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THE INFLUENCE OF AMINES ON VARIOUS PLATELET RESPONSES

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Four amines, galactosamine, mannosamine, histamine and arginine were studied for their effects on platelet aggregation, platelet morphological changes, platelet protein phosphorylation and platelet secretion. Galactosamine inhibited platelet aggregation in response to arachidonic acid and ionophore A23187 but did not inhibit changes in platelet morphology, or in platelet protein phosphorylation in response to these agents and only partially inhibited platelet secretion. The results suggest that galactosamine can be used as a selective inhibitor of platelet-platelet attachment without having a significant effect on intracellular processes. Mannosamine was similar to galactosamine except that it partially suppressed phosphorylation of myosin light chain. Histamine was similar to mannosamine except that some platelet damage was seen in platelets exposed to histamine and arachidonic acid or ionophore A23187. Arginine was non-selective: it suppressed platelet aggregation, secretion and phosphorylation of myosin light chain and a 40 kDa protein (40P) in response to arachidonic acid and ionophore A23187. Arginine was also potent in supressing platelet morphological changes. When the same four amines were evaluated for their effects on thrombin-induced aggregation; secretion was inhibited concomitantly with inhibition of aggregation. Inhibition of myosin light chain and 40P phosphorylation was evident with galactosamine, suggesting that when thrombin is used as the agonist, galactosamine is not a specific inhibitor of platelet-platelet attachment. These amines therefore have various effects on platelet responses. Under some conditions and with arachidonic acid or ionophore A23187 as agonist, one of them, galactosamine, can be used as a selective inhibitor of platelet-platelet attachment.

Introduction

A considerable advance has been made in recent years in the understanding of the process by which platelets stick together. Research by Gartner and co-workers suggested that the attachment of platelets to one another is similar to the attachment of a lectin to its receptor [1]. At about the same time Mustard and co-workers demonstrated that fibinogen binds to platelets when they are activated [2], and others confirmed this finding, providing evidence for a specific receptor for fibrinogen on activated platelets [3,4]. Gartner then showed that fibrinogen had the capacity to act as the lectin receptor activity [5], and subsequent studies by several investigators have shown that fibrinogen can bind to a complex of two platelet membrane glycoproteins (IIb and III) [6–8], providing support for the concept that fibrinogen is a cross-bridging molecule between adjacent platelets. The attachment of platelets to one another may be strengthened by secreted substances such as thrombospondin [9] which can interact as a

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lectin with fibrinogen and platelet glycoproteins [10,11]. Certain amines have been shown to interfere with lectin-lectin receptor interaction, with platelet aggregation with platelet-fibrinogen binding and with fibrinogen binding to the glycoprotein IIb-III complex [1,11–14].

In the present study, we have examined the selectivity of four amines, galactosamine, mannosamine, histamine and arginine for their effects on platelet-platelet stickiness. If it can be shown that these amines have effects on platelets other than the inhibition of cell-cell attachment, then this would bring into question the use of these agents as selective inhibitors of stickiness. To this end, we have evaluated the effects of these four amines on aggregation, secretion, morphological changes and protein phosphorylation in response to several agonists which activate platelets by different mechanisms.

Materials and Methods

Collection and washing of platelets

Blood was drawn from normal adult donors who were not on any medication following informed consent, from the antecubital vein into a syringe containing 0.1 vol. citrate anticoagulant to achieve final concentrations of 9.3 mM sodium citrate/0.7 mM citric acid/14 mM dextrose. Blood was centrifuged at $100 \times g$ for 20 min at room temperature to obtain platelet-rich plasma. To obtain washed platelets, the platelet-rich plasma was added to an equal volume of citrate anticoagulant containing 93 mM sodium citrate, 7 mM citric acid, 105 mM dextrose and 5 mM potassium chloride (pH 6.5) and the platelets pelleted by centrifugation at $500 \times g$ for 10 min at 4°C [5]. The supernatant was removed and the platelets resuspended. The resuspending buffer used was 10% platelet poor plasma from the same donor in Hanks' balanced salt solution (pH 7.4) containing dextrose, $NaHCO_3$ and $MgCl_2$ but not $CaCl_2$ for studies employing epinephrine or ADP as agonists. When thrombin, arachidonic acid or ionophore A23187 were used as agonists, the platelets were resuspended in Hanks' balanced salt solution containing 1 mg/ml bovine serum albumin.

