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INDUCTION OF THE PLATELET RELEASE REACTION BY CONCANAVALIN A—RELATION OF CON A BINDING TO RELEASE AND MODIFICATION OF RELEASE BY ATP, PGE_1 AND AMANTADINE

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Abstract—The binding of concanavalin A (con A) to washed human platelets was demonstrated with [^{63}Ni] and [^{3}H] labeled preparations. When con A binding was inhibited by α-methyl-D-mannoside, the con A-induced platelet release reaction was inhibited. PGE_1 markedly inhibited the release of nucleotides, serotonin and α-mannosidase (α-man) produced by both con A and thrombin but caused only a moderate decrease in con A binding. ATP, which inhibited release of serotonin by con A and thrombin, also caused a moderate decrease in con A binding. Amantadine, 5 mM, potentiated release of serotonin and most glycosidases from thrombin-treated platelets, but did not affect nucleotide or α-man release. Although 1 mM amantadine also enhanced serotonin release by con A-treated platelets without affecting release of nucleotides, 5 mM amantadine inhibited con A-stimulated release of serotonin, nucleotides and α-man and produced a 30 per cent reduction in con A binding. These data suggest that: (1) con A and thrombin activate release receptors by different mechanisms, (2) activation by con A may require binding to the receptors, (3) the mechanism for release after activation is similar for both, and (4) receptors governing the release of serotonin are different from those involved in release of nucleotides and α-man.

The jack bean lectin, concanavalin A (con A), like thrombin, causes the release of serotonin, adenine nucleotides and α-mannosidase (α-man) from washed human platelets [1–3]. In contrast to thrombin, however, con A does not release other glycosidases [β-galactosidase (β-gal), *N*-acetyl-β-glucosaminidase (NA-glu), or β-glucuronidase (β-glucur)] [1]. Further, con A blocks thrombin-induced release of β-gal, NA-glu and β-glucur from washed platelets [1].

These studies suggest that the platelet membrane may have specific receptors which are acted upon by agents such as con A and thrombin to initiate the release reaction. The manner in which these agents activate the release receptors is unknown. It is known, however, that con A binds to glycoproteins of cell membranes [4]. Thus, the initial step in con A stimulation of platelets could involve its binding to membrane receptors. The following studies were designed, first, to determine whether con A binds to platelets; second, to discover whether this binding is in any eay correlated with the function of con A as a release inducer; and third, to compare further the effects of con A and thrombin on platelets.

MATERIALS AND METHODS

Platelet concentrates in acid citrate-dextrose (ACD) were obtained from a commercial blood bank. EDTA was added to the concentrates at a final concentration of 1 mM. Platelets then were isolated and washed twice with Tris–saline–glucose–EDTA solution (0.03 M Tris–HCl, pH 7.4; 0.12 M NaCl, 5 mM glucose, 1 mM Na_2 EDTA) as previously described [1, 5]. After washing, platelets were suspended in Tris–saline–glucose without EDTA. In studies of release of platelet components, 1 ml of the suspension was incubated at 37° with 0.2 ml of solutions containing various additives.

The release of glycosidases, serotonin and nucleotides was measured and calculated as reported previously [1, 5] after incubation of platelet suspensions at 37° for 2.5 min (serotonin and nucleotides) or 5 min (glycosidases). In these experiments, the concentrations of thrombin and con A used were the lowest ones which produced consistently maximal releases as determined in preliminary studies.

Platelet concentration was determined with a Coulter counter [6].

Con A, labeled with either [^{63}Ni] (Miles-Yeda, Rehovot, Israel; 0.03 μCi/mg) or [^{3}H]acetyl (New England Nuclear, Boston, Mass; 5 Ci/m-mole), was used in binding studies. Both of these preparations co-chromatographed with electrophoretically homogeneous unlabeled con A (Miles-Yeda) when adsorbed onto a Sephadex G-75 column and eluted with a solution containing 0.1 M glucose. In addition, on ultracentrifugation in a 5–20% sucrose density gradient, the radioisotopic and cold con A preparations sedimented identically. Finally, the labeled compounds were as potent as the nonradioactive con A in causing release of serotonin and nucleotides from washed human platelets.

