Br. J. Pharmac. (1981), **73**, 639–647

INHIBITION OF PROSTAGLANDIN E_1-RESPONSIVE PLATELET ADENYLATE CYCLASE BY HEPARIN: A STUDY OF THE MECHANISM OF INHIBITION AND ITS RELEVANCE TO PLATELET AGGREGATION

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1 Heparin can produce platelet aggregation *in vitro* and *in vivo*; it has been proposed that this may be due to the reported inhibition of the prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase of the platelet by heparin.

2 The effect of heparin on the cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) response to PGE_1 was measured in intact and broken platelets both *in vitro* and in platelets obtained from normal subjects during intravenous infusion with herapin.

3 In platelet lysates, heparin produced a dose-related inhibition of PGE_1-stimulated adenylate cyclase. The maximum response to PGE_1 was reduced, with half-maximal inhibition occurring at 3 μg/ml heparin. This inhibition could be prevented by protamine sulphate.

4 Heparin did not affect PGE_1-stimulated cyclic AMP production in intact platelets either *in vitro* or in platelets taken during the infusion of 5,000 iu heparin over 2 h to 2 normal volunteers. Similarly, preincubation of platelets with heparin for up to 3 h at 37°C did not affect platelet adenylate cyclase.

5 The effects of heparin were very similar to those of fluoride on the platelet adenylate cyclase: heparin and fluoride increased basal enzyme activity slightly (3–4 fold) but their effects were not additive; both inhibited the response to PGE_1 by approximately 50% when added directly to the assay and the inhibitory effects of the two were not additive; preincubation of membranes with either heparin or fluoride produced an irreversible state of inhibition.

6 As heparin inhibits PGE_1-stimulated adenylate cyclase activity only in broken platelets, we suggest that the aggregatory effects of heparin are probably independent of any action on cyclic AMP production.

Introduction

Heparin, in common with several other naturally occurring glycosaminoglycans, has been found to inhibit hormone stimulated adenylate cyclase enzymes in a variety of tissues (Salomon, Amir, Azulai & Amsterdam, 1978; Amsterdam, Reches, Amir, Mintz & Salomon, 1978). The inhibition of the human platelet adenylate cyclase response to prostaglandin E_1 (PGE_1) is of particular interest as it occurs at a range of heparin concentrations which have been found to antagonize the anti-aggregatory effect of PGE_1 on adenosine diphosphate-induced platelet aggregation *in vitro* (Reches, Eldor & Salomon, 1979a, b). The role played by heparin in platelet function is not well understood as heparin is a potent anticoagulant and anti-thrombotic agent and yet can produce platelet aggregation under certain conditions. The possibility that heparin may produce its platelet-aggregating activity by the inhibition of the PGE_1-sensitive adenylate cyclase was suggested by Reches *et al.* (1979a, b) and is examined further in this study; an examination of the mechanism by which heparin inhibits the platelet adenylate cyclase enzyme is also described.

Methods

Preparation of intact platelets, platelet lysate and a washed platelet particulate preparation

Fresh human blood was mixed with 0.1 volumes of ice-cold 3.8% sodium citrate, 100mM Na_4EDTA and immediately centrifuged at 200 *g* for 10 min at 4°C (MSE 6L centrifuge). The platelet-rich-plasma was

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removed and used immediately in the assay of cyclic AMP production from intact platelets. Platelet lysates were prepared by centrifuging platelet-rich-plasma at 600 *g* for 10 min at 4°C; the resultant platelet pellet was resuspended to the original blood volume with 15 mM Tris-HCL (pH 7.4), 1 mM Na_4EDTA, 140 mM NaCl at 4°C then centrifuged at 600 *g* for 10 min at 4°C. This washed platelet pellet was then resuspended in 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM Na_4EDTA to one tenth of the original blood volume and platelets were lysed by rapid freezing in a mixture of solid CO_2 and ethanol, followed by thawing to room temperature. This freeze/thawing was repeated and aliquots of the platelet lysate were used immediately in the assay of adenylate cyclase activity. A washed particulate preparation was prepared by centrifuging platelet lysate at 12,300 *g* for 10 min at 4°C (Sorvall RC2B) and the pellet was then resuspended in 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM Na_4EDTA and again centrifuged at 12,300 *g* for 10 min at 4°C. This final pellet was resuspended in the above buffer and used as a washed particulate preparation in the assay of adenylate cyclase activity. In some experiments pellets were preincubated in buffer containing heparin or sodium fluoride for 10 min at 20°C. After centrifugation, pellets were resuspended in buffer, centrifuged and finally resuspended in buffer (as described for washed particulate preparation) and used in the assay of adenylate cyclase activity.

