Proc. Nat. Acad. Sci. USA
Vol. 71, No. 6, pp. 2500–2504, June 1974

Cholera Toxin Mimics Melanocyte Stimulating Hormone in Inducing Differentiation in Melanoma Cells

(adenylate cyclase/membrane receptors/tyrosinase/morphology/cell cycle)

EDWARD O'KEEFE AND PEDRO CUATRECASAS

Department of Pharmacology and Experimental Therapeutics, and Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Communicated by Victor A. McKusick, April 17, 1974

ABSTRACT **Cholera toxin (choleragen) and melanocyte stimulating hormone alter within hours the morphology of melanoma cells in culture, and they slow the growth of serum-stimulated cells. After 7–10 days, cells exposed to choleragen or hormone show increased size and a fibroblastic growth pattern. Tyrosinase (EC 1.14.18.1; monophenol monooxygenase) activity increases after 3 days in the presence of 10^{-8} M hormone or 10^{-10} M choleragen. Binding studies with ^{125}I-labeled choleragen indicate that although a melanoma cell can bind a maximum of 10^6 molecules of cholera toxin, only about 4000 binding sites must be occupied to achieve maximum stimulation of tyrosinase activity. Melanocyte stimulating hormone and choleragen probably have different membrane-binding sites. After exposure to choleragen for 5 min, membrane adenylate cyclase (EC 4.6.1.1) activity increases dramatically upon further incubation of intact cells for several hours at 37° and falls slowly to basal values over a period of more than 10 days. Hormone stimulation of adenylate cyclase is rapidly reversed by washing the cells, but subsequent restimulation of cyclase by the hormone is impaired. These studies indicate that cAMP mediates the effects of melanocyte stimulating hormone on growth and morphology as well as on tyrosinase activity. Cholera toxin may permanently activate the available adenylate cyclase molecules, and the protracted decay of stimulation that follows may reflect the biological turnover of adenylate cyclase molecules in these cells.**

Most regulatory functions ascribed to cAMP involve the control of specific metabolic activities by hormones in target cells (1). Recent evidence indicates that cAMP and hormones regulating its levels may determine to a major extent the growth, size, and shape of cells and the degree of expression of specific differentiated functions in cultured cells. Dramatic morphologic alterations are seen in several cell types within hours of the addition of cAMP (2), and growth is inhibited in the presence of exogenous cAMP, high levels of endogenous cAMP, or hormones that stimulate adenylate cyclase (EC 4.6.1.1) (3). Specific differentiated functions may be stimulated in reciprocal fashion (4). Reciprocal relationships between cAMP and cGMP may control differentiated function and growth (5).

Melanocyte stimulating hormone (MSH), in addition to stimulating (6) a cAMP-mediated, specific differentiated function (tyrosinase and its product, melanin pigment), can produce early and delayed (6) morphologic changes, inhibition of growth, and stimulation of adenylate cyclase in melanomas (7) and cultured melanoma cells. We report here that these events are duplicated by cholera toxin (choleragen), a protein that specifically stimulates adenylate cyclase (8) as a result of specific interactions with the membrane glycolipid, ganglioside GM_1 (9, 10). The toxin stimulates adenylate cyclase in various tissues (8, 11); it induces steroidogenesis in an adrenal cell line (12), inhibits cytolysis by sensitized lymphocytes (11), inhibits histamine release from lymphocytes by antigen and IgE (11), and inhibits DNA and RNA synthesis in human fibroblasts (13) *in vitro*. The toxin's ability to bind strongly to the plasma membrane of most cells, to stimulate adenylate cyclase activity at picomolar concentrations for prolonged periods, to mimic specific cAMP-mediated physiologic effects without need for hormones, and probably to complex directly with adenylate cyclase suggest the use of choleragen as a novel tool in the study of differentiation and of the regulation of adenylate cyclase. The present studies strengthen the view that cAMP can regulate genetic expression in eukaryotes.

Abbreviation: MSH, melanocyte stimulating hormone.

