PLATELET PHOSPHORYLASE ACTIVITY IN THE PRESENCE OF ACTIVATORS AND INHIBITORS OF AGGREGATION

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Summary

Phosphorylase activity in suspensions of human platelets has been assayed before and after addition of thrombin and epinephrine which aggregate platelets, prostaglandins E_1 and E_2 and dibutyryl-3',5'-AMP which inhibit aggregation, or sodium fluoride and isoproterenol which alter adenine nucleotide metabolism. These various reagents did not change phosphorylase a activity in intact platelets, nor did cyclic-3',5'-AMP affect platelet phosphorylase activity in subcellular fractions. In contrast to many other tissues, platelet phosphorylase activity does not appear to be modulated by a direct effect of cyclic AMP.

Introduction

Thrombin, epinephrine and ADP, which cause platelet aggregation, have been reported to increase platelet glycogen depletion and lactate production, and to decrease platelet intracellular ATP and ADP (Karpatkin, 1967; Karpatkin and Langer, 1968). Thrombin stimulates glucose oxidation in platelets (Warshaw, Laster and Shulman, 1966), and also glycolysis (Mürer, 1969). However, these metabolic measurements have not been

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made at sufficiently short intervals to document the dependence of platelet aggregation on increased glycolytic and glycogenolytic activity. The findings that aggregation of human platelets induced by thrombin or ADP is inhibited by PGE_1 (Kloeze, 1966), that adenyl cyclase activity in particles from ultrasonically disrupted platelets is markedly increased by PGE_1 (Wolfe and Shulman, 1969), and that cyclic-3',5'-AMP and its dibutyryl derivative inhibit platelet aggregation (Marquis, Vigdahl, and Tavorimina, 1969), raised the question of the role of phosphorylase activity in the aggregation phenomenon. The present studies were undertaken to see whether agents promoting platelet aggregation (thrombin, epinephrine) or ones inhibiting aggregation (PGE_1, dibutyryl-3',5'-AMP) would alter phosphorylase activity within the one minute period after addition during which they affect aggregation. The percentage of phosphorylase a (phosphorylase activity which is 5'-AMP independent) compared to total phosphorylase activity was measured.

Materials and Methods

Prostaglandins PGE_1 and PGE_2 were donated by Dr. John Pike of the Upjohn Company; cyclic-3',5'-AMP and its dibutyryl derivative were obtained from both Sigma Chemical Company and Calbiochem; L-epinephrine from Parke-Davis Company; and isoproterenol from Winthrop Labs. Human thrombin was obtained from Dr. D. Aronson of the NIH Division of Biologic Standards.

Platelets were isolated at room temperature as described previously (Wolfe and Shulman, 1969). Acid citrate (0.02M) or EDTA (0.006M) anticoagulants gave equivalent results. Platelet suspensions contained fewer than 100 red and white cells per 10^5 platelets. Platelets sedimented from plate-rich plasma at 2250 x g for 15 minutes were resuspended in 0.34M sucrose containing 0.004M theophylline and 0.05M tris-HCl buffer, pH 6.8.

All experiments were performed on platelet suspensions which had been pre-incubated in 0.004M theophylline at 37° C. for 10 minutes to inhibit phosphodiesterase activity before various reagents were added, but suspensions without theophylline gave the same results. During the first minute after addition of reagents, 0.3 ml aliquots were taken at 10 second intervals and immediately blown into precooled plastic capsules containing liquid nitrogen. The frozen sample was then pulverized by shaking in a "Wig-L-Bug" dental amalgamator for 15 seconds, after the method of Danforth, et al. (1962). Then 0.7 cc of 60% glycerol with 0.02M NaF and 0.001 to 0.005M EDTA cooled to -14° C. was added to each capsule and buffered to pH 6.8 with either 0.05M tris or 0.04M glycerolphosphate. The capsule was then shaken 15 seconds and 0.5 cc of an aqueous solution at 4° C. was added and the capsule shaken 15 more seconds. Two different types of solution were used in this step with equivalent results: one solution contained 0.05M tris, pH 6.8, 0.001M EDTA, 0.02M NaF, and 0.3% bovine serum albumin; the other solution contained 0.02M glycerol phosphate buffer, pH 6.8, 0.01M mercaptoethanol, 0.005M EDTA, and 0.02M NaF. Extracts were then centrifuged at 10,000 x g in a model L Spinco ultracentrifuge at 4° C., exposed to 0.08 cc of an aqueous suspension of acid-washed 105,000 x g for 60 minutes and filtered through an 0.8 μ Millipore filter. The 105,000 x g pellet was resuspended in 0.34M sucrose, 0.05M tris, pH 6.8, containing 0.004M theophylline, and was incubated 10 minutes at 37° C. for equilibration. Activators to be tested were then added and, after incubation at 37° C. for an additional one to ten minutes, 0.2 cc aliquots were added to the phosphorylase assay system described above.