Cyclic AMP accumulation in intact platelets

Freshly isolated platelet-rich-plasma was diluted with oxygenated buffer (Larkins, MacAuley, Rappoport, Martin, Tulloch, Byfield, Matthews & MacIntyre, 1974) containing theophylline 10 mM, to a concentration of approximately 10^6 platelets/ml. Platelet incubations (at 37°C) of total volume 500 µl, were started by the addition of platelet suspension and terminated by transferring the tubes to a boiling water bath for 3 min. Preliminary experiments established that, at a platelet concentration of approx. 10^6 platelets/ml, 10^{-6} M PGE_1 produced a maximal stimulation of cyclic AMP production which occurred within 2 min but remained constant for up to 10 min in the presence of 10 mM theophylline. (All incubations were terminated at 6 min subsequently). Incubations were performed in triplicate. In some experiments platelets were pre-incubated with heparin (or buffer alone) for 3 h at 37°C and then washed and lysed as described in the preparation of platelet lysates and lysate adenylate cyclase was determined.

The cyclic AMP content of the intact platelets was assayed by the protein binding method of Brown, Albano, Ekins, Sgherzi & Tampion (1971) using a standard curve made up of the appropriate proportion of platelet-poor-plasma (produced by centrifuging blood at 800 *g* for 10 min in an MSE 6L) and platelet incubation buffer. Heparin at up to 40 µg/ml did not affect the binding of cyclic AMP to the binding protein. Assays were performed in triplicate.

Adenylate cyclase assay

The adenylate cyclase activity of platelet lysates and of washed platelet particulate preparations was measured by the conversion of [α-^{32}P]-ATP to cyclic [α-^{32}P]-AMP; details were as described by Mac Neil, Crawford, Amirrasooli, Johnson, Pollock, Ollis & Tomlinson (1980). Cyclic [α-^{32}P]-AMP was isolated according to the method of Salomon, Londos & Rodbell (1974). Assays were performed in triplicate.

Heparin infusion in vivo

Two normal volunteers (male S.T. age 35 years and female S.M. age 30 years) had an indwelling cannula placed in a vein in the antecubital fossa from which blood samples were taken every 30 min throughout 6 h. From 2 to 4 h an infusion of 5000 iu of heparin in 50 ml sterile saline (0.9% w/v NaCl solution) was given via a cannula into the other arm. Each blood sample was used for the preparation of intact platelets and platelet lysate. The measurements of cyclic AMP production in intact platelets and of adenylate cyclase activity in platelet lysates were performed immediately.

Protein determination

Proteins were determined by the method of Hartree (1972).

Statistics

Results are expressed as the mean ± s.e. mean of triplicate determinations and the difference between means was assessed for significance by Student's *t* test. Values of $P < 0.05$ were taken as statistically significant.

Drugs and chemicals

[α-^{32}P]-adenosine triphosphate (10–30 Ci/mmol) and [8-^{3}H]-cyclic AMP (20–30 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks. Cyclic AMP (crystallised free acid), the sodium salt of adenosine triphosphate (ATP), phosphoenolpyruvate and pyruvate kinase (sp. act. approx. 200 iu/mg) were obtained from Boehringer Mannheim, London. Bovine serum albumin (fraction V, powder) was obtained from Armour Pharmaceuticals, Eastbourne, Sussex. Heparin used *in vitro* was Grade 11 sodium salt from porcine intestinal mucosa (151 USP JA units/mg) obtained from Sigma Chemi-