Platelet aggregometry

Platelet aggregation studies were performed using a Payton dual-channel aggregometer (Payton Assoc., Scarborough, Ontario) at 37°C. The baseline of the recording pen was set using the washed platelet suspension, and 100% aggregation was set using the resuspending buffer without platelets. 1 ml aliquots of suspended platelets ($(2–4) \cdot 10^8$ platelets/ml) in siliconized glass cuvettes were warmed 5–10 min (having been kept at 4°C before use) and 0.5 mM $CaCl_2$ added when the specimen was introduced into the aggregometer. Amines (histamine, arginine, mannosamine, or galactosamine suspended in Hanks' balanced salt solution or H_2O with pH adjusted to 7.4) were added 40–50 s. before the aggregating agent. The agonist (arachidonic acid, thrombin, ADP, epinephrine or ionophore A23187) or vehicle control was then added and the aggregation evaluated. Percent aggregation was recorded 2 min after addition of the agonist. The pH of the washed platelet suspension was checked following the aggregation to be sure it was between 7.3 and 7.5. With high concentrations of amines, the volume and platelet concentration of the initial washed platelet suspension, and the volume of the amine (in H_2O) to be added were adjusted to maintain a platelet count of $2 \cdot 10^8$/l and an osmolarity of between 280 and 310 mosM in the final suspension.

Electron microscopic studies

To platelets to be studied by electron microscopy, 1 ml 0.1% glutaraldehyde in White's saline (pH 7.4) (a 10% solution of a 1 : 1 mixture of (a) 2.4 M NaCl, 0.1 M KCl, 46 mM $Mg50_4$, 64 mM $Ca(NO_3)_2 \cdot 4\ H_2O$ and (b) 0.13 M $NaHCO_3$, 8.4 mM $Na_2HPO_4 \cdot 7\ H_2O$, 3.8 mM anhydrous KH_2PO_4 and 0.1 g/l Phenol red) was added 2 min after addition of agonist or vehicle control. Samples were then processed according to previous procedures [16] with a 30 min fixation in 3% glutaraldehyde in White's saline followed by a 90 min fixation in 1% OsO_4, staining with uranyl acetate overnight, followed by dehydration and embedding in Spurr resin. Sections were further stained with lead citrate before study on a Philips 400 electron microscope. A semiquantitative scale to gauge the degree of platelet activation (pseudopod formation and granule centralization) was used as follows: Platelets which were discoid with no pseudopodia were considered grade zero. Platelets

which were discoid but with pseudopodia were considered grade I. Platelets with early granule centralization (the distance from one microtubule bundle to its opposite bundle on the other side of the platelet was estimated to be less than 75% of the total diameter of the body of the platelet) were considered to be grade II. Platelets in advanced granule centralization (the distance from one microtubule bundle to the other was less than 50% of the average diameter of the body of the platelet) were considered to be grade III. 100 platelets from each sample were tabulated as above. A morphological index (M.I.) was then calculated as follows: $M.I. = I + (2 \times II) + (3 \times III)$, where I, II and III represent the percentage of platelets in each of these categories, respectively. Theoretical limits would then be 0 for completely inactivated platelets and 300 for completely activated ones. In order that the formation of large platelet aggregates would not obscure these changes, all samples processed for electron microscopy were unstirred.

Platelet secretion

Platelet-rich plasma was incubated for 15 min at 37°C with [^{3}H]serotonin (1 μCi/ml) to allow the labelled serotonin to be taken up into the platelet granules. The platelets were then washed and resuspended as described above. Samples were then treated with buffer or amine solution followed 30 s later by the agonist. At 2 min after addition of agonist (or vehicle control) on the aggregometer, 1 ml of 0.1% glutaraldehyde in White's saline was added to stop further secretion. The platelet samples were then pelleted at $500 \times g$ for 10 min to separate the platelets and the supernatant. Serotonin secretion was expressed as a percentage of the radioactivity in the supernatant over the total radioactivity in the supernatant plus the pellet.