Results

Control levels of phosphorylase a were on the average 55-60% of the total activity (see Table 1), which is in contrast to the report

of Scott (1967) who found levels of phosphorylase a of about 10%. Scott observed an increase in these levels to 50% on incubation of the platelets in nonphysiological media. We observed that 50% of the total phosphorylase was phosphorylase a even when platelets were spun down from plasma and frozen directly without exposure to other incubation media. The percent phosphorylase a was independent of platelet concentration which varied from 1.5-4.4 x 10^6 platelets per mm^3 in various suspensions used. Our findings are in agreement with the reports of

Table 1

Effect on Platelet Phosphorylase a Activity of Various Reagents Which Influence Metabolism of Platelet Adenine Nucleotides

Agent Added	Exper. Total Number	Phosphorylase a Activity as Percentage of Total Phosphorylase Activity — Seconds After Addition of Agent							
		0	10	20	30	40	50	60	120
Controls from all experiments	17	62			58			56	
Human Thrombin (1-4 NIH units/cc)	5	63	68	62	61	63	56	59	57
Epinephrine ($1x10^{-3}M$)	2	60	60	41	47	37	39	45	33
Prostaglandin PGE_1 (10^{-4} to $10^{-6}M$)	5	61	63	62	59	62	59	54	49
Prostaglandin PGE_2 (0.035M)	1	65	60	57	57	60	57	52	46
Dibutyryl-3',5'-AMP (0.005M)	2	61	60	54	54	57	43	48	41
Isoproterenol ($1.5x10^{-5}M$)	1	55	47	51	52	50	53	36	41
NaF (0.02M)	1	66	55	53	64	56	51	43	44

All values listed are averages when more than one experiment was performed. The standard deviations from the mean of the control values at 0, 30, and 60 seconds were 12.5, 12.1, and 14.0%, respectively. None of the values obtained with the various reagents added differed significantly from the mean value of the controls.

Karpatkin and Langer (1967, 1969 a, b) and Yunis and Arimura (1967), in which the level of phosphorylase a was found to be 50-60% of the total.

Human thrombin added to platelet suspensions at a concentration of 1-4 NIH units per cc caused no change in the relative content of phosphorylase a during the first minute after addition, as is shown in Table 1. This was true both in the absence and presence of 0.002M $CaCl_2$. Epinephrine (0.001M), prostaglandin PGE_1 (10^{-6} to 10^{-4}M), PGE_2 (0.035M), isoproterenol (1.5 x 10^{-5}M), NaF (0.02M) and dibutyryl-3',5'-AMP (0.005M) all failed to produce any changes in active phosphorylase levels during the first minute after addition to platelet suspensions (Table 1). Theophylline itself had no effect on the level of phosphorylase a in control experiments.

As is shown by the data in Fig. 1, the total phosphorylase activity decayed with equal rates in all platelet fractions even in the presence of 0.02M NaF, an inhibitor of phosphorylase phosphatase. Addition of cyclic-3',5'-AMP (0.001M) to these fractions had no significant effect in preventing the decay of total phosphorylase activity.