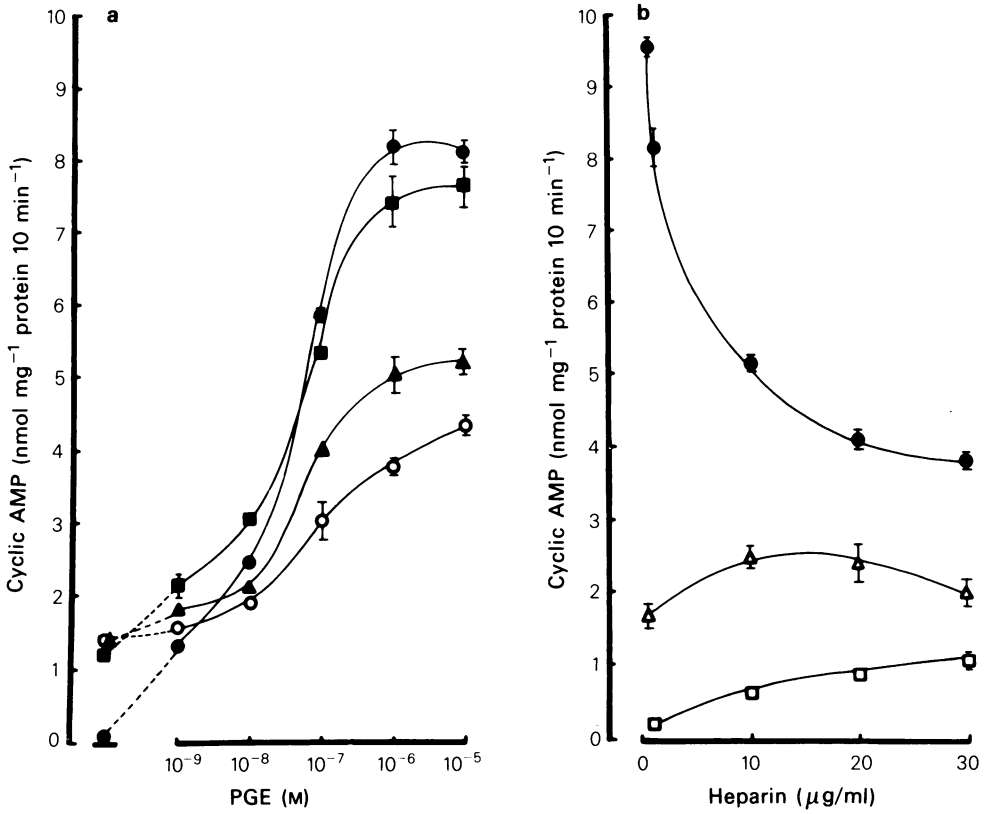

Figure 1 (a) The effect of heparin on prostaglandin E_1 (PGE_1)-stimulated adenylate cyclase activity in platelet lysate. Control (●), heparin 1 μg/ml (■), 20 μg/ml (▲) and 30 μg/ml (○). (b) The effect of heparin on basal (□), sodium fluoride (△) (10 mM)-stimulated and PGE_1 10^{-6}M(●)-stimulated adenylate cyclase activity in platelet lysate. All points are mean values of triplicates; vertical lines show s.e.mean.

Table 1 Effect of pre-incubating platelets with heparin on platelet adenylate cyclase activity

Heparin concentration (μg/ml)	*Cyclic AMP* (nmol mg^{-1} protein 10 min^{-1})	
	Basal	*PGE_1
0	0.3 ± 0.01	6.8 ± 0.02
5	0.4 ± 0.03	7.5 ± 0.4
10	0.5 ± 0.01	7.4 ± 0.2
25	0.3 ± 0.01	7.1 ± 0.4
50	0.3 ± 0.04	7.6 ± 0.3

Platelets were incubated with heparin (or buffer) for 3 h at 37°C and then washed and lysed as described in Methods.
*Maximal stimulatory concentration of PGE_1 (10^{-6}M). Values are mean ± s.e.mean of triplicate determinations.

cal Co. and *in vivo* was a solution of 1,000 iu/ml obtained from Weddel Pharmaceuticals Ltd., London. Protamine sulphate (10 mg/ml) was obtained from Evans Medical Ltd., Speke, Liverpool. All other chemicals were of A.R. grade.

Hormone preparations

Prostaglandin E_1 was a gift from Dr J.E. Pike, Upjohn Co., Kalamazoo, U.S.A.

