Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 2246–2250, April 1987
Biochemistry

Diphtheria toxin prevents catecholamine desensitization of A431 human epidermoid carcinoma cells

(refractoriness/β-adrenergic receptor/cAMP/protein synthesis/elongation factor 2)

MARIADELE DEBERNARDI AND GARY BROOKER*

Fidia-Georgetown Institute for the Neurosciences and Department of Biochemistry, Georgetown University Medical Center, Washington, DC 20007

Communicated by G. D. Aurbach, December 24, 1986 (received for review October 9, 1986)

ABSTRACT **We proposed that a rapidly turning over protein, induced in response to catecholamine stimulation of C6-2B rat astrocytoma cells, inhibits subsequent hormonal activation of adenylate cyclase. Studies upon which our hypothesis is based and confirmatory work in a variety of other cell lines and *in vivo* have utilized actinomycin D and cycloheximide to inhibit RNA and protein synthesis, respectively. These inhibitors, however, are not specific and have been reported also to interfere with other cellular processes. Diphtheria toxin is a specific protein synthesis inhibitor that acts only by ADP-ribosylating elongation factor 2, thus preventing peptide chain elongation. We thus tested whether diphtheria toxin could prevent catecholamine-induced desensitization in A431 human epidermoid carcinoma cells. The toxin inhibited protein synthesis and altered the time course of isoproterenol-stimulated cAMP accumulation as did the less-specific protein synthesis inhibitor cycloheximide. Cellular cAMP content after a 30-min exposure to isoproterenol was similar in control and in toxin-treated cells. However, after 4 hr of treatment with isoproterenol, toxin-treated cells accumulated up to six times more cAMP than controls. When cells or cell-free adenylate cyclase preparations were rechallenged with agonists, toxin-mediated inhibition of protein synthesis prevented desensitization. These results show that diphtheria toxin, a specific inhibitor of protein synthesis, can interfere with the normal physiological regulation of cAMP metabolism in eukaryotic cells and provide compelling evidence that catecholamine stimulation of adenylate cyclase promotes the synthesis of a protein(s) that, in some way, inhibits hormone-stimulated adenylate cyclase.**

The events and sites responsible for desensitization of the β-adrenergic receptor-coupled adenylate cyclase system are still poorly understood even though the three basic components needed for hormone-mediated activation of adenylate cyclase (receptors, GTP-binding proteins, and catalyst) have now been purified (1–6), and functionally reconstituted (7, 8). Furthermore, several of the components have now been cloned (9, 10). Desensitization could be related to changes in β-receptor localization (11), the extent of β-receptor phosphorylation (12), or reduced adenylate cyclase catalyst activity (13). Changes in guanine nucleotide-coupling proteins, however, have not been observed during desensitization (14). Our earlier studies in several cell systems, most notably the C6-2B rat astrocytoma cell line, showed that inhibitors of RNA or protein synthesis, such as actinomycin D or cycloheximide, reduce or reverse refractoriness induced by prolonged exposure of cells to β-adrenergic agonists (15) or N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate (Bt_2cAMP) (16). Subsequent studies showed that protein synthesis inhibitors prevent hormonal desensitization in diverse cell types activated by a variety of hormones (17–23).

The hypothesis, developed from our original experiments, that protein synthesis is required for cAMP induction of refractoriness was based on use of inhibitors lacking precise specificity. Some effects of cycloheximide and actinomycin D are unrelated to protein or RNA synthesis. Cycloheximide directly inhibits cathepsin D (24), DNA-dependent RNA polymerase I (25), UDP-galactosyltransferase (26), gluconeogenesis (27), glucose uptake (28), DNA synthesis (29), and even has been reported to stabilize mRNA coding for tyrosine aminotransferase and phospho*enol*pyruvate carboxykinase (30). There are several side effects of actinomycin D (31–38) including inhibition of rRNA synthesis, phospholipid synthesis, respiration, aerobic glycolysis, protein synthesis not related to inhibition of mRNA synthesis, and degradation of a number of enzymes.

