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# Adenylate Cyclase Stimulation by Trypsin (39109)

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In a previous investigation the level of adenylate cyclase of normal and transformed cells in culture was determined. Extremely high levels of adenylate cyclase were observed in F111 and F2412 cells shortly after cell transfer. Following this marked rise, the values fell and remained essentially level throughout the logarithmic and stationary phases of cell growth (1). The rise that follows cell transfer may be due to cell damage as a consequence of detachment from the floor of the flask or because of the trypsin that is used to detach the cells. The F111 cells may be detached by mechanical scraping or by trypsinization. This report describes the effects of these procedures on adenylate cyclase of F111 cells.

*Materials and methods. Cell culture.* The F111 rat embryo fibroblast cell line used in these experiments has been described previously (1). It was employed at Passages 40–43 and is a normal untransformed cell line. The cells were tested by the methods of Hayflick (2, 3) and found to be free of mycoplasma.

Cells were grown in 20 × 100 mm plastic culture dishes, seeded at a density of 5000/cm$^2$. The growth medium was Dulbecco's Modified Eagles Medium with glutamine containing 10% vol/vol fetal calf serum (Rehatuin). Additions to the medium were 13.5 g/liter of sodium bicarbonate, 2 mg/liter of Amphotericin B, and 50 μg/liter of Gentamicin. The cells were incubated in an atmosphere of 95% air and 5% $CO_2$. Cells were counted with a model Fn Coulter Counter which was calibrated daily. The cells were trypsinized with 1:300 trypsin (NBC) 2 g/liter in NaCl 6.0 g/liter, KCl 0.4 g/liter, glucose 1.0 g/liter, and Tris-HCl 0.6 g/liter, pH 7.5. If the cells were removed by scraping with a rubber policeman, the buffered saline–glucose solution without trypsin was used.

*Adenylate cyclase assay.* After removing the culture medium, the cells were washed once with 10 ml of the buffered saline–glucose and then removed either by scraping with a rubber policeman or by trypsinization. After removal they were centrifuged and prepared as described by Makman with the exception that 5 m*M* dithioerythritol was added to the buffer (4, 5). The incubation conditions were those described by Makman and Krishna and were determined to give linear rates of activity for 20 min at 30°C (4, 5, 6). Incubation was carried out in 10 × 75 mm test tubes containing 1.0 m*M* $^3$H ATP, 4.5 m*M* $MgCl_2$, 2.0 m*M* adenosine-3′,5′-monophosphate (cyclic-AMP), 2.5 m*M* phosphoenolpyruvate, 3.0 m*M* theophylline, 1.25 mg/ml pyruvate kinase, and 45 m*M* Tris-HCl, pH 7.6 in a final volume of 200 μl. In addition, the fluoride-stimulated samples contained 8 m*M* NaF. The reaction was started by addition of cell homogenate to give a final concentration ranging from 1.0–2.0 mg of protein/ml of assay. Incubation was at 30°C and was terminated by the addition of 0.2 ml of 50 m*M* Tris pH 7.6, 5 m*M* cyclic-AMP, and 3 m*M* ATP. The reaction mixtures were treated for 2 min in a boiling water bath after which 0.6 ml of distilled water was added to each tube. Cyclic-AMP was isolated and measured as described by Krishna (6).

*Results.* A comparison of adenylate cyclase levels in cells removed mechanically and those removed by trypsin is shown in Table I. The increase in basal level is particularly marked following trypsinization. However, both basal- and fluoride-stimulated levels of adenylate cyclase are increased by the treatment with trypsin.

