Vol. 121, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1984 Pages 266-270

THE EFFECT OF CARBONIC ANHYDRASE INHIBITION ON THE VELOCITY OF THROMBIN-STIMULATED PLATELET AGGREGATION UNDER PHYSIOLOGICAL CONDITIONS

Winfried Siffert*,Georg Fox and Gerolf Gros

Institut für Physiologie, Universitätsklinikum Essen, 4300 Essen, F.R.G.

Received April 9, 1984

SUMMARY : We have studied the effect of ethoxzolamide, a specific carbonic anhydrase inhibitor, on the velocity of thrombin-stimulated platelet aggregation. After preincubation of platelet rich plasma with 10^{-6} M ethoxzolamide the velocity of platelet aggregation was reduced by about 40%. Between 10^{-11} M and 10^{-10}M ethoxzolamide was necessary to achieve a half-maximal diminution of the aggregation velocity. An identical maximal reduction of the velocity of aggregation as with ethoxzolamide could be achieved by a nearly complete removal of CO_2 from the platelet rich plasma. These results suggest that the intracellular CO_2 hydration-dehydration reaction is involved in the activation of human platelets by thrombin. It is possible that the cytosolic carbonic anhydrase of platelets provides a rapid source of the protons that are transferred across the plasma membrane during the activation process.

We have recently described the presence of a cytosolic carbonic anhydrase[1] in human platelets (1). This enzyme, which catalyses the CO_2 hydration-dehydration reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$, has been reported to occur in a variety of H^+-secreting tissues (2,3). Since in the course of platelet activation movements of protons have been reported to occur across the platelet plasma membrane (4,5) the question arose whether CA might be involved in this process as it is in H^+ and HCO_3^- transport processes of other cells. In a first attempt to study the role of CA-catalysed hydration of CO_2 in platelet function, we measured the kinetics of thrombin-stimulated platelet aggregation in platelet rich plasma by means of light-transmission aggregometry before and after inhibition of CA.

* Present address: Institut für Physiologie, Ruhr-Universität, 4630 Bochum, F.R.G.

[1] CA, carbonic anhydrase; PRP, platelet rich plasma.

0006-291X/84 $1.50

MATERIALS and METHODS

Ethoxzolamide and bovine thrombin were purchased from Sigma (Munich, West Germany). Blood was drawn by venipuncture from healthy donors who had taken no drugs for at least 10 days. 9 parts of blood were gently mixed with 1 part of 3.8% sodium citrate solution. Platelet rich plasma[1] was isolated from the other blood components by centrifugation for 15 min. at 120 x g at room temperature. Platelet counts in PRP ranged between 2.5 and $5 \cdot 10^5$ μl^{-1}.

Platelet aggregation experiments were performed at 37°C with PRP in a light-transmission aggregometer (Lumi Aggro-Meter, Model 400, Coulter Electronics, Krefeld, West Germany) according to (6). Before aggregation was induced by the addition of bovine thrombin each sample was equilibrated with a mixture of 4% CO_2/ 20% O_2/76% N_2. Thereafter the pH of the PRP was always in the range of 7.42 ± 0.05. In one series of experiments PRP was freed from CO_2 by equilibrating the sample with 100% O_2 for 90 min. The increase in pH due to the loss of CO_2 was corrected by the addition of 0.1 N HCl readjusting a pH of 7.45. In the carbonic anhydrase inhibition experiments samples were allowed to equilibrate with ethoxzolamide for 30 min before used for aggregometry. Aggregation velocities were obtained from the initial (i.e. maximal) slopes of the recorded aggregation curves. Aggregation curves represent the change (increase) in light transmission with time occuring in the course of platelet aggregation. Here, 100% transmission is defined as the light transmission across platelet free plasma, 10% transmission as that observed across the platelet rich plasma before initiation of the aggregation. In some experiments clotting of the samples occurred; these were excluded from quantitative evaluation.