Platelet protein phosphorylation

Platelet-rich plasma was incubated for 1 h at 37°C with [^{32}P]orthophosphate (2 mCi/ml), washed and resuspended as described above and then treated with amine or vehicle control plus agonist or vehicle control. Aliquots of 100 μl were removed into 50 μl of boiling denaturation solution [7], boiled 3 min, stored overnight at −20°C and boiled 2 min before polyacrylamide gel electrophoresis. Samples of 40 μl were loaded on a 6–17.5% acrylamide gradient SDS slab gel with a 5% stack and electrophoresed for 1 h at 75 V and 4 h at 150 V. Gels were stained, destained and autoradiographed as described [7]. The autoradiographs were traced on a Beckman DU-8 spectrophotometer to evaluate the relative amount of radioactive phosphorus incorporated into the platelet proteins following stimulation by thrombin, arachidonic acid or ionophore A23187 either with or without previous exposure to the amines. The amount of phosphate incorporation is shown by absorbance at 600 nm, for myosin light chain and 40P using the DU-8 tracings.

Materials

Galactosamine, mannosamine, arginine, histamine, ADP, epinephrine, thrombin and fibrinogen were obtained from the Sigma Chemical Co. (St. Louis, Mo.). Ionophore A23187 was purchased from Calbiochem. Arachidonic acid was obtained from Nu Check Prep and made up as the sodium salt. [^{32}P]Orthophosphate and [^{3}H]serotonin were purchased from New England Nuclear and Hanks' balanced salt solution from Grand Island Biological Co.

Results and Discussion

Histamine, arginine, mannosamine and galactosamine all caused a concentration-dependent inhibition of platelet aggregation. In general, histamine and arginine were more effective inhibitors than galactosamine and mannosamine (Table I, Figs. 1, 2). However, for epinephrine and ADP, histamine was much more effective than arginine, mannosamine or galactosamine. With epinephrine, ADP and thrombin, aggregating agents whose activation is mediated by a cell-surface receptor, these amines inhibited secretion in close parallel to their inhibition of aggregation (Table I). Since the conditions used involved low levels of calcium, where secretion is not dependent on platelet-platelet attachment [8,19], the results suggest that all these amines have other effects on the stimulation of platelets by these agonists than just the inhibition of platelet-platelet attachment. A likely possibility with these three agonists is that their receptors involve amino groups or that receptor

TABLE I

INHIBITION BY AMINES OF THE EFFECTS OF ADP, EPINEPHRINE AND THROMBIN ON PLATELETS

	IC_{50} (mM)			
	Arginine	Histamine	Mannosamine	Galactosamine
ADP-(5 μM)				
induced aggregation	8.2	1.6	10.0	12.5
induced secretion	6.6	1.3	9.4	10.0
Epinephrine-(10 μM)				
induced aggregation	1.7	0.64	2.4	2.9
induced secretion	1.9	0.72	2.2	3.0
Thrombin-(0.2 U/ml)				
induced aggregation	5.2	4.4	43.0	50.0
induced secretion	5.7	4.7	45.0	52.0