We have now used diphtheria toxin, a specific inhibitor of protein synthesis to further test our hypothesis. The toxin specifically inhibits protein synthesis by catalyzing the transfer of ADP-ribose from NAD to elongation factor 2, thereby preventing peptide chain elongation during protein synthesis (39–41). We found that A431 cells (42) become desensitized after prolonged stimulation by isoproterenol, and this desensitization is blocked in cells incubated with diphtheria toxin. These experiments support the hypothesis that the synthesis of a protein(s) is required for development of catecholamine-induced refractoriness.

MATERIALS AND METHODS

Cell Culture. A431 cells were obtained from the American Type Culture Collection or were a generous gift of Stuart Aaronson (National Cancer Institute, Bethesda, MD). They were grown as monolayers in 24-well cluster plates in Ham's F-10 nutrient medium (GIBCO) buffered with 14 mM $NaHCO_3$, supplemented with 10% (vol/vol) donor calf serum (GIBCO) in a humidified atmosphere of 95% air/5% CO_2 at 37°C. Cells between passage 17 and 38 were plated in 2 ml of medium at 1.5×10^4 cells per ml and used at confluency. Diphtheria toxin (1 ng/ml) was added to cells in Ham's F-10 medium containing 10% (vol/vol) donor calf serum for 24 hr as indicated. The cells were then washed with serum-free Ham's F-10 containing 10 mM Hepes (pH 7.4) (Ham's F-10/Hepes) and treated as indicated to test the effect of diphtheria toxin pretreatment on catecholamine desensitization. The effect of cycloheximide upon isoproterenol-induced refractoriness was evaluated after coincubation of cells with 10 μM (−)-isoproterenol and cycloheximide at 5 μg/ml. 1-Methyl-3-isobutylxanthine (0.1 mM) was included in all 30-min or 4-hr incubation solutions.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: Bt_2cAMP, N^6,$O^{2'}$-dibutyryladenosine 3',5'-cyclic monophosphate.

*To whom reprint requests should be addressed.

cAMP Assay. The cAMP content of the cells was determined by rapidly aspirating the incubation medium and treating the cells with 0.1 M HCl containing 0.1 mM $CaCl_2$. The samples were agitated on an orbital shaker for 15 min at room temperature. The acid extracts were assayed for cAMP content using the Gamma-Flo automated radioimmunoassay system (43) after acetylation as described by Harper and Brooker (44). Protein precipitated with 0.1 M HCl was dissolved in 0.2 M NaOH and measured by the Bradford assay (45) using bovine serum albumin as standard.

Permeabilization Procedure. Cells were permeabilized by the method of Brooker and Pedone (46) with slight modification. After appropriate pretreatment, the cells were rendered permeable at 22°C by first washing once with Ham's F-10/Hepes. The cells were then changed to permeabilization buffer [1.3% (vol/vol) PEG 8000, 33 mM Pipes, 2 mM EGTA, 2 mM $MgCl_2$, 5.4 mM KCl, 0.44 mM KH_2PO_4, 137 mM NaCl, 0.34 mM Na_2HPO_4, and 5.6 mM glucose, pH 7.2], 1 ml per well, for 1 min. The buffer was then rapidly removed and replaced with 0.5 ml per well of a solution of 0.01% digitonin in permeabilization buffer. After 5 min, the cells were washed twice with 1 ml of digitonin-free permeabilization buffer and allowed to incubate for 15 min. Cells were washed once more with digitonin-free permeabilization buffer and then used for the adenylate cyclase assay.

Adenylate Cyclase Assay. The adenylate cyclase assay was initiated by replacing the last buffer wash with 0.5 ml per well of permeabilization buffer containing 0.1 mM ATP, 10 μM GTP, 0.1 mM 1-methyl-3-isobutyl-xanthine (to prevent the breakdown of cAMP), and 3 mM $MgCl_2$ with the appropriate drugs. It remained on the cells for 30 min at 22°C without agitation. The reaction was stopped by removing a 200-μl aliquot from each well and adding it to 1.8 ml of 0.1 M HCl containing 0.1 mM $CaCl_2$. The extracts were then assayed for cAMP as described above.

