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Loss and Restoration of Sensitivity to Epinephrine in Cultured BHK Cells: Effect of Inhibitors of RNA and Protein Synthesis

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SUMMARY

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Cultured baby hamster kidney fibroblasts were used to study the phenomenon of receptor-mediated tachyphylaxis. Exposure to epinephrine or other β-adrenergic agonists causes the adenylyl cyclase of these cells to become less responsive to stimulation by catecholamines without affecting the response to PGE_1 or NaF. Conversely, preincubation with PGE_1 leads to a selective loss of the response to PGE_1 but not to catecholamines. The loss of responsiveness to epinephrine is concentration dependent, can be prevented by propranolol, occurs rapidly (detectable within 2 min), requires approximately 2 h to reach maximum, and is not secondary to increased levels of cyclic AMP. Recovery from tachyphylaxis (resensitization) is ordinarily a slower process but may occur rapidly in certain circumstances. When tachyphylaxis is allowed to develop fully, as after exposure to epinephrine for 2 h, the cells remain poorly responsive for many hours and may require 24 h for complete resensitization. But if exposure to epinephrine is limited to 20 min or less, then recovery occurs much more rapidly (complete within 30 to 60 min). Inclusion of cycloheximide or actinomycin D during preincubation with epinephrine does not prevent tachyphylaxis but permits rapid recovery even after prolonged exposure to epinephrine. Both the development of tachyphylaxis and the recovery from it are temperature-dependent processes: Cells incubated with epinephrine at 6 instead of 30 or 37°C do not become tachyphylactic, and tachyphylactic cells incubated at low temperatures in the absence of epinephrine do not regain their sensitivity. The data suggest that the interaction of epinephrine with β-receptors in these cells leads to a third effect in addition to the stimulation of adenylyl cyclase and the development of tachyphylaxis. This third effect is slower than the other two and can be inhibited by cycloheximide or actinomycin D, but the nature of the effect is obscure.

INTRODUCTION

Tachyphylaxis, also commonly referred to as refractoriness or densensitization, has long been recognized as a potentially important determinant of drug action (1–3). Tachyphylaxis to catecholamines occurs in humans and laboratory animals and has also been demonstrated in isolated cells, using either the accumulation of cyclic AMP or the stimulation of adenylyl cyclase as the measured response (e.g., 4–14). We have been studying this phenomenon as it occurs in a line of cultured baby hamster kidney fibroblasts (BHK 21 c/13).[2] Although the characteristics of tachyphylaxis appear to differ somewhat from one type of cell to another, even for the same agonist, it was felt that a detailed study in a single type of cell might lead to some principles that could be applied more generally. BHK cells seemed attractive for this purpose because they had been used previously to study the metabolism of cyclic nucleotides (15–18), they possess an adenylyl cyclase system which can be stimulated by prostaglandins as well as by catecholamines (19, 20), and the accumulation of cyclic AMP in them is very small in the absence of a phosphodiesterase inhibitor. We here report our initial observations on the development of tachyphylaxis and the restoration of sensitivity in these cells, including a surprising effect of several

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[2] Abbreviations used: BHK, baby hamster kidney fibroblasts; MEM, minimal essential medium; MIX, 1-methyl-3-isobutylxanthine; PGE_1, prostaglandin E_1.

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drugs used to inhibit protein synthesis. Preliminary reports of some of this work have been presented (20, 21).

MATERIALS AND METHODS

Cell culture. BHK-21 c/13 (American Type Culture Collection) cells were grown in 150-cm^2 plastic culture flasks (Corning Products) containing 40 ml of Eagle's minimal essential medium (MEM) buffered to pH 7.4 with Hepes buffer and supplemented with 10% fetal calf serum (GIBCO). Cells were incubated at 37°C in a humidified atomsphere of air as described previously (19). Stock cultures were grown to densities of approximately 30–50 × 10^6 cells/150-cm^2 flask. For the experiments in which cyclic AMP levels were measured, the cells in the stock cultures were harvested with 0.25% trypsin, and a suspension of 3 × 10^6 cells was added to a constant volume of MEM containing 10% fetal calf serum in 75-cm^2 plastic culture dishes. Following 24 h of incubation at 37°C, the cultures were equilibrated with the fresh medium at 37°C for 1 h, prior to use in the experiment. Additional experimental details are provided in the legends to the figures. For the adenylyl cyclase experiments, the cells similarly harvested from the stock cultures were added to a constant volume of MEM containing 10% fetal calf serum in plastic culture dishes at a density of 6–8 × 10^6 cells/cm^2. Following 24 h of incubation at 37°C, the cultures were used for experiments. The cells were harvested by scraping with a rubber policeman and centrifuged at 1000*g* for 5 min. In some experiments confluent cultures were employed immediately after harvest by scraping.

Cyclic AMP assay. At the end of a challenge incubation with agonist, the medium was poured off and 3 ml of cold 0.4 N perchloric acid was added to cell cultures. Cells were scraped with a rubber policeman and homogenized with a Polytron homogenizer (Brinkman Instruments) at low speed. Denatured protein was removed by centrifugation and supernatant neutralized with 5 N KOH (4°C). After standing in a salted ice bath for 1 h, the precipitate was removed by centrifugation. To the supernatant fluid, 50 μl of 2 M Tris–HCl, pH 7.4, was added and the solution was applied to a small column packed with 1 g of dry alumina oxide (neutral, activity 1, E. Merck) followed by washing with 1 ml of 0.05 M Tris–HCl, pH 7.4. The eluate and washing were combined, the volume was measured, and an aliquot was used directly for determination of cyclic AMP by radioimmunoassay according to Steiner *et al.* (22). All assays were done in duplicate. Protein was measured by dissolving the cell debris in 0.1 N NaOH using the method described by Schacterle and Pollack (23). Bovine serum albumin was the protein standard.

Sonication and adenylyl cyclase assay. The cells harvested by scraping were pelleted by centrifugation, washed with 15 mM Hepes (pH 7.4)/150 mM NaCl (Hepes saline), and resuspended in 50 mM Tris–HCl (pH 7.4)/0.32 M sucrose (Tris–sucrose) in a 40-ml plastic centrifuge tube at 20–30 × 10^6 cells/ml (0.5–1.2 ml). The cells were sonicated using a Biosonic IV sonicator (Bronwill) fitted with a microprobe at a setting of 5 for 10 s as described previously (19). Adenylyl cyclase activity was measured by the method of Thompson *et al.* (24) using a reaction mixture containing 0.2 mM ATP, 1 μCi [α-^{32}P]ATP, 0.25 mg phosphocreatine, 5 units creatine phosphokinase, 5 mM $MgCl_2$, 1 mM cyclic AMP, 0.1% bovine serum albumin, 0.1 mM 3-isobutyl-1-methylxanthine (MIX), 50 mM Tris–HCl, pH 7.4, and sonicated cells in a total volume of 0.2 ml. Incubation was at 30°C for 10 min. Experimental details for the purification and validation of product were described elsewhere (24). All assays were in duplicate or triplicate.