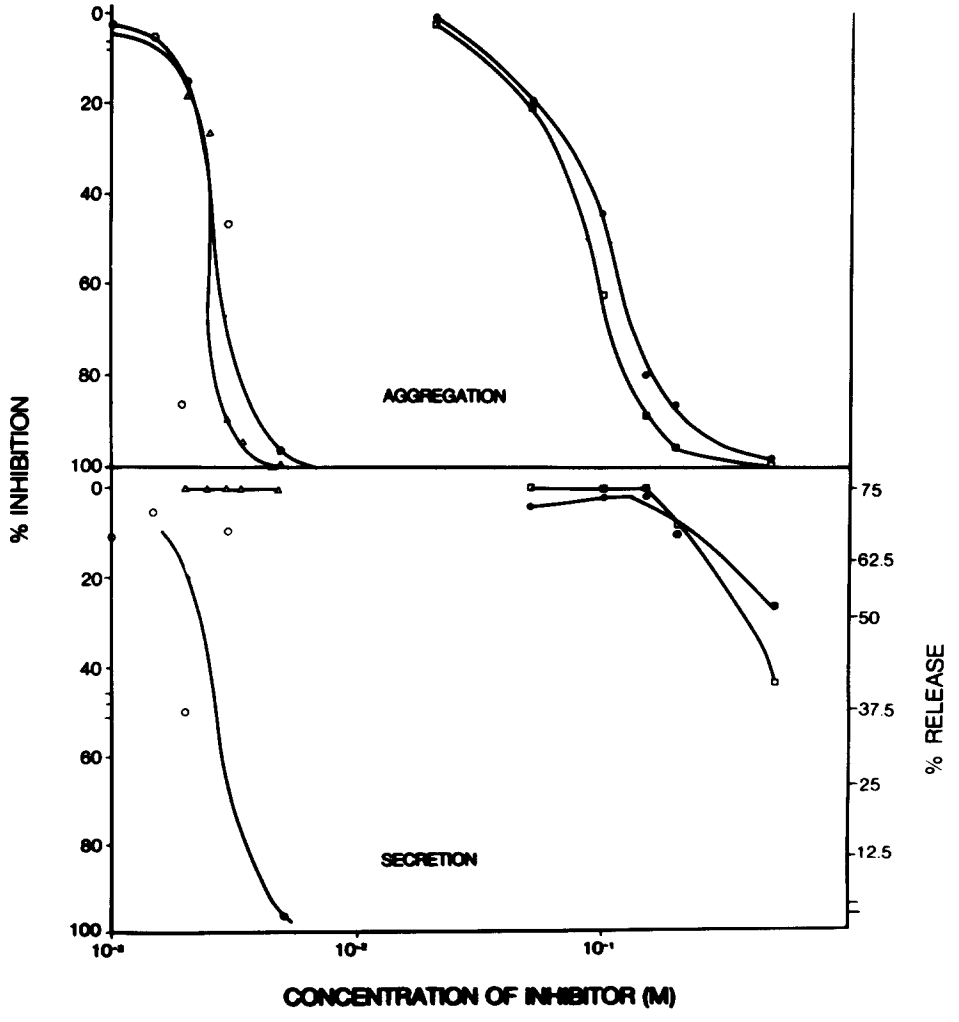

Fig. 1. The influence of amines on arachidonic acid (33 μM) induced platelet aggregation (top) and secretion of serotonin (bottom). △, histamine; ○, arginine; □, galactosamine; ●, mannosamine.

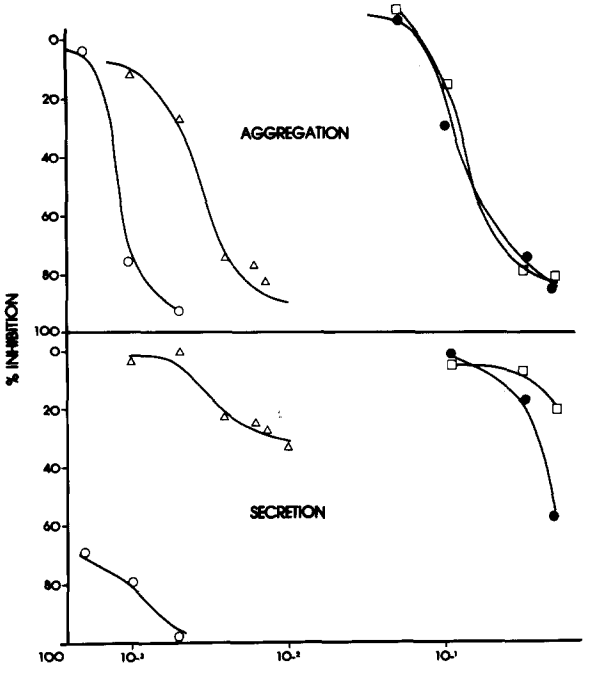

Fig. 2. The influence of amines on ionophore A23187-induced aggregation of platelets (top) and secretion of serotonin (bottom). Ionophore A23187 was used at a final concentration of 1 μM. △, histamine; ○, arginine; □, galactosamine; ●, mannosamine.