Protein Synthesis. Cells were labeled for 30 min at 37°C with [^{35}S]methionine (1 μCi/ml; 1 Ci = 37 GBq) in serum-free medium. Protein was precipitated with 10% (wt/vol) trichloroacetic acid, and the cells were solubilized in 1 ml of 0.2 M NaOH. Protein synthesis was measured by the incorporation of [^{35}S]methionine and expressed as a percent of control cells incubated under the same conditions.

Diphtheria toxin was purchased from List Biological Laboratories (Campbell, CA). Cycloheximide, 1-methyl-3-isobutylxanthine, ATP, GTP, and (−)-isoproterenol HCl were obtained from Sigma. Digitonin was purchased from BDH. [^{35}S]Methionine (>800 Ci/mmol) was obtained from Amersham. Other reagents were as described or were American Chemical Society quality or better.

RESULTS

Cycloheximide Prevents Isoproterenol-Induced Refractoriness in A431 Cells. Pretreatment of A431 cells with cycloheximide (5 μg/ml) for 4 hr does not significantly affect cAMP accumulation induced by a 30-min challenge with 10 μM (−)-isoproterenol (Fig. 1*A*). Protein synthesis was inhibited more than 90%. After a 4-hr isoproterenol treatment in the absence of cycloheximide, cells showed desensitization such that only 10% of the original response to a subsequent 30-min rechallenge with 10 μM (−)-isoproterenol is observed. In contrast, coincubation of cycloheximide with isoproterenol for 4 hr caused a 7-fold greater cAMP response after a subsequent 30-min rechallenge with isoproterenol (Fig. 1*B*). Cycloheximide-treated cells, in fact, retain 65% of their responsiveness to isoproterenol when rechallenged with the agonist. The data in Fig. 1*C* show that cycloheximide coincubation with 1 mM Bt_2cAMP for 4 hr totally protects the cells from Bt_2cAMP-induced refractoriness.

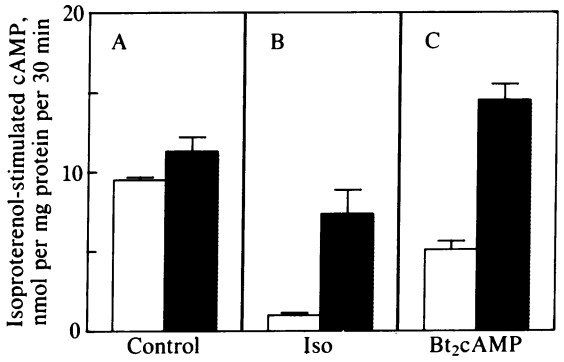

FIG. 1. Cycloheximide prevents isoproterenol and Bt_2cAMP-induced refractoriness. A431 cells were incubated for 4 hr, with (solid bars) or without (open bars) cycloheximide at 5 μg/ml in medium (control, *A*), in medium containing 10 μM (−)-isoproterenol (Iso, *B*), or in medium containing 1 mM Bt_2cAMP (*C*). Cells were then washed five times with Ham's F-10/Hepes (or with Ham's F-10/Hepes containing cycloheximide for cells treated with cycloheximide). cAMP was measured after a 30-min challenge with 10 μM (−)-isoproterenol. The data represent the mean ± SEM of three determinations. There was no difference ($P < 0.08$) in isoproterenol-stimulated cAMP accumulation between control cells with cycloheximide alone and cells incubated with cycloheximide plus Bt_2cAMP.

