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ENDOGENOUS INHIBITOR OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN CULTURED HUMAN EPITHELIAL CELLS

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Summary - We have identified an endogenous inhibitor of cyclic nucleotide phosphodiesterase (PDE) activity in cultured human epithelial cells. The inhibitor was non-dialyzable, inactivated by trypsin and boiling, but stable to a 60° C, 30 min. treatment. Separation of inhibitor from PDE was achieved by blue dextran affinity chromatography. PDE was eluted from this column by EDTA, while the inhibitor remained bound and was subsequently eluted with buffer containing cyclic GMP. The inhibitor was active against PDE from several sources including both Ca^{++} dependent and Ca^{++} independent forms from bovine brain and retina respectively. These characteristics differentiate the PDE inhibitor from human epithelial cells from those previously described from various bovine tissues.

Cyclic AMP metabolism has been studied intensively in cultured fibroblastic cells (1,2). In contrast, much less information is available concerning the cyclic AMP metabolism of cultured epithelial cells (3,4). Cyclic nucleotide phosphodiesterase (PDE) could play a potentially important role in regulating the extent and duration of cyclic AMP action. Indeed several studies have shown PDE to be an inducible enzyme system (5,6). Also heat-stable and heat-labile protein inhibitors of PDE from bovine brain and retina have been isolated and characterized (7-12). During the course of our studies on the regulation of cyclic AMP metabolism in cultured epithelial cells, we discovered an endogenous inhibitor of PDE activity. This report concerns the isolation and characterization of this inhibitor.

Materials and Methods

Human prostatic epithelial cells (MA-160) were obtained from Microbiological Associates, MD. These cells originally obtained from human hyperplastic prostatic tissue, subsequently became transformed in culture so that they now give rise to

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tumors in hamster cheek pouches (13), and athymic mice (unpublished observations). Stock cultures of MA-160 were maintained in Minimal Essential Medium with Earle's salts (EMEM), supplemented with nonessential amino acids, vitamin solution, 2mM L-glutamine, 1mM sodium pyruvate, 50µg streptomycin sulfate, 50U penicillin G/ml and 15% neonatal bovine serum (Biocell, Carson, Calif.).

For PDE assay cells were seeded onto 100mm tissue culture dishes. All cultures had their medium replenished the day before the experiment. Cells were processed for assay by removing the medium, washing the cells twice with cold saline, and then scraping the cells into 1 ml of cold 25mM Tris HCl buffer pH 7.4 containing 2mM $MgCl_2$. Cells were disrupted by 10 seconds of sonication at power setting 3.5 (Bronson Sonifier, Heat Systems, N. Y.). An aliquot of the homogenate was reserved, while the remainder was centrifuged at 20,000xg for 1 hr. The supernatant was saved for assay while the pellet was resuspended in the same buffer and recentrifuged. The washed pellet was resuspended in buffer and used for enzyme assays. The protein concentration of all fractions was adjusted so that no more than 25% of the substrate was hydrolyzed during the incubation. PDE activity of these fractions was assessed as described previously (14). Enzyme activity was measured in all fractions at 100µM and 0.1µM cyclic AMP concentrations.

The inhibitor was separated from PDE by a combination of ammonium sulfate precipitation followed by Cibicron-blue affinity chromatography. Ten large dishes of confluent cells were harvested and supernatant fractions prepared as described above. Ammonium sulfate was added to this fraction to a final concentration of 30%. The resultant slurry was stirred 30 min. in the cold (4° C) and centrifuged at 10,000xg for 30 min. The pellet was resuspended in 1 ml of buffer A (10mM PIPES pH 6.2 + 50mM NaCl + 2mM MgCl + 1mM dithiothreitol and 30% ethylene glycol), and dialyzed against buffer A. 1 ml of dialyzed enzyme was layered onto a 1 ml column of Cibicron blue-Sepharose 4B preequilibrated with buffer B (10mM PIPES pH 6.3 + 50mM NaCl, 1mM dithiothreitol, and 30% ethyleneglycol). After the enzyme had been absorbed onto the column, the resin was washed with 10 ml

Table 1. MA 160 PDE activity as a function of protein concentration

Protein (mg/ml)		Supernatant Specific Activity		Pellet Specific Activity	
Supernatant	Pellet	100μM	0.1μM	100μM	0.1μM
3.90	1.30	7.85	0.22	11.28	2.54
0.70	0.26	49.23	1.23	10.80	2.64
0.39	0.13	65.38	3.27	9.60	2.80

Cells were prepared and assayed for PDE activity as described in the text. Specific activity is defined as nmol adenosine/30min/mg protein. The experiment was repeated two additional times with similar quantitative results.

of buffer B and then the enzyme was eluted with 20 ml of buffer B + 1mM EDTA. Both wash and eluate fractions were assayed for PDE and inhibitor activity.