Results

The effect of herapin on PGE_1-stimulated adenylate cyclase activity in broken platelets

In platelet lysates, herapin produced a dose-related inhibition of platelet PGE_1-stimulated adenylate over the concentration range 1 to 30 μg/ml heparin as shown in Figure 1a. The maximum response to PGE_1 was reduced by heparin with half-maximal inhibition occurring at approx. 3 μg/ml of heparin as shown in Figure 1b. Basal activity was significantly increased by heparin but there was little effect on the activation of the enzyme by sodium fluoride as shown in Figure 1b. Heparin also inhibited PGE_1-stimulated adenylate cyclase activity in washed platelet particulate preparations to a similar degree.

The effect of heparin on PGE_1-stimulated adenylate cyclase activity in intact platelets

As shown in Table 1, preincubation of platelets with 0 to 50 μg/ml heparin for 3 h at 37°C had no significant effect on basal or PGE_1 (10^{-6}M)-stimulated adenylate cyclase activity when these platelets were subsequently washed and lysed. Similarly, preincubation of platelets with 25 μg/ml heparin for periods from 2 min to 3 h had no significant effect on basal or PGE_1-stimulated adenylate cyclase activity.

Cyclic AMP production by intact platelets in response to PGE_1 was also unaffected by heparin as shown in Table 2 where platelets were exposed to 0 to 50 μg/ml heparin for 6 min of incubation at 37°C. Neither basal nor PGE_1-stimulated cyclic AMP production were affected by heparin. Similar results were obtained after exposure of platelets to heparin for 30 min at 37°C.

Infusion of 5,000 iu of heparin over a 2 h period into two volunteers did not affect the cyclic AMP response to PGE_1 as determined by cyclic AMP production in intact platelets or by adenylate cyclase determination in the platelet lysate. Figure 2 shows basal and PGE_1-stimulated cyclic AMP production in the platelets of one of the subjects (S.M.) during the 6 h of the experiment. Neither were affected by heparin infusion. Similar results were obtained in the second volunteer S.T. Likewise the ability of a maximum stimulatory concentration of PGE_1 (10^{-6}M) to increase basal adenylate cyclase activity in

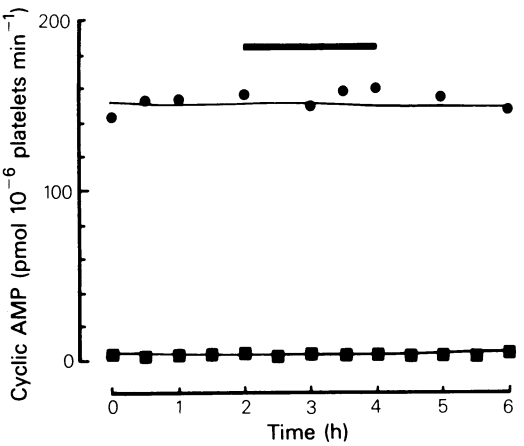

Figure 2 The effect of an infusion of 5,000 iu heparin (indicated by black bar) on basal (■) and prostaglandin E_1 (PGE_1) 10^{-6}M(●)-stimulated cyclic AMP production in intact platelets of subject S.M. Points are mean values of triplicates, s.e. means are all within the size of the symbols used.

Table 2 Effect of heparin on cyclic AMP production by intact platelets

Heparin concentration (μg/ml)	*Cyclic AMP* (pmol 10^{-6} platelets 6 min^{-1})	
	Basal	**PGE_1*
0	18.6 ± 3.0	87.0 ± 5.0
1	16.2 ± 2.4	101.4 ± 4.2
5	12.6 ± 0.1	93.0 ± 5.4
25	24.0 ± 3.0	119.4 ± 11.4
50	20.4 ± 2.0	105.0 ± 4.0

* Maximal stimulatory concentration of PGE_1 (10^{-6}M). Values are mean ± s.e. mean of triplicate determination.