Effect of Diphtheria Toxin on A431 Cell Protein Synthesis. The time course of cellular protein synthesis was determined in cells treated with the toxin at 1, 10, and 100 ng/ml. As shown in Fig. 2, after 24 hr, all the concentrations tested caused complete inhibition of [^{35}S]methionine incorporation into protein. Cell viability was tested with trypan blue after 24 hr. Cells treated with 10 or 100 ng/ml were not viable; however, greater than 90% of the cells treated with 1 ng/ml excluded trypan blue and were thus viable. At 4 and 6 hr, trypan blue was excluded from the cells even at the highest concentration of toxin used, although this concentration had inhibited protein synthesis more than 90%. Based on these results, we chose to conduct all subsequent studies after intoxication (toxin treatment) of cells for 20–24 hr with diphtheria toxin at 1 ng/ml. This produced protein synthesis-inhibited cells that remained viable during the time necessary to treat the cells for 4 hr with isoproterenol, to remove cAMP accumulated during this first incubation, and subsequently to measure cellular responsiveness to a 30-min rechallenge with isoproterenol.

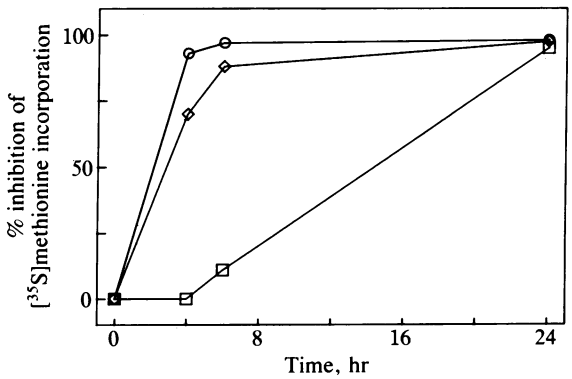

FIG. 2. Time course of diphtheria toxin-dependent inhibition of protein synthesis in A431 cells. A431 cells were incubated with Ham's F-10 medium containing 10% (vol/vol) calf serum and diphtheria toxin at 1 (□), 10 (◇), or 100 (○) ng/ml. Protein synthesis was measured for 30 min at 37°C at the indicated times and expressed as percent of control cell incorporation of [^{35}S]methionine into protein.

Table 1. Effect of 10 μM (−)-isoproterenol on A431 cell cAMP content

Time	cAMP, pmol/mg of protein	
	Control cells	Diphtheria toxin-treated cells
30 min	3441 ± 433	2731 ± 317
4 hr	190 ± 32	1208 ± 57

Basal level of cell cAMP was 5–15 pmol/mg of protein.

Diphtheria Toxin Prevents Isoproterenol-Induced Desensitization. As shown in Table 1, isoproterenol-stimulated cAMP accumulation was not appreciably different in control or diphtheria toxin-treated cells after 30 min, yet was about 6-fold higher in toxin-treated cells after a 4-hr isoproterenol exposure. Thus, preincubation with diphtheria toxin for 24 hr did not significantly affect acute cAMP accumulation stimulated by a 30-min challenge with 10 μM (−)-isoproterenol but retarded the decline in cellular cAMP at longer time points. When the cells were exposed to isoproterenol for 4 hr, the diphtheria toxin-pretreated cells showed an almost complete, and in some experiments total, retention of sensitivity to the agonist upon a 30-min isoproterenol rechallenge (Fig. 3*A*). Strikingly, after a 30-min rechallenge with 10 μM (−)-isoproterenol, desensitized control cells had only 7.6% of their original responsiveness while diphtheria toxin-treated cells had almost 95% of the response to isoproterenol.

Adenylate Cyclase Activity from Permeabilized A431 Cells. We performed preliminary experiments on 0.01% digitonin-permeabilized A431 cells and tested the adenylate cyclase activity of both control and intoxicated cells after different drug treatments. Fig. 3*B* shows that permeabilized cells (which now all take up trypan blue) do respond to isoproterenol if exogenous 0.1 mM ATP and 10 μM GTP are added to the adenylate cyclase reaction mixture. As in whole cells, isoproterenol-stimulated cyclase was similar in control and intoxicated cells, suggesting that diphtheria toxin treatment does not act directly to alter adenylate cyclase activity. However, membrane-bound adenylate cyclase from cells desensitized for 4 hr with isoproterenol showed only 14% of the initial isoproterenol-stimulated activity. In contrast, toxin-treated cells retained 49% of the original activity.