RESULTS

Cyclic AMP responses of cultured cells. Most of our studies involving the measurement of cyclic AMP in intact cells were carried out in the presence of 0.25 mM MIX. Under these conditions the addition of epinephrine caused the level of cyclic AMP to rise rapidly, reaching a maximum within 10 min, following which the level began to decline, even in the continued presence of the agonist. This was qualitatively similar to numerous previous observations, not only with cultured cells but also with more intact tissue preparations (25, 26). The concentration of epinephrine which gave a half-maximal increase after 10 min was approximately 3 × 10^{-7} M.

The level of cyclic AMP in control cells remained constant (at about 5 pmol/mg protein) for at least 4 h. The addition of a supramaximal concentration of epinephrine (10^{-4} M) caused an increase in this level of some 10- to 40-fold, depending on the cell preparation. When the epinephrine was then removed after .5 to 3 h, and the same concentration of epinephrine added again, the response was greatly reduced (usually to about 20% of the response in control cells).

By contrast, when the epinephrine was added in the absence of MIX, the increase in cyclic AMP was barely detectable. Epinephrine was still capable of producing tachyphylaxis under these conditions, however, as shown by the data in Table 1. This indicates that tachyphylaxis in these cells is not a result of increased levels of cyclic AMP.

In confirmation of numerous previous observations with other types of cells, the inclusion of propranolol in the incubation medium did not by itself cause tachyphylaxis but did prevent the rise in cyclic AMP and tachy-

TABLE 1

Development of tachyphylaxis to epinephrine in the absence of a phosphodiesterase inhibitor

Cultures of BHK cells (3 × 10^6 cells/75 cm^2) were incubated with or without 5 × 10^{-6} M epinephrine for 90 min in the absence of MIX. The cells were washed three times with fresh serum-free medium, and then fresh medium containing 10% FCS and 0.25 mM MIX was added to the dish. Following incubation for 30 min, the cells were challenged for 10 min with or without 10^{-4} M epinephrine. Each value is the mean ± SE of five experiments.

Preincubation conditions	Cyclic AMP (pmol/mg protein) challenge incubation	
	None	Epinephrine (10^{-4} M)
Vehicle	4.3 ± 0.4	152 ± 9
Epinephrine (5 × 10^{-6} M)	3.8 ± 0.4	23 ± 3

phylaxis in response to epinephrine. These data suggested that simple occupany of receptors is insufficient to produce tachyphylaxis. Instead, together with the previous data, they suggest that some result of the agonist–receptor interaction other than an increase in cyclic AMP is necessary in order for tachyphylaxis to occur.

The agonist specificity of this effect is shown in Fig. 1. Cells exposed to epinephrine for 2 h were subsequently exposed to a supramaximal concentration of either epinephrine or prostaglandin E_1 (PGE_1). These cells showed a response to PGE_1 comparable to that of control cells, whereas the response to epinephrine was only about 20% of control. The converse experiment, according to which the cells could be made tachyphylactic to PGE_1 with the retention of normal responsitivity to epinephrine, was also performed (data not shown). These data support the conclusion that cyclic AMP is not involved in the production of tachyphylaxis in these cells.

Effect of prior exposure to epinephrine on adenylyl cyclase activity. It was established in preliminary experiments that phosphodiesterase activity in tachyphylactic cells was not significantly greater than in control cells. By contrast, adenylyl cyclase activity was significantly reduced, suggesting that the reduced accumulation of cyclic AMP in tachyphylactic cells could be attributed entirely to lower cyclase activity. Accordingly, adenylyl cyclase activity was used as the endpoint in all subsequent experiments. Figure 2 shows that the maximum epinephrine-stimulated activities of cells pretreated with 10^{-6} and 10^{-4} M epinephrine were 31 and 17% of control, respectively, while the concentration of epinephrine required for half-maximal stimulation increased only slightly, if at all. Through experiments in which the concentrations of both ATP and magnesium were varied, it was established that the lower activity in the presence of epinephrine could be attributed entirely to a reduced velocity of the reaction, with no change in the apparent affinity for either ATP or Mg^{2+}. The stimulatory effects of NaF or PGE_1 were not appreciably altered in any way by prior exposure to epinephrine.

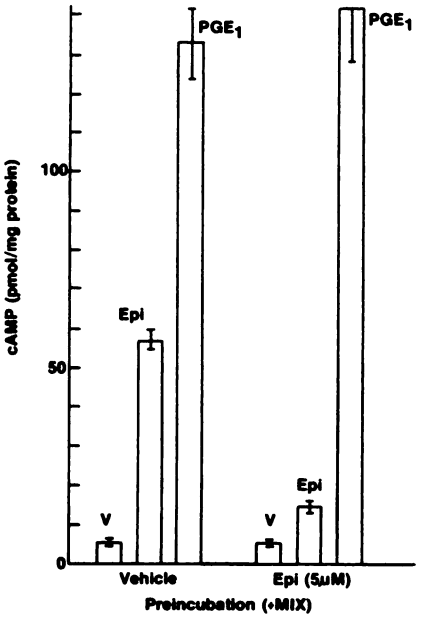

FIG. 1. *Selective loss of the response to epinephrine*

Cultures of BHK cells (3×10^6 cells/75 cm^2) were incubated with 5×10^{-6} M epinephrine or vehicle at 37°C for 90 min in the presence of 0.25 mM MIX. The cells were then washed three times with fresh serum-free medium. After incubating for an additional 30 min with fresh medium containing 10% fetal calf serum and 0.25 mM MIX, the cells were challenged for 10 min with vehicle (V), 10^{-4} M epinephrine (Epi), or 10^{-5} M PGE_1. Each value is the mean ± SE of six experiments.

