References and Notes

1. G. E. Tyson and R. E. Bulger, *Z. Zellforsch. Mikrosk. Anat.* **141**, 443 (1973).
2. K. G. Bensch and S. E. Malawista, *J. Cell Biol.* **40**, 95 (1969).
3. A. Krishan and D. Hsu, *ibid.* **48**, 407 (1971).
4. A. Nagayama and S. Dales, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 464 (1970).
5. S. Dales, D. C. Hsu, A. Nagayama, *J. Cell Biol.* **59**, 643 (1973).
6. R. Marantz and M. L. Shelanski, *ibid.* **44**, 234 (1970).
7. O. Behnke and A. Forer, *Exp. Cell Res.* **73**, 506 (1972); P. Filner and O. Behnke, *J. Cell Biol.* **59**, 99a (1973).
8. G. G. Borisy and E. W. Taylor, *ibid.* **34**, 525 (1967).
9. G. G. Borisy and J. B. Olmsted, *Science* **177**, 1196 (1972). At the suggestion of J. B. Olmsted a modification of the published procedure, omitting Mg^{2+}, increasing guanosine triphosphate concentration to 2.5 m*M*, and adjusting the *p*H of the assembly medium to 6.9, was utilized. Purification was achieved by microtubule assembly at 37°C followed by centrifugation and resuspension-disassembly of the pellet in fresh 4°C assembly medium.
10. J. Bryan, *J. Mol. Biol.* **66**, 157 (1972).
11. S. Granett, W. Dentler, G. B. Witman, J. L. Rosenbaum, *J. Cell Biol.* **59**, 119a (1973).
12. M. W. Kirschner, R. C. Williams, M. Weingarten, J. C. Gerhart, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1159 (1974).
13. A. V. Grimstone and A. Klug, *J. Cell Sci.* **1**, 351 (1966); F. D. Warner and P. Satir, *ibid.* **12**, 313 (1973).

14. Supported in part by PHS grant GM 19537.

5 June 1974

Reversal of Catecholamine Refractoriness by Inhibitors of RNA and Protein Synthesis

Abstract. *The generation of adenosine 3′,5′-monophosphate (cyclic AMP) in response to catecholamines in the 2B subclone of RGC6 rat glioma cells previously exposed to norepinephrine and refractory to further norepinephrine addition is substantially increased by addition of inhibitors of RNA and protein synthesis. The time course of the effect of these inhibitors on cyclic AMP concentration suggests that rapid protein synthesis and turnover are involved in catecholamine refractoriness. Norepinephrine induction of cyclic nucleotide phosphodiesterase is demonstrable in RGC6 cells but not in the 2B subclone. Thus, catecholamine refractoriness cannot be attributed to induction of phosphodiesterase. This implies that induction of a protein or proteins, important in catecholamine refractoriness, affects the synthesis rather than the degradation of cyclic AMP.*

The rat glial tumor cell line RGC6 is highly responsive to β-adrenergic agonists, which elicit a transient rise in the intracellular concentration of adenosine 3′,5′-monophosphate (cyclic AMP) (*1*). After the peak cyclic AMP concentration is obtained, the readdition of hormones does not lead to greater accumulation of intracellular cyclic AMP (*2*). The accumulation of an inhibitory substance in the medium has been ruled out, since the medium when added to untreated cultures elicits the same response as fresh medium containing norepinephrine (NE). Similar observations have been reported for human astrocytoma cells (*3*) and human fibroblasts (*4*). We have shown (*5*) that the catecholamine-mediated induction of lactic dehydrogenase (E.C. 1.1.1.27) in RGC6 cells is mediated by cyclic AMP and is blocked by certain inhibitors of RNA and protein synthesis. During these studies we observed that in the presence of actinomycin D (AD) and acetoxycycloheximide (CH), the cells maintained nearly peak levels of cyclic AMP for up to 12 hours. We now report that RNA and protein synthesis appear to be involved in the refractoriness of the cells to catecholamines once the initial peak of cyclic AMP is obtained (*6*).