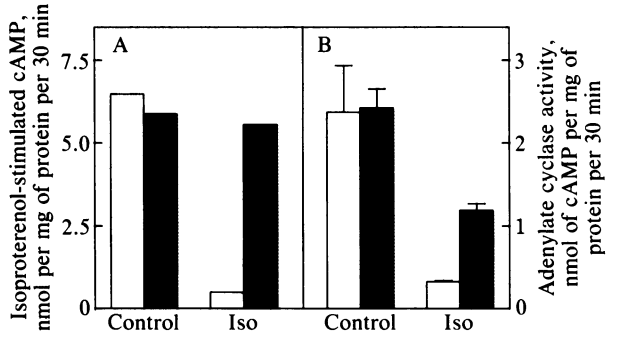

FIG. 3. (*A*) Diphtheria toxin prevents isoproterenol-induced refractoriness in A431 cells. Control (open bars) and toxin-treated (solid bars) cells were incubated for 4 hr with or without 10 μM (−)-isoproterenol (Iso), washed for 30 min, and rechallenged for 30 min with 10 μM (−)-isoproterenol. The level of isoproterenol-stimulated cAMP was measured. Three separate experiments on different passage-number cultures gave similar results except that the maximal responses varied. The data shown are from one typical experiment performed in duplicate. (*B*) Adenylate cyclase activity in digitonin permeabilized A431 cells. Control (open bars) or intoxicated (solid bars) cells were incubated 4 hr in Ham's F-10/Hepes with or without 10 μM (−)-isoproterenol. Cells were permeabilized for 5 min with 0.01% digitonin and then incubated for 30 min at 22°C with the reaction mixture for the adenylate cyclase assay containing 0.1 mM ATP, 10 μM GTP, and 10 μM (−)-isoproterenol. Data are expressed as the mean ± SEM of triplicate determinations.

DISCUSSION

Intracellular cAMP concentration increased more than 400-fold above basal after a 30-min treatment with 10 μM (−)-isoproterenol in the A431 epidermoid carcinoma cell line. The absolute cAMP response varied depending upon the passage number. Cells of lower passage number were generally more responsive (data not shown). A431 cells became desensitized after a 4-hr exposure to (−)-isoproterenol or Bt_2cAMP. This loss of responsiveness was significantly reduced in isoproterenol (or Bt_2cAMP)-treated cells incubated with the protein synthesis inhibitor cycloheximide. Desensitized cells rechallenged with isoproterenol for 30 min showed only 10% of the initial response to the agonist. Cycloheximide-treated cells with the same challenge showed 65% of the original response. Bt_2cAMP-induced refractoriness was not as great as isoproterenol-induced desensitization in A431 cells. This quantitative difference has also been observed in C6-2B cells (16).

The availability of this human cell line allowed us to use diphtheria toxin as a protein synthesis inhibitor. Treatment with the toxin at 1 ng/ml for 24 hr caused 80–90% inhibition of protein synthesis without affecting cell viability. Intoxicated cells appeared to be quite resistant to the desensitizing action of (−)-isoproterenol. After a 4-hr incubation with isoproterenol, cAMP content was always higher in diphtheria toxin-pretreated cells than in controls. Upon rechallenge with isoproterenol, intoxicated cells were almost completely, sometimes totally, protected from isoproterenol-induced desensitization. Using a method† for measuring hormone-stimulated adenylate cyclase, wherein the magnitude of hormone stimulation is as great as observed in whole cells, we found that (−)-isoproterenol stimulated adenylate cyclase activity from A431 cells, desensitized with (−)-isoproterenol, was markedly reduced. Comparable preparations from diphtheria toxin-treated cells showed much less refractoriness to isoproterenol, indicating that the protective effect of the toxin in whole cells must reflect an alteration of adenylate cyclase activity. This protein synthesis-dependent desensitization, therefore, represents a stable change in membrane-bound adenylate cyclase. These results support our working hypothesis that a rapidly turning over protein, induced in response to catecholamine-stimulated cAMP production, limits further agonist-stimulated activation of adenylate cyclase.

