Vol. 111, No. 1, 1983 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1983 Pages 112-119

DESENSITIZATION OF BETA$_2$-ADRENERGIC RECEPTORS AND ADRENOCORTICOTROPIN RELEASE

Seymour Heisler*, Terry Reisine and Julius Axelrod

Section on Pharmacology, Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20205

Received December 16, 1982

SUMMARY: Pre-exposure of mouse anterior pituitary tumor cells (A+T-20/D16-16) to (-) isoproterenol reduces the ability of this beta-adrenergic agonist to restimulate cyclic AMP synthesis or adrenocorticotropin hormone (ACTH) release from these cells. This beta-adrenergic receptor desensitization is time and dose-dependent, recoverable and specific for beta-receptors. Longer pretreatment times are required to decrease beta-receptor density than to induce receptor desensitization. This initial beta-receptor refractoriness involves an uncoupling of the receptor from adenylate cyclase since (-) isoproterenol treatment does not alter forskolin-activated cyclic AMP formation or ACTH release. In addition to diminishing beta-receptor responsiveness, (-) isoproterenol treatment induces a prolonged elevation of basal ACTH release. This finding indicates that the intracellular events leading to ACTH secretion may also be altered during the desensitization process.

Desensitization of beta-adrenergic receptors following their exposure to catecholamine agonists is believed to involve a multistep process (1-4). The first step is rapid in onset and characterized by a reduced ability of beta-receptor agonists to restimulate adenylate cyclase or to increase intracellular cyclic AMP levels. Beta-receptor density is not altered during this step although agonist affinity and the regulation of agonist binding by guanine nucleotides is diminished. The second step is much slower in onset and is associated with a loss of beta-receptors from the cell membrane. Despite the fact that this paradigm of desensitization has been explored in many cell systems few studies (5,6) have directly correlated these changes in beta-

*Visiting Scientist, MRC Canada, Unite de Bioregulation, Cellulaire et Moleculaire, Centre Hospitaler de l'Universite, Laval, Quebec, Canada GIV 4G2.

Abbreviations: ACTH, adrenocorticotropin hormone; Dulbecco's modified Eagle's medium, DMEM; ^{3}H-dihydroalprenolol, ^{3}H-DHA; corticotropin-releasing factor, CRF; vasoactive intestinal peptide, VIP.

0006-291X/83/040112-08$01.50/0

receptors and their coupling to adenylate cyclase with specific alterations in the functional responsiveness of the cell.

We have recently identified and characterized beta$_2$-adrenergic receptors on mouse anterior pituitary tumor cells (AtT-20/D16-16) (7-9). The cells are a homogenous population of corticotrophs releasing ACTH and beta-endorphin. Activation of beta$_2$ adrenergic receptors on AtT-20 cells leads to an increase in cyclic AMP formation followed by enhanced immunoreactive adrenocorticotropin (ACTH) secretion. We now report that short-term exposure of AtT-20 cells to (-) isoproterenol reduces the ability of the beta-adrenergic agonist to restimulate cyclic AMP formation and ACTH secretion, and that only after longer treatment is a loss of receptor density apparent. Our results indicate that the intracellular events leading to ACTH secretion are also altered during the desensitization process.

METHODS

Mouse AtT-20/D16-16 tumor cells (AtT-20) were grown and subcultured in Dulbecco's modified Eagle's medium (DMEM) as previously described (7-10). For cyclic AMP synthesis and immunoreactive ACTH release studies, cells were plated in 35 mm diameter culture dishes at a density of 2×10^5 cells/well and were used 5-6 days after subculturing (60-80% confluency). In receptor binding studies, the selective beta-adrenergic receptor antagonist [^{3}H]-dihydroalprenolol (^{3}H-DHA; 43 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was used to label receptors. Specific binding of [^{3}H]-DHA to AtT-20 cells was performed in the same manner as in previous studies (7,8).