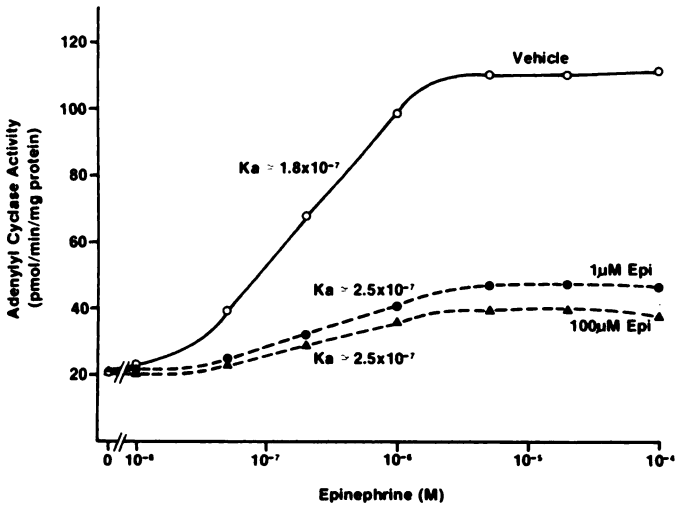

FIG. 2. *Stimulation by epinephrine of adenylyl cyclase from BHK cells preincubated with or without epinephrine*

Twenty-one cultures (5×10^6 cells/75 cm^2) were divided into three groups of 7 cultures each, and each group was incubated with 0, 1, or 100 μM epinephrine at 37°C for 2 h. The cells were then harvested by scraping and washed with Hepes–saline at 4°C to remove bound hormone. Adenylyl cyclase activities were assayed in the presence of graded concentrations of epinephrine or 10 mM NaF. NaF-stimulated activities of cells treated with 0, 1, and 100 μM epinephrine were 169, 171, and 153 pmol/min/mg, respectively.

The dose dependence of the tachyphylactic response is illustrated in Fig. 3. A maximal development of tachyphylaxis was evident at concentrations of epinephrine in the preincubation medium above 5×10^{-6} M, with no appreciable effect on either basal or NaF-stimulated activity. The concentration of epinephrine needed to produce half of the maximum degree of tachyphylaxis was approximately 10^{-7} M. Fortuitously or not, this value correlates closely with the concentration necessary for a half-maximum elevation of cyclic AMP in intact BHK cells (19) or for half-maximal stimulation of cyclase activity in sonicated cells (Fig. 2).

Tachyphylaxis to epinephrine developed very rapidly, as illustrated in Fig. 4. Upon exposure of the cells to 10^{-5} M epinephrine, a 40% reduction in cyclase activity was observed after 5 min, and the reduction was almost complete after 30 min. In another experiment exposure to 10^{-4} M epinephrine caused a 25% reduction of activity within 2 min.

Tachyphylaxis in these cells was also found to be temperature dependent. When the cells were incubated with 10^{-5} M epinephrine at 6°C for up to 2 h, no reduction of adenylyl cyclase responsiveness to the hormone was observed. This was in contrast to the 80% loss of responsiveness observed when the same cells were incubated

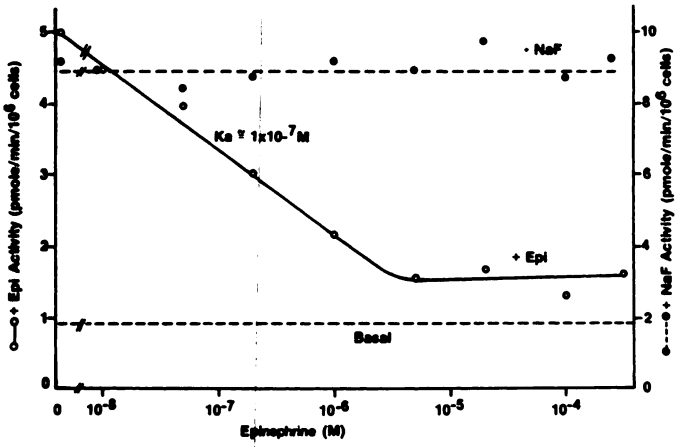

FIG. 3. *Dose dependence of epinephrine-induced tachyphylaxis*

Confluent cells (40–50 × 10^6 cells/175 cm^2) were harvested from T-flasks by scraping. The cells were washed with Hepes–saline at 40°C and resuspended in Hepes–saline containing 1% bovine serum albumin at a density of 30 × 10^6 cells/ml. The cell suspensions were then incubated at 30°C for 1 h in the presence of the indicated concentrations of epinephrine, following which the cells were washed three times with Hepes–saline at 40°C and resuspended in Tris–sucrose. Adenylyl cyclase was then assayed in the presence or absence of 10^{-4} M epinephrine (Epi) or 10 mM NaF.

with epinephrine at 37°C. Since catecholamines are known to bind to β-receptors at temperatures as low as 4°C (27, 28), this is further evidence that receptor occupancy per se is not sufficient to cause tachyphylaxis.

Despite several attempts to do so, we were unable to induce tachyphylaxis in broken cell preparations. If the cells were sonicated and then incubated with epinephrine, instead of the other way around, cyclase activity was the same as it was in control cells that were not incubated with epinephrine (data not shown).

The initial development of tachyphylaxis in these cells does not depend on protein synthesis, as shown by the

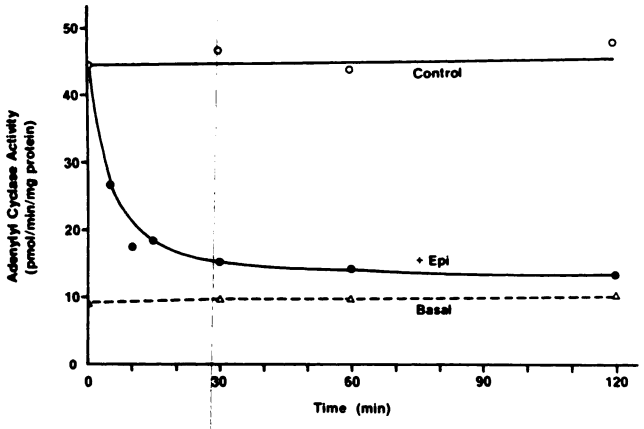

FIG. 4. *Time course of epinephrine-induced tachyphylaxis*

Cells were incubated at a density of 5 × 10^6 cells/75 cm^2 in the presence or absence of 10^{-5} M epinephrine at 37°C for the indicated periods of time. The cells were harvested by scraping, quickly chilled in an ice bath, and washed with Hepes–saline at 4°C. Cells from two plates were combined and used for adenylyl cyclase assay. Open circles, epinephrine (10^{-4} M)-stimulated activity of control cells; closed circles, epinephrine-stimulated activity of epinephrine-treated cells; open triangles, basal activity of control cells. Each value is the mean of three determinations.

results summarized in Table 2. When cultures of low cell density were incubated with cycloheximide for 2 h, basal cyclase activity was reduced to approximately 40% of control, epinephrine-stimulated activity to about 70%, and NaF-stimulated activity only slightly. However, cycloheximide did not prevent the development of tachyphylaxis. The concentrations of cycloheximide used, 2 × 10^{-5} and 10^{-4} M, were found to inhibit the incorporation of [^{3}H]leucine into cellular protein by 80 and 95%, respectively. Cells preincubated with epinephrine under these conditions showed reductions in epinephrine-stimulated cyclase activity of 66 and 65%, respectively, compared to cells preincubated with cycloheximide alone. This was less than the 86% reduction seen in control cells preincubated without cycloheximide, but, in view of the greater inhibitory effect of cycloheximide on basal than on stimulated activity, this difference is difficult to interpret. If it is interpreted as inhibition of tachyphylaxis, the important point is that it is relatively small compared to the almost complete inhibition of protein synthesis. The failure of cycloheximide to prevent tachyphylaxis was confirmed in four separate experiments using different batches of cells each time.