Results

When we initially examined cyclic nucleotide phosphodiesterase activity from MA-160 cells, we found that while the particulate fraction was linear with protein concentration, the soluble fraction was not (Table 1). These data suggested to us that there could be a soluble inhibitor of the PDE. A number of treatments were tested for their effect on this putative inhibitor. When the soluble fraction was heated at 60° C for 30 min., the PDE activity was inactivated, but the fraction was still able to inhibit the PDE activity in the particulate fraction (Table 2). However, boiling the soluble fraction for 5 min. eliminated all the inhibitory activity (Table 2). Extensive dialysis of the soluble fraction against Tris-HCl pH 7.4 buffer plus 2mM $MgCl_2$ did not eliminate the inhibitory activity of this fraction (Table 2), while a trypsin treatment did inactivate a large portion of the inhibitory activity.

In our attempts to fractionate the inhibitor from the PDE, we found that ammonium sulfate precipitation and DEAE-cellulose chromatography did not separate the two activities. However a modification of the procedure described by Morrill et al (15) using blue-dextran sepharose chromatography did separate the inhibitor from PDE. Fig. 1 illustrates a typical elution profile

Table 2. The effect of various treatments on PDE inhibitory activity

Treatment	% inhibition of PDE activity
-----	100
60° C, 30 min.	100
boiling (5 min.)	2
dialysis (48 hrs.)	100
trypsin (50µg, 30 min. at 30° C)	13

Inhibitor from MA-160 cells was prepared by blue-dextran affinity chromatography as described in the text. Control inhibition (100%) refers to that amount of inhibitor protein required to reduce the particulate PDE (20,000xg pellet) activity from MA-160 by 50%. The particulate PDE, assayed using 100µM cyclic AMP as substrate, was used for all of these inhibitor studies.

from this procedure. A 0-30% ammonium sulfate fraction of MA-160 20,000xg supernatant was dialyzed and placed on a 1 ml Cibicron-blue Sepharose column. The non-adherent proteins were washed off the column with buffer. Approximately 25-30% of the applied PDE activity did not adhere to the column and this fraction exhibited linearity with respect to protein concentration. The bound PDE was eluted from the column with buffer containing 1mM EDTA. These fractions

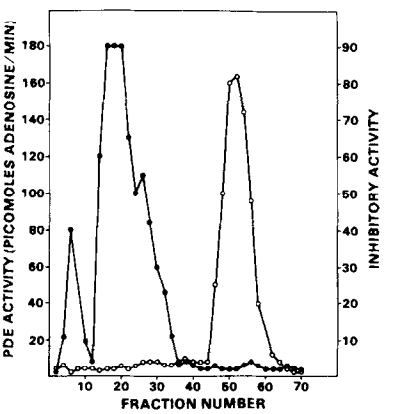

Fig 1. Separation of inhibitor from PDE using blue-dextran sepharose chromatography. A 30% ammonium sulfate fraction (dialyzed, 7 mg protein) from MA-160 cell cytosol was loaded onto a 1 ml column of Cibicron blue-Sepharose. 70, 1 ml fractions were collected and tested for PDE activity (.----.) and inhibitor activity (o---o). After fraction 12, 1mM EDTA was added to the buffer, while after fraction 42, 0.2mM cyclic GMP was added to the buffer.