The 2B subclone of RGC6 cells was derived from RGC6 cells obtained from G. Sato's laboratory and was used between passages 4 and 26. No differences in cyclic AMP accumulation elicited by catecholamines were observed between high-passage cells and frozen stocks of cells at passages 4 to 6. The 2B clone is diploid and free of mycoplasma (*7*). The RGC6 cells were obtained from the American Type Culture Collection. Cells were grown at 37°C for 10 days to a monolayer of stationary cells in T-30 Falcon plastic flasks in Ham's F-10 medium (Gibco) supplemented with 10 percent virus-free fetal calf serum (Reheis, Armour) without antibiotics. At the time of the experiments, cultures were provided with serum-free fresh medium containing appropriate hormones or drugs and kept at 37°C. Cell number and total protein remained unchanged during the experiments. The incubation was terminated by decanting the medium, the cells were immediately rinsed with 5 ml of 0.9 percent saline at 4°C, and 5 percent trichloroacetic acid at 4°C was added. The cells were scraped off the flask with a rubber policeman, and denatured proteins were removed by centrifugation. Cyclic AMP was purified by column chromatography and assayed either by high pressure liquid chromatography (*8*) or by the binding assay (*9*) as modified (*10*). Identical results were obtained by either method. Protein was measured by the method of Lowry *et al.* (*11*).

Figure 1A shows the effect of 3 μ*M* NE, alone and in the presence of CH (5 μg/ml), on cell cyclic AMP concentrations in the 2B subclone at 1 and 6 hours. Cyclic AMP concentration was high 1 hour after NE addition, and was reduced almost to the control value by 6 hours. Cyclic AMP concentration reached a peak 20 to 30 minutes after addition of NE and was 70 percent of the peak value at 1 hour. Addition of CH, an inhibitor of protein synthesis, along with NE had no significant effect on cell cyclic AMP concentrations at 1 hour but had a dramatic effect at 6 hours. The presence of CH markedly reduced the decline in cyclic AMP normally observed in the presence of NE alone. Inclusion of AD (1 μg/ml) gave results identical to those for CH (data not shown). Under these conditions, CH inhibited 97 percent of pro-

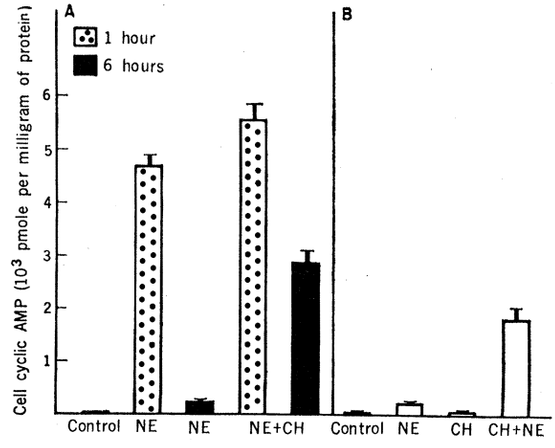

Fig. 1. (A) Effect of CH (5 μg/ml) on intracellular cyclic AMP in cells treated simultaneously with NE (3 μ*M*). Each bar represents the mean and standard error of the mean for four cultures. (B) Intracellular cyclic AMP in refractory cells treated with NE (3 μ*M*) or CH (5 μg/ml) for 1 hour. The combination of NE and CH relieved the refractory condition of the cells. Control cell cyclic AMP was 11 ± 1 pmole per milligram of protein. The NE was L-norepinephrine bitartrate.

Table 1. Phosphodiesterase activity in RGC6 and RGC6-2B cell homogenates after the cells had been treated with NE. Norepinephrine (3 μM) and CH (5 μg/ml) were added in serum-free fresh media to cells (T250 Falcon plastic flasks) as indicated. After 6 hours the medium was poured off and the cells were rinsed with saline at 4°C. Cells were scraped off in 40 mM tris(hydroxymethyl)-aminomethane hydrochloride, pH 8.0, containing 10.9 percent sucrose and 3.75 mM mercaptoethanol. The cells were homogenized in a Teflon-glass homogenizer for seven strokes at 1100 rev/min. Phosphodiesterase was assayed according to the method of Thompson and Appleman (*14*). The assay was performed at 30°C in the presence of 1 μM cyclic AMP, 5 mM $MgCl_2$, 40 mM tris-HCl (pH 8.0), and 3.75 mM mercaptoethanol; EGTA (3 mM) was present where indicated. Each value is the mean ± standard error of the mean for three obtained by pooling two cultures. Both lines were at same cell density (10-day confluent stationary phase cultures, approximately 10^7 cells per 75 cm^2). The RGC6 cells were from the American Type Culture Collection.