RESULTS AND DISCUSSION

(-) Isoproterenol was shown to stimulate cyclic AMP formation and ACTH secretion from AtT-20 cells in a time and dose-dependant manner by activating beta$_2$-adrenergic receptors (7-9). Pretreatment with (-) isoproterenol reduced the ability of a maximally effective concentration of the beta-adrenergic agonist to restimulate either cyclic AMP formation or immunoreactive ACTH release (Fig. 1). Cyclic AMP formation and ACTH release were significantly reduced after only 15 min of agonist exposure. Following 20 hr of treatment with (-) isoproterenol, stimulation of cyclic AMP formation and ACTH secretion were less than 20% of control values.

To determine the effect of exposure of (-) isoproterenol on receptor density, the binding of the beta-adrenergic antagonist, [^{3}H]-DHA, was examined.

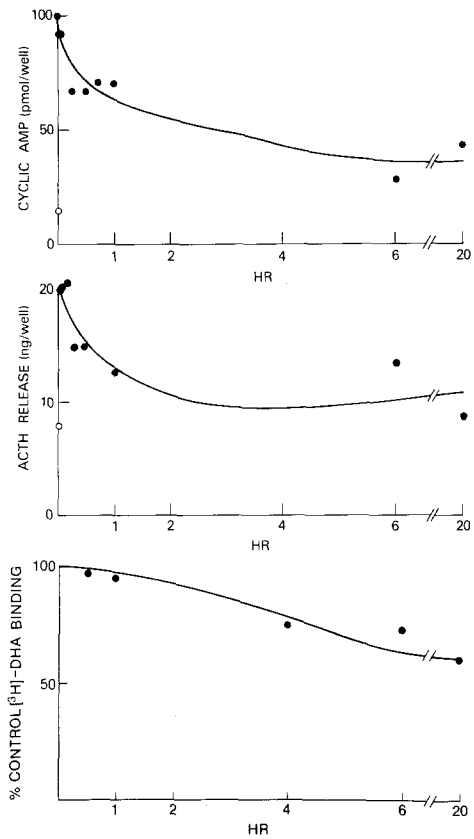

Figure 1. Time dependent reduction of (-) isoproterenol-induced cyclic AMP synthesis (top panel), immunoreactive ACTH secretion (middle panel), and beta-receptor density (lower panel), following desensitization with 10^{-6}M (-) isoproterenol. For cyclic AMP and ACTH studies, AtT-20 cells were equilibrated for 30-60 min at 37°C in DMEM containing 25 mM Hepes (pH 7.4), 2% fetal calf serum and 3 ug/ml bacitracin. For desensitization the medium was aspirated and the cells incubated at 37°C with fresh medium containing 10^{-6}M (-) isoproterenol for the indicated times. At the end of incubation the medium was withdrawn and the cells were washed with agonist-free medium. For cyclic AMP studies, cells were first preincubated for 15 min at 37°C in culture medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and then post-incubated 15 min in IBMX containing medium with or without 10^{-6}M (-) isoproterenol. The medium was aspirated and 1 ml of 10 mM acetic acid containing 0.5 mM IBMX was added to the cells. The suspension was sonicated and an aliquot removed for cyclic AMP measurement. For ACTH secretion studies, following desensitization the washed cells were incubated for 60 min with or without 10^{-6}M (-) isoproterenol. After completion of incubation, an aliquot of the medium was removed and centrifuged and ACTH in the supernatant was measured. Closed circles represent (-) isoproterenol-pretreated, (-) isoproterenol-challenged cells. The open circle represents basal cyclic AMP levels and ACTH secretion in nonpretreated cells. Cyclic AMP and ACTH were determined as previously described (7,10). For receptor binding studies, AtT-20 cells were grown in 75 cm^2 flasks and desensitized by the addition of 10^{-6}M (-) isoproterenol. At the times indicated the cells were washed with agonist-free medium and a membrane fraction prepared as previously described (8). [^{3}H]-Dihydroalprenolol ([^{3}H]-DHA) (25 nM) was incubated with about 50 ug protein for 30 min at 25°C and bound [^{3}H]-DHA was separated from free radioligand by filtration over GF-C glass fiber filters. Specific binding (displaced by 10 uM d,l-propranolol) accounted for about 70% of total tissue binding. Nonspecific, nontissue binding was subtracted from total binding in calculating tissue specific [^{3}H]-DHA binding. Values represent the means of 3-6 separate experiments.