It is important to point out that inhibition of protein synthesis by either cycloheximide for 4 hr or diphtheria toxin for 24 hr did not affect activation of adenylate cyclase by isoproterenol in whole cells or adenylate cyclase assayed in permeabilized cells. Inhibiting protein synthesis thus does not interfere with adenylate cyclase activity or trypan blue exclusion in the cells. Other investigators have confirmed and extended our findings (15, 16) that either cycloheximide or actinomycin D prevents hormone-induced desensitization in diverse hormone-receptor systems [dog thyroid cell, thyrotropin (17); murine epidermis *in vivo*, isoproterenol (18);

†Our laboratory has developed a procedure involving permeabilization of cells and the subsequent measurement of adenylate cyclase activity after the addition of hormone, GTP, and ATP (46). In contrast to more traditional preparations of homogenized cells, the permeabilized-cell adenylate cyclase is highly responsive to hormonal agonists, often increasing hormone-stimulated adenylate cyclase activity 100-fold above the basal level—comparable to the extent of stimulation seen in cellular cAMP after hormonal stimulation.

rat ovary, lutropin, follitropin, and prostaglandin E_2 (19); murine thyroid cell, thyrotropin (20); rat Leydig tumor cell, lutropin (21); rat ovarian cell, lutropin (22); rat thyroid cell line, thyrotropin (23)].

Our previous studies with cycloheximide and actinomycin D implied hormone-mediated induction of mRNA coding for synthesis of a putative "refractoriness protein(s)" rather than a nonspecific effect of inhibiting protein synthesis. In C6-2B cells (15) the RNA synthesis inhibitor, actinomycin D, prevented catecholamine refractoriness only if added during the initial phase of catecholamine-stimulated cAMP production, while cycloheximide prevented refractoriness from the inception of hormone stimulation and could even reverse refractoriness within several hours once refractoriness had developed. This suggests that hormone-stimulated mRNA production was needed for subsequent synthesis of the putative inhibitory protein and that the protein is short lived. Furthermore, in other experiments (16), we observed that the prevention of catecholamine refractoriness (and inhibition of protein synthesis) by cycloheximide was readily reversible. Removal of cycloheximide from cells pretreated for 5 hr with isoproterenol and cycloheximide causes "super-refractoriness" (less responsiveness than in cells incubated with isoproterenol alone) within an hour. This suggests that removal of the protein synthesis blockade caused a burst of protein synthesis from mRNA accumulated during the period of blocked protein synthesis.

Although we have not yet identified the putative protein(s), these experiments with diphtheria toxin provide the strongest evidence that such a protein does indeed exist. It is possible that the protein is involved in the regulation or modification of receptors analogous to phosphorylation of the β-adrenergic receptor by the β-adrenergic receptor kinase (12). On the other hand, protein synthesis-sensitive refractoriness is heterologous wherein stimulation of cAMP synthesis through one receptor causes general refractoriness to cyclase agonists, be they heterologous receptors, cholera toxin (47), or forskolin (48). The generality of protein synthesis-sensitive refractoriness makes it likely that the putative protein involved acts at a postreceptor site.

The effect of diphtheria toxin on cAMP metabolism is presumably mediated by the specific action of the toxin to inhibit all protein synthesis and consequently block synthesis of the putative protein involved in refractoriness. The similarity of mechanism of diphtheria toxin to ADP-ribosylate elongation factor 2 with the ADP-ribosylation by cholera or pertussis toxin of other GTP-binding proteins known to regulate hormone-stimulated adenylate cyclase is worthy of consideration. Thus, the possibility that diphtheria toxin is involved in the ADP-ribosylation of known or even unidentified GTP-binding proteins involved in the regulation of adenylate cyclase must be considered. Elongation factor 2, however, is the sole substrate for diphtheria toxin. Even if devoid of direct effect on the component proteins of hormone-stimulated adenylate cyclase, the toxin might alter a possible common function shared by elongation factor 2 and the adenylate cyclase system. Reddy *et al.* (49) reported that one of the elongation factors involved in prokaryotic protein synthesis, elongation factor Tu, actively regulates *Escherichia coli* adenylate cyclase. We, however, found no change in acute hormone-stimulated cAMP accumulation in cells pretreated with diphtheria toxin. Thus, it seems unlikely that the toxin directly interacts with an adenylate cyclase component as do other bacterial toxins. For the same reason, it is unlikely that the change in elongation factor 2 acts indirectly, for example, by sparing GTP normally consumed in protein synthesis and thereby providing more GTP for adenylate cyclase. The best interpretation of our results is that inhibition of general cellular protein synthesis by diphtheria toxin prevents synthesis of an isoproterenol-induced protein that inhibits adenylate cyclase.

