissociation constants were designated K_H for the high-affinity state and K_L for the low-affinity state, respectively. The ratio of these constants, K_L/K_H, could then be calculated. We have previously shown that K_L/K_H is a correlate of high-affinity state (HRN) formation and reflects "coupling" of receptor occupation with adenylate cyclase activation (13). Thus, the ratio of K_L/K_H reflects the extent to which the agonist stabilizes the ternary complex HRN (9, 11, 13).

Statistical comparisons of parameter estimates were conducted as described previously (9, 13). The curves were first analyzed together, allowing parameter estimates for each curve to reach their optimal values. The curves were then reanalyzed with the corresponding parameters constrained to be estimated as a common value. The effect of this sharing of parameters among the curves upon the goodness-of-fit was tested. The shared parameters were considered statistically indistinguishable when the sharing process did not significantly worsen the fit. Therefore, the reported binding parameters and the statistical comparisons represent analysis of the composite data and are not simple means of individual experiments.

Cyclic AMP. Neutrophils incubated as described above were collected and resuspended in a buffer consisting of 75 mM Tris, 5 mM $MgCl_2$, 1 mM EGTA, and 0.5 mM isobutylmethylxanthine at pH 7.65. The ability of these cells to accumulate cAMP was assessed by incubation for 2 min at 37°C in the presence or absence of 10^{-4} M (−)-isoproterenol. The radioimmunoassay for cAMP was performed with cAMP kits obtained from New England Nuclear, Boston, MA.

RESULTS

Beta adrenergic receptor desensitization. Neutrophils exposed to isoproterenol for 3 h underwent desensitization of catecholamine-stimulated adenylate cyclase. The ability of neutrophils preexposed to isoproterenol to accumulate cAMP in response to subsequent catecholamine stimulation was reduced by 86% (Fig. 1). This desensitization was associated with a 40% reduction in receptor density (down-regulation, Table I). There was also a small though statistically significant increase in the dissociation constant of [^{3}H]DHA for the receptor not observed in previous studies in other systems. The biological significance (if any) of this small change in antagonist affinity is unknown.

To determine whether the desensitization process also involves "uncoupling," the ability of isoproterenol to induce or stabilize the formation of a high-affinity
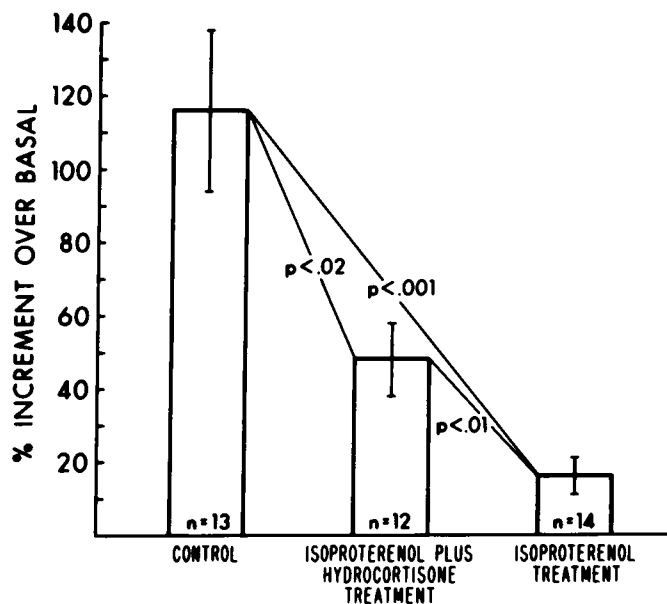

FIGURE 1 Isoproterenol-stimulated accumulation of cAMP in human neutrophils previously incubated under control, isoproterenol plus hydrocortisone treatment, and isoproterenol treatment conditions. Values represent percent increase in cAMP over basal levels; the bars represent SEM values. Basal cAMP levels in picomoles per 10^7 cells were 1.4±0.17 for control, 3.1±0.63 for isoproterenol plus hydrocortisone treatment, and 3.5±0.77 for isoproterenol treatment conditions. *P* represent comparisons between the groups noted.