Actinomycin D at a concentration of 0.5 μg/ml, sufficient to inhibit 80–90% of the incorporation of [^{3}H]uridine into intracellular RNA, had no significant effect on ad-

TABLE 2

Lack of effect of inhibitors of protein and RNA synthesis on epinephrine-induced tachyphylaxis

Cultures of BHK cells (5 × 10^6 cells/75 cm^2) were preincubated with the inhibitors at 37°C for 1 h and then further incubated with or without 10^{-5} M epinephrine for 2 h. The cells from two plates that had been incubated under the same conditions were combined and washed with Hepes-saline by centrifuging at 4°C. Adenylyl cyclase activity was assayed in the presence or absence of 10^{-4} M epinephrine or 10 mM NaF. Values are the means of duplicate determinations. The column headed "% Inhibition" indicates the reduction in specific activity of epinephrine-treated cells relative to that of cells not treated with epinephrine.

Inhibitor	Epinephrine	Adenylyl cyclase activity (pmol/min/mg protein)			
		Basal	+ Epinephrine (10^{-4} M)	% Inhibition	+ NaF (10^{-2} M)
Expt 1					
None	−	20.1	55.5		145
None	+	20.7	25.6	86.2	149
Cycloheximide (2 × 10^{-5} M)	−	8.3	37.7		125
Cycloheximide (2 × 10^{-5} M)	+	8.0	18.1	65.6	119
Cycloheximide (10^{-4} M)	−	7.1	34.7		121
Cycloheximide (10^{-4} M)	+	8.1	17.8	64.9	124
Expt 2					
None	−	14.1	50.4		134
None	+	14.4	20.5	83.2	143
Actinomycin D (0.5 μg/ml)	−	13.5	49.3		130
Actinomycin D (0.5 μg/ml)	+	13.4	19.8	82.1	132

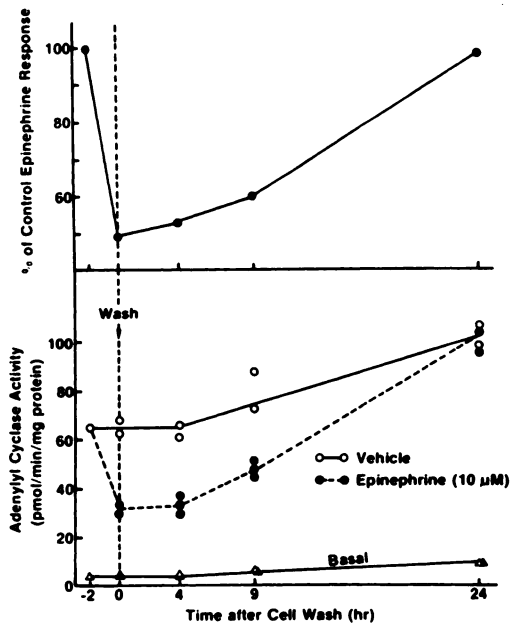

FIG. 5. *Time course of recovery from epinephrine-induced tachyphylaxis*

Cultures of BHK cells (10×10^6 cells/150 cm^2) were incubated with or without 10^{-5} M epinephrine for 2 h at 37°C. The cells were washed four times with fresh medium to remove epinephrine and then further incubated in fresh medium at 37°C. Adenylyl cyclase activity in the presence or absence of 10^{-4} M epinephrine was measured at various intervals thereafter, as indicated. The upper panel displays the epinephrine-stimulated activity from cells that had been exposed to epinephrine as a percentage of the corresponding activity from control cells. The actual data are displayed in the lower panel.

enylyl cyclase and likewise did not affect the development of tachyphylaxis.

Recovery of adenylyl cyclase activity after tachyphylaxis. The recovery of β-adrenergic sensitivity following densensitization of cultured human fibroblasts had been shown to be a slow process, requiring 20–24 h for full recovery (4, 6), and a similar slow recovery was observed with BHK cells (Fig. 5). Only partial restoration of epinephrine sensitivity was observed at 9 h after washing the cells free of hormone; complete recovery was evident after 24 h. The increased activities detected during the recovery incubation were related to cell growth. An increase in the specific activity of adenylyl cyclase during the growth of BHK cells had been reported previously (29).

Although neither cycloheximide nor actinomycin D inhibited the development of tachyphylaxis, the inclusion of these drugs in the incubation medium during the recovery phase, after tachyphylaxis had been induced by preincubating the cells for 2 h in the presence of epinephrine, did markedly inhibit the recovery of epinephrine sensitivity (Table 3). Incubating BHK cells for 24 h with these drugs caused a significant loss of protein from the cell sheets and had a depressing effect on both basal and epinephrine-stimulated cyclase activity (compared to control cells incubated in drug-free medium). Activity in the presence of NaF was less affected, however, and not affected at all by actinomycin D.

When these drugs were included in the preincubation medium during the development of tachyphylaxis, and then removed along with the epinephrine, the surprising discovery was made that the subsequent recovery from tachyphylaxis was greatly facilitated. Figure 6 shows that cells exposed to epinephrine plus cycloheximide during the 2-h preincubation period recovered within minutes after cell washing (instead of hours, as in the case of cells exposed to epinephrine by itself), and similar results were obtained with actinomycin D. Recovery of epinephrine sensitivity under these conditions was usually complete within 30 min, in marked contrast to the slow recovery seen when tachyphylaxis was induced in the absence of RNA or protein synthesis inhibitors (Fig. 5).

This quick recovery process was found to be temperature dependent. When the cells were desensitized to epinephrine in the presence of cycloheximide and then further incubated without epinephrine or cycloheximide at 4°C for 2 h, only a slight recovery was observed,

TABLE 3

Effect of inhibitors of protein and RNA synthesis on recovery from tachyphylaxis

Subcultures of BHK cells were grown to confluency by incubation for 3 days in 75-cm^2 plastic dishes with medium changes every day. The confluent monolayers were preincubated with or without 10^{-5} M epinephrine for 2 h at 37°C. The cells were then washed with the medium to remove the catecholamine and further incubated in fresh medium with various additions for 24 h at 37°C. Adenylyl cyclase activities were measured in the presence or absence of 10^{-4} M epinephrine or 10^{-2} M NaF. Each value is the mean and SE of duplicate determinations on each of three experiments. The column headed "% Inhibition" indicates the reduction in specific activity of epinephrine-treated cells relative to that seen in cells not previously exposed to epinephrine.