To further characterize the effect of trypsin on stimulation of adenylate cyclase, the cells were treated for 5, 10, 15, and 20 min. Fig. 1 shows that the increase in both basal and fluoride stimulated levels is maximal at 10 min, after which further treatment decreases the level of adenylate cyclase. Cells vary considerably in the time required to detach them from the culture dish. The F111 cells require 5–10 min to bring about detachment, which

TABLE I. SCRAPING VERSUS TRYPSINIZATION ON ADENYLATE CYCLASE OF F111 CELLS[a]

| | Scraped | Trypsin |
|---|---|---|
| Basal | 7.7 ± 2.0 | 48.0 ± 13.1 |
| Fluoride-stimulated | 67.0 ± 8.4 | 124.0 ± 14.0 |

[a] Values are pmoles cyclic-AMP/min/mg protein. The cells were washed with 10 ml of the buffered saline-glucose; 1 ml of 0.25% trypsin, 1:300 in buffered saline-glucose at 37° for 5 min was used to detach the cells; and 2.0 ml of fetal calf serum was added to stop trypsin proteolysis. The scraped cells were treated with 1 ml of buffered saline-glucose without trypsin. Cells were detached with a rubber policeman and 2.0 ml of fetal calf serum were added. Results are mean ± SD of three experiments.

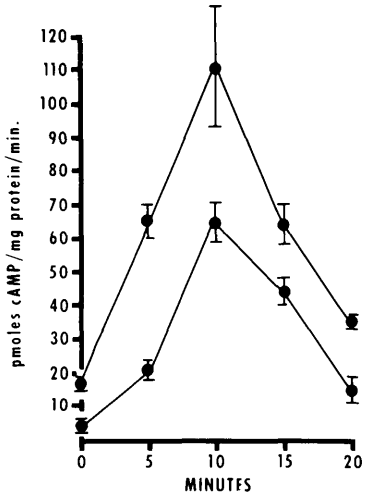


FIG. 1. Effect of preincubation with trypsin on adenylate cyclase activity. F111 cells were washed with buffered glucose saline and incubated with 25 μg/ml of 1:300 trypsin at 37° for the indicated time. The upper curve is the fluoride stimulated level and the lower curve the basal level of adenylate cyclase. Proteolysis was stopped by the addition of 2.0 ml of fetal calf serum. Results are mean ± SD of three experiments.

correlates with the time required for maximum stimulation.

In the next experiment the effect of different concentrations of trypsin on adenylate cyclase was measured. The time of incubation was held constant at 5 min (Table II).

TABLE II. STIMULATION OF ADENYLATE CYCLASE WITH DIFFERENT CONCENTRATIONS OF TRYPSIN[a]

| Trypsin (μg/ml) | Cyclic-AMP/min/mg of protein (pmoles) | |
|---|---|---|
| | Basal | Fluoride |
| 0.0 | 8.0 ± 1.8 | 42.0 ± 3.1 |
| 0.5 | 11.0 ± 2.1 | 42.0 ± 4.0 |
| 1.0 | 28.0 ± 1.8 | 40.0 ± 3.7 |
| 5.0 | 35.0 ± 2.6 | 48.0 ± 4.6 |
| 25.0 | 64.0 ± 5.1 | 130.0 ± 8.2 |
| 2500.0 | 54.0 ± 4.8 | 205.0 ± 9.3 |

[a] F111 cells were treated with 1.0 ml of the indicated level of 1:300 trypsin in buffered saline-glucose for 5 min. Results are the mean ± SD of three determinations.

TABLE III. TRYPSIN STIMULATION OF ADENYLATE CYCLASE AND INHIBITION BY SOYBEAN TRYPSIN INHIBITOR[a]

| | Cyclic-AMP/min/mg of protein (pmoles) | |
|---|---|---|
| | Basal | Fluoride |
| Control | 6.7 ± 1.4 | 46.0 ± 3.1 |
| SBI | 6.1 ± 1.6 | 53.0 ± 4.2 |
| Trypsin 1:300 | 51.0 ± 4.2 | 185.0 ± 7.6 |
| Trypsin 1:300 + SBI | 5.6 ± 1.1 | 32.0 ± 2.8 |
| Crystalline trypsin | 13.9 ± 2.0 | 87.0 ± 6.3 |

[a] Trypsin 1:300, 2500 μg/ml, and the soybean trypsin inhibitor (SBI), 1350 μg/ml, were incubated at 5°C for 18 hr and then added to the cells for 5 min at 37°. Trypsin was added to the cells at 2500 μg/ml for 5 min at 37°. Crystalline trypsin, bovine pancreas Type III (Sigma), was used at 50 μg/ml for 5 min at 37°. Each result is the mean ± SD of three determinations.