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To determine con A binding, platelet suspensions were incubated with either [^{63}Ni]con A (used undiluted at 0.03 μCi/mg) or [^{3}H]acetyl con A (diluted to a specific activity of 0.1 μCi/mg) at 37°. At the completion of the experiments, the suspensions were diluted with 4 ml of buffer solution (Tris–saline–EDTA), and platelets were immediately pelleted by centrifugation at 4°. The platelets were washed twice with 4-ml aliquots of buffer, dissolved in 15 ml Aquasol (New England Nuclear Corp.), and assayed for radioactivity in a Beckman LS-150 liquid scintillation counter.

In all experiments, statistical significance of paired observations (control vs experimental) was determined by Student's t-test.

Purified thrombin (BT 2000), ATP, amantadine and α-methyl-D-mannoside (α-MM) were obtained from the Sigma Chemical Co., St. Louis, Mo. The four methylumbelliferyl glycosides used in the measurement of glycosidase activity were brought from Schwarz-Mann, Orangeburg, N.Y., and Pierce Chemical Co. Rockford, Ill. PGE_1 was the gift of Upjohn Laboratories. Con A, three times crystallized, desalinated, lyophilized powder, was obtained from Miles-Yeda.

RESULTS

Preliminary studies showed that binding of isotopic compounds was maximum at about 20 min. About 70–80 per cent of the binding occurred within the first 2.5 min, and by 5 min 80–90 per cent of the total binding had occurred. Con A-stimulated releases were completed within 5 min after addition of the lectin [1]. It is therefore at this early binding that we must look if we are to seek a correlation between binding and release since binding occurring after completion of release can obviously not be involved in initiation of release. Hence a 5-min incubation period was used in all binding experiments.

The amount of con A bound was directly proportional to platelet concentration over the range of 0.5 to 2.5 × 10^9 cells/ml.

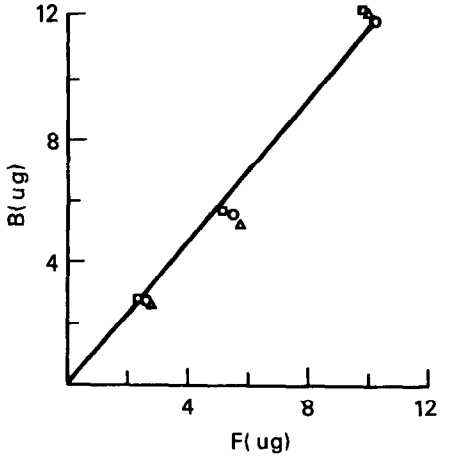

Fig. 1. Binding of [^{3}H]acetyl con A of different specific activities to human platelets. Platelet suspensions (1 ml) were incubated for 5 min in various concentrations of labeled con A using an undiluted preparation (5 Ci/mmole) (O——O); [^{3}H]acetyl con A diluted 4-fold with unlabeled con A (△——△); and [^{3}H]acetyl con A diluted 8-fold with unlabeled con A (□——□). B = bound con A; F = free con A.

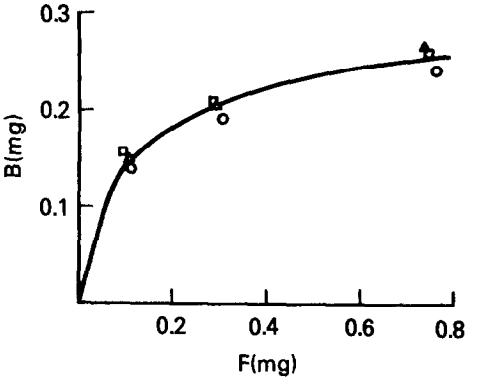

Fig. 2. Binding of [^{63}Ni]con A of different specific activities to human platelets. Platelet suspensions (1 ml) were incubated for 5 min in various concentrations of labeled con A using an undiluted preparation (0.03 μCi/mg) (O——O); [^{63}Ni]con A diluted 2-fold with unlabeled con A (△——△); and [^{63}Ni]con A diluted 4-fold with unlabeled con A (□——□). B = bound con A, F = free con A.

