REFERENCES AND NOTES

1. S. M. Broder and R. C. Gallo, *N. Engl. J. Med.* **311**, 1292 (1984).
2. J. J. Goedert *et al.*, *Lancet* **1984-II**, 711 (1984); J. Laurence *et al.*, *N. Engl. J. Med.* **311**, 1269 (1984); H. C. Lane *et al.*, *ibid.* **313**, 79 (1985); J. K. A. Nicholson *et al.*, *Ann. Intern. Med.* **103**, 37 (1985).
3. A. S. Fauci *et al.*, *Ann. Intern. Med.* **102**, 800 (1985).
4. M. Popovic *et al.*, *Science* **224**, 497 (1984).
5. F. Barré-Sinoussi *et al.*, *ibid.* **220**, 868 (1983); R. C. Gallo *et al.*, *ibid.* **224**, 500 (1984); J. Schüpbach *et al.*, *ibid.* p. 503; M. G. Sarngadharan, M. Popovic, L. Bruch, J. Schüpbach, R. C. Gallo, *ibid.* p. 506.
6. D. Klatzman *et al.*, *ibid.* **225**, 59 (1984); M. Popovic, E. Read-Connole, R. C. Gallo, *Lancet* **1984-II**, 1472 (1984).
7. D. Klatzman *et al.*, *Nature (London)* **312**, 767 (1985).
8. A. G. Dalgleish *et al.*, *ibid.*, p. 763.
9. J. Zavada, *J. Gen. Virol.* **15**, 183 (1972); P. Clapham, K. Nagy, R. A. Weiss, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 2886 (1984).
10. J. S. McDougal *et al.*, *J. Immunol.* **135**, 3151 (1985).
11. T. C. Fuller *et al.*, *Human Immunol.* **9**, 89 (1984).
12. C. Terhorst, A. van Agthoven, E. Reinherz, S. Schlossman, *Science* **209**, 520 (1980).
13. J. S. Allan *et al.*, *ibid.* **228**, 1091 (1985); F. D. Veronese *et al.*, *ibid.* **229**, 1402 (1985).
14. J. Schüpbach *et al.*, *ibid.* **224**, 503 (1984).
15. L. Ratner *et al.*, *Nature (London)* **313**, 277 (1985).
16. M. B. Breuner, I. S. Trowbridge, J. L. Strominger, *Cell* **40**, 183 (1985).
17. J. S. McDougal *et al.*, *J. Immunol. Methods* **76**, 171 (1985).
18. R. A. Weiss *et al.*, *Nature (London)* **316**, 69 (1985); M. Robert-Guroff, M. Brown, R. C. Gallo, *ibid.* p. 72.
19. F. Barin *et al.*, *Science* **228**, 1094 (1985).
20. G. M. Shaw *et al.*, *ibid.* **226**, 1165 (1984); F. Wong-Staal *et al.*, *ibid.* **229**, 759 (1985).
21. O. Narayan, J. E. Clements, D. E. Griffin, J. S. Wolinsky, *Infect. Immun.* **32**, 1045 (1981).
22. Monoclonal antibodies and paraproteins used in this study were T4A, T4, T11 (OKT4A, OKT4, OKT11, Ortho Pharmaceutical) and the murine plasmacytoma paraprotein MOPC 141 (Litton Bionetics).
23. The HTLV-III/LAV preparation (LAV1 prototype strain) was derived from culture supernatants of HTLV-III/LAV–infected, PHA-stimulated lymphocytes (*17*), which were harvested by sequential centrifugation (300*g* for 7 minutes, followed by 1500*g* for 20 minutes) and concentrated 1000-fold by ultracentrifugation at 90,000*g* for 90 minutes over a cushion of 15 percent Renografin-60 (Squibb).
24. O. Acuto *et al.*, *Cell* **34**, 717 (1983). Precise conditions were 5×10^7 cells per milliliter in phosphate-buffered saline (PBS), *p*H 7.4, containing 1 mCi of $Na^{125}I$ per milliliter and 20 μg of lactoperoxidase per milliliter. At times 0, 1, 5, and 10 minutes, 10 μl of 0.03 percent H_2O_2 was added. Reaction was at 23°C and was stopped at 15 minutes by two centrifugations in 50 volumes of cold PBS containing 10 m*M* NaI.
25. Lysing buffer is 0.02*M* tris, 0.12*M* NaCl, *p*H 8.0, containing 0.2 m*M* phenylethylsulfonyl fluoride, aprotinin (5 μg/ml), 0.2 m*M* EGTA, 0.2 m*M* NaF, 0.2 percent sodium deoxycholate, and 0.5 percent Nonidet P-40 (by volume).
26. The human sera (nonimmune and HTLV-III/LAV–immune) were absorbed with CEM cells, and IgG fractions were prepared (*17*). Human IgG was coupled to Sepharose 4B (Pharmacia) by using CNBr [J. Porath, R. Axen, S. Ermback, *Nature (London)* **215**, 1491 (1967)]. These absorbents contained 4 to 5 mg of IgG per milliliter of Sepharose. Monoclonal antibody and the MOPC 141 absorbents are washed preparations of Sepharose CL–Protein A (2 mg of Protein A per milliliter of Sepharose; Pharmacia) that had been incubated with ≥5 mg of monoclonal protein per milliliter of Sepharose-Protein A.
27. D. M. Neville and H. Glossman, *Methods Enzymol.* **32**, 92 (1979).
28. We thank J. Curran, B. L. Evatt, F. Murphy, J. Bennett, and W. Dowdle for reviewing the manuscript, L. Leathers for editorial assistance, and J. Carter for typing the manuscript.