binding involves processes which can be inhibited by amine groups. Thus, for epinephrine, inhibition by amines could be due to interference with binding of the methylamine group [20] on epinephrine to the platelet receptor. Similarly, ADP binding to its receptor may involve an amine group [21], as may the binding or action of thrombin at its receptor site.

For thrombin, an inhibitory effect of galactosamine at a site other than inhibition of platelet-platelet attachment was confirmed by studying platelet protein phosphorylation in response to thrombin (Fig. 3). Galactosamine showed significant inhibition of phosphorylation of myosin light chain and 40P stimulated by thrombin. The amines (except for arginine) were less effective inhibitors of morphological changes induced by thrombin, (Table II), but the lack of effect of amines on the morphological changes may well have come about because the sensitivity of the electron microscopic evaluation is less than the sensitivity of the biochemical evaluation of protein phosphorylation.

With the thromboxane A_2 precursor, arachidonic acid and the calcium ionophore A23187, arginine inhibited secretion and aggregation in parallel; however, histamine, mannosamine and galactosamine inhibited aggregation but largely failed to inhibit secretion (Figs. 1, 2). The inhibition of arachidonic acid and ionophore A23187-induced platelet aggregation by galactosamine could be overcome by addition of 1 mg/ml fibrinogen to the washed platelets. Similar to the studies shown in Fig. 3 with thrombin, galactosamine was studied for its effect on arachidonic acid induced protein phosphorylation. Galactosamine did not inhibit protein phosphorylation in response to

TABLE II

PLATELET MORPHOLOGY INDEX

10–20% of platelets in samples of platelets pretreated with histamine and then exposed to thrombin, arachidonic acid or ionophore A23187 showed some damage. Such platelets were not counted in the morphological index assessment.

	Agonist			
	None	Thrombin (0.2 U/ml)	Arachidonic acid (33 μM)	Ionophore A23187 (1 μM)
Inhibitor				
None	46	244	251	266
Galactose (150 mM)	55	252	244	272
Galactosamine (150 mM)	51	242	249	276
Mannosamine (150 mM)	49	271	245	256
Histamine (10 mM)	39	218 *	215 *	252 *
Arginine (10 mM)	30	110	118	160

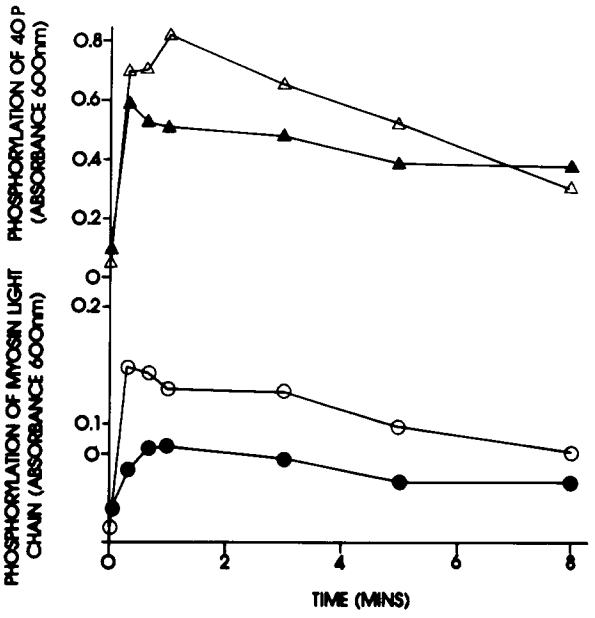

Fig. 3. The influence of 100 mM galactosamine on the phosphorylation of two proteins (40P and myosin light chain) induced by 0.2 U/ml thrombin. The samples were stirred. Open symbols: control without galactosamine. Solid symbols: 100 mM galactosamine added.

