INHIBITION OF PLATELET AGGREGATION AND THE PLATELET RELEASE REACTION BY α, ω DIADENOSINE POLYPHOSPHATES

Margaret J. HARRISON and R. BROSSMER

Institut für Biochemie II (Med. Fakultät) der Universität, D-69 Heidelberg, Im Neuenheimer Feld 328, German Federal Republic

and

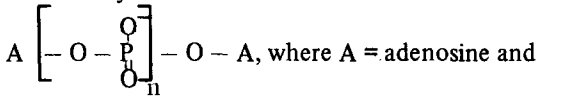
R. S. GOODY

Max-Planck-Institut für Medizinische Forschung, D-69 Heidelberg, Jahnstrasse 29 German Federal Republic

Received 12 March 1975

1. Introduction

The aggregation of human blood platelets by adenosine diphosphate (ADP) is specifically inhibited by adenosine and a few related compounds [1–3]. While the mode of action of adenosine remains controversial, investigations have been made with various adenosine analogues [4]. The present report is of studies with a series of adenosine derivatives which have not been tested previously with this system. This group of substances contain adenosine molecules joined in pairs, by different numbers of phosphate groups, at position 5′ of the two ribosyl moieties. They may be represented by the formula:



A $\left[-O-\underset{\underset{O}{\|}}{\overset{O^-}{P}}\right]_n-O-A$, where A = adenosine and n = the number of phosphate groups.

Evidence is presented which is consistent with the idea that adenosine and its derivatives compete with ADP at the platelet surface. The inhibitory power of these derivatives increases with the value of n up to a limit of n = 5.

2. Material and methods

The preparation of the adenosine phosphate derivatives AP_2A, AP_3A, AP_4A, AP_5A and AP_6A will be described elsewhere [5]. No compound showed any ADP or adenosine impurities i.e. no more than 1% present. The solutions of the derivatives were frozen quickly and held at −20°C when not in use. All other chemicals were from commercial sources and of analytical grade. All solutions were adjusted to pH 7.4 and to isosmolarity before use. Both washed platelets and platelet rich plasma (PRP) were prepared as previously described [6]. Glucose 0.1% (w/v) was included in the medium if the platelets were to be incubated as long as 30 min. Human fibrinogen at a final concentration of 0.5 mg/ml was used with washed platelets. A solution of thrombin containing 6 N.I.H. units/ml was made up daily. To label platelets, 10 ml plasma were incubated with [^{3}H] serotonin to give a final concentration of 0.008 μCi/ml and gently shaken at 20°C for 20 min. The platelets were then washed. Release experiments were performed by a modification [7] of the method of Massini and Lüscher [8], but with [^{3}H] serotonin instead of [^{14}C] serotonin. The aggregation and inhibition of aggregation of stirred platelets was measured by the turbidimetric method [9,10]. Adenylate kinase activity was measured by the method of Adam [11]. One unit of adenylate kinase is defined as 2 μmol ADP formed per min at 25°C.

3. Results

The time dependence and relative activities of

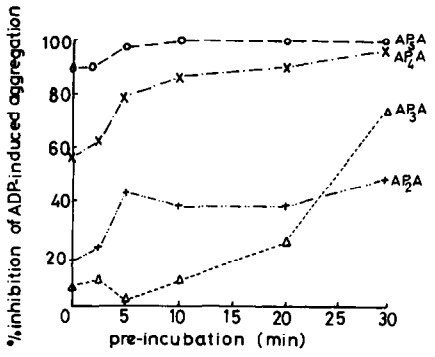


Fig.1. 100 μM concentrations of each of the inhibitors were incubated with PRP for different times before 10 μM ADP was added. The extent of aggregation was measured by the △OD. The extent of inhibition of aggregation by the inhibitors was calculated by comparison with control platelets, to which only ADP was added.

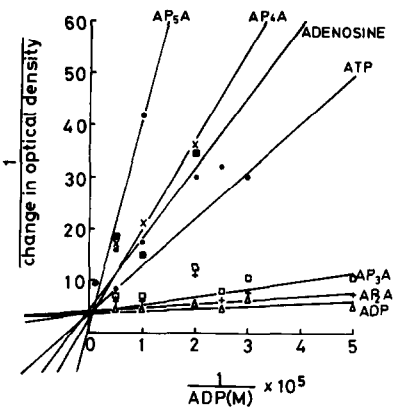


Fig.2. 30 μM concentrations of each of the inhibitors were incubated for 10 min with washed platelets. Fibrinogen and variable concentrations of ADP were added and the changes in optical density were measured. Double reciprocal plots were made, taking the change in optical density (△OD) in five min as the velocity (v) of the inhibition of ADP aggregation. For the sake of clarity, only a few values of 1/ADP less than 1.0, and no standard deviations are shown on the figure. With 1/ADP at 0.2×10^5 and 0.5×10^5 the SDs of 1/△OD for AP_5A were 7.7 and 9.2 respectively; for AP_3A they were 1.4 and 0.4. (○)AP_5A; (x)AP_4A; (■)adenosine; (●)ATP; (□)AP_3A; + AP_2A; (△)ADP.