Treatment	Cyclic AMP hydrolyzed (picomoles per milligram per minute)	
	− EGTA	+ EGTA
RGC6		
Control	24.1 ± 1.1	19.7 ± 0.5
$1 \times 10^{-4} M$ NE	36.8 ± 1.9	33.6 ± 1.5
$1 \times 10^{-4} M$ NE + CH (5 μg/ml)	26.8 ± 1.6	22.6 ± 0.3
RGC6 2B clone		
Control	67.4 ± 0.4	51.0 ± 1.7
$3 \times 10^{-6} M$ NE	63.1 ± 0.4	52.0 ± 1.1
$3 \times 10^{-6} M$ NE + CH (5 μg/ml)	71.7 ± 0.2	48.3 ± 1.0
$1 \times 10^{-4} M$ NE	59.1 ± 4.5	

tein synthesis, and AD inhibited 95 percent of RNA synthesis but did not inhibit total protein synthesis (*5, 7*). These results suggest that de novo synthesis of RNA and protein occurs rapidly after application of NE to the cells and that this protein synthesis is important in the regulation of cell cyclic AMP concentrations.

The addition of CH at the time of NE readdition dramatically restored the capacity of the cells to accumulate cyclic AMP. Cells from the 2B subclone were initially exposed to 3 μM NE for 4 hours, by which time the cells were refractory to subsequent NE addition. Fresh medium containing NE, CH, or both, was added, and cyclic AMP was measured 1 hour later (Fig. 1B). One hour after addition of NE alone, cyclic AMP content had increased only to the refractory level (300 pmole per milligram of protein), that is, the level observed before the cells were washed. When used alone, CH had no effect on cyclic AMP content. However, when CH and NE were added together, high levels of cyclic AMP were found 1 hour later. This experiment demonstrates that blockade of protein synthesis allowed refractory cells to respond to NE.

We investigated the effect of CH and AD added to cultures at various times after administration of NE (Fig. 2). Cell cyclic AMP levels were measured 6 hours after addition of NE. The maximal response was defined as the concentration of cyclic AMP at 6 hours when the inhibitor was added together with NE at zero time. When AD was added at 1 hour, the protective effect was reduced by 50 percent, a result indicating that newly made RNA is involved in the synthesis of proteins that render the cell refractory to NE and that this RNA is made soon after NE addition. In contrast, when CH was added at 3 hours, its protective effect was almost maximal, and its effect at 5 hours was 50 percent of maximal. Since refractoriness is fully established by 3 hours after initial NE treatment, this result and the results of Fig. 1B can only be interpreted to mean that the protein that is involved in refractoriness is turning over very fast, certainly with a half-time less than 1 hour.

We have tried to identify the nature of the protein whose synthesis is induced by NE and is important in regulating the cyclic AMP concentration in this clone. The cells could become refractory due to reduced cyclic AMP production or increased cyclic AMP destruction. Other possibilities include induction of enzymes that accelerate the destruction of the catecholamine or extrusion of an inhibitory substance into the medium. However, the latter two possibilities can be ruled out since medium taken from refractory cells elicits the same response in untreated cells as does fresh medium containing NE.

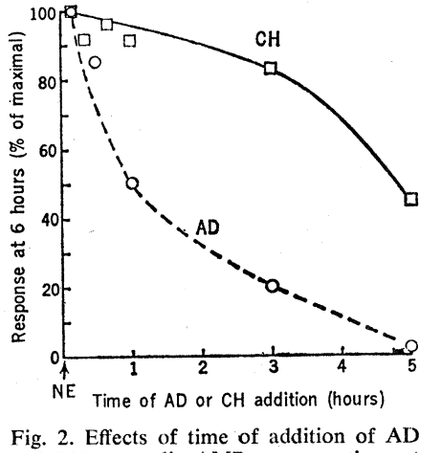

Fig. 2. Effects of time of addition of AD and CH on cyclic AMP concentrations at 6 hours in NE-treated cells. All cultures received serum-free fresh medium containing 3 μM NE and either CH (5 μg/ml) or AD (1 μg/ml). The abscissa represents the time of addition of these compounds after NE was added. The maximal response is the concentration of cyclic AMP at 6 hours when the inhibitor (AD or CH) was added at zero time (with NE). Each point represents the mean of four cultures; the largest standard error of one mean was ± 7 percent.

Extensive unpublished studies in our laboratories also suggest that the protein whose synthesis is induced by NE and blocked by CH or AD is not cyclic nucleotide phosphodiesterase (*12*). This enzyme exists in two kinetic forms (*13*). Extensive kinetic, chromatographic, and density gradient centrifugation studies (*14–16*) have failed to indicate induction of any specific molecular form of phosphodiesterase in cells of the 2B subclone treated with NE. Table 1 shows that phosphodiesterase, measured by the method of Thompson and Appleman (*14*) in the presence or absence of ethylene glycol bis(aminoethyl ether) tetraacetic acid (EGTA), is induced in the RGC6 cells after addition of NE but is not induced in the 2B subclone. Cyclic nucleotide phosphodiesterase is induced in other cell lines including RGC6 (*17*) and has been suggested to be a molecular factor in catecholamine refractoriness.