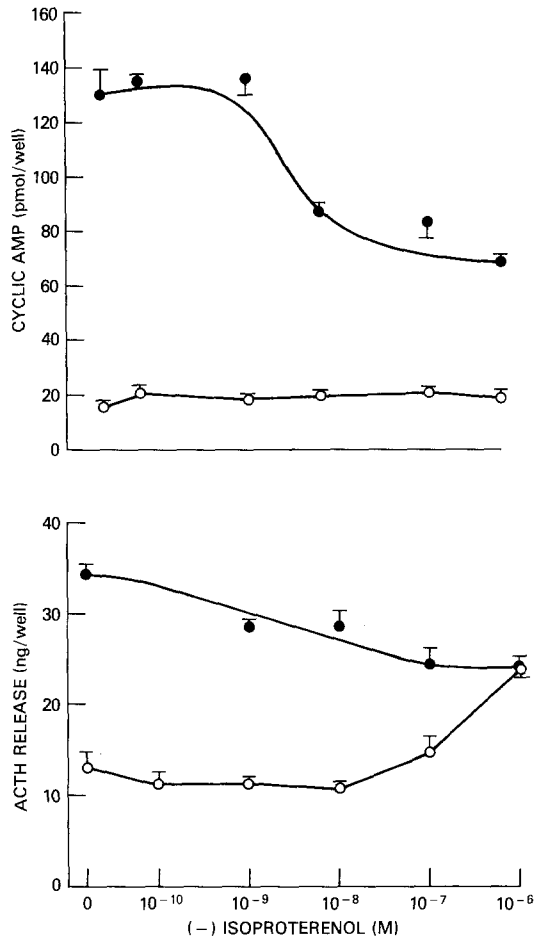

Figure 2. Concentration-dependent reduction of (-) isoproterenol-induced cyclic AMP synthesis and ACTH secretion. Cells were equilibrated and pretreated with 10^{-10}M to 10^{-6}M (-) isoproterenol for 1 hr at 37°C. After washing, cells were incubated with or without 10^{-6}M (-) isoproterenol for 60 min at 37°C and cyclic AMP formation (upper panel) and ACTH release (lower panel) then measured as described in Fig. 1 legend. Closed circles represent (-) isoproterenol-pretreated, (-) isoproterenol-challenged cells. Open circles represent (-) isoproterenol-pretreated nonrechallenged cells. Values represent the means of three separate experiments.

[^{3}H]-DHA binding to beta-receptors on AtT-20 cell membranes was found to be saturable, specific and of high affinity (8). Decreases in [^{3}H]-DHA binding following agonist treatment occurred relatively late, but binding was reduced to 60% of control after 20 hours (Fig. 1). This diminished binding (after 20 hr treatment) was associated with a decreased density of beta-receptors (control Bmax = 61 $\pm$ 55; treated Bmax = 38.5 $\pm$ 7.7; n = 3, $P < 0.05$) with no appreciable change in receptor-ligand affinity (Control K_D = 10.7 $\pm$ 2.4,

treated K_D = 8.7 ± 0.9, n = 3, P > 0.05). Nonspecific binding and protein content were the same in control and treated cells.

(-) Isoproterenol-induced desensitization of beta-receptors on AtT-20 cells with respect to cyclic AMP formation was dose-dependant with half-maximal inhibition (IC_{50}) of stimulated cyclic AMP formation occurring at 5 nM (Fig. 2). Higher concentrations of (-) isoproterenol were needed to reduce ACTH-stimulated release. The desensitization of beta-receptors linked to adenylate cyclase and ACTH secretion was also produced by epinephrine, norepinephrine and the selective $beta_2$-adrenergic agonist, salmefamol (data not shown). Furthermore, the (-) isoproterenol-induced desensitization was blocked by propranolol but not by the $beta_1$-receptor antagonist, practolol (not shown).