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	Medium n = 16	Hydrocortisone* n = 9	Isoproterenol + hydrocortisone n = 17	Isoproterenol n = 7
Receptor density				
(fmol/mg protein)	42±4.0	49±4.6	17±3.6‡§	25±2.5‡
K_D (nM)	0.75±0.17	0.33±0.8	1.8±0.59‖§	1.5±0.17‡

Data represent mean±SEM. An unpaired t test was used to derive P values.
* Data from reference 13.
‡ $P < 0.005$ vs. medium or hydrocortisone value.
§ P, NS vs. isoproterenol value.
‖ $P < 0.05$ vs. medium or hydrocortisone value.

state of beta adrenergic receptors was examined. Beta adrenergic receptors obtained from cells incubated under control conditions demonstrated complex, shallow isoproterenol competition curves very similar to our prior experience (13) (Fig. 2). The binding parameters derived from the computer modelling are contained in Table II. High- and low-affinity states of the receptor were detected by curve modelling procedures
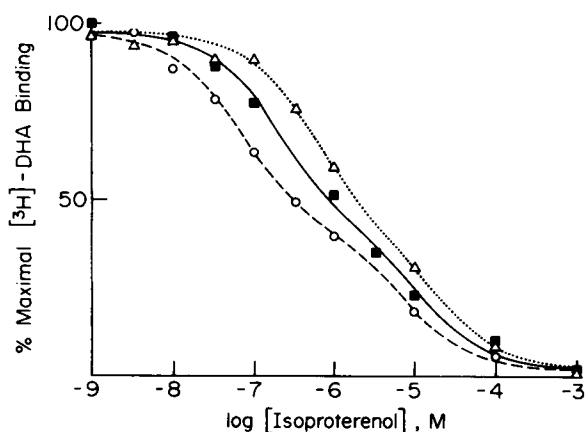

FIGURE 2 Composite curves for the competition of isoproterenol for [³H]DHA binding to beta adrenergic receptors derived from cells incubated as noted. The abscissa represents the concentration of isoproterenol competing for [³H]DHA binding to the beta adrenergic receptor. The ordinate represents [³H]DHA bound expressed as the percentage of maximal [³H]DHA bound in the absence of added isoproterenol. Data points represent composite means from 6 experiments for control (O), 7 experiments for isoproterenol plus hydrocortisone treatment (■), and 13 experiments for isoproterenol treatment groups (△). The computer-drawn lines represent the best fits to the data. In the absence of competitor, ~0.04 nM [³H]DHA was bound in control cells, and ~0.02 nM [³H]DHA was bound in the other treatment groups.

with distinct K_H and K_L values quite similar to those previously reported under similar conditions (13). In contrast, the curves from cells incubated with isoproterenol alone (Fig. 2) were shifted to the right of the control curve. This shift to the right was quantitated as an eightfold increase in the EC_{50} (Table II). Curve modelling reveals that two states of the receptor were again observable. However, these states of the receptor displayed altered dissociation constants, K_H and K_L, which were statistically significantly different from the corresponding control values. Although both K_H and K_L rose, the change in K_H was predominant, resulting in a reduction in K_L/K_H from 120 to 39 ($P < 0.002$). We have previously demonstrated that a fall in K_L/K_H correlates with decreased stabilization of the high-affinity state of the receptor by agonists (13). Thus, exposure of human neutrophils to isoproterenol in vitro results in diminished ability of agonists to stabilize the high-affinity state of the beta adrenergic receptor.

The effect of steroid hormones upon the process of desensitization was examined by exposing neutrophils to isoproterenol and hydrocortisone simultaneously. As seen in Fig. 1, the ability of isoproterenol plus hydrocortisone-treated cells to accumulate cAMP in response to catecholamine is statistically significantly greater than that of cells exposed to isoproterenol alone. However, the steroid effect did not extend to the down-regulation induced by isoproterenol. Neither the beta adrenergic receptor density nor antagonist affinity in the isoproterenol plus hydrocortisone treatment group are different from that of the isoproterenol group (Table I). Thus, the presence of hydrocortisone attenuates the isoproterenol-induced desensitization of the beta adrenergic receptor without altering isoproterenol-induced down-regulation.