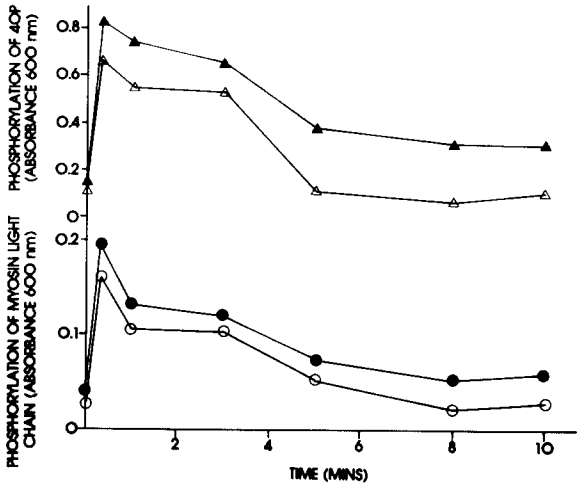

Fig. 4. The influence of 150 mM galactosamine on the phosphorylation of 40 P and myosin light chain induced by arachidonic acid. Samples were stirred. Open symbols: control without galactosamine. Closed symbols: 150 mM galactosamine added.

arachidonic acid, and indeed there was slight enhancement (Fig. 4). Since the formation of large aggregates may under some circumstances be associated with a decrease in the extent of phosphorylation [17], this would account for the slight enhancement in phosphorylation seen when archidonic acid was added to galactosamine-treated platelets. We next studied phosphorylation induced by arachidonic acid and ionophore A23187 in unstirred platelets and evaluated the effects of the amines. Arginine strongly inhibited phosphorylation of both 40P and myosin light chain in response to both agonists (Table III). Mannosamine and histamine had no effect on phosphorylation of 40P by ionophore A23187 and arachidonic acid, but partially suppressed phosphorylation of myosin light chain by these agonists. Galactosamine had no effect on phosphorylation by either agonist. The results suggest that galactosamine affects solely the platelet-platelet attachment occurring at the platelet surface and does not affect internal reactions stimulated by arachidonic acid and ionophore A23187. Mannosamine and histamine appear to affect primarily the surface reactions involved in platelet-platelet

TABLE III

THE INFLUENCE OF AMINES ON PROTEIN PHOSPHORYLATION IN UNSTIRRED PLATELETS EXPOSED TO ARACHIDONIC ACID AND IONOPHORE A23187

Agonist	Percent of maximum phosphorylation achieved with agonist alone				
	Protein	Galactosamine	Mannosamine	Histamine	Arginine
Arachidonic	40P	127	87	88	8
acid (33 μM)	myosin light chain	103	58	42	47
Ionophore A23187	40P	96	114	108	32
(1 μM)	myosin light chain	88	57	43	13

attachment, but also have some inhibitory effect on the internal protein phosphorylation. Arginine has major effects on internal as well as surface processes stimulated by arachidonic acid and ionophore A23187, as evidenced by its inhibition of internal morphological changes (Table II) as well as protein phosphorylation events.

The results of this study show the variety of effects which amines can produce. With ADP, epinephrine and thrombin amines may have effects on agonist-membrane receptor interactions. With ristocetin (studied by Scott et al. [22], amine inhibition could result from ristocetin or Von Willebrand protein interaction with their receptors. Caution must therefore be used in interpreting studies using these amines. However, our results suggest that when arachidonic acid or ionophore A23187 is used as the agonist, selective inhibition of platelet-platelet attachment can be achieved using galactosamine. The selective effect is in all likelihood due to galactosamine preventing the binding of fibrinogen to its receptor on the glycoprotein IIb-III complex [11], a step currently believed to the essential for the platelet-platelet attachment required for aggregate formation.

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