Additions		Adenylyl cyclase activity (pmol/min/mg protein)			
Preincubation	Recovery incubation	Basal	+ Epinephrine (10^{-4} M)	% Inhibition	+ NaF (10^{-2} M)
None	No recovery period	5.6 ± 0.3	51.3 ± 1.6		79.9 ± 2.9
Epinephrine	No recovery period	5.8 ± 0.1	20.5 ± 0.1*	67.8	81.1 ± 0.8
None	None	4.4 ± 0.1	40.3 ± 0.8	—	51.7 ± 1.8
Epinephrine	None	4.8 ± 0.4	38.0 ± 2.2	7.5	53.3 ± 0.9
None	Cycloheximide (0.02 mM)	1.4 ± 0.1	19.0 ± 0.1	—	41.8 ± 0.2
Epinephrine	Cycloheximide (0.02 mM)	1.2 ± 0.0	10.6 ± 0.7*	46.6	42.9 ± 2.1
None	Actinomycin D (0.5 μg/ml)	2.7 ± 0.1	20.1 ± 0.4	—	53.3 ± 1.4
Epinephrine	Actinomycin D (0.5 μg/ml)	2.7 ± 0.1	11.7 ± 0.9*	48.3	52.1 ± 0.7

* $P < 0.001$, compared to activity from control cells not incubated with epinephrine; values not marked with an asterisk were not significantly different from control Values ($P > 0.21$).

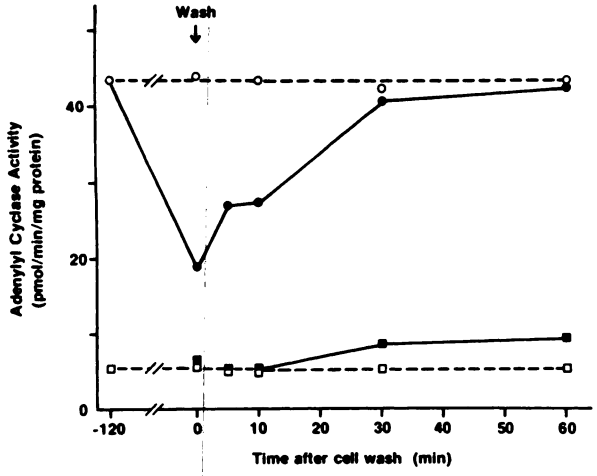

FIG. 6. *Recovery from tachyphylaxis induced by epinephrine in the presence of cycloheximide*

Cultures of BHK cells (5×10^6 cells/75 cm^2) were incubated with 0.02 mM cycloheximide for 1 h and then with cycloheximide plus either vehicle or 10^{-5} M epinephrine for an additional 2 h. The cells were then washed with MEM containing % fetal calf serum and further incubated with fresh medium for various times, as indicated. Two paired cultures were combined and used for the assay of adenylyl cyclase in the presence of absence of 10^{-4} M epinephrine. (○- - -○) Epinephrine-stimulated activity of control cells; (●——●) epinephrine-stimulated activity of epinephrine-treated cells; (□- - -□) basal activity of control cells; (■——■) basal activity of epinephrine-treated cells.

although the control cells which were incubated at 37°C were almost completely recovered (Table 4). The results of other experiments, in which cycloheximide was included in the washing and recovery medium as well as during preincubation, showed that this quick recovery process was not blocked by the inhibitor.

Recovery after short-term exposure to epinephrine. The results presented to this point suggested that while *de novo* protein synthesis was not required for the initial development of tachyphylaxis in response to epinephrine, it might be required for the maintenance of tachyphylaxis. If a protein does exist for this purpose, it might be supposed that a finite period of time would be required in order for it to be synthesized, and the following experiments were performed with this hypothesis in mind.

When cells were exposed to 10 μM epinephrine for 10 min, the stimulation of adenylyl cyclase by epinephrine was reduced to 68% of control. However, as shown in Fig. 7, the response quickly recovered to 100% of control after incubating for just 10 min in the absence of epinephrine. When the cells were exposed to epinephrine for 30 and 60 min, and the epinephrine then removed, they recovered partially in 10 min but no further recovery was evident even after 3 h of incubation (in line with the data shown in Fig. 5). In another experiment not shown in Fig. 7, cells were exposed to 270 μM epinephrine for 20 min, reducing the epinephrine response to 58% of control, but the responsiveness of these cells, like those exposed for only 10 min, was restored completely after incubating for just 10 min without the hormone. Taken together, these data suggest that if a factor is synthesized in response to epinephrine to maintain the cells in an unresponsive state, its accumulation is substantial after 30 min but not after 20 min.

To test this possibility further, cells were incubated with 10 μM epinephrine for various time periods, washed free of hormone, and then incubated in fresh medium for an additional 45 min. Figure 8 shows that cells exposed to epinephrine for up to 15 min recovered almost completely within the 45-min recovery period, whereas cells exposed for 30 min or longer exhibited only a partial recovery. The degree of recovery in these cells was reduced as a function of the time of exposure to epinephrine. Little or no recovery was observed after exposure for 2 h, and, as shown in Fig. 5, complete recovery of these cells required up to 24 h.

TABLE 4

Effect of temperature on restoration of sensitivity from tachyphylaxis induced by epinephrine in the presence of cycloheximide

Cultures of BHK cells (10×10^6 cells/175 cm^2) were preincubated with 0.02 mM cycloheximide for 1 h at 37°C and then with cycloheximide plus either 10^{-5} M epinephrine or vehicle for an addition 2 h. The cells were then washed five times with cold serum-free medium and further incubated with fresh medium at either 4 or 37°C for a 2-h recovery period. Cyclase activity was then measured in the presence or absence of 10^{-4} M epinephrine. Values are the means of two paired experiments ± half the range of variation. The column headed "% Inhibition" indicates the reduction in specific activity of epinephrine-treated cells relative to the activity of control cells.

Preincubation	Recovery incubation	Adenylyl cyclase activity (pmol/min/mg protein)		
		Basal	+ Epinephrine	% Inhibition
Cycloheximide	No recovery period	2.0 ± 0.0	23.0 ± 2.1	—
Epinephrine + cycloheximide	No recovery period	2.7 ± 0.2	11.7 ± 1.5	57.1
Cycloheximide	4°C, 2 h	2.3 ± 0.4	22.5 ± 0.1	—
Epinephrine + cycloheximide	4°C, 2 h	2.6 ± 0.1	13.8 ± 0.7	44.6
Cycloheximide	37°C, 2 h	2.0 ± 0.2	27.4 ± 1.7	—
Epinephrine + cycloheximide	37°C, 2 h	2.4 ± 0.4	26.0 ± 1.6	7.1