Table 3 Effect of increasing substrate concentration on heparin inhibition of prostaglandin E_1 (PGE_1) stimulated adenylate cyclase

Substrate concentration (mM)		*Control*		*+ Heparin I*		*% inhibition of*
ATP	*Mg*	*Basal*	**PGE_1*	*Basal*	**PGE_1*	*PGE_1 response*
1	5	0.2 ±0.01	10.2 ±0.2	0.5 ±0.2	5.5±0.01	45%
1	10	0.5 ±0.1	10.3 ±0.2	0.4 ±0.1	6.0±0.6	43%
1	15	0.4 ±0.03	9.6 ±0.5	0.3 ±0.1	5.3±0.2	46%
1	20	0.4 ±0.04	9.3 ±0.5	0.3 ±0.05	5.6±0.2	41%
1	25	0.5 ±0.1	8.3 ±0.2	0.3 ±0.1	4.3±0.3	49%
1	4	0.2 ±0.01	10.5 ±0.4	0.3 ±0.1	6.0±0.2	45%
3	4	0.2 ±0.4	3.3 ±0.4	0.5 ±0.02	1.8±0.1	44%
5	4	0.03±0.04	1.05±0.1	0.01±0.01	0.7±0.5	33%
8	4	0.01±0.01	0.4 ±0.2	0.01±0.01	0.2±0.05	51%

* Maximal stimulatory concentration of PGE_1 (10^{-6}M).
[1]30 μg/ml heparin.
Values are mean ± s.e. mean of triplicate determinations.

the platelet lysate was unchanged during or after the period of heparin infusion: in subject S.M., PGE_1 increased basal adenylate cyclase activity by 26.3 ± 1.0 fold (mean ± s.e.mean), (n = 3) in the 2 h pre-heparin, by 27.2 ± 1.9 fold (n = 3) during the 2 h of heparin infusion and by 28.4 ± 1.2 fold (n = 4) during the 2 h post-heparin; in subject S.T., PGE_1 increased basal adenylate cyclase activity by 14.1 ± 0.3 fold (n = 3) pre-heparin, by 11.7 ± 0.7 fold (n = 4) during heparin infusion and by 13.2 ± 1.0 fold (n = 4) post-heparin.

Investigation of the mechanism of the inhibition of PGE_1-stimulated adenylate cyclase by heparin in platelet lysates

Interaction of heparin with the ATP Mg^{2+} substrate complex of the adenylate cyclase enzyme was investigated in experiments in which either the ATP concentration or the Mg^{2+} concentration of the incubation were increased. Although these procedures affected both basal and PGE_1-stimulated adenylate cyclase activity, the degree of inhibition produced by 30 μg/ml heparin was unaffected as shown in Table 3.

However, the inhibition produced by heparin could be completely prevented or reversed by the addition of protamine sulphate to the adenylate cyclase assay as shown in Figure 3. Protamine had no significant effect on basal or PGE_1-stimulated adenylate cyclase activity on its own, but 30 μg/ml added to the assay completely opposed the inhibitory effect of 30 μg/ml heparin on the PGE_1 response. In two other experiments between 30 and 40 μg/ml protamine sulphate was required to completely block the inhibitory effects of 30 μg/ml heparin.

Protamine was only effective in preventing this inhibition if present in the assay or with the enzyme preparation before exposure to heparin. The inhibitory effect of heparin, once established, could not be reversed by subsequent addition of protamine.

Finally, the inhibition of the PGE_1 response produced by heparin was compared with that produced

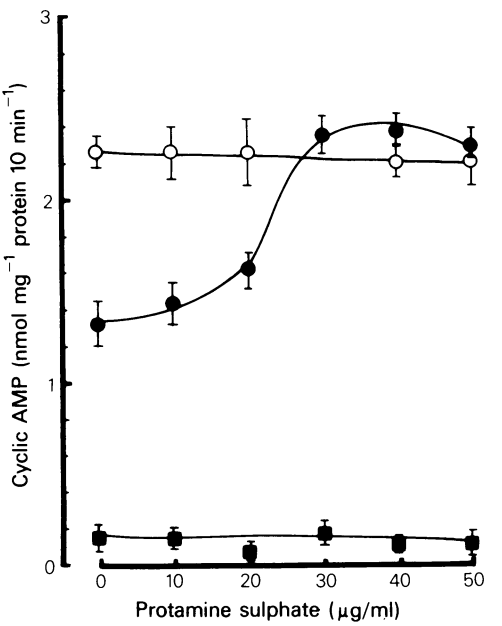

Figure 3 The effect of protamine sulphate on the inhibition of prostaglandin E_1 (PGE_1) produced by heparin in the platelet lysate. The effect of protamine sulphate on basal (■) and PGE_1 10^{-6}M(○)-stimulated adenylate cyclase activity in the absence of heparin and the effect of protamine sulphate on the response to PGE_1 (10^{-6}M) when 30 μg/ml heparin was present (●). Points are mean values of triplicates; vertical lines show s.e. mean.