AP_2A, AP_3A, AP_4A and AP_5A (100 μM) in the inhibition of ADP (10 μM) aggregation in PRP are shown in fig.1. At 30 μM AP_6A inhibits ADP aggregation in PRP slightly less than does AP_5A at the same concentration. The adenosine derivatives at 30 μM inhibit the aggregation of washed platelets by ADP (1–10 μM) and thrombin (0.006–0.06 NIH/ml) and by dextran sulphate (1 nM). They do not affect DEAE-Dextran (6 nM) aggregation. The extent of the inhibition of aggregation is dependent on the dose of the inhibitors. For AP_2A and AP_3A, inhibition of aggregation is also dependent on the time of their incubation with the platelets prior to the addition of ADP. Fig.2 is typical of several experiments. It shows double reciprocal plots of the change in optical density of the platelet suspensions with variable ADP concentration and constant concentrations of the different inhibitors. The graph is highly suggestive of similar V_{max} values in the control and inhibition experiments, indicating competitive inhibition. The figure does not give absolute values for V_{max} but it indicates the relative efficiency of the various compounds to produce inhibition. At a given concentration, AP_5A is the strongest inhibitor of ADP aggregation. The inhibitors in order of strength are $AP_5A > AP_4A >$ adenosine $>$ ATP $> AP_3A > AP_2A$. From the intercept on the X axis, an approximate value for the apparent dissociation constant of the receptors and ADP of 1.25 μM is obtained. For AP_5A at the concentration used, the inhibition is so strong that a reliable value of the intercept can not be obtained. Due to the weaker inhibition of AP_4A, it is possible to obtain an apparent value of its inhibition constant of 700 nM. Since AP_5A inhibits considerably more strongly, it appears to have much higher affinity for receptor sites than ADP itself or than ATP or adenosine.

Enzymatic breakdown of AP_nA compounds to adenosine could cause inhibition of platelet aggregation. However, incubation of the AP_nA compounds (at 1 mM) for 10 min with washed platelets or PRP does not lead to detectable breakdown as judged by thin layer chromatography. Also, incubation of adenosine deaminase (40 ng/ml) for 10 min with the AP_nA compounds at 10–100 μM in the presence of washed platelets results in no reduction in the inhibition of ADP aggregation. This concentration of deaminase hinders the inhibition produced by 20–200 μM adenosine.

It is possible that the calcium necessary for platelet aggregation might be removed by binding with the AP_nA compounds. To test this, the experiments were

repeated with five times the usual concentration of calcium, i.e. with 5 mM calcium. The extent of inhibition of aggregation by the AP_nA compounds was not altered.

Washed platelets incubated with 100 μM AP_5A give a slow release of serotonin. There was no significant release at 1 min but 24 ± 5% release in 30 min (control 17 ± 5%). The reason for this slow release is not known. Lower concentrations of AP_5A do not give a release in this time. Without preincubation, 30 μM AP_5A inhibits ADP-release in washed platelets and PRP by 44% and 55% respectively. At 30 μM, AP_nA derivatives inhibit the ADP release reaction in PRP with diminished strength as n decreases from 6 (91 ± 8% inhibition) to 2 (4 ± 4% inhibition).

4. Discussion

The AP_nA derivatives inhibit aggregation in the same order of efficiency as they inhibit the enzyme activity of adenylate kinase [5,12] i.e. up to n = 5 the more phosphat groups, the more inhibitory the activity. AP_5A inhibits ADP aggregation more strongly than does AP_4A or AP_6A. This suggests that the distance between the bases in the AP_5A molecule corresponds most satisfactorily to the distance between the ATP and AMP binding sites of the enzyme. Adenylate kinase occurs in high concentrations in muscle cells where a part of it is probably closely associated with actomyosin. The nature of this association is not clear (Schirmer, 1974, personal communication). On the platelet surface, the binding site for AP_5A might be structurally related to adenylate kinase. This enzyme has not yet been found on the platelet surface [13]. However it is possible that adenylate kinase itself is associated with the contractile protein thrombosthenin, which occurs partly on the platelet membrane [14]. This adenylate kinase could be the target of the diadenosine polyphosphates. We found the enzyme in the supernatant of washed platelets, after stirring them for 5 min, at a concentration of 70 mU/10^9 platelets. This platelet adenylate kinase is inhibited by AP_5A in the same way as the muscle enzyme. The results of Haslam and Mills [13] agree well with ours. They found adenylate kinase activity at a concentration of 90 mU/10^9 platelets in the suspending medium of washed platelets which were stirred for 15 min. Due to slightly different conditions used for the assay, the values are not directly comparable but are in the same range.