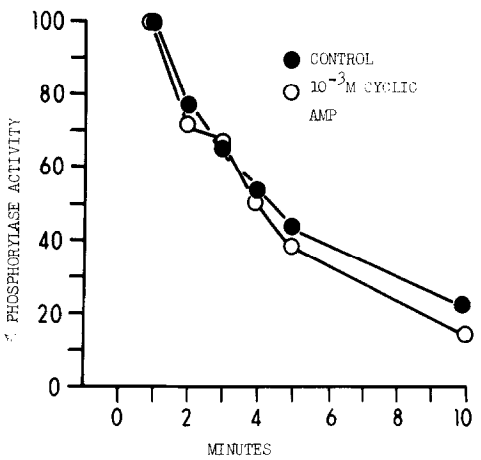

Fig. 1.--Effect of incubation at 37.5° C. on total phosphorylase activity in presence and absence of 0.001M cyclic-3',5'-AMP. The data represents the mean of 5 experiments performed on pellets obtained at 105,000 x g (see Methods). Similar results were obtained on supernatants of the 105,000 x g and 48,000 x g sediments.

Discussion

The results reported in this paper indicate that agents which cause platelet aggregation do not increase phosphorylase a levels before or during the aggregation process. These same agents, nevertheless, increase glycogen depletion and glucose oxidation when measurements are made subsequent to aggregation. Activators of platelet adenyl cyclase as well as cyclic-3',5'-AMP itself had no effect on the level of phosphorylase a in platelets. If cyclic-3',5'-AMP activated phosphorylase kinase in experiments with subcellular fractions, less phosphorylase decay might have been observed. However, there was no prevention of decay on adding cyclic-3',5'-AMP, even in the presence of NaF. The high endogenous level of phosphorylase a activity not dependent on 5'-AMP, as well as the inability to activate platelet phosphorylase with cyclic-3',5'-AMP, is consistent with the possibility that control of phosphorylase activity may be modulated by intracellular concentrations of noncyclic adenine nucleotides as previously suggested by Karpatkin and Langer (1969 a). It must be remembered that the treatment of platelet extracts with activated charcoal during the phosphorylase assay removed other adenine nucleotides such as ATP and ADP, in addition to removing AMP. Thus, changes in intracellular levels of these compounds occurring prior to disruption of platelets could not affect the amount of active or total phosphorylase under conditions of this assay. Moreover, although prostaglandin PGE_1 markedly increases platelet cyclic-3',5'-AMP through increases in adenyl cyclase activity, the effects of PGE_1 on platelet aggregation do not appear to be mediated through the formation of phosphorylase a by cyclic-3',5'-AMP.

References

Danforth, W. H., Helmrich, E., and Cori, C.F., (1962), P.N.A.S. 48, 1191.
Karpatkin, S., (1967), J. Clin. Invest. 46, 409.
Karpatkin, S., and Langer, R. M. (1967), Abstracts N.Y. Heart Assoc. 43, 1193.

Karpatkin, S., and Langer, R. M., (1968), J. Clin. Invest. 47, 2158.
Karpatkin, S., and Langer, R. M., (1969a), Biochim. Biophys. Acta, 185, 350.
Karpatkin, S., and Langer, R. M., (1969b), J. Biol. Chem. 244, 1953.
Kloeze, J., (1966), in "Proceedings of Nobel Symposium II, Stockholm, 1966", Almquist & Wiksell, Stockholm, p. 241.
Marquis, N. R., Vigdahl, R. L., and Tavorimina, P. A., (1969), Biochem. & Biophys. Res. Commun. 36, 965.
Mürer, E. H., (1969), Biochim. Biophys. Acta 172, 266.
Scott, R. B., (1967), Blood 30, 321.
Taussky, H. H., and Shorr, E., (1953), J. Biol. Chem. 202, 675.
Warshaw, A. L., Laster, L., and Shulman, N. R., (1966), J. Clin. Invest. 45, 1923.
Wolfe, S., and Shulman, N. R., (1969), Biochem. & Biophys. Res. Commun. 35, 265.
Yunis, A. A., and Arimura, G. K., (1967), Blood 30, 859.