To show that the labeled compounds were bound in a manner indistinguishable from unlabeled con A and that they contained no radioactive contaminants which might cause errors in the binding measurements, the following experiments were done. Cells were incubated with different concentrations of undiluted radioactive con A, and the amounts bound were determined. These binding measurements then were repeated using isotopic con A diluted with the stable preparation. If binding properties were identical, solution with the cold compound would not alter the number of molecules bound at any given free concentration of con A. As can be seen in Figs. 1 and 2, both labeled compounds fulfilled this criterion.

Con A binding which could not be blocked by the haptene inhibitor, α-MM, was considered nonspecific. Figure 3 shows that 5 mM α-MM inhibited 50 per

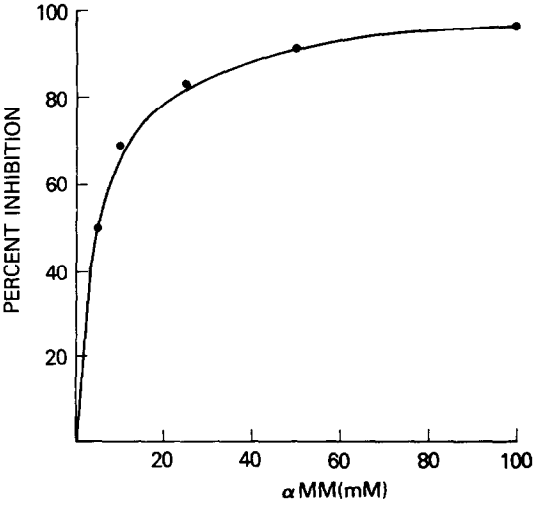

Fig. 3. Inhibition of [^{63}Ni]con A binding to human platelets by α-MM. Platelet suspensions (1 ml) were incubated for 5 min with [^{63}Ni]con A (0.03 μCi/mg, 500 μg/tube) and binding was determined as described in Methods.

cent of the binding, while at higher concentrations (50–100 mM) more than 90 per cent of con A binding was eliminated. Further, if 0.1 M α-MM was added to cells after they had been incubated with con A for 5 min, 84 per cent of the binding was reversed within 5 min, and by 10 min more than 90 per cent of the binding was lost. Results were similar with both isotopic compounds.

The affinity constant (K_a) of con A for release receptor sites and the number of release receptors can theoretically be obtained by determining binding at equilibrium of con A at various concentrations and analyzing the data by Scatchard plots [7]. Under these conditions, $K_a = -\text{slope}$, and the number of binding sites is approximated by the intercept of the plot with the abscissas. There are, however, two problems. First, it is not necessarily true that all con A binding sites are release receptors; and second, there is no way of proving that con A is in equilibrium with the release receptors after 5 min, although on the basis of the kinetics of the release reaction, this seems to be a reasonable assumption. Furthermore, the calculation of cell binding sites by the Scatchard analysis assumes that each molecule reacts with only one cell receptor. Since the con A used in this study possesses four potential binding sites, per molecule, a molecule of con A could combine with more than one cell site. The Scatchard analysis, therefore, can provide only an estimate of the minimum number of platelet receptors present. Thus, the figures derived below for K_a and number of release receptor sites can only be considered rough approximations. The molecular weight of con A in neutral solution was assumed to be 110,000 [8, 9]. The mean K_a (three experiments) was 1.67×10^6 M^{-1} (range 1.39 to 1.87) for the [^{63}Ni] compound and 1.27×10^6 M^{-1} (range 1.22 to 1.35) for the tritiated preparation. With [^{63}Ni]con A, 5.87×10^5 (range 5.79 to 5.93) sites/platelet were found, while with the [^{3}H] compound there were 5.31×10^5 (range 5.06 to 5.53) sites. Typical plots are shown in Fig. 4.