3 September 1985; accepted 20 November 1985

Prostacyclin Stimulation of the Activation of Blood Coagulation Factor X by Platelets

ASIM K. DUTTA-ROY, TARUN K. RAY, ASRU K. SINHA*

When platelets were incubated with prostacyclin, prostaglandin E_1, or prostaglandin D_2 at concentrations insufficient to increase the level of adenosine 3′,5′-monophosphate (cyclic AMP), coagulation factor X was activated by a platelet cysteine protease. Prostacyclin or prostaglandin E_1 at higher concentrations increased the cyclic AMP level and inhibited the activation of factor X by platelets. Inhibition of platelet adenylate cyclase by 2′,5′-dideoxyadenosine allowed the activation of the protease at higher concentrations of the autocoids. Prostaglandins A_1, A_2, B_1, B_2, E_2, $F_{2\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$, and thromboxane B_2, which do not affect platelet cyclic AMP level, did not stimulate the protease.

THE BLOOD COAGULANT FACTOR Xa plays a pivotal role in the blood coagulation process through the formation of the prothrombinase complex (*1*). We showed earlier that not only is the activation of factor X a critical step in coagulation, but the activated factor is a potent inhibitor of thromboxane A_2 synthesis in platelets (*2*) and prostacyclin (PGI_2) synthesis in endothelial cells (*3*). During blood coagulation, factor Xa is produced by a limited proteolysis of factor X by two different but interrelated pathways known as the intrinsic and extrinsic pathways (*4*). Factor X is also reported to be activated by a Ca^{2+}-dependent activator present on the platelet surface (*5*).

Prostacyclin, prostaglandin E_1 (PGE_1), and PGD_2 are known for their ability to inhibit platelet aggregation by increasing the intracellular level of adenosine 3′,5′-monophosphate (cyclic AMP) but do not seem to have any effect on the activation of blood coagulation factors (*5, 6*). Now, we report that the exposure of human blood platelets to amounts of these prostaglandins that are insufficient to increase cyclic AMP levels results in rapid stimulation of a protease or proteases that activate factor X. Higher concentrations of these prostaglandins inhibit platelet-dependent activation of factor X as a result of the increase in cyclic AMP. These findings indicate the existence of a pathway by which prostaglandins induce the activation of factor X in platelets.

Addition of 2 n*M* PGI_2 or PGE_1 to a mixture of gel-filtered platelets and purified bovine factor X resulted in the generation of factor Xa in the presence of purified bovine factor Va, as indicated by hydrolysis of S-2222 (Fig. 1) or coagulation assay (*7*). Human factor X was similarly activated. No activity was observed if prostaglandin, platelets, or factor X was omitted. There was no effect on the activity of factor Xa (0.4 unit/ml) when either 2 n*M* or 10 n*M* PGI_2 or PGE_1 was added to the assay mixture. Other investigators have shown that the catalytic activity of factor Xa in the conversion of prothrombin to thrombin is enhanced by the interaction of protease with the platelet surface factor Va, which acts as the receptor of factor Xa (*8*). We found that the addition of factor Va increased by approximately tenfold the amidolytic activity of factor Xa, as compared with controls containing no factor Va. Factor Va itself has no effect either on the hydrolysis of S-2222 or on the activation of factor X. The addition of factor Xa inhibitors like soybean trypsin inhibitor (23 μ*M*) or phenylmethylsulfonyl fluoride (2 m*M*) to the reaction mixture inhibited the hydrolysis of S-2222 by 100 and 95 percent, respectively, indicating the generation of factor Xa in the assay mixture.