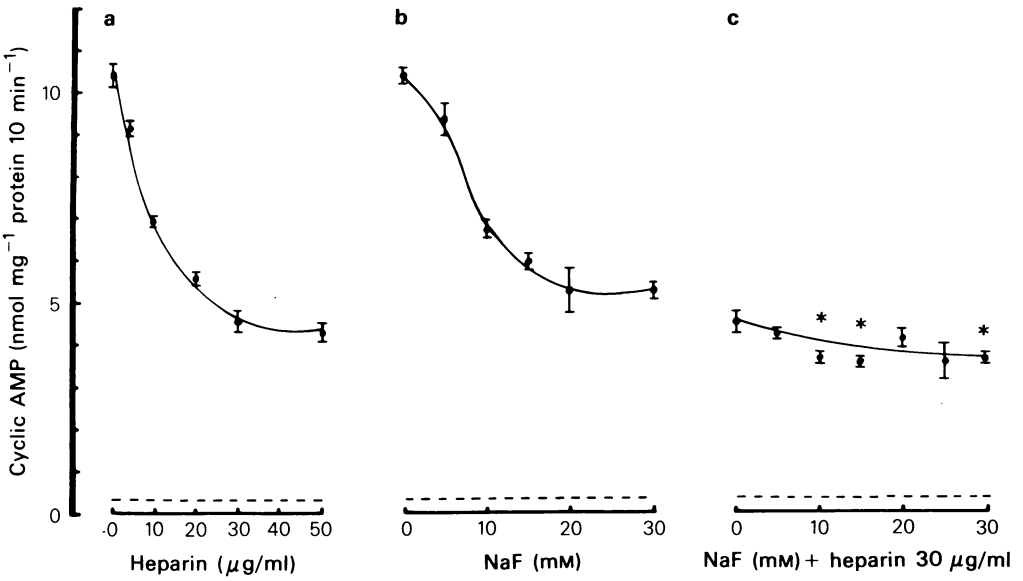

Figure 4 A comparison of the effect of (a) heparin and (b) sodium fluoride on the response to a maximally stimulating concentration of prostaglandin E_1 (PGE_1) (10^{-6}M) in the platelet lysate. (c) Addition of heparin (30 μg/ml) to fluoride reduced the response to PGE_1 only slightly (6%). Dotted line indicates basal enzyme activity in absence of heparin and fluoride. Points are mean values of triplicates; vertical lines show s.e.mean. *Values which are significantly ($P<0.05$) lower than amount of cyclic AMP produced in the presence of 30 μg/ml heparin alone.

Table 4 Effect of preincubation of membranes with heparin and fluoride on the adenylate cyclase response to prostaglandin E_1 (PGE_1)

Preincubation conditions	*Additions to assay*	*Cyclic AMP* (nmol mg^{-1} protein 10 min^{-1})	*% inhibition of PGE_1 response*
Buffer	PGE_1	2.23 ± 0.07	
Buffer	PGE_1, heparin	1.19 ± 0.03	47%
Buffer	PGE_1, NaF	1.21 ± 0.05	46%
Heparin	PGE_1	0.11 ± 0.01	95%
Heparin	PGE_1, heparin	0.12 ± 0.01	95%
Heparin	PGE_1, Naf	0.12 ± 0.02	95%
NaF	PGE_1	0.53 ± 0.03	76%
NaF	PGE_1, heparin	0.55 ± 0.05	75%
NaF	PGE_1, NaF	0.65 ± 0.09	71%

Platelet particulate preparations were preincubated with buffer, heparin (30 μg/ml) or NaF (20 mM) for 10 min at 20°C. Preparations were then centrifuged, washed and resuspended in buffer (approximately 35 min) and used in the assay of adenylate cyclase as described in methods. Additions to assay: PGE, 10^{-6}M; heparin 30 μg/ml; NaF 20 mM. In this experiment preincubation of membranes with heparin also reduced the response to fluoride by 92%. Basal activity was 0.12 ± 0.01 nmol mg^{-1} cyclic AMP protein 10 min^{-1}. Values are mean ± s.e. mean of triplicate determinations.