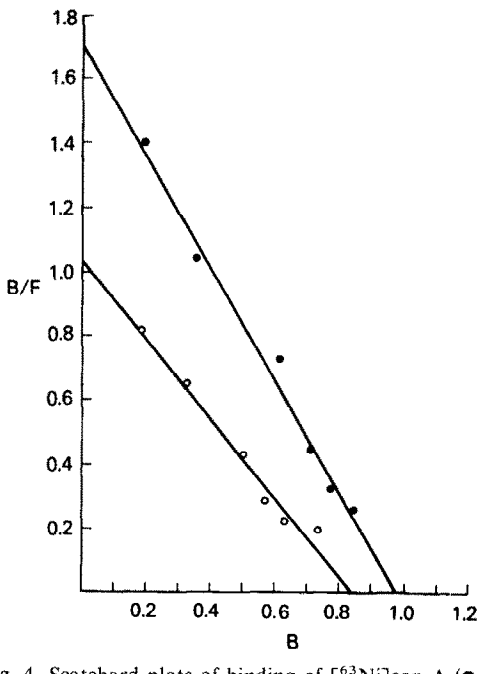

Fig. 4. Scatchard plots of binding of [^{63}Ni]con A (●—●) and [^{3}H]acetyl con A (○—○) to washed human platelets. B, nmoles con A bound/10^9 platelets. F, concentration of free con A (nmoles/ml). The association constants derived from these plots are 1.75×10^6 M^{-1} using the [^{63}Ni] compound and 1.22×10^6 M^{-1} with the [^{3}H]acetyl preparation. Binding sites are 5.9×10^5 and 5.06×10^5 for [^{63}Ni]con A and [^{3}H]acetyl con A, respectively, when data from this figure are used for the calculations.

PGE_1 and ATP produced moderate inhibition of binding of con A, while thrombin had no effect (Table 1). PGE_1 inhibited the release by both con A and thrombin of serotonin, nucleotides and α-man (Table 2). Release by con A was inhibited to a greater extent than was that by thrombin. In contrast, ATP inhibited release of serotonin by con A and thrombin to about the same extent. Alpha methyl-D-mannosidase inhibited release of serotonin, α-man and nucleotides induced by con A (Table 2).

Treatment of platelets with 5 mM amantadine produced a moderate inhibition of the binding of con A, while 1 mM amantadine had no effect (Table 3). (In all amantadine studies, platelet suspensions were preincubated for 5 min with the appropriate concentration of this compound before addition of the isotopic con A.) Amantadine, 1 mM, produced an increase in the release of serotonin from washed platelets by both con A and thrombin, but had no effect on release of nucleotides (Table 4). Amantadine, 1 mM, also partially reversed the inhibition of thrombin-stimulated serotonin release by PGE_1 and ATP without affecting nucleotide release (Table 5). At a higher concentration (5 mM), amantadine inhibited release of serotonin, nucleotides and α-man by con A, but increased release of serotonin, β-gal, NA-glu and β-glucur by thrombin (Tables 4 and 6). Release of α-man by thrombin was not affected. The effect of amantadine alone on platelet release was very slight. Mean release of serotonin was 2.1 ± 0.2 per cent with 5 mM and 0.6 ± 0.16 per cent with 1 mM amantadine, while glycosidase was unchanged.

DISCUSSION

Nachman *et al.* [10] demonstrated binding of concanavalin A to a partially purified surface glycoprotein of platelets. We have shown above that con A readily binds to intact platelets with a high affinity

Table 1. Effects of various compounds on binding of [^{63}Ni]con A to human platelets*

	Amount bound (nmoles/10^9 cells)
Control	0.97 ± 0.035 (10)
Thrombin (1 unit)	0.94 ± 0.034 (10)
ATP (5 mM)	0.79 ± 0.037† (9)
PGE_1 (5 μg)	0.71 ± 0.02† (9)

* Binding was determined as described in Methods after incubation of cells for 5 min at 37° with [^{63}Ni]con A (500 μg/ml). Concentrations of additives are per 1.0 ml. In the above experiments and those presented in the following tables, platelet concentrations ranged from 1.0 to 2.5 × 10^9 cells/ml. Values are means ± S.E.M. The number of experiments is indicated in parentheses.

† $P < 0.001$.