MATERIALS AND METHODS

Cloudman S-91 melanoma cells (CCL 53, NCTC 3960, American Type Culture Collection) were maintained in Ham's F-10 medium with 10% fetal-calf serum containing 50 units/ml of penicillin and streptomycin in an atmosphere of 5% CO_2 in humidified air at 37° in monolayers in plastic tissue-culture flasks and were subcultured by scraping. Purified α-MSH was a gift from Drs. S. Lande and A. Lerner of Yale University, and synthetic α-MSH was given by Dr. W. Rittel of Ciba Ltd., Basel, Switzerland. Purified (14) cholera toxin (choleragen) was obtained from Dr. R. S. Northrup, SEATO Cholera Research Program. Tyrosinase (EC 1.14.18.1; monophenol monooxygenase) was assayed by a modification of the method of Pomerantz (16) (see Fig. 1). [α-^{32}P]ATP was synthesized by the method of Symons (15), adenylate cyclase was assayed essentially by the method of Pohl *et al.* (17) (see Fig. 6), and ^{125}I-labeled choleragen was prepared as described (9).

RESULTS

Effects of MSH and Choleragen on Growth and Morphology. MSH induces gross morphologic changes in melanoma cells after 30 min at 37°, and identical changes develop more slowly (1.5 hr) with choleragen. Cells become more refractile and spherical (see refs. 2 and 12) and many cells become detached from the monolayer. The loss of cells (by detachment) depends on the toxin concentration in the same way as does tyrosinase stimulation (see below). For as long as three weeks, cells exposed to MSH (added every 2–3 days) or to choleragen (every 5–7 days) show an altered growth pattern, larger cell size, and increased pigmentation.

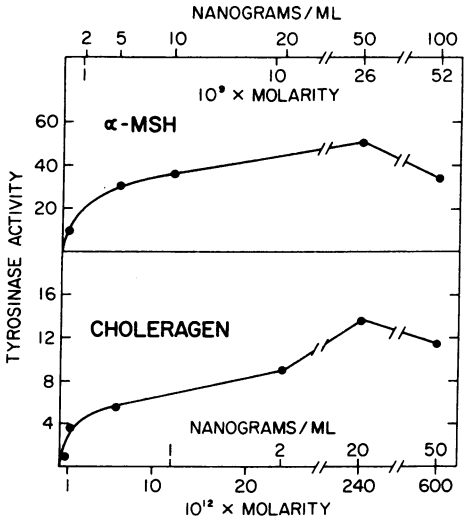

FIG. 1. Stimulation of tyrosinase activity of melanoma cells by MSH and choleragen. Cells were treated with MSH or choleragen, harvested after 5 days, counted in a Coulter Counter, and centrifuged for 10 min at 1000 × *g*. The pellet was sonicated for 2 sec, and triplicate 20-μl samples were assayed (15) for conversion of L-[3,5-^{3}H]tyrosine (purified on AG50W-X8, lyophilized just before use) to ^3H_2O in a mixture of 0.4 mM tyrosine, 0.12 mM L-β-3,4-dihydroxyphenylalanine, 2 to 4 × 10^5 cpm [^{3}H]tyrosine (1 Ci/mmole), and 100 mM sodium phosphate buffer, pH 6.9, in a volume of 50 μl. The reaction (20 min, 37°) was terminated with 0.5 ml of 0.17 M acetic acid. The mixture was chromatographed on a 3 × 0.7-cm column of Bio-Rad AG50W-X8, 100–200 mesh (H^+ form) and eluted with 1.6 ml of distilled water. MSH was added and the medium was changed every second day; choleragen was added only on the first day and in absence of serum. Activity of control cultures is subtracted from the experimental values. Results expressed as 10^5 × pmol of ^3H_2O produced per min/cell. Differences in activity between experiments reflect varying age and cell density of cultures. Cell number is used for comparisons of activity, since protein determination by the Folin–Ciocalteu reagent is altered by melanin in MSH- or toxin-stimulated cultures (6).

Cells partially synchronized by depleted medium show a 2- to 4-fold increase in the uptake of [^{3}H]thymidine 24 hr after the addition of fresh medium containing serum. These quiescent cells show the same pronounced morphologic changes after addition of MSH or choleragen as do rapidly growing cells. Also, MSH and toxin inhibit serum-stimulated [^{3}H]thymidine incorporation into DNA in these cells (Table 1).

The tendency of MSH- or toxin-treated cells to round up and detach from plastic surfaces is reflected by a decrease in the number of cells in treated monolayers. This can be mistakenly interpreted as an effect on cell division in cultures treated for several days; this apparent decrease in cell counts is also found after treatment for only several hours. [^{3}H]Thymidine incorporation, autoradiography, or other methods must be used to document inhibition of growth.