Altered coupling could explain the steroid effect on desensitization while leaving down-regulation unaltered. Coupling was again examined by constructing

TABLE II

Binding Parameters Derived from Beta Adrenergic Receptors Exposed to Control, Hydrocortisone, Isoproterenol plus Hydrocortisone, or Isoproterenol Treatment

	Medium $n = 6$	Hydrocortisone* $n = 8$	Isoproterenol + hydrocortisone $n = 7$	Isoproterenol $n = 13$
EC_{50} (nM)	340±75	360±61	930±140‡	2,600±400‡
K_H (nM)	9.4±2.1	10±3.5	26±11‡	260±43‡
K_L (nM)	1,100±300§	1,800±270§	1,500±570§	9,900±2600
K_L/K_H	120±3.7∥	180±5.0∥	57±4.2∥	39±1.9∥

Data represent mean±SEM. Statistical evaluations were performed as summarized in the text and references 9, 11, and 13. The number of curves analyzed is noted by n.
* Data from reference 13.
‡ Isoproterenol and isoproterenol + hydrocortisone values distinct from each other and from medium or hydrocortisone values, $P < 0.002$.
§ $P < 0.002$ vs. isoproterenol treatment value.
∥ Each value in row different from all other values in row, $P < 0.002$.

isoproterenol competition curves for receptors derived from neutrophils simultaneously exposed to isoproterenol and hydrocortisone. As seen in Fig. 2, the composite agonist competition curve is shallow and complex, and lies between the control and isoproterenol treatment curves. The resultant EC_{50} for the isoproterenol plus hydrocortisone treatment curve is intermediate between the EC_{50} values for the control and the isoproterenol treatment curves (Table II). Two states of the beta adrenergic receptor were also observed after combined treatment, with dissociation constants, K_H and K_L, as noted in Table III. The presence of hydrocortisone markedly attenuated the isoproterenol-induced change in K_L such that a statistically significant difference from the control K_L was no longer found. As a result, the K_L/K_H for the isoproterenol plus hydrocortisone treatment curve is intermediate between the control and the isoproterenol treatment values. Thus, isoproterenol-induced desensitization reduces the stability of the agonist-induced high-affinity state formation, while the presence of hydrocortisone attenuates this reduction. The ability of hydrocortisone to stabilize high-affinity state formation is further indicated by the observation that pretreatment of cells with hydrocortisone alone impedes the ability of the guanine nucleotide 5'-guanylyl imidodiphosphate [GPP(NH)P] to shift the competition curve to the right, resulting in a competition curve whose EC_{50} is threefold lower than that for control curves performed in the presence of GPP(NH)P.

If the alterations induced by hydrocortisone were typical of steroid-mediated mechanisms, one would expect several characteristics. First, one would not expect to see a hydrocortisone effect with brief incubations. Second, steroid congeners might induce similar alterations. We examined these two characteristics in a second series of desensitization experiments. In this series of experiments the competition curve derived from the isoproterenol treatment group was shifted to the right with an EC_{50} of 2,100±230 nM (nine experiments), similar to the value of 2,600 nM in Table II. In these experiments two site fits to the competition curves from the desensitized cells could not be obtained, presumably due to the marked uncoupling. When hydrocortisone (2.7 µM final concentration) was added for the last 15 min of the 3-h isoproterenol treatment the resultant competition curve was very similar to the isoproterenol treatment curve. The EC_{50} for the 15-min treatment was indistinguishable from the isoproterenol treatment value (five experiments). Thus, no effect of hydrocortisone was observed with brief incubations.

Prednisolone was chosen as a hydrocortisone congener to determine whether other steroids induced similar effects. Prednisolone was examined in a manner parallel to hydrocortisone. The two concentrations chosen were designed to correspond to 2 and 1.2 µM hydrocortisone, both well within physiologically achievable levels. After exposure of neutrophils to 20 µg/dl prednisolone sodium succinate (equivalent to 2 µM hydrocortisone) + isoproterenol, competition curves were more shallow and shifted to the left compared to isoproterenol treatment curves (EC_{50} 930±100 nM vs. 2,100±230 nM, $P < 0.001$), as seen after hydrocortisone exposure. The value of K_L/K_H for this prednisolone + isoproterenol curve was 80±4.6 ($n = 5$), and was distinct from the isoproterenol treatment value in Table II ($P < 0.001$). However, addition of prednisolone at a lower concentration, 12 µg/dl or the equivalent of 1.2 µM hydrocortisone, resulted in competition curves indistinguishable from the isoproterenol treatment curves. Thus, the attenuation of isoproterenol-induced uncoupling appears to proceed by a mechanism typical of steroid-mediated effect.