RESULTS and DISCUSSION

Fig. 1 shows two original aggregometer records obtained with PRP (pH 7.45) that had been equilibrated with 4% CO_2, in the absence and presence of $1 \cdot 10^{-6}$ M ethoxzolamide. Platelet aggregation was induced by the addition (arrows) of thrombin (final

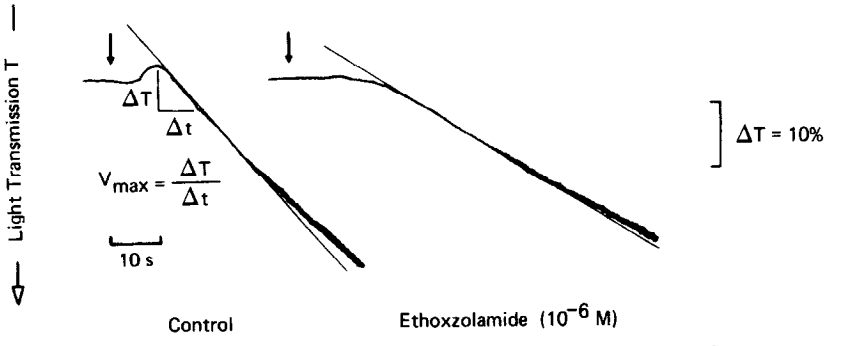

Fig. 1: Aggregation of human platelets in PRP at 37°C initiated by the addition (↓) of thrombin (1.5 U/ml). PRP was preequilibrated with 4% CO_2 (pH 7.45). Inhibition of carbonic anhydrase in the presence of $1 \cdot 10^{-6}$ M ethoxzolamide reduces the velocity of platelet aggregation.

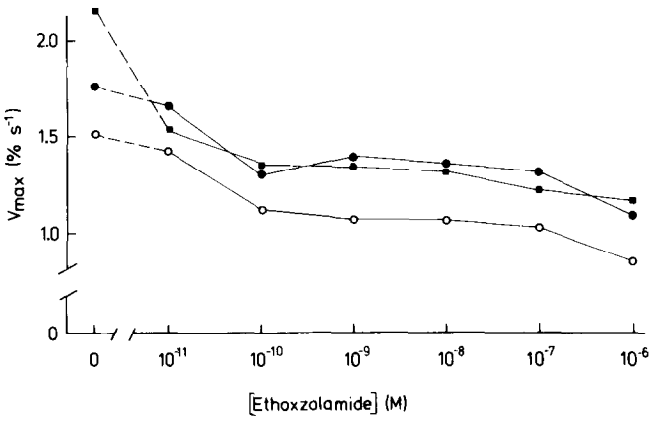

Fig. 2: Effect of carbonic anhydrase inhibition on the initial velocity of platelet aggregation, v_{max}. The velocity of platelet aggregation is given as the rate of change of light transmission through PRP, $\Delta T/\Delta t$ ($\%\cdot s^{-1}$), at various ethoxzolamide concentrations. Each data point (■, ●, ○) represents the mean of six determinations.

concentration 1.5 U/ml). It may be seen in Fig. 1 that after CA inhibition (i) the onset of aggregation is delayed and (ii) the initial slope of the downward deflection of the record is reduced by about 40%. Fig. 2 shows the aggregation velocities in PRP from three different preparations (■, ●, ○) expressed as initial rates of increase in light transmission, v_{max} ($\%\cdot s^{-1}$), plotted versus the concentration of the CA inhibitor. Each point represents the mean of six determinations, S.E. was always less than 4%. Platelet aggregation velocities clearly decrease with increasing inhibitor concentration. At 10^{-10} M ethoxzolamide the rate of aggregation is reduced by about 27% (24% - 34%) as compared with the control values. Increasing the inhibitor concentration from 10^{-10} M to 10^{-7} M did not lead to a significant further reduction of platelet aggregation velocity, whereas at 10^{-6} M ethoxzolamide an additional slight reduction, yielding a total reduction by 40%, could be observed.

The ethoxzolamide concentration producing 50% of the maximum inhibitory effect (IC_{50}) is between 10^{-10} and 10^{-11}M. The inhibition constant of platelet CA for ethoxzolamide has been determined to be $2\cdot10^{-10}$ M (1). Considering that these latter measurements have been done in platelet lysates containing 0.15 M Cl^- (which tends to increase K_I for sulfonamide) the agreement between IC_{50} and K_I may be considered satisfactory. From this and from the properties of platelet CA reported earlier (1) we conclude that it is a high-activity (CA II-like) enzyme that is

involved in platelet aggregation. This CA isoenzyme is essentially fully inhibited by 10^{-7} M ethoxzolamide; it is therefore unclear why a minor, but statistically significant, reduction of aggregation velocity is seen between 10^{-7} and 10^{-6} M ethoxzolamide.