Stimulation of Tyrosinase Activity. Tyrosinase activity, which closely parallels changes in pigmentation, increases slightly after 1–3 days and more markedly after 4–6 days to levels 5- to 10-fold initial levels with MSH concentrations up to 5 × 10^{-8} M (100 ng/ml), at which stimulation is maximal (Figs. 1 and 2). Tyrosinase activity falls when MSH is not added repeatedly, presumably because of degradation of the

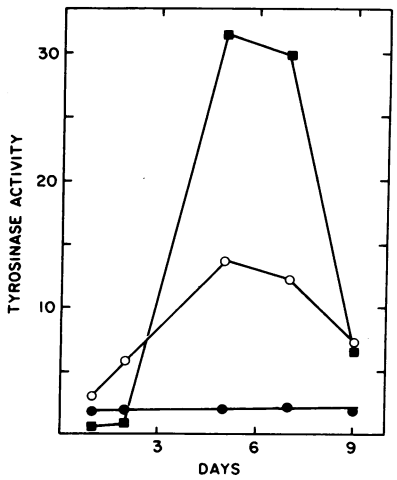

FIG. 2. Time course of tyrosinase stimulation by MSH and choleragen. Confluent melanoma cells were treated with 5 × 10^{-11} M choleragen (■) on day 0 or with 10^{-8} M MSH (○) on day 0 and day 3. Medium was changed every other day. Results are expressed as 10^5 × pmol of ^3H_2O produced per min/cell. Control cells are (●).

hormone, but with continued stimulation the activity continues to rise for at least 9 days (6). Choleragen produces up to 30-fold increases in enzyme activity at about 3 × 10^{-10} M (20 ng/ml). Since serum and gangliosides present in serum inhibit the binding of choleragen to cells (9), greater stimulation is obtained if the toxin is added to the cells before serum.

The tyrosinase of crude melanoma-cell sonicates is enzymatically similar to purified melanoma tyrosinase (15). With 0.12 mM L-dopa as cofactor, the K_m for tyrosine is 0.2 mM and substrate inhibition is seen at 0.8–1.0 mM tyrosine. The apparent K_m for tyrosine is the same in control, MSH-stimulated, and choleragen-stimulated cells, suggesting that stimulation involves synthesis or activation of new enzyme rather than a change in affinity for substrate.

Binding of ^{125}I-Labeled Choleragen to Melanoma Cells. Binding of cholera toxin to melanoma cells, like binding to fat cells and liver membranes (9), is complete within 5 min and

TABLE 1. *Inhibition of DNA synthesis in melanoma cells by MSH and cholera toxin*

MSH			Cholera toxin	
ng/ml	Exp. 1 (cpm)	Exp. 2 (cpm)	ng/ml	cpm
0	29,250 ± 4,750*	11,450 ± 1,680	0	27,100 ± 5,780
200	11,540 ± 220	2,290 ± 330	100	14,800 ± 3,570
20	14,500 ± 2,980	4,230 ± 1,085	1	18,430 ± 3,760
2	26,230 ± 2,910	4,840 ± 1,370	0.1	27,570 ± 3,600

* cpm ± standard deviation.

Melanoma cells were cultured in 16-mm dishes on Linbro Multi-Dish Disposo-Trays until confluent. Medium depleted for at least 4 days was changed 30 min after additions; MSH was added again after the medium change. One microcurie of [^{3}H]-thymidine was added 24 hr later, and after 1 hr at 37°, the radioactivity in trichloroacetic acid-precipitable material was determined (13).