In a separate experiment, diisopropyl phosphofluoridate derivative of factor X was prepared as described previously except that factor Xa was replaced by the zymogen (*2*). When factor X was replaced by the inhibitor-treated zymogen in the incubation mixture, no factor Xa activity was seen, as compared with the controls. Since diisopropyl phosphofluoride is a covalent inhibitor of factor Xa (*1*), the prostaglandin-mediated

Thrombosis Research Center and Department of Medicine, Temple University, Philadelphia, PA 19140.

*To whom correspondence should be addressed.

activation of factor X by platelets cannot be due to autocatalytic reaction.

The proteolytic activity of the prostaglandin-treated platelets was directly demonstrated by a radiometric assay in which [^{14}C]azocasein (*9*) was used instead of factor X in the incubation mixture in the absence of added factor Va. Platelets treated with 2 n*M* of either PGE_1 or PGI_2 hydrolyzed approximately 4.43 ± 0.26 μg of azocasein per minute per 10^8 cells in 1.0 mg of the substrate at 23°C. In the absence of the prostaglandins, no hydrolysis of [^{14}C]azocasein by platelets could be demonstrated in the incubation mixture under identical conditions.

The concentration of factor X used in the incubation mixture was approximately half the amount present in normal plasma. A twofold increase of factor X concentration in the assay mixture increased the hydrolysis of S-2222 by approximately 20 percent.

Concentrations of PGI_2 greater than 2 n*M* resulted in a decrease in the activation of the zymogen with a narrow optimum (Fig. 2). The decrease of activation of factor Xa occurred with a concomitant rise of cyclic AMP in platelets. For PGE_1, which is less potent than PGI_2 in increasing the cyclic AMP level, the maximal activation of factor X occurred over a broader range of the autocoid (2 to 8 n*M*) when compared to PGI_2. As in the case of PGI_2, the activation of factor X began to decrease only when the cyclic AMP level began to increase at higher concentrations of the prostaglandin.

Table 1. Effects of cyclic AMP on PGI_2- and PGE_1-stimulated activation of factor X by platelets. The experiments were run as described in Fig. 1. When Bt_2-cyclic AMP was used, the platelets were incubated with the nucleotide for 20 minutes at 23°C before the addition of the prostaglandins. The effects of DDA on the prostaglandin stimulation of factor X activation by platelets were studied by incubating the platelets with the inhibitor for 1.0 minute at 23°C before the addition of the prostaglandins. The results are means ± SEM of three experiments.

Addition to platelets	Factor Xa [U · (10^8 platelets)$^{-1}$ · min^{-1}]	Cyclic AMP [pmol · (10^8 platelets)$^{-1}$]
None	0.05 ± 0.01	2.0 ± 0.55
PGI_2 (2.0 n*M*)	0.61 ± 0.25	2.1 ± 0.75
PGI_2 (2.0 n*M*) + Bt_2–cyclic AMP (1.0 m*M*)	0.04 ± 0.01	
PGI_2 (2.0 n*M*) + forskolin (3 μ*M*)	0.03 ± 0.01	35 ± 5.58
PGI_2 (10 n*M*)	0.05 ± 0.01	20 ± 3.66
PGI_2 (10 n*M*) + DDA (500 μ*M*)	0.62 ± 0.24	2.0 ± 0.67
PGI_2 (1.0 μ*M*)	0.05 ± 0.01	56 ± 8.55
PGI_2 (1.0 μ*M*) + DDA (500 μ*M*)	0.66 ± 0.27	2.0 ± 0.64
PGI_2 (2.0 n*M*) + DDA (500 μ*M*)	0.65 ± 0.27	2.0 ± 0.57
PGE_1 (2.0 n*M*)	0.50 ± 0.15	2.1 ± 0.56
PGE_1 (2.0 n*M*) + Bt_2–cyclic AMP (1.0 m*M*)	0.04 ± 0.01	
PGE_1 (2.0 n*M*) + forskolin (3 μ*M*)	0.04 ± 0.01	30 ± 3.64
PGE_1 (1.0 μ*M*)	0.04 ± 0.01	17.5 ± 1.66
PGE_1 (1.0 μ*M*) + DDA (500 μ*M*)	0.55 ± 0.27	2.2 ± 0.42
DDA (500 μ*M*)	0.05 ± 0.01	2.0 ± 0.42
Forskolin (3 n*M*)	0.04 ± 0.01	2.2 ± 0.43