The double reciprocal plots in fig.2 suggest that adenosine, the adenosine derivatives and ATP act competitively with ADP. Born [15] suggested that inhibition by adenosine and its derivatives was due to competition between adenosine and ADP at a specific receptor site on the platelet membrane. Rozenberg and Holmsen [16] proposed that the inhibition was associated with the process of adenosine uptake by platelets with concurrent ATP hydrolysis. This ATP is thought to be necessary for the aggregation mechanism. However Born and Mills [1] showed that drugs that inhibited uptake of adenosine by platelets did not inhibit the action of adenosine on ADP-induced aggregation. Later, Born [17] suggested that inhibition of ADP by adenosine might involve competition for a third component, not necessarily at the same binding site. Holmsen [18] contended that adenosine binds to some area of the membrane before it exert its inhibitory effect. Once taken up and converted to AMP, adenosine might interfere sterically with adenylate kinase, which in some way could inhibit ADP-induced aggregation. Adenosine may also exert its action through adenylate cyclase and the cyclic AMP system [19,20]. One would not expect our group of adenosine derivatives to penetrate platelet membrane since all are multivalent-negatively charged compounds. Furthermore, the immediate effect of these derivatives on some aspect of platelet function suggests that they act at the membrane level. It is possible that the adenosine polyphosphates could act competitively with ADP, and at the same time affect the cyclic AMP system. The latter effect would not require penetration of the platelet membrane. Further studies which are concerned with the effects of these adenosine derivatives on the cyclic AMP system, thrombosthenin or ADP receptor sites are required. They may help to elucidate the precise nature of the inhibitory mechanisms of these dinucleotide phosphates on ADP-induced platelet aggregation.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 90 'Cardiovasculäres System', Bad Godesberg, GFR. Thanks are due to Dr R. H. Schirmer (Max-Planck-

Institut für Medizinische Forschung, Heidelberg) for helpful discussions and to Miss R. Jesper of the Biochemistry Institute and to Mrs M. Isakov of the Max-Planck-Institut for skillful technical assistance.

References

[1] Born, G. V. R. and Mills, D. C. B. (1969) J. Physiol. (London) 202, 41–42P.
[2] Kikugawa, K., Itzuka, K. and Ichino, M. (1973) J. Med. Chem. 16, 358–364.
[3] Kikugawa, K., Itzuka, K., Higuchi, Y., Hirayama, H. and Ichino, M. (1972) J. Med. Chem. 15, 387–390.
[4] Haslam, R. J. (1967) in: Physiology of Hemostasis and Thrombosis (Johnson, S. A. and Seegers, W. H., eds.), pp. 88–112, C. C. Thomas, Springfield.
[5] Goody, R. S., Fröhlich, P., Walter, B. and Schirmer, R. H., to be submitted to Eur. J. Biochem.
[6] Patscheke, H. and Brossmer, R. (1974) Thromb. Diath, Haemorrh. (Stuttg.), in press.
[7] Wörner, P. and Brossmer, R. (1974) Thromb. Res., in press.
[8] Massini, P. and Lüscher, E. F. (1971) Thromb. Diath. Haemorrh. (Stuttg.) 25, 13.
[9] O'Brien, J. R. (1962) J. Clin. Pathol. 15, 446–449.
[10] Born, G. V. R. and Cross, M. J. (1963) J. Physiol. (London) 168, 175–195.
[11] Adam, H. (1963) in: Methods of Enzymatic Analysis Bergmeyer, H. U., ed.), pp 539, 573, Academic Press (Inc.) Ltd., London.
[12] Lienhardt, G. V. and Secemski, I. I. (1973) J. Biol. Chem. 248, 1121–1123.
[13] Haslam, R. J. and Mills, D. C. B. (1967) Biochem. J. 103, 773–784.
[14] Booyse, F. M., Hoveke, T. P., Zschoke, D. and Rafelson, M. E. (1971) J. Biol. Chem. 246, 4291–4297.
[15] Born, G. V. R. (1965) Ann. Roy. Coll. Surg. Engl. 36, 200–206.
[16] Rozenberg, M. C. and Holmsen, H. (1968) Biochim. Biophys. Acta 155, 342–352.
[17] Born, G. V. R. (1970) Acta Med. Scand., Suppl. 525, 173–174.
[18] Holmsen, H. (1970) Acta Med. Scand., Suppl. 525, 169–172.
[19] Salzman, E. W., Kensler, P. C. and Levine, L. (1972) Ann. N. Y. Acad. Sci. 201, 61–71.
[20] Rozenberg, M. C. and Walker, C. M. (1973) Brit. J. Haematol. 24, 409–418.