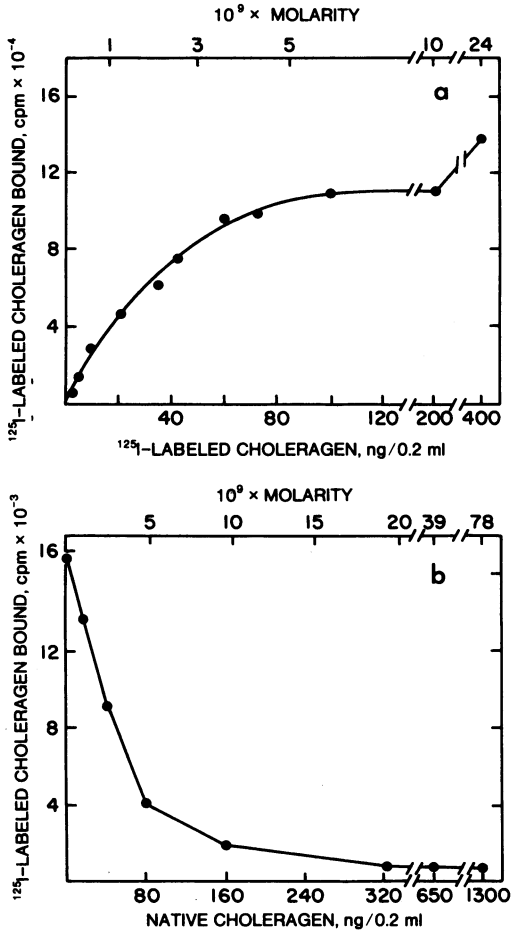

FIG. 3. (*a*) Specific binding of ^{125}I-labeled cholera toxin to melanoma cells. 10^5 cells in 0.2 ml of Hanks' buffer–0.1% albumin were incubated (30 min, 24°) with ^{125}I-labeled choleragen (14 μCi/μg), diluted with 3 ml of cold 0.1% albumin-buffer, and filtered and washed on EAWP (Millipore) filters (9). Specific binding was determined (9) by subtracting those counts not displaced by the prior addition of native toxin; 97% of iodotoxin can be displaced. (*b*) Competition of binding of ^{125}I-labeled choleragen to melanoma cells by native toxin. Native toxin was present for 10 min (24°) before addition of 6 ng of ^{125}I-labeled choleragen (82,800 cpm).

becomes progressively irreversible with time, while the binding of peptide hormones to receptors is slower and reversible (18). Saturation of binding does not occur until the cells bind an average of about 10^6 molecules per cell (Fig. 3*a*) and requires concentration of choleragen of 6×10^{-9} M or greater. The biological effects (morphologic change, tyrosinase stimulation, and adenylate cyclase stimulation) are complete at one-twentieth this concentration. MSH and choleragen do not compete for the same binding site, since 5×10^{-7} M MSH (added first) does not decrease the binding of 10^{-10} M ^{125}I-labeled choleragen. Half-maximal displacement of ^{125}I-labeled toxin occurs with about 2×10^{-9} M native toxin (Fig. 3*b*).

The very strong nature of toxin binding is shown by the fact that melanoma cells exposed to toxin (10 ng/ml) for 5 min and then washed twice show stimulation of tyrosinase activity equal to that of unwashed cells exposed to toxin every second day (Fig. 4). In contrast, MSH apparently dissociates very significantly since cells washed after a 30-min exposure show no stimulation of tyrosinase. Cells treated with MSH every

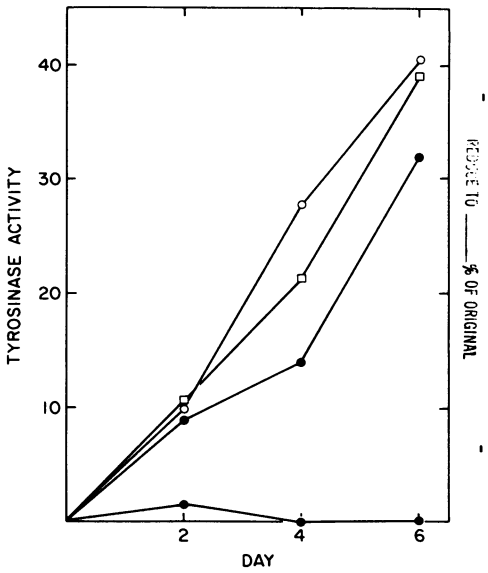

FIG. 4. Stimulation of melanoma-cell tyrosinase by brief or continuous exposure to MSH and choleragen. Cells were treated with 10^{-8} M α-MSH continuously (●) or for 30 min followed by removal (washing twice) of the medium (⊙), or with 10^{-10} M choleragen for 5 min (□) or continuously (○). Serum was added after the addition and washing procedures. Fresh MSH and choleragen were added every second day for continuously exposed cultures, and medium was changed every second day. Control activity has been subtracted for each day of assay. Activity is 10^5 × pmol of ^3H_2O produced per min/cell.

second day (and not washed) are stimulated as effectively as toxin-treated cells.