Our results suggest that in the 2B subclone, synthesis of a protein or proteins is important in catecholamine refractoriness. This protein is rapidly synthesized after addition of NE and acts in some way to reduce the synthetic rate of cyclic AMP since phosphodiesterase activity is unaltered.

Such a mechanism also is suggsted by the work of Su and Perkins (*3*), who reported evidence for a decreased rate of cyclic AMP formation in human astrocytoma cells after treatment with NE, and by that of Franklin and Foster (*4*), who showed that refractoriness to catecholamines in human lung fibroblasts did not prevent the cells from responding normally to prostaglandin E_1.

JEAN DE VELLIS

Department of Anatomy, Laboratory of Nuclear Medicine and Radiation Biology, and *Mental Retardation Center, School of Medicine, University of California, Los Angeles 90024*

GARY BROOKER

Department of Pharmacology, University of Virginia School of Medicine, Charlottesville 22903

References and Notes

1. A. G. Gilman and M. Nirenberg, *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2165 (1971); J. de Vellis and G. Brooker, *Fed. Proc.* **31**, 513 (1972).
2. J. Schultz, B. Hamprecht, J. W. Daly, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1266 (1972); E. T. Browning, J. P. Schwartz, B. McL. Breckenridge, *Mol. Pharmacol.* **10**, 162 (1974).
3. Y. F. Su and J. P. Perkins, *Fed. Proc.* **33**, 493 (1974).
4. T. J. Franklin and S. J. Foster, *Nat. New Biol.* **246**, 146 (1973).
5. J. de Vellis and G. Brooker, *Fed. Proc.* **31**, 513 (1972); J. de Vellis, D. Inglish, R. Cole, J. Molson, in *Influence of Hormones on the Nervous System*, D. Ford, Ed. (Karger, Basel, 1971), pp. 25–39; J. de Vellis and D. Inglish, *In Vitro* **7**, 247 (1972).
6. A preliminary communication has been presented [J. de Vellis, D. Inglish, G. Brooker, *Fed. Proc.* **33**, 507 (1974)].
7. J. de Vellis and G. Brooker, in *Tissue Culture of the Nervous System*, G. Sato, Ed. (Plenum, New York, 1973), pp. 231–245.
8. G. Brooker, *J. Biol. Chem.* **246**, 7810 (1971).
9. A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305 (1970).
10. G. Brooker, in *Biochemical Methods of Analysis*, D. Glick, Ed. (Interscience, New York, in press).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
12. R. W. Butcher and E. W. Sutherland, *ibid.* **237**, 1244 (1962).
13. G. Brooker, L. J. Thomas, M. M. Appleman, *Biochemistry* **7**, 4177 (1968).
14. W. J. Thompson and M. M. Appleman, *ibid.* **10**, 311 (1971).
15. W. J. Thompson, S. A. Little, R. H. Williams, *ibid.* **12**, 1889 (1973).
16. W. J. Thompson and M. M. Appleman, *J. Biol. Chem.* **246**, 3145 (1971).
17. P. Uzunov, H. M. Shein, B. Weiss, *Science* **180**, 304 (1973); V. Manganiello and M. Vaughan, *Proc. Natl. Acad. Sci. U.S.A.* **69**, 269 (1972); M. D'Armiento, G. S. Johnson, I. Pastan, *ibid.*, p. 69; J. P. Schwartz and J. V. Passonneau, *Fed. Proc.* **33**, 1391 (1974).

18. Supported by AEC AT(04-1)Gen-12, NIH grants HD-04612, HD-05615, and HL-15985 and the American Heart Association. We thank D. Inglish and D. Farson who meticulously performed most of these experiments.

14 May 1974; revised 26 August 1974

β-Adrenergic Receptor: Stereospecific Interaction of Iodinated β-Blocking Agent with High Affinity Site

Abstract. *An iodine-labeled β-adrenergic inhibitor (*^{125}*I-hydroxybenzylpindolol) binds specifically to a site on turkey erythrocyte membranes. A series of β-adrenergic agonists and inhibitors compete for this binding site, with apparent affinities paralleling biological effectiveness as activators or inhibitors of catecholamine-stimulated adenylate cyclase. The activity of* d-(+) *agonists or inhibitors was 1 percent (or less) than that of the corresponding* l-(−) *isomers in competing for binding of the iodinated blocker as well as in affecting catecholamine-stimulated adenylate cyclase.* l-(−)-*Norepinephrine was about one-tenth as active as* l-(−)-*isoproterenol in competing for the β-blocking agent site. The stereospecificity of the interaction with the iodinated β-blocking agent and the correspondence between affinity for site and biological potency of analogs suggested that this interaction is involved in function of the β-adrenergic receptor.*