by fluoride in the platelet. Fluoride is effective only in broken cell preparations and as shown in the present study, heparin affects the adenylate cyclase enzyme only in broken platelet preparations. Heparin and fluoride increased basal adenylate cyclase activity to a similar degree in the platelet: in a comparison of 6 experiments, fluoride produced a 3.7 ± 1.0 fold (mean ± s.e.mean) and heparin a 3.8 ± 1.1 fold stimulation of basal activity but their combined effects on basal were not additive (Figure 1b). In a comparison of 6 experiments, heparin inhibited the response to PGE_1 by $50.8 \pm 4.3\%$ (mean ± s.e.mean) and fluoride inhibited the response by $49.9 \pm 1.4\%$ and as shown in Figure 4, when maximally inhibitory concentrations of heparin and fluoride were combined, there was little further increase in the inhibition of PGE_1. In this particular experiment the maximum inhibition of the response to PGE_1 was 58% produced by heparin alone, 48% produced by fluoride alone and 64% produced by heparin and fluoride together. Although this inhibition produced by heparin and fluoride together was significantly greater ($P < 0.05$) than that produced by heparin alone, this increase of 6% is much less than the additive effect which might have been expected to occur if fluoride and heparin were acting via entirely separate mechanisms on the adenylate cyclase enzyme. (The inhibition of the PGE_1 response produced by fluoride could not be prevented by the addition of up to 50 μg/ml protamine sulphate).

Table 4 shows that preincubation of washed particulate membranes with heparin or fluoride increased the degree of inhibition of the PGE_1 response from 47% and 45% respectively, seen when heparin and fluoride were added directly to the assay, to 95% and 79%. This inhibition persisted despite the washing of membrane preparations before addition to the assay and the inhibition was not further increased by addition of heparin or fluoride to the assay. An investigation of the time course of the development of the inhibition produced by heparin and fluoride showed an immediate onset of this inhibition (within 30 s) which did not develop further throughout the 10 min of the adenylate cyclase assay. However, platelet lysate preparations pre-incubated with heparin or fluoride showed a progressive decrease in their response to PGE_1 when subsequently used to initiate the adenylate cyclase assay. Platelet lysates pre-incubated with either 30 μg/ml heparin or 20 mM NaF became completely unresponsive to PGE_1 after 30 to 40 min at 20°C.

Discussion

In addition to its well-known anticoagulatory action, paradoxically heparin can also induce platelet aggregation manifested by increased intravascular coagulation in man (Klein & Bell, 1974) and in the rat (Bell, Anderson & Anderson, 1977) and by thrombocytopenia (Bell, Tomasulo, Alving & Duffy, 1976). It can, in addition, produce aggregation of washed platelets (Eika, 1972) and it can augment ADP-induced platelet aggregation in citrated platelet rich plasma (Thomson, Forbes & Prentice, 1973). Reches *et al.* (1979a) suggested that these aggregatory effects of heparin could be due to the ability of heparin to inhibit the PGE_1 responsive platelet adenylate cyclase. This suggestion is examined in the present study.

In vivo the platelet adenylate cyclase is stimulated by prostacyclin produced by the blood vessel wall and the resultant increase in platelet cyclic AMP has an anti-aggregatory effect on circulating platelets (Best, Martin, Russell & Preston, 1977). The mechanism of the anti-aggregatory effect of platelet cyclic AMP has been further elucidated by Haslam, Davidson, Fox & Lynham (1978) who showed that PGE_1 increased the cyclic AMP-dependent phosphorylation of a membrane-bound polypeptide and they suggest that this polypeptide is itself inhibitory, via the mobilisation of cytosolic calcium, to the phosphorylation of two other polypeptides involved in platelet aggregation. Against this background of the current understanding of the role of cyclic AMP in platelet aggregation, any inhibitory effect of heparin on the platelet PGE_1 (and prostacyclin)-stimulated adenylate cyclase would tend to lead to an increase in platelet aggregation.