In order to verify the hypothesis that CA-catalysed CO_2 hydration is involved in the activation process of platelets by thrombin, we compared platelet aggregation velocities obtained with PRP incubated with CA inhibitor to those obtained with CO_2-depleted PRP. The results are shown in Fig. 3. Platelet aggregation velocities are given in per cent of the values found for control preparations (4% CO_2, no other additives). Each column represents the mean of ten determinations, the bars represent S.D. It can be seen that inhibition of platelet CA by 10^{-6} M ethoxzolamide and removal of CO_2 reduce the aggregation velocity by almost the same extent (∿ 40%). Reequilibrating CO_2-depleted samples of PRP with CO_2 leads to reestablishment of the velocities observed in the control experiments.

The results presented here suggest that CA-catalysed CO_2 hydration-dehydration is involved in the activation of platelets by thrombin. Inhibition of platelet CA reduces the initial velocity of platelet aggregation and thus reveals that the, uncatalysed, CO_2 hydration-dehydration can be a rate-limiting step

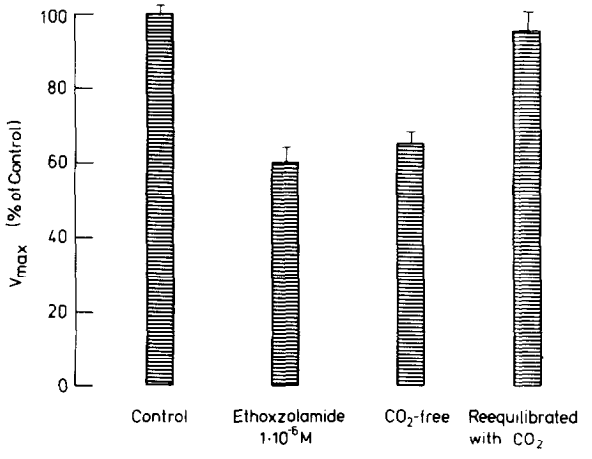

Fig. 3: Effect of CA inhibition or CO_2-depletion on the velocity of platelet aggregation. The initial aggregation velocity v_{max} is given in % of the control value (presence of CO_2, no CA inhibitor). Aggregation was induced by 1.5 U/ml thrombin. Each column represents the mean of ten determinations (+ S.D.).

in platelet activation. That it is indeed the CO_2 hydration-dehydration reaction which is involved here and not any other enzymic function of carbonic anhydrase, is confirmed by the results obtained with CO_2-depleted PRP: removal of CO_2 (reversibly) decreases the initial velocity of platelet aggregation to a similar degree as does inhibition of the catalysis of CO_2 hydration.

Recent investigations have shown (4,5) that the stimulation of human platelets with thrombin leads to a rapid extrusion of protons from the cytoplasm to the extracellular space. This movement of H^+ ions seems to be associated with a rise of intracellular pH in thrombin stimulated platelets (7). In addition, it is known that initiation of platelet aggregation by ADP stimulates the uptake of Na^+ into platelets (8,9). These latter ionic movements appear to be electroneutral (10) and it was postulated that stimulation of a Na^+-H^+ exchange could be the triggering event for the activation of human platelets (11). Platelet CA might be involved in this process by making rapidly available protons which can then be transferred from the cytoplasm to the extracellular space in exchange for Na^+. Further studies are necessary to define the exact role of CA in the process of platelet aggregation.

REFERENCES

1. Siffert, W. and Gros, G. (1984) Biochem.J. 217, 727-730
2. Maren, T.H. (1967) Physiol.Rev. 47, 595-781
3. Carter, M.J. (1972) Biol.Rev. 47, 465-513
4. Akkerman, J.W.N., Holmsen, H., and Loughnane, M. (1979) Anal. Biochem. 97, 387-393
5. Akkerman, J.W.N., and Holmsen, H. (1981) Blood 57, 956-966
6. Michal, F., and Born, G.V.R. (1971) Nature 231, 220
7. Horne, W.C., Norman, N.E., Schwartz, D.B., and Simons, E.R. (1981) Eur.J.Biochem. 120, 295-302
8. Feinberg, H., Sandler, W.C., Scorer, M., Le Breton, G.C., Grossman, B. and Born, G.V.R. (1977) Biochim.Biophys.Acta 470, 317-324
9. Sandler, W.C., Le Breton, G.C., and Feinberg, H. (1980) Biochim.Biophys.Acta 600, 448-455
10. MacIntyre, D.E. and Rink, T.J. (1982) Thromb.Haemostasis 47, 22-26
11. Leven, R.M., Gonnella, P.A., Reeber, M.J., and Nachmias, V.T. (1983) Thromb.Haemostasis 49, 230-234