Interaction of β-adrenergic agonists with specific receptors accounts for a variety of biological phenomena in diverse tissues; and in each, adenylate cyclase is activated with consequent generation of adenosine 3′,5′-monophosphate (cyclic AMP), which in turn produces the ultimate physiological response (*1*). The generality of this sequence has prompted many investigators to analyze directly for the receptor. These several studies (*2*, *3*) to date have depended on determining binding of ligands of low specific activity (tritiated catecholamines, 2 to 15 c/mmole) and have detected a site that recognizes primarily the catechol function of the molecule. This site does not distinguish between active or inactive stereoisomers of the agonist (*2*, *3*). Conversely, the typical β-adrenergic blocking agents (β-blockers) show low affinity for the catechol-specific site but high affinity as inhibitors of adenylate cyclase and biological activity (*2*, *3*). The β-blockers are ethanolamine analogs, not catechol analogs, and show the same required stereoconfiguration as do the catecholamines for biological activity. Thus it appeared (*3*) that receptor recognition of the stereospecific ethanolamine function was common to both the β-agonists and β-blockers. We now report identification of such a stereospecific site by determining binding of an iodine-labeled β-blocker to a high affinity site on turkey erythrocyte membranes. The apparent affinities of either β-adrenergic agonists or blockers for this site parallel biological effectiveness of the compounds as activators or inhibitors of catecholamine-responsive adenylate cyclase in the turkey erythrocyte membrane.

Hydroxybenzylpindolol (HYP) (*4*) was iodinated by a modification (*5*) of the Hunter-Greenwood technique (*6*) and purified by extraction (equal volumes in upper and lower phases) into ethyl acetate from a mixture of 0.02*M* potassium iodide and 1*M* acetic acid. Specific activity of the labeled compound in the ethyl acetate phase was in the range of 200 to 300 c/mmole. The preparation and storage of turkey erythrocyte membranes were as described earlier (*3*). Binding of ^{125}I-labeled HYP was determined by sedimenting the membranes in microcentrifuge tubes (*7*). The amount of ^{125}I-labeled HYP bound to membranes was more than ten times that in the blank (that is, the radioactivity trapped in the centrifuge tube in the absence of added membranes). Adenylate cyclase was assayed (Fig. 1, legend) according to Salomon *et al.* (*8*).

The iodine-labeled inhibitor bound rapidly to erythrocyte membranes. Binding was maximal within 8 minutes at 25°C, and within 4 minutes at 37°C. The biological significance and specificity of binding were determined with catecholamines, analogs, and inhibitors by comparing apparent affinities for the receptor with potency as inhibitors of isoproterenol-stimulated adenylate cyclase. These studies were carried out in parallel under virtually identical conditions of incubation (see Fig. 1). The compounds recognized as β-blockers were effective as inhibitors of isoproterenol-stimulated adenylate cyclase and showed an order of apparent affinity for the binding site on the membranes virtually identical to the order of potencies as inhibitors of isoproterenol-stimulated adenylate cyclase (Fig. 1). The *dl*-HYP is more potent than *l*-(−)-propranolol in adenylate cyclase inhibition as well as in inhibition of binding of ^{125}I-labeled HYP (*9*). Inhibition of binding and inhibition of adenylate cyclase were both dependent on stereochemical configuration; *l*-(−) analogs were greater than 100 times more effective than *d*-(+) analogs. The following compounds tested at $10^{-4}M$ did not inhibit iodine-labeled HYP binding: dihydroxyphenylglycol, dihydroxymandelic acid, and octopamine. Dopamine, metanephrine, and phentolamine (all at $10^{-4}M$) inhibited binding by 7, 13, and 17 percent, respectively. All β-blockers that were effective as inhibitors of isoproterenol-stimulated adenylate cyclase activity as well as inhibitors of specific binding of the iodinated β-blocker were derivatives of ethanolamine. Conversely, nonagonist catechols—for example, dihydroxyphenylglycol or dihydroxymandelic acid—which interact strongly with a catechol-specific site on the membrane (*3*) were ineffective as inhibitors of iodine-labeled HYP binding. On the other hand, biologically effective catecholamines *l*-(−)-isoproterenol or *l*-(−)-