In the present study, heparin inhibited the response to PGE_1 in broken platelets but failed to affect the response to PGE_1 in intact platelets either *in vitro* or during the infusion of 5,000 iu heparin in two normal volunteers. This latter experiment was undertaken to mimic the exposure to heparin which platelets receive when heparin is given clinically, when concentrations of 3 iu/ml heparin may occur in the circulation (Gillet & Besterman, 1973). Although Reches *et al.* (1979b) noted that heparin failed to affect basal or PGE_1-stimulated cyclic AMP production by intact platelets, these authors suggest that the size of the cyclic AMP pool relevant to platelet-aggregation may be small compared to total platelet cyclic AMP, so that any changes may not be detectable. However, other agents which are known to inhibit platelet aggregation via an effect on platelet adenylate cyclase activity seem to be effective only when platelet cyclic AMP is initially elevated (e.g. by PGE_1-stimulation) and they then produce large (70 to 80%) decreases in platelet-cyclic AMP (Haslam, Davidson & Desjardins, 1978). For these reasons we suggest that the lack of effect of heparin on the cyclic AMP response to PGE_1 in intact platelets argues strongly against heparin producing its observed aggregatory effects via an inhibitory action on the platelet adenylate cyclase. An alternative explana-

tion for the aggregatory effects of heparin could be that put forward by Anderson, Mohammed, Chuang & Mason (1980), that heparin potentiates the synthesis of thromboxane A_2, a potent platelet aggregating agent whose stimulatory actions on the platelet release reaction (leading to aggregation) can be divorced from its inhibitory actions on the platelet adenylate cyclase (Gorman, 1979). Hence, heparin could increase platelet aggregation independently of platelet cyclic AMP levels.

The mechanism by which heparin inhibits PGE_1-stimulated adenylate cyclase in broken platelets is itself of interest, however, as heparin is the most potent of several glycosaminoglycans which have been demonstrated to inhibit hormone and prostaglandin-stimulated adenylate cyclases (Salomon *et al.*, 1978; Amsterdam *et al.*, 1978). Salomon *et al.* (1978) concluded that heparin affected hormone binding but that this could not solely explain the degree of inhibition found. In the present experiments we failed to demonstrate any effect of heparin on the response to PGE_1 in intact platelets suggesting that heparin does not significantly affect PGE_1 binding but does require access to the inner platelet membrane for its inhibitory effect on adenylate cyclase. The was no evidence that heparin affected the binding of ATP or magnesium to the enzyme. However, there were marked similarities between the action of heparin and that of fluoride on the platelet adenylate cyclase; both were effective only in broken platelets, both increased basal activity to a similar degree but their effects were not additive (Figure 1b), both inhibited PGE_1-stimulated activity, again to a similar degree (50%) and they produced no further inhibition when combined. Because of these similarities we suggest that heparin may be acting in a similar manner, or on similar components to fluoride in the platelet. In the case of heparin, the effects could be completely prevented, but not reversed, by protamine sulphate, suggesting that the protamine-heparin complex is inactive.

Although fluoride has been reported to inhibit the agonist stimulation of the fat cell adenylate cyclase, while stimulating basal enzyme activity, its mechanism of action is still unknown (Vaughan, 1976). In the present study the inhibition of the response to PGE_1 produced by heparin and fluoride rarely exceeded 55% when these agents were added directly to the adenylate cyclase assay. This inhibition occurred immediately. However, enzyme preparations preincubated with heparin or fluoride gradually became totally unresponsive to PGE_1 and this inhibition was irreversible. The inhibitory effects of both heparin and fluoride occurred at concentrations of these agents which were themselves stimulatory in the platelet (higher concentrations of fluoride became inhibitory in the platelet, with 100 mM NaF returning enzyme activity to basal levels). As a result of these findings, we propose that fluoride and heparin activate the adenylate cyclase of the platelet by a similar mechanism which involves some action with both the catalytic unit and the enzyme coupling mechanism (nucleotide regulatory unit).

In conclusion, heparin inhibits the PGE_1-stimulated adenylate cyclase in broken platelets in a manner very similar to fluoride. Since heparin was without effect on the cyclic AMP response to PGE_1 in intact platelets it is unlikely that the aggregatory effects of heparin *in vitro* or *in vivo* can be explained by an inhibitory effect of heparin on platelet adenylate cyclase.

We are grateful to The Welcome Trust for financial support. S.T. was in receipt of a Wellcome Senior Research Fellowship in Clinical Science.

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(*Received October 24, 1980. Revised January 9, 1981.*)