The basal levels of adenylate cyclase gradually increase with an increase in trypsin concentration whereas the fluoride stimulated levels increase only if the trypsin concentration is 25 μg/ml or higher. The fluoride-stimulated levels continue to increase at 2500 μg/ml, but the basal levels of adenylate cyclase are not changed by this large increase in trypsin.

Trypsin (1:300) contains several enzymes such as α-chymotrypsin and elastase as well as trypsin (7, 8). To determine if trypsin is responsible for the stimulation of adenylate

cyclase, the effects of soybean trypsin inhibitor on the stimulation of adenylate cyclase was investigated. In addition, crystalline trypsin was also tested for its ability to stimulate adenylate cyclase (Table III). The activation by crystalline trypsin is not as great as that of 1:300 trypsin; however, no attempt was made to find an optimum concentration of crystalline trypsin. This evidence together with the complete inhibition by soybean trypsin inhibitor suggests that the stimulation of adenylate cyclase is due to the trypsin content of 1:300 trypsin.

The stimulation of adenylate cyclase by trypsin may be due to an increased synthesis of enzyme or to a direct effect of trypsin on the enzyme. For example, trypsin might possibly expose or react with the adenylate cyclase receptors. Because of the short incubation time required to stimulate adenylate cyclase, the most probable explanation would be activation of the receptor rather than new enzyme synthesis. The enzyme might be stimulated by exposure of the receptor portion of the enzyme following proteolysis. If more receptors are exposed, then there should be a greater response to those compounds that react with the receptors. Prostaglandin $E_1$ increases cyclic-AMP in some cells, and most fibroblasts have an adenylate cyclase that is activated by $PGE_1$ (9, 10). It is seen from Table IV that the response to $PGE_1$ is increased more in the cells that are not trypsinized. These data suggest that trypsinization does not prevent the response to $PGE_1$, and they do not provide any evidence for exposure of additional $PGE_1$ receptors; rather, it would seem that trypsin is acting on the catalytic unit since the effect of fluoride is augmented.

TABLE IV. TRYPSIN ON ADENYLATE CYCLASE STIMULATION BY PROSTAGLANDIN $E_1$[a]

| | Cyclic-AMP/min/mg protein (pmoles) | |
|---|---|---|
| | Basal | Prostaglandin $E_1$ |
| Control | 7.3 ± 3.4 | 20.6 ± 4.0 |
| Trypsin | 42.8 ± 6.3 | 57.3 ± 7.8 |

[a] Prostaglandin $E_1$ was added to the cell homogenates at 10 μg/ml. Cells were treated with 2500 μg/ml 1:300 trypsin for 5 min at 37°C, and the reaction was stopped by addition of fetal calf serum. The results are the mean ± SD of six determinations.

*Discussion.* The evidence presented clearly indicates that the increased adenylate cyclase that we had previously reported following transfer of F111 and related cells is a response to trypsin. This is a considerable effect and one that lasts for several hours following trypsinization of the cells (1). Although the effect that we have described is due to trypsin, this does not exclude the interesting possibility that other proteolytic enzymes may cause increased adenylate cyclase activity. The considerable literature on density-dependent inhibition suggests that limited proteolysis can increase mitosis and initiate cell division (11, 12, 13). Burger *et al.* have examined the cyclic-AMP levels of cells stimulated to divide by trypsin and found lower levels of cyclic-AMP (14). Similarly, Otten *et al.* added trypsin to 3T3-42 cells and observed decreases in cyclic-AMP (9). It would be expected that increases in adenylate cyclase such as we have reported would result in an *increase* in cellular cyclic-AMP. What appears to be a discrepancy in the findings reported here and those of others may be due to different conditions of treatment. As shown in Fig. 1 and Table II, the concentration of trypsin and the time of treatment determine the degree of stimulation of adenylate cyclase. A decrease in cellular cyclic-AMP may result from increased permeability produced by the trypsin treatment. Trypsin increases the penetration of Erythrosin B into cells (15), and Phillips has reported a loss of lactic dehydrogenase from cells treated with trypsin (16). Cell surfaces are altered by trypsin, and this may lead to cells which are "leaky" (17). Thus, cyclic-AMP may be synthesized at a more rapid rate, but it may be lost from the cell so that no increase in cellular cyclic-AMP occurs.