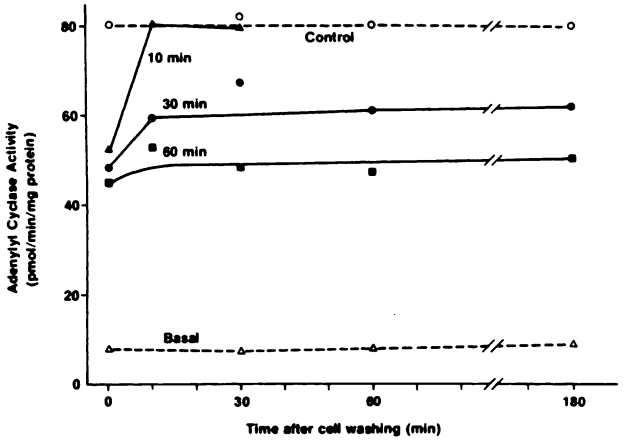

FIG. 7. *Restoration of sensitivity after short-term exposure to epinephrine*

Cells were preincubated with 10^{-5} epinephrine for 10, 30, and 60 min, following which they were washed four times to remove hormone. They were then incubated in fresh medium for various times, as indicated, and adenylyl cyclase activity was measured in the presence or absence of 10^{-4} M epinephrine. Dashed lines indicate basal (△) and epinephrine-stimulated (○) activities of control cells; solid lines indicate epinephrine-stimulated activities of cells that had been preincubated with epinephrine for 10 (▲), 30 (●), and 60 (■) min.

The existence of a protein which is synthesized in response to epinephrine, and which acts to delay the restoration of sensitivity to epinephrine, was also suggested by the data shown in Table 5. In these experiments the cells were incubated with epinephrine for 2 h, and cycloheximide was added either simultaneously with or at various times after the addition of epinephrine. In the cells which received cycloheximide at 30 or 60 min after epinephrine, the recovery after a 1-h incubation in the absence of hormone was not much different from that of control cells which were not exposed to cycloheximide at all. By contrast, in cells which received the inhibitor at the same time as or within 10 min of the addition of epinephrine, the recovery was between 77 and 91% complete, approaching the 100% recovery seen when cycloheximide was added 1 h before the epinephrine, as in the experiment of Fig. 5.

The rate of recovery after tachyphylaxis had been induced by incubating with epinephrine for 2 h in the absence of an inhibitor was quite variable, as noted previously in the case of human fibroblasts incubated with isoproterenol (6). In some experiments no recovery was observed even after 4 h (as in Fig. 5), whereas in other experiments between 10 and 30% recovery was seen after as little as 1 h (as in Table 5).

TABLE 5

Recovery of sensitivity to epinephrine as a function of the time of addition of cycloheximide

BHK cells (5×10^6 cells/75 cm^2) were cultured for 3 days, with medium changed on the second day and 1 h before the experiment. Epinephrine (10^{-5} M) was added at time 0 and the cultures were incubated at 37°C for 2 h. Cycloheximide (2×10^{-3} M) was added either simultaneously with or at various times after the epinephrine, as indicated. After the cells had been exposed to epinephrine for 2 h, they were washed four times and incubated for an additional 1 h with fresh medium. Adenylyl cyclase was assayed in the presence or absence of 10^{-4} M epinephrine. "% Resensitization" is the ratio of (recovered activity − desensitized activity) to (control activity − densensitized activity) × 100. In this experiment, epinephrine-simulated activity of control cells was 44.8 ± 0.5 and that of desensitized cells was 20.2 ± 1.8 pmol/min/mg protein. Cycloheximide had essentially no effect on epinephrine sensitivity of control cells or on the development of tachyphylaxis. Values are the means ± half the range of two paired experiments in duplicate.

Time of addition (min)	% Resensitization
No cycloheximide added	33.8 ± 1.4
0	77.2 ± 2.0
10	91.2 ± 8.5
30	39.8 ± 7.3
60	31.5 ± 2.9

DISCUSSION

There are a number of similarities between BHK cells and other cells in which tachyphylaxis to epinephrine or other β-adrenergic agonists has been studied. As in the human fibroblasts studied by Franklin and his colleagues (4, 6), tachyphylaxis occurs rapidly, is agonist specific, and can be prevented by β-adrenergic blocking agents; recovery is a slower process which can be inhibited by drugs that inhibit RNA or protein synthesis. It seems likely, on the basis of the available data, that human fibroblasts and BHK cells respond to catecholamines in a fundamentally similar way. We have also established that tachyphylaxis in BHK cells is associated with a selective diminution of the maximal rate of catecholamine-stimulated adenylyl cyclase activity, rather than with a change in phosphodiesterase activity, in which regard these cells seem superficially similar to frog erythrocytes (7, 30), rat glioma cells (12), and mouse lymphoma cells (9). Agonist specificity has not been demonstrated in all of these cells, due to the lack of suitable alternate agonists, but tachyphylactic S49 cells were shown to be capable of responding to cholera toxin (9). There seems little doubt that it is the ability of the β-receptors to mediate the stimulation of adenylyl cyclase, rather than the adenylyl cyclase itself, which is defective in these cells.

There are also some differences between BHK and other cells, notably frog erythrocytes. Neither the induction of tachyphylaxis nor the restoration of sensitivity

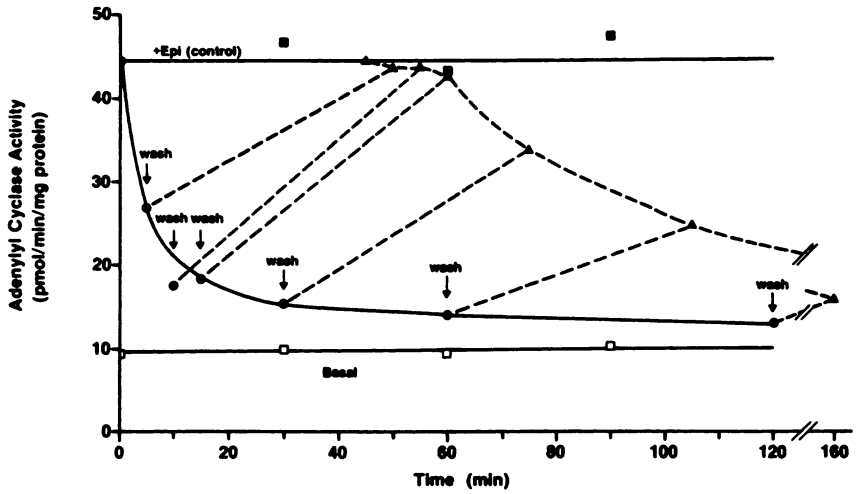

FIG. 8. *Restoration of sensitivity as a function of the time of exposure to epinephrine*

Cells were incubated with 10^{-5} M epinephrine for various times. They were then washed and either harvested immediately or incubated for an additional 45 min in fresh medium. Activities were measured in the presence of 10^{-4} M epinephrine before (●) and after (▲) the recovery incubation. Basal (□) and epinephrine-stimulated (■) activities of control cells that were not exposed to epinephrine are also indicated.

depended on temperature in frog erythrocytes (31), whereas both processes were temperature dependent in BHK cells. However, this difference might reflect differences between poikilothermic and mammalian cells rather than fundamental differences in the actual processes involved. A temperature dependence for tachyphylaxis similar to that seen with BHK cells has recently been reported for both rat glioma cells (32) and S49 lymphoma cells (33).