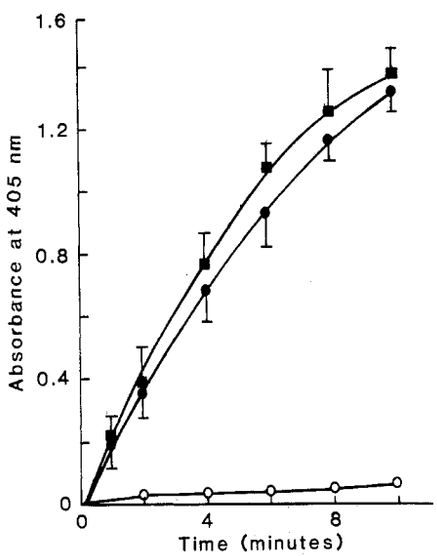

Fig. 1. PGE_1- and PGI_2-stimulated activation of factor X by platelets. Factor X was assayed by using chromogenic substrate S-2222 (*16*). Blood donors had not taken any medication for at least 2 weeks before blood donation. Venous blood was collected in 13 m*M* sodium citrate. Details of the preparation of gel-filtered platelets have been described except that the Tyrode's buffer did not contain any glucose (*2*). Bovine factor X was prepared by the method of Bajaj and Mann (*17*) and had specific activity of 100 to 150 units per milligram of protein. S-2222 (*N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-*p*-nitroanilide hydrochloride and its methyl ester) was the product of Kabi Diagnostica, Stockholm, Sweden. Typically, the formation of factor X was measured by incubating 0.21 unit of factor X with gel-filtered platelets (4.6×10^6) suspended in Tyrode's buffer (without glucose, *p*H 7.4), containing 0.03 unit of factor Va, 1.2 m*M* $CaCl_2$, 5 m*M* $MgCl_2$, and 0.4 m*M* S-2222. Incubation was carried out in the presence or absence of 2 n*M* PGI_2 or PGE_1 in a total volume of 0.4 ml in polypropylene centrifuge tubes (Eppendorf) for various times at 23°C. After incubation, the reaction mixture was centrifuged at 8000*g* at 4°C. The supernatant was collected and the released *p*-nitroaniline from S-2222 was determined in a Gilford spectrophotometer at 405 nm. The rate of factor Xa production was calculated by determining the kinetics of *p*-nitroaniline formation during the first minute of the incubation. Control experiments were run in an identical reaction mixture except that they did not contain any prostaglandin. The means ± SEM (n = 10) are indicated by vertical lines. PGI_2 (■); PGE_1 (●); control (○).

The inhibitory effect of cyclic AMP was demonstrated directly by incubating the platelets with 1.0 m*M* dibutyryl cyclic AMP (Bt_2–cyclic AMP) before the addition of 2.0 n*M* PGI_2 or PGE_1. Addition of forskolin (3 μ*M*) to the reaction mixture also completely inhibited the activation of factor X by low concentrations of PGI_2 or PGE_1 and increased the cyclic AMP level 15 times above the basal level (Table 1). However, unlike the prostaglandins, forskolin at a low concentration (3 n*M*), which did not increase cyclic AMP level in platelets, had no effect on the activation of the protease. Since forskolin stimulates the formation of cyclic AMP through its interaction with the catalytic subunit of adenylate cyclase (*10*), the failure of a low concentration of the diterpene to stimulate the cysteine protease indicated the probable involvement of prostaglandin receptors in the stimulatory process. Experiments with dideoxyadenosine (DDA), a potent inhibitor of platelet adenylate cyclase (*11*), provided additional evidence that cyclic AMP, and not high concentrations of prostaglandins as such, blocked factor X activation. Addition of DDA to the platelet suspension not only inhibited the formation of cyclic AMP in these cells, but allowed the activation of factor X even in the presence of 1 μ*M* PGI_2 or PGE_1, concentrations that previously completely suppressed the activator (Table 1). Dideoxyadenosine itself has no effect on the activation of factor X by platelets in the presence or absence of the prostaglandins.

