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Cyclic Adenosine Monophosphate Production by Embryonic Chick Cells

Abstract. *Cells dissociated from 1-day-old chick embryos produce a pulse of cyclic adenosine monophosphate (cyclic AMP) when stimulated with cyclic AMP. There is a stimulus threshold concentration of about 10^{-8} molar cyclic AMP and an upper limit, above which the response is suppressed, of about 6×10^{-6} molar. The response occurs within 5 seconds of stimulation and corresponds to an average pulse size in the range of 10^7 molecules per cell.*

A microelectrode releasing small amounts of cyclic adenosine monophosphate (cyclic AMP) continuously can divert the axis of a 1-day [Hamburger and Hamilton (*1*) stages 4 and 5] chick embryo (*2*). Cells dissociated from such an embryo agglutinate in suspension; the agglutination is enhanced by added cyclic AMP and its dynamics have been interpreted as implying that it is controlled by a propagated cyclic AMP signal (*3*). It therefore seemed important to establish whether cells dissociated from the chick embryo do, indeed, produce cyclic AMP in response to stimulation by cyclic AMP. We report here the results of direct assays performed to settle this point.

Fertile White Leghorn eggs were incubated for 1 day at 38°C. The embryos, at Hamburger and Hamilton stages 4 and 5, were removed and dissociated into single cells by agitation in $10^{-4}M$ EDTA in chick Ringer solution (*3, 4*). The cells so obtained have been described previously (*4*). The cells were incubated, in suspension in Ringer solution, at 38°C for 1 hour, having been adjusted to the required cell density after a sample was counted in a hemocytometer. Stimulation with cyclic AMP was accomplished, at 38°C, by adding 0.2 ml of cell suspension, with an Eppendorf pipette, to 1.8 ml of cyclic AMP solution in Ringer solution. The mixture was vortexed to ensure complete mixing and samples (500 μl) were transferred to test tubes, which had been preheated in boiling water to inhibit further cellular responses. The cells were removed by centrifugation at 4500*g* for 15 minutes since we had found that their nonspecific cyclic AMP binding capacity interfered with the subsequent assay. Samples were taken at times up to 40 seconds after stimulation; each experiment was performed in triplicate. For high stimulus concentrations the samples were diluted with Ringer solution to bring the added cyclic AMP concentration to $10^{-7}M$, within the preferred range for assay. The assay used depended on the capacity of human erythrocyte ghosts to bind cyclic AMP with high specificity. Ghosts were prepared by the method of Fairbanks *et al.* (*5*) and the assay was performed as described by Hesse *et al.* (*6*). The technique is essentially that of isotope dilution wherein tritiated cyclic AMP, bound to the ghosts, is displaced by cyclic AMP in the sample to be assayed, and the amount displaced is compared with amounts on a calibration curve for the batch of ghosts used. The input cyclic AMP concentration is subtracted from the total found, giving the amount produced by the stimulated cells.

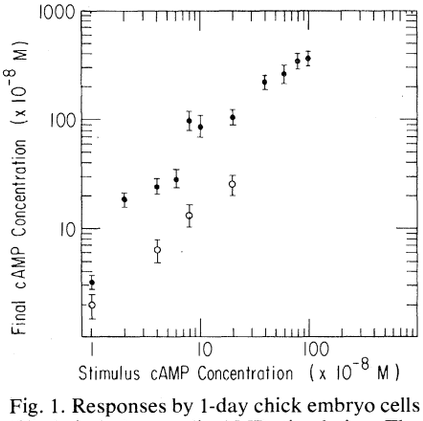

Fig. 1. Responses by 1-day chick embryo cells (filled circles) to cyclic AMP stimulation. The cell density was 7.7×10^6 per milliliter. Samples, in triplicate for each point, were collected 40 seconds after stimulation. A cyclic AMP phosphodiesterase (PDE) inhibitor, IBMX (3-isobutyl-1-methyl xanthine), was present at a concentration of $10^{-5}M$. Diminished responses were found after incubation for 40 seconds without IBMX, suggesting the presence of an extracellular PDE (*3*). Open circles show responses at room temperature.