Table 2. Inhibition of platelet release reaction*

	Serotonin (% release)	Nucleotide (nmoles released/ 10^9 cells)	α-man (% release)
Con A (500 μg)	66.5 ± 3.2 (14)	72.9 ± 4.5 (13)	13.8 ± 1.1 (13)
+PGE_1 (5 μg)	4.8 ± 3.1† (7)	8.2 ± 4.0† (8)	0.08 ± 0.4† (8)
+ATP (5 mM)	10.6 ± 6.7† (5)		9.2 ± 3.4 (5)
+α-MM (5 mM)	0.08 ± 0.2† (5)	8.6 ± 1.6† (5)	1.2 ± 0.6† (5)
Thrombin (1 unit)	75.9 ± 3.3 (11)	83.8 ± 3.6 (8)	18.3 ± 1.9 (8)
+PGE_1 (5 μg)	25.0 ± 7.2† (9)	44.3 ± 8.8‡ (8)	8.6 ± 1.5‡ (8)
+ATP (5 mM)	9.1 ± 4.7† (5)		12.9 ± 2.4 (5)

* One-ml aliquots of platelet suspension were incubated at 37° with indicated additions in a total volume of 1.2 ml. Releases were determined as indicated in Methods. Concentrations of additives are per 1.2 ml of reaction mixture. The number of observations is indicated in parentheses. Values are means ± S.E.M.
† $P < 0.001$.
‡ $P < 0.01$.

Table 3. Effect of amantadine on binding of [^{63}Ni]con A to human platelets*

	Amount bound (nmoles/10^9 cells)
Control	0.89 ± 0.03 (10)
Amantadine (1 mM)	0.97 ± 0.05 (8)
Amantadine (5 mM)	0.70 ± 0.05† (10)

* Platelet suspensions were incubated for 5 min at 37° with the indicated concentration of amantadine (0–5 mM) prior to the addition of [^{63}Ni]con A. Incubations were continued for an additional 5 min. Binding was determined as described in Methods. The number of observations is indicated in parentheses. Values are means ± S.E.M.
† $P < 0.01$.

(K_a) and that the number of binding sites is relatively numerous. The slight decrease in K_a and number of receptor sites found when [^{3}H] acetyl con A was used could have been due to modification of the tertiary structure of con A resulting from acetylation.

When the binding of con A was inhibited by α-MM, the release reaction was also inhibited, so that binding appears to be required before release is initiated. The observation that α-MM entirely inhibited the release reaction at a concentration (5 mM) which inhibited con A binding by only 50 per cent suggests that release occurs once a critical number of receptors are activated. It is not known whether all of the con A binding sites are release receptors and whether the receptors acted upon by thrombin and con A are the same. Thrombin did not affect the binding of con A to platelets, nor did it affect the release reaction induced by con A [1]. Con A, however, blocked thrombin-stimulated glycosidase release without affecting serotonin and nucleotide release, suggesting that there may be competition for at least one class of release receptors [1].

We have previously reported inhibition of thrombin-stimulated glycosidase release from platelets by PGE_1 and ATP [5], and others have reported that PGE_1 inhibited release of serotonin, nucleotides and calcium [11]. In the current study, we found that PGE_1 prevented release by both thrombin and con A of nucleotides, serotonin and α-man and that ATP also inhibited both con A- and thrombin-stimulated release of serotonin. Also, 1 mM amantadine potentiated serotonin release by both con A and thrombin. These similarities suggest that the mechanism for

Table 4. Effect of amantadine on serotonin and nucleotide release by con A and thrombin*

	Serotonin (% release)	Nucleotides (nmoles released/ 10^9 cells)
Con A (500 μg)	55.9 ± 3.5	80.6 ± 3.9
+Amantadine (1 mM)	77.4 ± 2.5†	82.3 ± 5.6
+Amantadine (5 mM)	27.9 ± 9.5‡	24.1 ± 6.9§
Thrombin (1 unit)	64.0 ± 3.5	86.6 ± 4.8
+Amantadine (1 mM)	83.7 ± 1.1§	90.1 ± 4.7
+Amantadine (5 mM)	94.9 ± 2.4§	86.5 ± 3.7

* Conditions and procedures were the same as in Table 2 except that platelet suspensions were preincubated for 5 min at 37° with the indicated concentration of amantadine (0–5 mM) prior to the addition of thrombin or con A. Concentrations of additives are per 1.2 ml. Values are means ± S.E.M. for eight experiments.
† $P < 0.01$.
‡ $P < 0.02$.
§ $P < 0.001$.