The early onset of beta-receptor refractoriness to stimulation of cyclic AMP synthesis or ACTH release despite little or no change in receptor density could be due to an uncoupling of the beta-receptor from adenylate cyclase, a decreased activity of the adenylate cyclase, or an alteration in the ACTH secretory process. To determine which of these possibilities were operative, the ability of forskolin, a compound that directly stimulates adenylate cyclase (12,13) to generate cyclic AMP and to stimulate the release of ACTH was examined. Forskolin increases cyclic AMP synthesis and ACTH secretion in a dose-dependent manner (9, in preparation). Pretreatment of AtT-20 cells with (-) isoproterenol for 6 hr, at which time (-) isoproterenol stimulated cyclic AMP synthesis and ACTH release were reduced, did not alter the extent to which forskolin stimulated cyclic AMP formation and ACTH release (Fig. 3). This finding indicates that the desensitization of the beta-receptor to stimulation of cyclic AMP formation and ACTH release is most likely due to uncoupling of the receptor from the adenylate cyclase and not to changes in the adenylate cyclase or changes in ACTH secretory mechanisms.

Short-term beta-receptor desensitization was reversible. The loss of beta-receptor responsiveness following 15 min exposure to (-) isoproterenol (10^{-6}M) was reversed after 1 hr of drug withdrawal in the case of ACTH secretion and

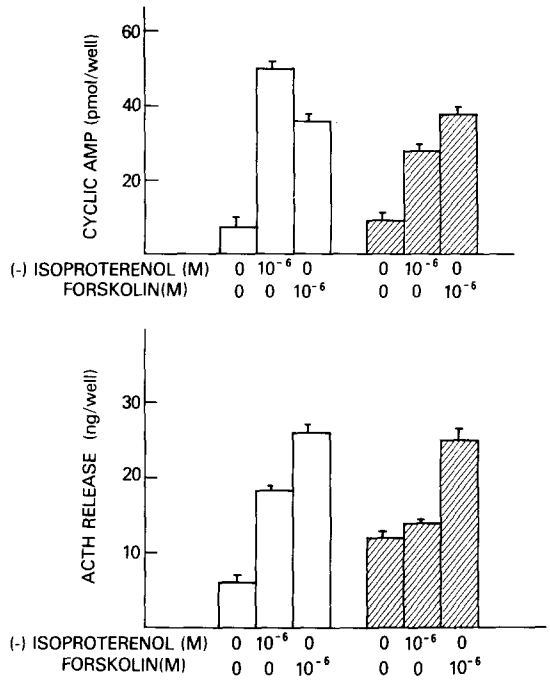

Figure 3. The effect of (-) isoproterenol pretreatment on forskolin stimulated cyclic AMP synthesis and ACTH secretion. AtT-20 cells were treated with or without (-) isoproterenol (10^{-6}M) for 6 hours. The medium was then removed and the cells were washed once with fresh medium. For cyclic AMP (upper panel) studies, AtT-20 cells were incubated for 15 min with medium containing 0.5 mM IBMX and then post-incubated 15 min in IBMX containing medium with or without (-) isoproterenol (10^{-6}M) or forskolin (10^{-6}M). Cyclic AMP was analyzed as described in Fig. 1. Cross-hatched figures represent treated condition and clear figures are nontreated. ACTH secretion studies were performed as described in Fig. 1 except that forskolin (10^{-6}M) was also used to stimulate secretion. Values represent the mean $\pm$ S.E.M. of 3-6 separate experiments.

partially recovered (85% of control) for cyclic AMP synthesis (data not shown). Following pretreatment with lower concentrations of the beta-adrenergic agonist (10^{-8}M), complete recovery of the cyclic AMP response was observed.

We have previously found that corticotropin-releasing factor (CRF) and vasoactive intestinal peptide (VIP) increase cyclic AMP synthesis and ACTH release (7,9,10). The desensitizing action of (-) isoproterenol treatment appeared to be limited to beta-adrenergic receptors since exposure of AtT-20 cells to (-) isoproterenol (1 uM) for 0.25 - 20 hr did not alter CRF or VIP's stimulation of cyclic AMP formation or ACTH secretion (data not shown).