Figure 1 shows the results of a typical experiment. Cyclic AMP production was caused by stimulus concentrations over a range of 10^{-8} to $10^{-6}M$. A control experiment, in which samples of cells were added to Ringer solution only, showed no cyclic AMP release; the graph shows a much reduced production by cells at room temperature (22° to 23°C). The amount of cyclic AMP produced per cell may be readily calculated; it reached a maximum of 2×10^8 molecules for a stimulus concentration of $10^{-6}M$. For a stimulus of $8 \times 10^{-8}M$, which produced the greatest input/output ratio, the response corresponded to 6×10^7 molecules per cell. In further experiments we found that no response could be detected, with our assay, for stimulus concentrations above $6 \times 10^{-6}M$, and that all the cyclic AMP was released within 5 seconds of stimulation, the shortest time we could employ reliably. No responses were found to dibutyryl cyclic AMP over a concentration range of 10^{-8} to $10^{-5}M$.

These results show that cells from the early chick embryo can produce cyclic AMP very rapidly, when stimulated with cyclic AMP, and thus provide further evidence for a role of cyclic AMP in intercellular communication during development. While the results are consistent with rapid extracellular release of cyclic AMP, this must be established by further experiment. Nonetheless, our results show that there are interesting parallels with signaling in the cellular slime mold *Dictyostelium discoideum* (*7*) and suggest that responsiveness to low concentrations of extracellular cyclic AMP might be a usually unnoticed but nonetheless widespread phenomenon in metazoan cells and tissues. Indeed, Whitfield *et al.* (*8*) came to a similar conclusion when examining the effects of cyclic AMP concentrations between 10^{-8} and $10^{-6}M$ on mitogenesis in rat thymic lymphoblasts.

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Platelet Factor 4: An Inhibitor of Collagenase

Abstract. *Human platelet factor 4 (PF4) is known to bind to heparin and inhibit its anticoagulant effect. This factor also inhibits the enzyme collagenase derived from cultured human skin and collagenase extracted from human granulocytes. The addition of heparin to the PF4-collagenase assay system has no effect on the observed inhibition of collagenase. Thus PF4 inhibits collagenase, in addition to neutralizing heparin.*

Platelet factor 4 (PF4) is a cationic protein of low molecular weight obtained from platelets either during the release reaction (*1*) or by mechanical damage (*2*). Its purification by affinity chromatography (*3*) and amino acid sequence have been reported (*4*). The only biological activity of PF4 described so far is that of neutralizing the anticoagulant effect of heparin (*5*), and the physiological significance of this activity is unknown. We report a new role for PF4, its ability to inhibit human skin and granulocyte collagenases.

The enzyme collagenase is found in many tissues (*6*) and has also been isolated from human granulocytes (*7*). Collagenases degrade the protein collagen at neutral *p*H and physiological temperature, and their regulation of collagen metabolism is important in both normal and disease states. We have observed inhibition of the enzyme from normal human skin (*8*) and tumor cells (*9*) by cationic proteins of low molecular weight isolated from cartilage and aorta. In order to ascertain whether collagenase inhibition by cationic proteins is a general phenomenon, we examined the role of another positively charged protein, highly purified PF4.

Platelet factor 4 was isolated from human platelets by affinity chromatography (*3*). The protein displayed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis. No phenylalanine or methionine was found by amino acid analysis. These data were taken as evidence for homogeneity.

Human facial skin was cultured, and the medium containing collagenase was treated as described (*10*). Granulocytes were isolated either by Ficoll-Paque centrifugation of freshly drawn defibrinated whole blood (*11*) or by dextran sedimentation of the white cell-rich fraction of human blood (*12*).