Treatment of platelets with aspirin (1.0 m*M*) before the addition of either PGI_2 or PGE_1 produced no effect on the activation of factor X, although the aspirin-treated platelets did not show secondary aggregation with 0.5 n*M* thrombin or 4 μ*M* of adenosine diphosphate. These results indicate that platelet cyclooxygenase (*12*) is not involved in the activation of the platelet protease (or proteases) responsible for activation of factor X.

The activated protease that catalyzed the conversion of factor X to Xa was not released into the medium after centrifugation (8000*g* for 10 minutes) but remained associated with the platelet pellet.

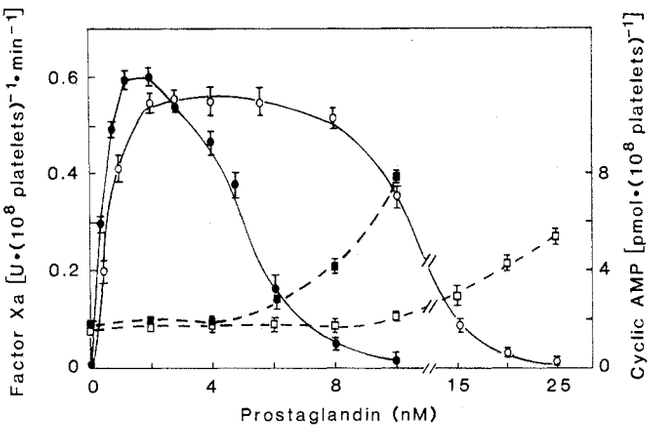

Fig. 2. Effect of PGI_2 or PGE_1 on the activation of factor X by platelets and the increase of cyclic AMP level. Activation of factor X by gel-filtered platelets with varying concentrations of PGI_2 or PGE_1, as indicated, was determined by using S-2222. The rate of factor Xa–formation was calculated from the kinetics of formation of *p*-nitroaniline. Parallel experiments were run to determine the cyclic nucleotide level. Cyclic AMP contents of platelets were determined by an affinity elution technique (*18*) by use of the protein kinase binding method (*19*) described previously. As little as 0.1 pmol of cyclic AMP could be measured by this method. Results shown here are means ± SEM ($n = 6$). Factor Xa formation is shown in the presence of PGI_2 (●) and PGE_1 (○); cyclic AMP level is shown in the presence of PGI_2 (■) and PGE_1 (□).

Both Ca^{2+} (optimal concentration, 1.2 m*M*) and Mg^{2+} (optimal concentration, 5 m*M*) are needed for the prostaglandin-stimulated activation of the coagulation factor. The prostaglandin-stimulated protease activity in platelets was decreased by 25 percent in the presence of 1.5 m*M* Mg^{2+}. No activity could be demonstrated in the absence of either of the metal ions, and the addition of 5 m*M* EDTA or EGTA totally inhibited the activation of factor X in the complete assay system. The effects of these metal ions on the activation of factor X by platelets are apparently related to the process of activation of the zymogen in the presence of the autocoids, since they produced no effect on the activity of purified bovine factor Xa as determined by amidolytic assay.

In an attempt to understand the nature of the protease in the activation of factor X, we added the inhibitors that did not inhibit factor Xa (and thereby interfere with the assay) in the incubation mixture. The generation of factor Xa activity in the complete assay system containing 2 n*M* PGI_2 was completely inhibited by iodoacetamide and *p*-chloromercuribenzoic acid (Table 2). These compounds, which are cysteine protease inhibitors, produced no effect on the activity of the purified bovine factor Xa, a serine protease (*1*). The inhibitors produced identical effects when PGI_2 was replaced by PGE_1 (2 n*M*) in the reaction mixture. The differential effects of the protease inhibitors not only indicate the generation of factor Xa in the assay mixture, but also suggest that the prostaglandin-stimulated protease of platelets activating the zymogen is probably a cysteine protease.