DISCUSSION

Exposure of human neutrophils to isoproterenol in vitro leads to a state of diminished catecholamine responsiveness manifest as a decrease in isoproterenol-induced cAMP accumulation. This state of hyporesponsiveness is associated with down-regulation of the beta adrenergic receptors ($\downarrow$R), and a diminished stability of the agonist-induced high-affinity state of receptors (reflected in $\downarrow K_L/K_H$). Thus, exposure of beta adrenergic receptors to isoproterenol for several hours results in a condition in which receptor occupation by agonist is partially uncoupled from enzyme activation. This uncoupling is highly analogous to that demonstrated for the homologous desensitization of frog erythrocyte beta adrenergic receptors (8, 9, 11).

In humans, diminished antagonist binding to beta adrenergic receptors has been demonstrated after in vitro (13) or in vivo (15–20) exposure to catecholamines. Diminished cAMP accumulation or adenylate cyclase activity has also been observed in human leukocytes exposed for prolonged periods to catecholamines (21–29). However, these prior studies with human leukocytes have not investigated the status of uncoupling of the beta adrenergic receptors as assessed by detailed competition curves. Thus, the present studies are the first to indicate that the desensitized human leukocyte beta adrenergic receptor is uncoupled as a result of diminished high-affinity state formation by agonists.

This study indicates that catecholamine exposure results in at least two alterations of the beta adrenergic receptor in human neutrophils: down-regulation (reduction in receptor density) and uncoupling (reduction in high-affinity state formation). Under the conditions tested, hydrocortisone appears to diminish the desensitization primarily by attenuating the uncoupling process without affecting down-regulation. This attenuation appears to be associated with a stabilizing effect on high-affinity state formation. Harden et al. (30) have demonstrated that there may be different time courses for the processes of down-regulation and receptor uncoupling due to catecholamine exposure. Thus, the beta adrenergic receptor may undergo uncoupling before measureable down-regulation. Since down-regulation and uncoupling have separate time courses and different responses to hydrocortisone, they may be distinct yet related processes. Further examination of the molecular events underlying these processes will better determine how they might be related. Separate control of these regulatory processes could allow overall beta adrenergic responsiveness to be very finely tuned according to physiologic need.

The observed steroid-induced attenuation of catecholamine-induced desensitization agrees well with other data regarding the relationship of steroid hormones to catecholamine action. Steroid hormones in-

crease beta adrenergic receptor density in human (14, 31, 32) and animal (33, 34) tissues. Further, steroid hormones enhance adenylate cyclase action (assessed either by enzyme activity or cAMP accumulation) (14, 28, 35–37). Finally, hydrocortisone induces a tightened coupling of receptor and enzyme in neutrophils exposed in vitro (13). Thus, steroids exert a positive effect, in general, upon the beta adrenergic receptor-adenylate cyclase system in either the presence or absence of desensitizing influences. The best physiologic correlate of our in vitro observations is tachyphylaxis (desensitization) to isoproterenol action in bronchi. Repeated exposure to isoproterenol results in progressively less bronchodilation in response to subsequent isoproterenol administration. Hydrocortisone administration has been shown to return desensitized human bronchial tissue to a normal degree of relaxation (38). A similar effect of methylprednisolone exists in dogs (7). Thus, our observations provide an insight into the physiologically observable attenuating effects of steroids upon catecholamine-induced desensitization.

Our present observations are consistent with our prior demonstration of tightened coupling when neutrophils are exposed to steroid alone (13). Whether the observed alterations in coupling in the combined treatment group represent a direct steroid effect upon desensitization alone, or a combination of steroid-induced supersensitization and catecholamine-induced desensitization is not known at this time. The biochemical basis for the effects of steroids on the agonist-induced uncoupling of receptors remains to be determined.

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