After granulocytes were obtained, collagenase was isolated (*13*). The enzyme was partially purified by $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added to 20 percent saturation at 4°C, and centrifuged at 25,000*g* for 15 minutes at 2°C. The supernatant was brought to 50 percent saturation and centrifuged as above. Most of the enzymatic activity was present in the dialyzed 50 percent $(NH_4)_2SO_4$ pellet as determined by the following assay.

Collagenase activity was measured by the release of [^{14}C]glycine peptides from guinea pig skin collagen (*14*). The PF4 was first incubated for 30 minutes at 22°C with human skin or human granulocyte collagenase. This mixture was then incubated with the collagen substrate for 4 hours at 37°C. The reaction was terminated by centrifugation for 5 minutes in a Beckman 152 Microfuge. Portions of the supernatant were placed in Bray's solution containing 4 percent Cab-O-Sil, and radioactivity was measured in a liquid scintillation counter. Polyacrylamide disc gel electrophoresis of reaction products confirmed that proteolysis had occurred.

Inhibition of human skin collagenases was observed when as little as 0.82 μg of PF4 were first incubated with collagenase in a total volume of 250 μl (Table 1). Comparable inhibition of granulocyte collagenase was also found.

Platelet factor 4 does not appear to be a general enzyme inhibitor because at a concentration of 10 μg/ml it does not inhibit plasma lipoprotein lipase (*15*). Moreover, it does not affect coagulation factors IXa, Xa, or XIa (*16*).

The concentration of PF4 utilized in these studies was higher than the 5 to 50 ng/ml in human plasma, but below the level of 8000 to 15,000 ng/ml in human serum (*17*). These concentrations therefore are in the physiological range.

In view of the known binding of heparin to PF4 and neutralization of its anticoagulant effect, the influence of heparin on PF4 inhibition of collagenase was examined. When 0.1 to 10.0 units of heparin were incubated with PF4 prior to incubation with collagenase, no effect on the inhibition pattern was observed (Table 2).

The demonstration that PF4 possesses

Table 1. Inhibition of collagenase by human platelet factor 4.

Reaction mixture*	Enzyme activity†	Inhibition (%)
Human skin collagenase (782 μg)	788	
Human skin collagenase plus 2.1 μg of PF4	410	48.0
Human skin collagenase plus 0.82 μg of PF4	472	40.0
Human granulocyte enzyme (477 μg)	486	
Human granulocyte enzyme plus 4.1 μg of PF4	124	74.5

*Protein values were determined by the Warburg and Christian method (*18*) for collagenase and for PF4 by the Lowry method (*19*). All solutions were buffered in 0.05*M* tris, 0.005*M* $CaCl_2$, *p*H 7.4. † Enzyme activity is expressed as the number of counts per minute above trypsin blank. Total number of counts per minute was 1620 per 400 μg of collagen. The assay was performed at 37°C for 4 hours.

Table 2. Platelet factor 4 inhibition of human skin collagenase in the presence of heparin.

Reaction mixture*	Enzyme activity†	Inhibition (%)
Human skin collagenase (889 μg)	598	
Human skin collagenase plus 2.0 μg of PF4	398	33.6
Human skin collagenase plus 2.0 μg of PF4 plus 0.1 unit of heparin	416	30.5
Human skin collagenase plus 2.0 μg of PF4 plus 1.0 unit of heparin	377	37.0
Human skin collagenase plus 2.0 μg of PF4 plus 10.0 units of heparin	412	31.0

*Protein values were determined by Warburg and Christian method (*18*) for collagenase and by the Lowry method (*19*) for platelet factor 4. Heparin was first incubated with PF4 for 15 minutes at 22°C. Collagenase was then added and the mixture was allowed to stand for 30 minutes. A portion of the reaction mixture was then pipetted into [^{14}C]glycine-labeled collagen and incubated for 4 hours at 37°C. All solutions were buffered in 0.05*M* tris, 0.005*M* $CaCl_2$, *p*H 7.4. †Enzyme activity is expressed as counts per minute above trypsin blank. The total number of counts per minute was 1540 per 400 μg of collagen.

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