Stimulation of Adenylate Cyclase Activity. When cells exposed to choleragen are incubated for several hours, crude membrane preparations of these cells show increased adenylate cyclase activity (Fig. 5). After a lag of 20–30 min, enzyme activity increases progressively during the first day and then falls slowly over a period of several days (Fig. 6).

During the initial, accelerating period of toxin stimulation, MSH and fluoride become progressively less effective stimulators of adenylate cyclase activity in membranes of toxin-treated cells (Fig. 5). Toxin treatment for 5 hr or more results in the loss of sensitivity to MSH, and the ratio of MSH-stimulated to basal activity becomes 1. Stimulation by fluoride is also not observed in the toxin-treated membranes, and this compound, instead, inhibits enzyme activity; the ratio of fluoride-stimulated to basal activity becomes less than 1. Stimulation by toxin added in the presence of serum is less effective (compare Figs. 6 and 7); this may be attributable to the presence in serum of gangliosides (9).

While adenylate cyclase activity falls very significantly after the first day (Fig. 6), it is still about four times greater than the unstimulated values after 4 days. Attempts to restimulate the falling cyclase activities by further incubation of intact cells with toxin for 4 hr were unsuccessful as late as 7 days after initial toxin exposure (Fig. 7). Thirteen days later, however, basal activity had fallen to control values, fluoride-stimulation was comparable to that in control cells, and exposure to choleragen for 4 hr stimulated activity as in cells newly exposed to choleragen.

MSH, which results in the same morphological changes and tyrosinase stimulation described for cholera toxin, does not cause persistent elevations of adenylate cyclase activity unless

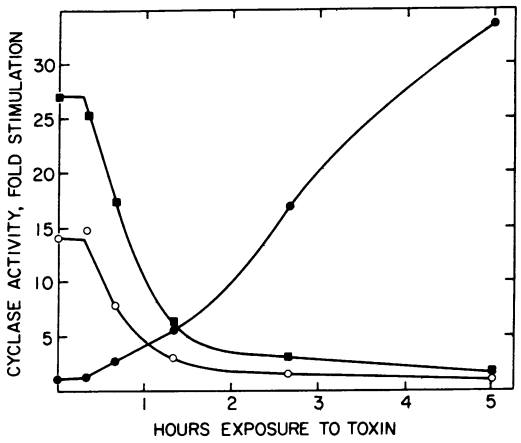

FIG. 5. Time course of stimulation of melanoma-cell adenylate cyclase by cholera toxin. Cells were harvested and chilled at various times after addition of 10^{-10} M toxin. The pellet was suspended in 10 ml of 5 mM Tris·HCl (pH 8.0) with 0.2 mM $CaCl_2$ and 0.2 mM $MgCl_2$ at 4°, homogenized for 45 sec (Polytron), centrifuged (30 min, 40,000 × *g*), and resuspended in 50 mM Tris·HCl (pH 8.0). The incubation mixture contained 0.25 mM GTP, 0.5 mM ATP, 5 mM $MgCl_2$, 10 mM aminophylline, 50 μg per ml of pyruvate kinase, 5 mM phospho*enol*pyruvate, 100 cpm/pmol of [α-^{32}P]ATP, and 20 μl of membrane suspension in a total volume of 100 μl. 5 × 10^{-6} M MSH or 10 mM NaF were added at 4° and the mixture was incubated for 12 min at 33°, heated to 100° for 1 min, and diluted with 1 ml of 25 mM Tris·HCl and 2 × 10^4 cpm of [^{3}H]cAMP. [^{32}P]cAMP was eluted with 2 ml of 25 mM Tris·HCl from 1 g of neutral alumina. Ratio of activities of toxin-treated to control cells (●); ratio of NaF (○) and MSH-stimulated (■) activities to basal activity of toxin-treated cells for each time of incubation with toxin.

the hormone is present continuously in the medium. When cells are washed, harvested, and assayed within 1 hr of a 4-hr period of incubation with MSH, basal enzyme activity is

