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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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collagenase inhibitory activity suggests a possible physiological role for this protein in connective tissue metabolism. Platelets are normally activated during in vivo clot formation or tissue injury. Such injury induces an inflammatory reaction with attraction of granulocytes to the injured area. The precise nature of the interactions among platelets, collagen, released PF4, granulocytes, and collagenase remains to be elucidated.

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Temperature Coupling in the Vocal Communication System of the Gray Tree Frog, *Hyla versicolor*

Abstract. *The gray tree frog mates over a temperature range of at least 9°C. Gravid females, tested at two different temperatures, preferred synthetic mating calls with temperature-dependent temporal properties similar to those produced by a male at about the same temperature as their own. Thus, the vocalization system and the temporal pattern recognition system are affected by temperature in a qualitatively similar fashion.*

Several properties of the mating calls of the gray tree frog, *Hyla versicolor*, are temperature-dependent (*1*). Since these frogs call over a temperature range of at least 13°C (*2*), the question of whether females respond differentially to vocalizations produced over this temperature range arises (*3*). In my experiments, females discriminated between synthetic mating calls on the basis of temperature-dependent temporal properties; furthermore, the females preferred calls with temporal properties similar to those produced by a conspecific male at about the same body temperature as their own. Despite the widespread implicit assumption that this phenomenon, which I term temperature coupling, occurs in the sound communication systems of poikilothermic animals (*4, 5*) my experiments are, to my knowledge, the first demonstration in vertebrates and the first corroboration of the classic study of crickets published more than 20 years ago by Walker (*6*).

Synthetic mating calls were generated by summing two phase-locked sinusoids of 1.1 kHz (−6 dB) and 2.2 kHz (0 dB), modulating this waveform to produce pulses of variable pulse-repetition rate (PR) and duration (PD), and then superimposing another stage of modulation on trains of such pulses to produce calls. The PR's were chosen on the basis of a linear regression analysis of the mating calls of 39 males with body temperatures between 12.8° and 26°C (Fig. 1) (*7*). Further analysis indicated that PD was inversely correlated with temperature so that the duty cycle (PD divided by pulse period) tended to remain constant. [Although there was a slight tendency for the number of pulses per call (PN) to decrease with rising temperature, PN was variable within and among individuals; therefore I decided to hold PN (14 pulses) as well as the duty cycle (~0.6) constant in the two principal experimental stimuli (Fig. 2. A and B).] I used three additional experimental stimuli: a typical natural call (Fig. 2C), recorded at 23°C, and two synthetic versions. One synthetic call (Fig. 2D) was composed of frequency-modulated (FM) pulses, and the other (Fig. 2E) lacked FM pulses (*8*). Each kind of call was recorded on magnetic tape on one channel of a stereophonic recorder (Nagra) and had a fixed timing relationship to another call on the other channel (Fig. 2A) (*9*).

Discrimination experiments took place in a dimly lit semianechoic, temperature-regulated (±1°C) chamber (4 m by 2 m by 1.8 m). A stereophonic recorder (Nagra) drove two speakers (Nagra) placed 2 m apart on a plywood floor. Sound pressure levels (SPL's) of the two sounds were equalized midway between the speakers (*10*). Twenty-six amplexed pairs were collected in Boone County, Missouri, in early May 1977. Each gravid female was separated from the male and either refrigerated at 4°C (to inhibit oviposition) for as long as 18 hours before testing or placed directly into the chamber. After a minimum of 20 minutes of acclimation in the chamber, each female was placed in a petri dish midway between the speakers and restrained with a hardware cloth cage until both sounds had been played several times. After the female was freed, a response was tabulated if she touched a speaker or came within 5 cm of it. The cloacal temperature of the animal was taken immediately with a quick-reading thermometer (Schultheis).

Eighteen females responded in at least one discrimination experiment. Twelve females, tested at 75 dB, chose the synthetic call with a PR of 15 sec^{-1} when their body temperatures were about 16°C. Ten females, tested at 75 dB, chose the synthetic call with a PR of 24 sec^{-1} and one chose the synthetic call of 15 sec^{-1} when their body temperatures were about 24°C (Table 1). These responses were obtained from a total of 14 females. Six that first responded at 24°C later responded at 16°C; three that first responded at 16°C later responded at 24°C. The one female that chose the 15 sec^{-1} call at 24°C initially chose the 15 sec^{-1} at 16°C. At least 6 hours elapsed between the first test at 75 dB at one temperature and the second test at 75 dB at the other temperature. (Females were refrigerated at 4°C until 20 minutes before the second test.) Clearly, the females preferred the calls with temperature-dependent temporal properties similar to those produced by a conspecific male at about the same temperature as their own.

Tests at 85 dB were conducted within a few minutes of the 75-dB test. Each female was replaced at the central release point, and the stimuli were usually ex-

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