Another possibly trivial difference was that we were unable to induce tachyphylaxis by incubating broken cell preparations with epinephrine, whereas this appears to be possible with membrane preparations of frog erythrocytes (34) as well as several mammalian tissues (35). In the case of the frog erythrocytes, it is possible that the tachphylaxis produced in the cell-free preparation was fundamentally different from that produced in intact cells, since the refractoriness of adenylyl cyclase to stimulation by catecholamines could be reversed by the addition of guanyl nucleotides in the former case (34) but not the latter (30). In the case of the mammalian preparations, it should be noted that very high concentrations of Mg^{2+} were required (35) and also that the process was very slow compared to what happens in BHK cells (Fig. 4). Perhaps the most reasonable explanation for our failure to produce tachyphylaxis in sonicated cells is that the concentrations of Mg^{2+} and probably other factors become so diluted upon cell breakage that the reaction(s) responsible for tachyphylaxis cannot occur. The restoration of sensitivity has not to our knowledge been observed in a broken cell preparation under any conditions.

Still another difference between BHK fibroblasts and frog erythrocytes is that doses of cycloheximide capable of inhibiting protein synthesis by up to 75% in rat liver did not inhibit the restoration of sensitivity to catecholamines in frog erythrocytes (31). An inability of cycloheximide or actinomycin D to interfere with the restoration of sensitivity after tachyphylaxis was also noted in the case of Ehrlich ascites cells (8). These results may or may not reflect fundamental differences in the mechanisms by which different cells regain their sensitivity to agonists after the induction of tachyphylaxis. It is interesting to note in this regard that BHK cells rendered tachyphylactic in the presence of cycloheximide resemble Ehrlich ascites cells quite closely, in that the rapid restoration of sensitivity seen after removal of the agonist is not prevented by cycloheximide or actinomycin D in either case.

Indeed one of our most interesting observations was that when tachyphylaxis was induced in the presence of either cycloheximide or actinomycin D, the subsequent recovery from tachyphylaxis was greatly facilitated. This finding is reminiscent of observations made previously by other investigators studying tachyphylaxis to catecholamines in C6 glioma cells (12, 36) and to TSH in cultured thyroid cells (37), but there are also some differences. For example, the development of tachyphylaxis in both the glioma and the thyroid cells could actually be prevented by the addition of cycloheximide, whereas this was not the case in BHK cells. It was also shown that tachyphylaxis in glioma cells could be rapidly reversed by cycloheximide, even when the cycloheximide was added many hours after the agonist, which is in further contrast to the behavior of BHK cells (which are in this respect similar to thyroid cells rendered tachyphylactic to TSH).

One conclusion which can be drawn from these results is that the interaction of a catecholamine with β-adrenergic receptors in BHK cells leads to at least two events which are partially independent of each other. One is the stimulation of adenylyl cyclase, while the other is an event or events leading to the initial development of tachyphylaxis. Our initial interpretation of the results with the protein synthesis inhibitors was that there might also be a third event, namely, the induction of a protein which acts to maintain the cells in a refractory state even after the hormone has been removed. The results of our experiments to this point seem compatible with this interpretation, and yet we now feel that an alternate and possibly simpler interpretation should be considered. This is based on the evidence that in some cells tachyphylaxis is followed by a loss of receptors (14) and that the loss or internalization of receptors (38, 39) occurs by an endocytotic process involving the action of transglutaminase (39). It seems possible that in BHK cells the receptors are converted by the agonist first to a form capable of stimulating adenylyl cyclase and then to a tachyphylactic form incapable of doing this, following which they become susceptible to endocytosis. If the latter step were inhibited by cycloheximide or actinomycin D, then it is easy to see how these drugs might facilitate the restoration of sensitivity following the removal of the agonist. Experiments to distinguish between these two hypotheses are currently in progress.

An additional conclusion which is now more obviously true than it was (40) is that there are some β-adrenergic effects which are not mediated by cyclic AMP. One is the development of tachyphylaxis, while another appears to be the inhibition of magnesium uptake (41).

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REFERENCES

1. Barsoum, G. S. and J. H. Gaddum. The pharmacological estimation of adenosine and histamine in blood. *J. Physiol.* **85**: 1–14 (1935).
2. Cantoni, G. L., and G. J. Eastman. On the response of the intestine to smooth muscle stimulants. *J. Pharmacol. Exp. Ther.* **87**: 392–399 (1946).
3. Katz, B., and S. Thesleff. A study of the desensitization produced by acetylcholine at the motor end-plate. *J. Physiol.* **138**: 63–80 (1957).
4. Franklin, T. J., and S. J. Foster. Hormone-induced desensitization of hormonal control of cyclic AMP levels in human diploid fibroblasts. *Nature New Biol.* **246**: 146–148 (1973).
5. Strada, S. J., and B. Weiss. Increased response to catecholamines of the cyclic AMP system of rat pineal gland induced by decreased sympathetic activity. *Arch. Biochem. Biophys.* **160**: 197–204 (1974).
6. Franklin, T. J., W. P. Morris and P. A. Twose. Desensitization of beta adrenergic receptors in human fibroblasts in tissue culture. *Mol. Pharmacol.* **11**: 485–491 (1975).
7. Mickey, J., R. Tate and R. J. Lefkowitz. Subsensitivity of adenylate cyclase and decreased β-adrenergic receptor binding after chronic exposure to isoproterenol in vitro. *J. Biol. Chem.* **250**: 5727–5729 (1975).
8. Lauzon, G. J., S. Kulshrestha, L. Starr and H. P. Bar. On the accumulation of cyclic AMP in Ehrlich cells and adenylate cyclase desensitization in response to epinephrine. *J. Cyclic Nucleotide Res.* **2**: 90–114 (1976).
9. Shear, M., P. A. Insel, K. L. Melmon and P. Coffino. Agonist-specific refractoriness induced by isoproterenol: Studies with mutant cells. *J. Biol. Chem.* **251**: 7572–7576 (1976).