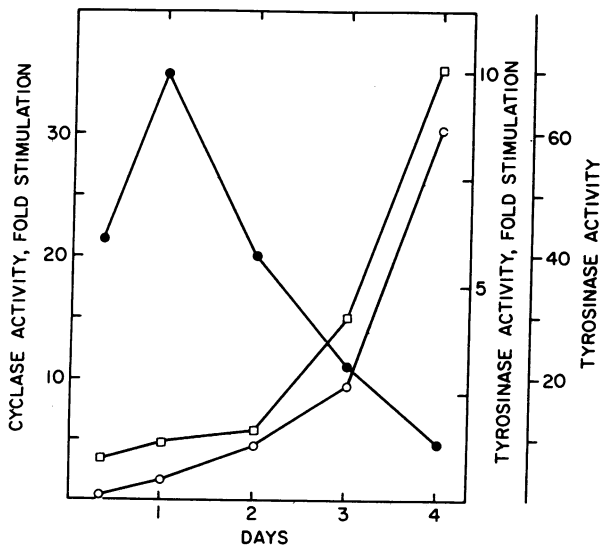

FIG. 6. Adenylate cyclase and tyrosinase activities of cholera toxin-stimulated melanoma cells. Confluent monolayers were exposed to 10^{-10} M choleragen on day 0 only and harvested after 6 hr and on each successive day for 4 days. Serum was added 15 min after addition of toxin. Adenylate cyclase activity is expressed as toxin-treated/control (●). Tyrosinase activity is expressed as toxin-treated/control (□), or as 10^5 × pmol of ^3H_2O/min per cell (○).

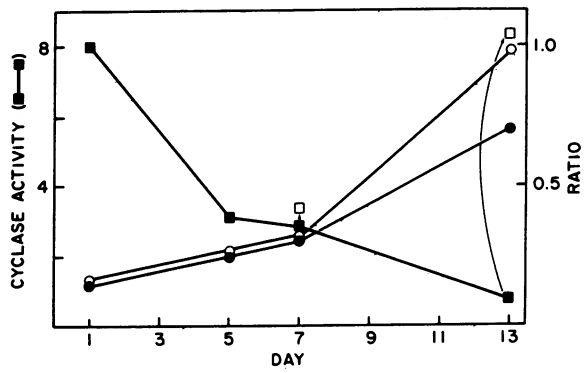

FIG. 7. Relationship of basal adenylate cyclase activity to MSH- and fluoride-stimulated activities after exposure to 10^{-10} M toxin (in 10% fetal-calf serum). *Left ordinate:* ratio of basal activity of toxin-treated to control cells (■). Similar ratio for cells re-exposed to toxin on days 7 and 13 [(□) with *arrows*]. *Right ordinate:* ratio of MSH stimulation of toxin-treated cells to MSH stimulation of control cells (●); similar ratio for fluoride-stimulated activity (○). MSH was 5 × 10^{-6} M; NaF was 10 mM.

only minimally elevated. Furthermore, direct addition of fresh MSH to such membranes results in impaired stimulation (Fig. 8). Half-maximal stimulation by MSH is still seen at 2 μg/ml, suggesting that the impaired MSH response is not the result of altered affinity for MSH. Thus, continuous exposure of cells to MSH is accompanied by a change ("resistance") in the responsiveness of the adenylate cyclase of these cells to MSH. These studies also show that MSH binding is rapid and reversible. Choleragen (10 ng/ml) added directly to membranes in the assay does not stimulate or affect MSH stimulation of cyclase activity, supporting the point that MSH and choleragen have different binding sites.

DISCUSSION

The demonstration that choleragen imitates precisely the effects of MSH on growth, morphology, and differentiated function in melanoma cells strongly suggests that all observed effects of MSH are basically mediated by cAMP. Tyrosinase activity returns to normal values in the absence of a continuous stimulus of adenylate cyclase (Fig. 2). The equal stimula-

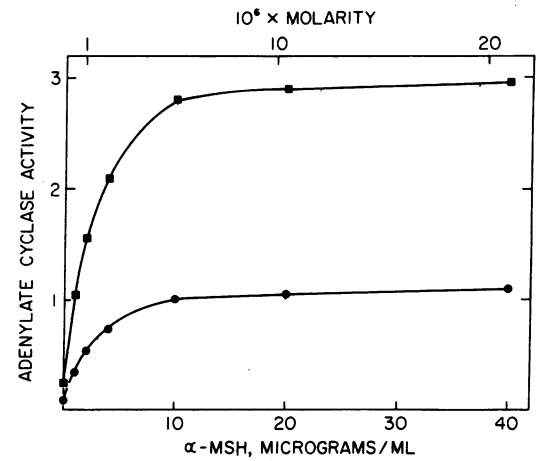

FIG. 8. Effect of incubating melanoma cells with MSH on the activity of adenylate cyclase of membrane preparations. Cells were incubated (4 hr, 37°) with (●) or without (■) 10^{-8} M α-MSH. MSH was added 15 min (4°) before assay. Results are expressed as 10^5 × pmol of cyclic AMP produced/min per cell. Medium had been depleted for 6 days.

tion of tyrosinase seen after continuous exposure to MSH or a single exposure to toxin suggests that moderate adenylate cyclase stimulation present several days after toxin stimulation is sufficient for maximal tyrosinase stimulation.