Table 5. Amantadine reversal of PGE_1 and ATP inhibition of thrombin-stimulated release*

	Nucleotides (nmoles released/ 10^9 cells)	Serotonin (% release)
Thrombin (1 unit)	71.4 ± 9.1	66.0 ± 6.5
+ PGE_1 (5 μg)	46.0 ± 3.6†	22.7 ± 6.0‡
+ PGE_1 + amantadine (1 mM)	46.9 ± 5.2	47.4 ± 5.4§
+ ATP (5 mM)		13.6 ± 6.3‡
+ ATP (5 mM) + amantadine (1 mM)		36.9 ± 4.7‡

* Conditions and procedures were the same as in Table 4. Concentrations of additives are per 1.2 ml. Values are means ± S.E.M. for five experiments.
† $P < 0.02$.
‡ $P < 0.001$.
§ $P < 0.01$.

Table 6. Effect of amantadine on release of glycosidases by thrombin and con A*

	% Release			
	β-gal	α-man	NA-glu	β-glucur
Thrombin (1 unit)	26.4 ± 2.0	23.1 ± 2.4	39.3 ± 1.9	23.2 ± 1.9
+ Amantadine (5 mM)	37.1 ± 1.6†	23.9 ± 2.7	48.9 ± 2.0†	30.0 ± 1.7†
Con A (500 μg)		15.9 ± 1.8		
+ Amantadine (5 mM)		5.7 ± 1.3‡		

* Conditions and procedure for determinations of releases were the same as in Table 4. Concentrations of additives are per 1.2 ml. Values are means ± S.E.M. for nine experiments.
† $P < 0.001$.
‡ $P < 0.01$.

release (subsequent to activation of the "release receptor") is similar for both con A and thrombin.

Amantadine at a concentration of 5 mM inhibited release caused by con A but not by thrombin. Binding of con A was moderately decreased by this level of amantadine, although again the degree of inhibition of binding does not correlate with the degree of inhibition of release. The effects of 5 mM amantadine suggest that thrombin and con A may activate release receptors by different mechanisms. Whereas con A perhaps must bind to platelets in order to activate the release reaction, thrombin may produce an enzymatic alteration in the receptor. Tollefsen *et al.* [12], in fact, showed that di-isopropyl phosphoryl thrombin inhibited the binding of native thrombin to platelets and at the same time potentiated the release by native thrombin of serotonin.

Amantadine has been found to release serotonin from and inhibit uptake of serotonin by unwashed platelets in plasma [13]. We have found, in washed platelets that 1 mM amantadine, while having no effect alone, produced an apparent increase in the release of serotonin caused by thrombin or con A without affecting the release of nucleotides and α-man. With this same level of amantadine, inhibition of thrombin-induced serotonin release by PGE_1 and ATP was partially reversed, while the inhibition of nucleotide release was unaffected. Amantadine also seemed to increase the thrombin-activated release of all glycosidases studied except α-man. The apparent potentiation of release could conceivably be explained by inhibition of re-uptake of released substances, but there is no evidence that proteins such as glycosidases can be accumulated by platelets. Also, the fact that release of one of the glycosidases (α-man) was not increased suggests that the increase in release of the other glycosidases was truly a potentiation effect of amantadine. Therefore, it seems reasonable to assume, also, that the effect of amantadine on serotonin release is due, at least in part, to potentiation. We have shown previously [1] that the glycosidase release receptors are apparently different from the receptors related to release of serotonin, nucleotides and α-man. The apparent segregation of serotonin release from that of α-man and nucleotides, as demonstrated by the amantadine studies, suggests that the serotonin release receptors are different from those for nucleotides and α-man. Hence, there may be at least three different types of release receptors: those for glycosidases, those for serotonin, and those for nucleotides and α-man.

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