10. Conolly, M. E., and J. K. Greenacre. The lymphocyte β-adrenoceptor in normal subjects and patients with bronchial asthma. *J. Clin. Invest.* **58:** 1307–1316 (1976).
11. Perkins, J. P., G. L. Johnson and T. K. Harden. Drug-induced modification of the responsiveness of adenylate cyclase to hormones. *Advan. Cyclic Nucleotide Res.* **9:** 19–32 (1978).
12. Terasaki, W. L., G. Brooker, J. deVellis, D. Inglish, C.-Y. Hsu and R. D. Moylan. Involvement of cyclic AMP and protein synthesis in catecholamine refractoriness. *Advan. Cyclic Nulceotide Res.* **9:** 33–52 (1978).
13. Barber, R., R. B. Clark, L. A. Kelly and R. W. Butcher. A model of desensitization in intact cells. *Advan. Cyclic Nucleotide Res.* **9:** 507–516 (1978).
14. Su, Y.-F., T. K. Harden and J. P. Perkins. Isoproterenol-induced desensitization of adenylate cyclase in human astrocytoma cells. *J. Biol. Chem.* **254:** 38–41 (1979).
15. Pledger, W. J., W. J. Thompson and S. J. Strada. Effects of growth conditions on cyclic nucleotide phosphodiesterases of cultured fibroblasts. *J. Cyclic Nucleotide Res.* **1:** 251–259, 1975.
16. Pledger, W. J., W. J. Thompson and S. J. Strada. Mediation of serum-induced changes in cyclic nucleotide levels in cultured fibroblasts by cyclic nucleotide phosphodiesterase. *Nature* **256:** 729–731 (1975).
17. Pledger, W. J., W. J. Thompson and S. J. Strada. Serum modification of cyclic nucleotide phosphodiesterase independent of protein synthesis. *Biochem. Biophys. Res. Commun.* **70:** 58–65 (1976).
18. Strada, S. J. and W. J. Thompson. Multiple forms of cyclic nucleotide phosphodiesterase: Anomalies or biologic regulators? *Advan. Cyclic Nucleotide Res.* **9:** 265–283 (1978).
19. Achar, S. B., S. J. Strada, R. B. Sanders, W. J. Pledger, W. J. Thompson and G. A. Robison. Sonication as a tool for the study of adenylyl cyclase activity. *J Cyclic Nucleotide Res.* **3:** 189–198 (1977).
20. Morishima, I., G. A. Robison, W. J. Thompson and S. J. Strada. Mechanism of catecholamine desensitization of cultured fibroblasts. *Pharmacologist* **18:** 186 (1976).
21. Morishima, I., S. J. Strada, W. J. Thompson and G. A. Robison. Studies on the loss and restoration of sensitivity of BHK cells to stimulation by beta adrenergic agonists. *Advan. Cyclic Nucleotide Res.* **9:** 780 (1978).
22. Steiner, A. L., R. E. Wehmann, C. W. Parker and D. M. Kipnis. Radioimmunoassay for the measurement of cyclic nucleotides. *Advan. Cyclic Nucleotide Res.* **2:** 51–61 (1972).
23. Schacterle, G. R. and R. L. Pollack. A simplified method for the quantitative assay of small amounts of protein in biologic material. *Anal. Biochem.* **51:** 654–655 (1973).
24. Thompson, W. J., R. J. Williams and S. A. Little. Studies on the assay and activities of guanyl and adenyl cyclases of rat liver. *Arch. Biochem. Biophys.* **159:** 206–213 (1973).
25. Robison, G. A., R. W. Butcher, I. Øye, H. E. Morgan and E. W. Sutherland. The effect of epinephrine on cyclic AMP levels in the isolated perfused rat heart. *Mol. Pharmacol.* **1:** 168–177 (1965).
26. Kakiuchi, S., and T. W. Rall. The influence of chemical agents on the accumulation of cyclic AMP in slices of rabbit cerebellum. *Mol. Pharmacol.* **4:** 367–378 (1968).
27. Brown, E. M., G. D. Aurbach, D. Hauser and F. Troxler. β-Adrenergic receptor interactions: characterization of iodohydroxybenzylpindolol as a specific ligand. *J. Biol. Chem.* **251:** 1232–1238 (1976).
28. Maguire, M. E., E. M. Ross and A. G. Gilman. The β-adrenergic receptor: Ligand binding properties and the interaction with adenylyl cyclase. *Advan. Cyclic Nucleotide Res.* **8:** 1–83 (1977).
29. Achar, S. B., S. J. Strada, R. B. Sanders, G. A. Robison and W. J. Thompson. Hormonal regulation of adenylyl cyclase of cultured fibroblasts. *Fed. Proc.* **35:** 423 (1976).
30. Williams, L. T. and R. J. Lefkowitz. Slowly reversible binding of catecholamine to a nucleotide-sensitive state of the beta adrenergic receptor. *J. Biol. Chem.* **252:** 7207–7213 (1977).
31. Mukherjee, C., M. G. Caron and R. J. Lefkowitz. Regulation of adenylate cyclase coupled β-adrenergic receptors by β-adrenergic catecholamines. *Endocrinology* **99:** 347–357 (1976).
32. Nickols, G. A. and G. Brooker. Temperature sensitivity of cyclic AMP production and catecholamine-induced refractoriness in a rat astrocytoma cell line. *Proc. Nat. Acad. Sci. USA* **75:** 5520–5524 (1978).
33. Insel, P. A. and M. Sanda. Temperature-dependent changes in binding to beta adrenergic receptors of intact S49 lymphoma cells. *J. Biol. Chem.* **254:** 6554–6559 (1979).
34. Mukherjee, C., and R. J. Lefkowitz. Regulation of beta adrenergic receptors in isolated frog erythrocyte plasma membranes. *Mol. Pharmacol.* **13:** 291–303 (1977).
35. Bockaert, J., M. Hunzicker-Dunn and L. Birnbaumer. Hormone-stimulated desensitization of hormone-dependent adenylyl cyclase. *J. Biol. Chem.* **251:** 2653–2663 (1976).
36. DeVellis, J. and G. Brooker. Reversal of catecholamine refractoriness by inhibitors of RNA and protein synthesis. *Science* **186:** 1221–1223 (1974).
37. Rapoport, B., and R. J. Adams. Induction of refractoriness to thyrotropin stimulation in cultured thyroid cells. *J. Biol. Chem.* **251:** 6653–6661 (1976).
38. Chuang, D.-M., and E. Costa. Evidence for internalization of the recognition site of β-adrenergic receptors during receptor subsensitivity induced by isoproterenol. *Proc. Nat. Acad. Sci. USA* **76:** 3024–3028 (1979).
39. Davies, P. J. A., D. R. Davies, A. Levitzki, F. R. Maxfield, P. Milhaud, M. C. Willingham and I. H. Pastan. Transglutaminase is essential in receptor-mediated endocytosis of α_2-macroglobulin and polypeptide hormones. *Nature* **283:** 162–167 (1980).
40. Robison, G. A., R. W. Butcher and E. W. Sutherland. *Cyclic AMP.* Academic Press, New York (1971).
41. Maguire, M. E. and J. J. Erdos. Inhibition of magnesium uptake by adrenergic agonists and prostaglandin E_1 is not mediated by cyclic AMP. *J. Biol. Chem.* **225:** 1030–1035 (1980).

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