Tyrosinase stimulation is complete when less than 1% of the available toxin-binding sites are occupied. On the basis of binding, melanoma cells appear to have a relatively large amount of GM_1 ganglioside in the membrane; this glycolipid is the presumed receptor for choleragen (9). All these binding sites, however, cannot be strictly categorized as superfluous (or "in excess") for the biological effects of the toxin. The primary biological activity of the toxin (stimulation of adenylate cyclase) may depend principally on the number of ganglioside and cyclase molecules in the membrane, while the more "distant" effects (e.g., tyrosinase stimulation) may in addition depend on the sensitivity of the intervening events to the protracted and continuously elevated levels of cAMP. Similar general considerations may apply to MSH, and may in part explain why the MSH concentrations required for tyrosinase stimulation (Fig. 1) are so much lower than those required for adenylate cyclase stimulation (Fig. 8). As implied earlier, maximal stimulation of tyrosinase may occur by the continuous stimulation of only a small proportion of the total adenylate cyclase molecules.

Cholera toxin becomes irreversibly incorporated into the cell membrane (9), where it possibly binds to or otherwise perturbs all available adenylate cyclase molecules and thus effects a fixed stimulation of an enzymatic activity which is usually responsive to modification by hormones (ref. 9; and V. Bennett and P. Cuatrecasas, in preparation). Activation increases over a period of hours while hormonal and fluoride stimulation is lost; fluoride becomes an inhibitor of adenylate cyclase. After 1 day, there is a progressive fall in basal activity over more than 10 days.

Possible explanations for the fall in adenylate cyclase activity after choleragen stimulation (Fig. 6) are the development of a specific inhibitor, inactivation or loss of the toxin or of toxin-cyclase complexes, or altered affinity of the enzyme for GTP, ATP, or Mg^{2+} in a progressive fashion. The fall may reflect in part the normal turnover of adenylate cyclase molecules; newly synthesized cyclase molecules may thus escape the action of the toxin. However, toxin may persist within the membrane, or other yet unrecognized adjustments may operate, since subsequent restimulation does not reach control values as long as 7 days after initial exposure to toxin (Fig. 7). The possibility that certain adjustments may occur upon protracted stimulation of the enzyme, and which are independent of the nature of the stimulus, is suggested by the altered response of the enzyme to MSH after incubation of the intact cells with MSH (Fig. 8). Clearly, however, the cell is eventually able to recover from its encounter with choleragen; it may be possible, therefore, to determine the biological half-life of adenylate cyclase in this system. The role of cell division in the recovery process is probably minimal, since stimulated cells divide very infrequently and become markedly hypertrophied.

Unlike toxin stimulation of adenylate cyclase, MSH stimulation in intact cells returns promptly to basal values upon removal of MSH from the medium. The relative resistance of membranes of MSH-treated cells to further stimulation by MSH (Fig. 8) may be pertinent to the desensitization of cAMP accumulation in cultured fibroblasts upon hormonal stimulation (19), or to the relative refractoriness of hormonally stimulated fat cells (20). It will be important to determine the mechanism of this altered sensitivity of adenylate cyclase.

We thank V. Bennett for assistance with the assay of adenylate cyclase, E. Richelson for helpful discussions, and L. Hernaez for expert technical assistance. Supported by grants from The American Cancer Society (BC-63), National Institutes of Health (AM 14956), National Science Foundation (GB 34300), and The Kroc Foundation. E.O'K. is the recipient of an American Cancer Society Postdoctoral Fellowship. P.C. is the recipient of a USPHS Research Career Development Award (AM31464).