To determine the specificity of PGE_1 and PGI_2, we substituted various prostaglandins for them in the assay mixture. Of all the compounds tested, only PGD_2 showed a similar effect on the platelet-mediated activation of the coagulation factor. At 2.0 n*M* PGD_2, 0.48 ± 0.2 unit of factor Xa per 10^8 platelets per minute was formed in the incubation mixture. Prostaglandins A_1, A_2, B_1, B_2, E_2, $F_{1\alpha}$, $F_{2\alpha}$, 6-keto-prostaglandin $F_{1\alpha}$, or thromboxane B_2 at 2.0 n*M* or at 10 n*M* produced no effect on the stimulation of platelet-dependent activation of factor X. Other investigators have shown that among these prostanoids only PGI_2, PGE_1, and PGD_2 inhibit platelet aggregation and increase cyclic AMP levels in these cells (*13*).

Platelet-dependent activation of factor X is stimulated when prostaglandins that inhibit platelet aggregation through increased cyclic AMP are present in the assay mixture in concentrations insufficient for the activation of adenylate cyclase. The stimulation of prostaglandin-dependent platelet cysteine protease, which activates factor X, is counterregulated through the increase of intracellular cyclic AMP. However, factor Xa itself might provide an additional regulatory control on the activation. Since factor Xa inhibits its PGI_2 synthesis in endothelial cells (*3*), the protease itself might provide a negative feedback on the activation of the zymogen by platelets.

We showed earlier that PGE_1 is capable of inhibiting platelet aggregation through its action on plasma factors (*14*). However, concentrations of the autocoid needed for its effects on the plasma factors are much higher (micromolar ranges) than the amounts of the prostaglandin required for the activation of factor X through its action on platelets.

It is believed that, as a result of tissue injury, the initiation of blood coagulation occurs through the activation of factor X by intrinsic and extrinsic pathways (*4*). However, tissue injury would also produce PGI_2 in endothelial cells (*15*). The prostaglandin might then initiate blood coagulation through activation of factor X and augment the effects of intrinsic and extrinsic pathways on the coagulation process.

Table 2. Effects of protease inhibitors on the PGI_2-stimulated activation of factor X by platelets and on purified bovine factor Xa. Gel-filtered platelets (4.6×10^6) suspended in Tyrode's buffer, *p*H 7.4, containing 2 n*M* PGI_2, 0.21 unit of factor X, 0.03 unit of factor Xa, 1.2 m*M* $CaCl_2$, 5.0 m*M* $MgCl_2$, and 0.4 m*M* S-2222 in a total volume of 0.4 ml were incubated with the above inhibitors for 5 minutes at 23°C. After incubation, the platelets were separated by centrifugation and the release of *p*-nitroaniline was determined as described in Fig. 1. The results are means ± SEM for four experiments.

Inhibitor	PGI_2-stimulated factor X activation (%)	Bovine factor Xa activity (%)
None	100	100
Iodoacetamide (10 m*M*)	0	99 ± 0.56
p-Chloromercuribenzoic acid (10 m*M*)	0	98 ± 0.50

REFERENCES AND NOTES

1. C. M. Jackson, in *Hemostasis and Thrombosis*, R. W. Colman, J. Hirsh, V. J. Marder, E. W. Salzman, Eds. (Lippincott, Philadelphia, 1982), pp. 100–111; C. Hougie, *Proc. Soc. Exp. Biol. Med.* **101**, 132 (1952); M. P. Esnouf and W. J. Williams, *Biochem. J.* **84**, 62 (1962); W. H. Seegers, E. R. Cole, C. R. Harmison, E. Marciniak, *Can. J. Biochem. Physiol.* **41**, 1047 (1963); R. L. Lunblad and E. W. Davie, *Biochemistry* **4**, 113 (1965).
2. A. K. Sinha, A. K. Rao, J. Willis, R. W. Colman, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6088 (1983).
3. A. K. Sinha, A. K. Dutta-Roy, H. C. Chiu, G. J. Stewart, R. W. Colman, *Arteriosclerosis* **5**, 244 (1985).
4. R. G. Macfarlane, *Nature (London)* **202**, 498 (1964); E. W. Davie and O. D. Ratnoff, *Science* **145**, 1310 (1964).
5. N. Semeraro and J. Vermylen, *Br. J. Haematol.* **36**, 107 (1977).
6. J. Kloeze, *Experientia* **26**, 307 (1970); G. S. Duboff, J. A. Penner, J. Rohwedder, *Nature (London)* **251**, 430 (1974).
7. R. W. E. Denson, in *Human Blood Coagulation*, R. Biggs, Ed. (Blackwell Scientific, Oxford, 1972), pp. 631–634. The coagulation assay of factor Xa was