Table 3. The effect of inhibitor from MA-160 cells on PDE activity from various sources

Cell or Tissue	PDE activity no inhibitor	PDE activity + inhibitor
A549	68.4	30.1
K-16	51.0	19.8
Bovine Brain Ca^{++}-dependent	86.3	42.1
Bovine Retina Ca^{++}-independent	77.9	39.5

Supernatant PDE was prepared from A549 and K16 cells as described for MA-160 cells. Bovine brain Ca^{++}-dependent PDE was prepared as described by Cheung (16), while bovine retinal Ca^{++}-independent PDE was prepared as described by Liu and Wong (8). The source of the inhibitor was a 60° C, 30 min. heat treated 20,000xg supernatant from MA-160 cells and was used at a concentration that suppressed 50% of MA-160 particulate PDE activity. PDE activity was measured using 100μM cAMP and is expressed as nanomoles adenosine/30 min./mg protein.

also had enzyme activity which was proportional to protein concentration. When the column was then washed with buffer containing 0.2mM cyclic GMP, no substantial additional PDE was eluted, but a sharp peak of inhibitor was recovered (Fig. 1).

The ability of the inhibitor from MA-160 cells to suppress PDE activity from other sources was examined (Table 3). Crude homogenate PDE activities from a human alveolar carcinoma cell line (A549) and a rat liver epithelial cell line (K16) were both inhibited in a concentration dependent fashion by the inhibitor from MA-160. Neither A549 or K16 cells displayed any evidence of an endogenous PDE inhibitor. In addition Ca^{++}-activated and Ca^{++}-independent PDE activity from bovine brain and retina respectively was depressed by the inhibitor from MA-160 cells.

Discussion

Several PDE inhibitors from various tissues have been described (7-12). However most of these are heat stable molecules (95° C, 5 min.) which clearly differentiate them from the inhibitor which we have described. Wang and Desai (12) as well as Klee and Krinks (11) have reported finding a heat labile PDE

inhibitor from bovine brain. It appears that this inhibitor specifically suppresses the activity of the Ca^{++}-dependent PDE. We have demonstrated that the inhibitor from MA-160 cells inhibits the activity of both Ca^{++} dependent and independent forms of PDE, thus differentiating it from the heat labile bovine inhibitor. The inhibitory molecule is probably a protein, since it is relatively large (non-dialyzable) and susceptible to trypsin digestion. Although the mechanism of action of the inhibitor is not known, the finding that the inhibitor binds more avidly to blue-dextran than PDE suggest that the inhibitor might be sequestering the substrate and preventing it from being hydrolyzed by PDE. Kinetic studies (data not presented) have shown that the inhibition is competitive with respect to substrate concentration. The physiological significance of this inhibitor for the regulation of cyclic nucleotide metabolism awaits further investigation.

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REFERENCES

1. Kelly, L. A., and Butcher, R. W. (1974) J. Biol. Chem. 249, 3098-3102.
2. Chlapowski, F. J., Kelly, L. A., and Butcher, R. W. (1975) Advances in Cyclic Nucleotide Research. 6, 245-338, Raven Press, N. Y.
3. Niles, R. M., Makarski, J. S., Kim, J. H., and Rutenburg, A. M. (1977) In Vitro 13, 467-472.
4. Makarski, J. S., and Niles, R. M. (1978) Exp. Cell Res. 114, 191-195
5. Russell, T. R., and Pastern, I. H. (1974) J. Biol. Chem. 249, 7764-7769.
6. Schwartz, J. P., and Passonneau, J. V. (1974) Proc. Natl. Acad. Sci. USA 71, 3844-3848.
7. Dumler, I. L., and Etingof, R. N. (1976) Biochem. Biophys. Acta 429, 474-484.
8. Liu, Y. P., and Wong, V. G. (1979) Biochem. Biophys. Acta 583, 273-278.

9. Sharma, R. K., Wirch, E., and Wang, J. H. (1978) J. Biol. Chem. 253, 3575-3580.

10. Beale, E. G., Dedman, J. R., and Means, A. R. (1977) J. Biol. Chem. 252, 6322-6327.

11. Klee, C. B., and Krinks, M. H. (1978) Biochemistry 17, 120-126.

12. Wang, J. H., and Desai, R. (1976) Biochem. Biophys. Res. Comm. 72, 926-932.

13. Fraley, E. F., Eckers, S., and Vincent, M. (1970) Science 170, 530-533.

14. Chlapowski, F. J., and Butcher, R. W. (1973) Biochem. Biophys. Acta 309, 138-148.

15. Morrill, M. E., Thompson, S. T., and Stellwagen, E. (1979) J. Biol. Chem. 254, 4371-4374.

16. Cheung, W. Y. (1969) Biochem. Biophys. Acta 191, 303-315.