Exposure of AtT-20 cells to (-) isoproterenol also increased basal ACTH release during subsequent incubation. Basal ACTH secretion was greater than

untreated controls at all times examined (data not shown). The increased basal ACTH release induced by (-) isoproterenol (Fig. 2) and salmefamol was dose-dependent and blocked by propranolol. This elevated basal ACTH release may be due to a rapid stimulation of ACTH synthesis since total ACTH content was increased after only 15 min of (-) isoproterenol exposure (data not shown).

The diminished stimulation of cyclic AMP and ACTH secretion following (-) isoproterenol treatment may have been the result of an activation of phosphodiesterase. This possibility seems unlikely in light of the normal ability of forskolin to stimulate cyclic AMP formation after 6 hr of (-) isoproterenol treatment. Furthermore, all the cyclic AMP studies were done in the presence of a phosphodiesterase inhibitor and at no time after the (-) isoproterenol pretreatment were basal cyclic AMP levels reduced.

The uncoupling of beta-adrenergic receptors from adenylate cyclase following agonist pretreatment has previously been reported in several cell systems (1-5). However, it is not clear whether this uncoupling results in a desensitization of functional beta-receptors since in few cell systems can the biological response and biochemical characteristics of such receptors be measured. The similar time course and magnitude for the diminished cyclic AMP and ACTH secretory responses observed here indicates that most if not all of the beta-adrenergic receptors on AtT-20 cells which were desensitized were functional receptors. This close relationship between adenylate cyclase activity and ACTH secretion should allow a further elucidation of the mechanism of regulation of functional beta-adrenergic receptors.

ACKNOWLEDGEMENTS: The authors would like to thank Drs. M. Zatz, J. Tallman and A. Hoffman for careful review of the manuscript and Ms. Bobbi Holcomb for her secretarial assistance. S. Heisler was a visiting scientist of the Canadian MRC.

REFERENCES

1. Su, Y.-F., Harden, T.K. and Perkins, J.P. (1979) J. Biol. Chem. 254, 38-41.
2. Su, Y.-F., Harden, T.K. and Perkins, J.P. (1980) J. Biol. Chem. 255, 7410-7419

3. Harden, T.K., Su, Y.-F. and Perkins, J.P. (1979) J. Cyclic Nucleotide Res. 5, 99-106.
4. Doss, R.C., Perkins, J.P. and Harden, T.K. (1981) J. Biol. Chem. 256, 12281-12286.
5. Catt, K.J., Harwood, J.P., Aguilera, G. and Dufau, M.L. (1979) Nature 280, 109-116.
6. Kebabian, J.W., Zatz, M., Romero, J.A. and Axelrod, J. (1975) Proc. Natl. Acad. Sci. USA 72, 3735-3739.
7. Heisler, S., Reisine, T.D., Hook, V.Y.H. and Axelrod, J. (1982) Proc. Natl. Acad. Sci. USA 79, 6502-6506.
8. Reisine, T.D., Heisler, S., Hook, V.Y.H. and Axelrod, J., J. Neurosci. (in press).
9. Reisine, T., Heisler, S., Hook, V.Y.H. and Axelrod, J. (1982) Biochem. Biophys. Res Commun. 108, 1251-1257.
10. Hook, V.Y.H., Heisler, S., Sabol, S. and Axelrod, J. (1982) Biochem. Biophys. Res. Commun. 106, 1364-1371.
11. Minneman, K.D., Hegstrand, L. and Molinoff, P.B. (1979) Mol. Pharmacol. 16, 21-33.
12. Seamon, K.D. and Daly, J.W. (1981) J. Biol. Chem. 256, 9799-9801.
13. Seamon, K.D., Padgett, W. and Daly, J.W. Proc. Natl. Acad. Sci. USA (1981) 78, 3363-3367.
14. Law, P.Y., Hon, D.F. and Loh, H. (1982) Mol. Pharmacol. 22, 1-4.
15. Sharma, S.K., Klee, W. and Nirenberg, M. (1975) Proc. Natl. Acad. Sci. USA 72, 3092-3096.