Our observations of amelioration of catecholamine desensitization by the toxin may have relevance to clinical *Corynebacterium diphtheriae* infection. Desensitization is a natural process that protects cells from excessive hormonal input. Thus, if desensitization is prevented, the hormonal action is potentiated or prolonged. One serious, and often fatal, sequel to diphtheria infection is cardiac arrhythmia (50). This might reflect potentiated catecholamine action due to the toxin's blockade of the naturally occurring protective desensitization process.

This research was supported by Grant HL 28940 from the National Institutes of Health.

1. Caron, M. G., Cerione, R. A., Benovic, J. L., Strulovici, B., Staniszewski, C., Lefkowitz, R. J., Codina-Salada, J. & Birnbaumer, L. (1985) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, eds. Greengard, P. & Robison, G. A. (Raven, New York), Vol. 19, pp. 1–12.
2. Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M. & Gilman, A. G. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6516–6520.
3. Hildebrandt, J. D., Sekura, R. D., Codina, J., Iyengar, R., Manclark, C. R. & Birnbaumer, L. (1983) *Nature (London)* **302**, 706–709.
4. Bokoch, G. M., Katada, T., Northup, J. K., Ui, M. & Gilman, A. G. (1984) *J. Biol. Chem.* **259**, 3560–3567.
5. Pfeuffer, E., Dreher, R. M., Metzger, H. & Pfeuffer, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 3086–3090.
6. Smigel, M. D. (1986) *J. Biol. Chem.* **261**, 1976–1982.
7. Cerione, R. A., Sibley, D. R., Codina, J., Benovic, J. L., Winslow, J., Neer, E. J., Birnbaumer, L., Caron, M. G. & Lefkowitz, R. J. (1984) *J. Biol. Chem.* **259**, 9979–9982.
8. May, D. C., Ross, E. M., Gilman, A. G. & Smigel, M. D. (1986) *J. Biol. Chem.* **260**, 15829–15833.
9. Robishaw, J. D., Russel, D. W., Harris, B. A., Smigel, M. D. & Gilman, A. G. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 1251–1255.
10. Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. G., Frielle, T., Bolanowski, M. A., Bennet, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J. & Strader, C. D. (1986) *Nature (London)* **321**, 75–79.
11. Hertel, C., Coulter, S. J. & Perkins, J. P. (1985) *J. Biol. Chem.* **260**, 12547–12553.
12. Benovic, J. L., Strasser, R. H., Caron, M. G. & Lefkowitz, R. J. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 2797–2801.
13. Harper, J. F. (1986) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11**, 167–176.
14. Green, D. A. & Clark, R. B. (1981) *J. Biol. Chem.* **256**, 2105–2108.
15. DeVellis, J. & Brooker, G. (1974) *Science* **186**, 1221–1223.
16. Terasaki, W. L., Brooker, G., De Vellis, J., Inglish, D., Hsu, C. Y. & Moylan, R. B. (1978) in *Advances in Cyclic Nucleotide Research*, eds. Greengard, P. & Robison, G. A. (Raven, New York), Vol. 9, pp. 33–52.
17. Rapoport, B. & Adams, R. J. (1976) *J. Biol. Chem.* **251**, 6653–6661.
18. Marks, F. (1980) *FEBS Lett.* **113**, 206–210.
19. Bergh, C. & Ahren, K. (1980) *Acta Endocrinol.* **94**, 251–258.
20. Nilsson, H., Ahren, B., Gustafson, A. & Hedner, P. (1981) *Acta Endocrinol.* **97**, 202–206.
21. Dix, C. J. & Cooke, B. A. (1981) *Biochem. J.* **196**, 713–719.
22. Hedin, L. & Ahren, K. (1983) *Mol. Cell. Endocrinol.* **29**, 335–347.
23. Hirayu, H., Magnusson, R. P. & Rapoport, B. (1985) *Mol. Cell. Endocrinol.* **42**, 21–27.
24. Wildenthal, K. & Griffin, E. E. (1976) *Biochim. Biophys. Acta* **444**, 519–524.
25. Timberlake, W. E., Hagen, G. & Griffin, D. H. (1972) *Biochem. Biophys. Res. Commun.* **48**, 823–827.
26. Mitranic, M., Sturgess, J. M. & Moscarello, M. A. (1976) *Biochim. Biophys. Acta* **421**, 272–279.