performed in the absence of added Russell's viper venom.
8. W. H. Kane and P. W. Majerus, *J. Biol. Chem.* **257**, 3963 (1982).
9. [^{14}C]Azocasein was prepared by reductive alkylation with [^{14}C]formaldehyde [D. Dottavio-Martin and J. M. Ravel, *Anal. Biochem.* **87**, 522 (1978)]. Reaction mixture containing 400 μg of [^{14}C]azocasein (21,525 dpm per milligram of protein), 1.2 m*M* $CaCl_2$, 5.0 m*M* $MgCl_2$, 2 n*M* PGE_1 or PGI_2, and 4.6×10^6 platelets in Tyrode's buffer were incubated for 20 minutes at 23°C. After incubation, 0.5 mg of unlabeled azocasein was added to the reaction mixture followed by the addition of 0.3 ml of 10 percent trichloroacetic acid. The trichloroacetic acid–treated reaction mixture was allowed to stand for 3 hours at 23°C and then the supernatant was collected by centrifugation at 8000*g* at 4°C. The radioactivity of the supernatant was measured by using ACS-II solvent in the liquid scintillation counter.
10. K. Seamon and J. W. Daly, *J. Biol. Chem.* **256**, 9799 (1981).
11. J. N. Fain *et al.*, *ibid.* **247**, 6866 (1973).
12. M. Hamberg, J. Svensson, B. Samuelsson, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2994 (1975).
13. E. W. Salzman and L. Levin, *J. Clin. Invest.* **50**, 131 (1971); R. J. Haslam, *Ciba Found. Symp.* **35**, 121 (1975); D. C. B. Mills and D. E. Macfarlane, *Thromb. Res.* **5**, 401 (1974); S. Moncada and J. R. Vane, *Pharmacol. Rev.* **30**, 293 (1979).
14. A. K. Sinha and R. W. Colman, *Science* **200**, 202 (1978).
15. J. H. Brox and A. Nordoy, *Haemostasis* **12**, 345 (1982); J. M. Ritter, S. E. Barrow, I. A. Blair, C. T. Dollery, *Lancet* **1983-I**, 317 (1983).
16. L. Aurell, P. Friberger, G. Karlsson, G. Claeson, *Thromb. Res.* **11**, 595 (1977).
17. S. P. Bajaj and K. G. Mann, *J. Biol. Chem.* **248**, 7729 (1973). Human factor X was kindly provided by the late Jeannette Piperno.
18. A. K. Sinha and R. W. Colman, *Eur. J. Biochem.* **73**, 367 (1977).
19. A. G. Gilman, *Proc. Natl. Acad. Sci. U.S.A.* **67**, 305 (1970).
20. Supported in part by a grant from the American Heart Association, Keystone Chapter, Pennsylvania. We are grateful to B. Ashby, R. W. Colman, and P. N. Walsh for their helpful suggestions and critical review of the paper. Human factor Va and [^{14}C]azocasein used in these studies were kindly provided by A. Annamali and T. Vitto, respectively.

11 July 1985; accepted 12 November 1985

A Parathyroid Hormone–Like Protein from Cultured Human Keratinocytes

J. J. MERENDINO, JR., K. L. INSOGNA, L. M. MILSTONE, A. E. BROADUS, A. F. STEWART*

Parathyroid hormone–like factors have been found in extracts of tumors associated with humoral hypercalcemia of malignancy, many of which are of squamous epithelial origin. Cultured, nonmalignant human keratinocytes were examined for the production of similar factors. Keratinocyte-conditioned medium from ten cultures stimulated the production of cyclic adenosine monophosphate in clonally derived rat osteosarcoma cells sensitive to parathyroid hormone. Bovine [Nle^{8,18}, Tyr^{34}]PTH-(3-34)NH_2, a competitive inhibitor of parathyroid hormone, stopped the adenylate cyclase production stimulated by keratinocyte-conditioned medium, but antisera to parathyroid hormone had no effect on such adenylate cyclase activity. The active component of keratinocyte-conditioned medium has a molecular weight exceeding that of native parathyroid hormone. These characteristics are shared by the parathyroid hormone receptor agonists associated with humoral hypercalcemia of malignancy, which suggests that normal human keratinocytes may produce a factor related to that produced by malignant tumors associated with humoral hypercalcemia of malignancy.