1. Robinson, G. A., Butcher, R. W. & Sutherland, E. W. (1971) *Cyclic AMP* (Academic Press, New York).
2. Hsie, A. W. & Puck, T. T. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 358–361; Johnson, G. S., Friedman, R. M. & Pastan, I. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 425–429; Prasad, K. N. & Hsie, A. W. (1973) *Nature New Biol.* **233**, 141–142.
3. Otten, H., Johnson, G. S. & Pastan, I. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1192–1198; Sheppard, J. R. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 1316–1320; Burger, M. M., Bombik, B. M., Breckenridge, B. & Sheppard, J. R. (1972) *Nature New Biol.* **239**, 161–163; Froelich, J. E. & Rachmeler, M. (1972) *J. Cell Biol.* **55**, 19–31.
4. Masui, H. & Garren, L. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 3206–3210; Pawelek, J., Wong, G., Sansone, M. & Morowitz, J. (1973) *Yale J. Biol. Med.* **46**, 430–443; Richelson, E. (1973) *Nature New Biol.* **242**, 175–177.
5. Hadden, J. W., Hadden, E. M., Haddox, M. K. & Goldberg, N. D. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 3024–3027; Strom, T. B., Carpenter, C. B., Garovoy, M. R., Austen, K. F., Merrill, J. P. & Kaliner, M. (1973) *J. Exp. Med.* **138**, 381–393; Kram, R. & Tomkins, G. M. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 1659–1663.
6. Johnson, G. S. & Pastan, I. (1972) *Nature New Biol.* **237**, 267–268; Wong, G. & Pawelek, J. (1973) *Nature New Biol.* **241**, 213–215; Kreider, J. W., Rosenthal, M. & Lengle, N. (1973) *J. Nat. Cancer Inst.* **50**, 555–558.
7. Kreiner, P. W., Gold, C. J., Keirns, J. J., Brock, W. A. & Bitensky, M. J. (1973) *Yale J. Biol. Med.* **46**, 583–591.
8. Finkelstein, R. A. (1973) *CRC Critical Rev. Microbiol.* **2**, 553–623; Field, M. (1971) *N. Engl. J. Med.* **284**, 1137–1144; Carpenter, C. C. J., Jr. (1971) *Amer. J. Med.* **50**, 1–7; Pierce, N. F., Greenough, W. B. & Carpenter, C. C. J., Jr. (1971) *Bacteriol. Rev.* **35**, 1–13.
9. Cuatrecasas, P. (1973) *Biochemistry* **12**, 3547–3558; 3558–3566; 3567–3577; 3577–3581.
10. Pierce, N. F. (1973) *J. Exp. Med.* **137**, 1009; Holmgren, J., Lonroth, I. & Svennerholm, L. (1973) *Scand. J. Inf. Dis.* **5**, 77; King, C. A. & van Heyningen, W. E. (1973) *J. Inf. Dis.* **127**, 639; van Heyningen, W. E. (1974) *Science* **183**, 656.
11. Sharp, G. W. G. (1973) *Annu. Rev. Med.* **24**, 19–28; Lichtenstein, L. M., Henney, C. S., Bourne, H. R. & Greenough, W. B. (1973) *J. Clin. Invest.* **52**, 691–697.
12. Wolff, J., Temple, R. & Cook, G. H. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2741–2744; Donta, S. T., King, M. & Sloper, S. (1973) *Nature New Biol.* **243**, 246–247.
13. Hollenberg, M. D. & Cuatrecasas, P. (1973) *Proc. Nat. Acad. Sci. USA* **70**, 2964–2968.
14. Finkelstein, R. A. & LoSpalluto, J. J. (1969) *J. Exp. Med.* **130**, 185–202.
15. Pomerantz, S. H. (1966) *J. Biol. Chem.* **241**, 161–168.
16. Symons, R. H. (1968) *Biochim. Biophys. Acta* **155**, 609–610.
17. Pohl, S. L., Birnbaumer, L. & Rodbell, M. (1971) *J. Biol. Chem.* **246**, 1849–1856; Siegel, M. & Cuatrecasas, P. (1974) *Mol. Cell. Endocrinol.*, in press.
18. Cuatrecasas, P. (1974) *Annu. Rev. Biochem.* **43**, in press.
19. Franklin, T. J. & Foster, S. J. (1973) *Nature New Biol.* **246**, 146–148.
20. Ho, R. J. & Sutherland, E. W. (1971) *J. Biol. Chem.* **246**, 6822–6827.