Some of the metabolic effects that have been attributed to the action of proteases on cells are increased glucose uptake (hemidiaphragm), stimulation of glucose oxidations, inhibition of lipolysis in fat cells, and increased respiration in kidney cells (18, 16). This report expands the number of biochemical changes that follow the commonly

used procedure of removing cells from culture flasks with trypsin.

*Summary.* Adenylate cyclase activity of a rat embryo fibroblast cell line (F111) is markedly increased by brief treatment with 1:300 trypsin. The degree of stimulation depends upon the length of time the cells are treated and the concentration of trypsin. Crystalline trypsin produced a stimulation similar to that obtained with 1:300 trypsin. Further, the addition of soybean trypsin inhibitor blocked the stimulation of adenylate cyclase by 1:300 trypsin. Trypsin-treated adenylate cyclase responds to $PGE_1$, but there is no increase over that of untreated enzyme. This result and the increase in fluoride-stimulated levels of activity suggest that the trypsin is acting upon the catalytic unit of the enzyme.

1. Ryan, W. L., and Curtis, G. L., *in* "The Role of Cyclic Nucleotides in Carcinogenesis" (J. Schultz and H. G. Gratzner, eds.), p. 1. Academic Press, New York (1973).
2. Hayflick, L., and Stanbridge, E., *Ann. NY Acad. Sci.* **143,** 608 (1967).
3. Hayflick, L., *Tex. Rep. Biol. Med. Suppl.* 1, **23,** 285 (1965).
4. Makman, M. H., *Proc. Nat. Acad. Sci. USA* **68,** 2127 (1971).
5. Makman, M. H., *Science* **170,** 1421 (1970).
6. Krishna, G., Weiss, B., and Brodie, B. B., *J. Pharmacol. Exp. Ther.* **163,** 379 (1968).
7. Pine, L., Taylor, G. C., Miller, D. M., Bradley, G., and Wetmore, H. R., *Cytobios* **1,** 197 (1969).
8. Shotton, David M., *in* "Methods in Enzymology" (G. Perlmann and L. Lorand, eds.), Vol. 19, p. 113. Academic Press, New York (1970).
9. Otten, J., Johnson, G. S., and Pastan, I., *J. Biol. Chem.* **247,** 7082 (1972).
10. Peery, C. V., Johnson, G. S., and Pastan, I., *J. Biol. Chem.* **246,** 5785 (1971).
11. Rubin, H., *Science* **167,** 1271 (1970).
12. Burger, M. M., *Nature* **227,** 170 (1970).
13. Sefton, B. M., and Rubin, H., *Nature* **227,** 843 (1970).
14. Burger, M. M., Bombik, B. M., Breckenridge, B. M., and Sheppard, J. R., *Nature New Biol.* **239,** 161 (1972).
15. Phillips, H. J., and Terryberry, J. E., *Exp. Cell Res.* **13,** 341 (1957).
16. Phillips, H. J., *Can. J. Biochem.* **45,** 1495 (1967).
17. Moscona, A., Trowell, O. A., and Willmer, E. N., *in* "Cells and Tissues in Culture, Methods Biology and Physiology," Vol. 1, pp. 55–59. Academic Press, New York (1965).
18. Sakai, T., Lavis, V. R., Ensinck, J. W., and Williams, R. H., *Proc. Soc. Exp. Biol. Med.* **145,** 1096 (1974).

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