HYPERCALCEMIA IS THE MOST common paraneoplastic syndrome and affects up to 40 percent of patients with certain types of cancer (*1*). Although some cases result from direct destruction of bone by metastatic tumor, hypercalcemia frequently arises in patients without skeletal involvement (*1*). There is substantial evidence that in such instances the elevated serum calcium results from the secretion by tumors of a circulating factor (or factors) that leads to bone resorption (*1–3*). In one series of 50 unselected patients with cancer and hypercalcemia, nearly 80 percent suffered from this humoral hypercalcemia of malignancy (HHM) (*2*).

Certain factors derived from tumors associated with HHM share functional properties with parathyroid hormone (PTH). For example, we reported that extracts of four of five tumors from patients with HHM stimulated the PTH-sensitive adenylate cyclase in canine renal cortical membranes, whereas extracts of tumors from patients without HHM did not (*4*). This activity was competitively inhibited by PTH receptor antagonists but not by an antiserum directed against PTH. Similar findings have been reported by Strewler and co-workers (*5*) who used an HHM-associated human renal carcinoma cell line. In addition, Rodan *et al.* (*6*) demonstrated that medium conditioned by cells derived from HHM-associated tumors stimulated formation of cyclic adenosine monophosphate (AMP) by PTH-sensitive rat osteosarcoma (ROS) cells in a dose-dependent fashion (*6*). This activity was related to bone resorption in vitro.

Tumors with squamous epithelial features, including those of the oropharynx, lung, esophagus, cervix, vulva, and skin, account for the largest single subset of HHM-associated tumors (*1, 2*). We therefore examined nonmalignant human keratinocytes to determine whether nonneoplastic squamous epithelial cells might produce a factor (or factors) similar to the PTH receptor agonists derived from HHM-associated tumors. Our findings indicate that cultured human keratinocytes do produce an adenylate cyclase–stimulating protein that acts through the PTH receptor but that is distinct from native PTH.

Cultures of human keratinocytes were established from ten neonatal human foreskins (*7*). First passage keratinocytes were grown 1 week past confluence either in dishes containing irradiated murine 3T3 fibroblasts in complete medium [Dulbecco's modified Eagles medium (DMEM) containing fetal bovine serum (20 percent; KC Biologicals), hydrocortisone (0.4 μg/ml), and antibiotics (*7*)] or in dishes coated with type I collagen (Collagen Corp.) in calcium-free DMEM containing fetal bovine serum (10 percent), hydrocortisone (0.4 μg/ml), and antibiotics (*8*).

Keratinocyte-conditioned medium (KCM) harvested from confluent, first-passage cultures 48 hours after medium change was tested for parathyroid hormone–like bioactivity in the ROS assay with a modification of the method of Rodan and co-workers (*6, 9*). This assay measures cyclic AMP production (as an index of adenylate cyclase activity) by clonal ROS 17/2.8 cells in response to PTH receptor agonists. The assay has a detection limit of $5 \times 10^{-11}M$ PTH and shows a linear response over the range of 1×10^{-10} to $2.5 \times 10^{-9}M$. Normal PTH is believed to circulate at 10^{-12} to $10^{-11}M$. The only other known agonists in this system are β-adrenergic agonists such as isoproterenol.

Conditioned medium from each of the ten keratinocyte cultures stimulated adenylate cyclase activity in the ROS assay (Table 1). Four cultures of irradiated 3T3 cells, three cultures of neonatal foreskin dermal

J. J. Merendino, Jr., and A. E. Broadus, Yale University School of Medicine, New Haven, CT 06510.
K. L. Insogna, L. M. Milstone, and A. F. Stewart, West Haven Veterans Administration Medical Center, West Haven, CT 06516, and Yale University School of Medicine, New Haven, CT 06510.

*To whom correspondence should be addressed.