27. Jomain-Baum, M., Garber, A. J., Farber, E. & Hanson, R. W. (1973) *J. Biol. Chem.* **248,** 1536–1543.
28. Timberlake, W. E. & Griffin, E. E. (1973) *Biochem. Biophys. Res. Commun.* **54,** 216–221.
29. Sullia, S. B. & Griffin, D. H. (1977) *Biochim. Biophys. Acta* **475,** 14–22.
30. Ernest, M. J. (1982) *Biochemistry* **21,** 6761–6767.
31. Penman, S., Vesco, C. & Penman, M. (1968) *J. Mol. Biol.* **34,** 49–69.
32. Pastan, I. & Friedman, R. M. (1968) *Science* **160,** 316–317.
33. Laszlo, J., Miller, D. S., McCarthy, K. S. & Hochstein, P. (1966) *Science* **151,** 1007–1010.
34. Honig, G. R. & Rabinovitz, M. (1965) *Science* **149,** 1504–1506.
35. Grossman, A. & Mavrides, C. (1977) *J. Biol. Chem.* **242,** 1398–1405.
36. Reel, J. R. & Kenney, F. T. (1968) *Proc. Natl. Acad. Sci. USA* **61,** 200–206.
37. Shambaugh, G. E., Balinsky, J. B. & Cohen, P. P. (1969) *J. Biol. Chem.* **244,** 5295–5308.
38. Balinsky, J. B., Shambaugh, G. E. & Cohen, P. P. (1969) *J. Biol. Chem.* **245,** 128–137.
39. Gill, D. M. & Dinius, L. L. (1973) *J. Biol. Chem.* **248,** 654–658.
40. Pappenheimer, A. M., Jr. (1977) *Annu. Rev. Biochem.* **46,** 69–94.
41. Wregget, K. A. (1986) *J. Receptor Res.* **6,** 95–126.
42. Hoebeke, J., Durieu, O., Delavier, C., Schmutz, A. & Strosberg, A. D. (1984) in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research*, eds. Greengard, P. & Robison, G. A. (Raven, New York), Vol. 17, pp. 73–80.
43. Brooker, G., Terasaki, W. L. & Price, M. G. (1976) *Science* **194,** 270–276.
44. Harper, J. F. & Brooker, G. (1975) *J. Cyclic Nucleotide Res.* **1,** 207–218.
45. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
46. Brooker, G. & Pedone, C. (1986) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **11,** 113–121.
47. Nickols, G. A. & Brooker, G. (1979) *J. Cyclic Nucleotide Res.* **5,** 435–447.
48. Barovsky, K., Pedone, C. & Brooker, G. (1983) *J. Cyclic Nucleotide Protein Phosphorylation Res.* **9,** 181–189.
49. Reddy, P., Miller, D. & Peterkofsky, A. (1986) *J. Biol. Chem.* **261,** 11448–11451.
50. Rocha, H. (1982) in *Cecil, Textbook of Medicine*, eds. Wyngaarden, J. B. & Smith, L. H., Jr. (Saunders, Philadelphia), 16th Ed., pp. 1490–1493.