INFECTION AND IMMUNITY, Aug. 1994, p. 3108–3114
0019-9567/94/$04.00+0

Vol. 62, No. 8

Pertussis Toxin Activates Platelets through an Interaction with Platelet Glycoprotein Ib

KATHLEEN A. SINDT,[1]* ERIK L. HEWLETT,[1,2] GERALD T. REDPATH,[3] RINO RAPPUOLI,[4] LLOYD S. GRAY,[3] AND SCOTT R. VANDENBERG[3]

Departments of Pharmacology,[1] Pathology,[3] and Internal Medicine,[2] University of Virginia, Charlottesville, Virginia 22908, and Research Institute of Biocine Sclavo, 53100 Siena, Italy[4]

Received 31 January 1994/Returned for modification 9 March 1994/Accepted 3 May 1994

Platelets present a unique model to study the B-oligomer effects of pertussis toxin because they become activated in response to the B oligomer but are not suspectible to ADP-ribosylation by the holotoxin. In these studies, the B oligomer of pertussis toxin caused concentration-dependent platelet activation, as determined by increases in intracellular calcium concentration, dense granule secretion, and platelet aggregation. Stirring was required for pertussis toxin to increase intracellular calcium. A monoclonal antibody against platelet glycoprotein Ib abolished increases in intracellular calcium concentration and increased the latency and reduced the slope of the aggregation response elicited by the B oligomer. Pertussis toxin also evoked [^{14}C]serotonin release from platelets, and this effect was inhibited, though not eliminated, by an antibody against platelet glycoprotein Ib. Binding of pertussis toxin to glycoprotein Ib was observed after nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These data suggest that the B oligomer of pertussis toxin induces platelet activation mediated, at least in part, by an interaction with platelet glycoprotein Ib.

Pertussis toxin (PT), a major virulence factor produced by *Bordetella pertussis*, is composed of five different subunits, S_1 through S_5, in a 1:1:1:2:1 ratio. The S_1 or A subunit contains the ADP-ribosyltransferase activity of PT, while the B-oligomer portion of the molecule is responsible for the binding of the holotoxin to the target cell surface (29). Despite the 70% amino acid sequence similarity between the S_2 and S_3 subunits (16), their binding specificities differ. Saukkonen et al. (24) demonstrated S_2 binding to lactosylceramide and S_3 binding to gangliosides, indicating that PT can interact with both sialylated and nonsialylated glycoconjugates.

In addition to its role in delivering the A subunit to cells, the B oligomer of PT elicits biological effects, including increased intracellular calcium concentration in and proliferation of T lymphocytes (9, 17, 26–28) and platelet aggregation (1). These B-oligomer effects are presumed to be elicited by the binding of the B oligomer to a specific receptor and require significantly higher concentrations of toxin than are necessary for the enzymatic activity of the A subunit. In lymphocytes, both 43- and 70-kDa binding proteins have been identified. Rogers et al. (21) showed that the presence of a 43-kDa PT binding protein is dependent on the expression of a CD3-TCR complex. These authors proposed that the binding of PT to a receptor activates a TCR-coupled phospholipase C, leading to phosphatidylinositol turnover and increased intracellular calcium (22). The 70-kDa PT binding protein has yet to be further characterized (5), though it may be the p73 lipopolysaccharide (LPS) receptor identified by Lei and Morrison (14) that also binds PT. Although the lack of PT-mediated ADP-ribosyltransferase activity with intact platelets has fostered the idea that platelets do not have a surface membrane receptor for PT (30), the rise in intracellular inositol phosphates during B-oligomer-induced aggregation (1) suggests the presence of a signal transducing receptor. The nature of such a receptor, however, is not known.

Platelet activation is a complex response initiated by metabolic changes, such as increases in intracellular calcium and protein phosphorylation, culminating in physical alterations, such as shape change, aggregation, and granule release. One mechanism by which platelets become activated is by binding of von Willebrand factor (vWF) to the platelet glycoprotein GPIb-IX complex (32). Consisting of two disulfide-linked subunits, GPIbα (145 kDa) and GPIbβ (17 kDa), GPIb is a major sialoglycoprotein on the platelet membrane (13). GPIX, a small (20-kDa) integral membrane protein, is found in noncovalent association with GPIb. Platelet adhesion studies suggest that GPIb is linked to a signaling pathway that activates protein kinase C, which in turn activates the platelet glycoprotein receptor for fibrinogen (GPIIbIIIa), leading to platelet aggregation (25). In this study, we have characterized PT B-oligomer-induced platelet activation as measured by an increase in intracellular calcium, aggregation, and granule release and examined PT binding to platelet membrane proteins.

MATERIALS AND METHODS

PT preparation. *B. pertussis* 165 was grown in 7.2 liters of modified Stainer-Scholte medium for 48 h. Culture supernatant was obtained by centrifugation at 14,000 × *g* for 60 min at 4°C. The pH of the supernatant was adjusted to 9, and the 7.2 liters was passed over a 200-ml hydroxyapatite column (HOAP; Calbiochem) equilibrated with 10 mM KPO_4, pH 9. The void volume was collected, adjusted to pH 6, and passed over a second 200-ml HOAP column equilibrated with 10 mM KPO_4, pH 6. The column was washed with 500 ml of 100 mM KPO_4 (pH 6) and then 500 ml of 100 mM KPO_4 (pH 7) and eluted with 100 mM KPO_4 (pH 7)–0.5 M NaCl. The eluate was passed over a 35-ml fetuin-Sepharose column (made by coupling 8 mg of fetuin per g of CNBr-activated Sepharose 4B) equilibrated with 100 mM KPO_4 (pH 7)–0.5 M NaCl. After the column was washed, the PT was eluted with 3 M KSCN in 100

* Corresponding author. Mailing address: University of Virginia, Division of Clinical Pharmacology, Box 419 HSC, Charlottesville, VA 22908. Phone: (804) 924-9633. Fax: (804) 982-3830. Electronic mail address: kas4e@Virginia.EDU.

mM KPO_4 (pH 7)–0.5 M NaCl. The PT was dialyzed and concentrated, using a YM-10 membrane (Amicon), to approximately 100 ml in 154 mM NaCl–1 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and stored at 4°C. This procedure yields 8 to 12 mg of purified PT. The 9K/129G ADP-ribosyltransferase-deficient mutant was prepared as previously described (20).

B-oligomer preparation. B oligomer was prepared by the method of Burns et al. (4) on the basis of the dissociation of S1 and B oligomer in the presence of 3 M urea, ATP, and 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) and separation on carboxymethyl-Sepharose CL-6B (Pharmacia). This material contains 0.1 to 0.5% contamination with holotoxin as determined by the CHO assay (11).

PRP. Platelet-rich plasma (PRP) was obtained as previously described (3, 31). Briefly, venous whole blood from healthy volunteers was collected without trauma in trisodium citrate (0.38% final concentration). The first 5 ml was discarded. The sample was centrifuged at 200 × *g* for 5 min at 20°C. For aggregation studies, PRP was supplemented with 0.2 mg of glucose per ml to help maintain the biological responsiveness of the platelets. The platelet concentration was adjusted to 200,000 platelets per μl with platelet-poor plasma, and the platelet suspension was rotated at 25°C in full containers for 30 min prior to use.

GFP. Gel-filtered platelets (GFP) were prepared from PRP in which the initial whole blood was collected in acid-citrate-dextrose solution (130 mM sodium citrate, 11 mM citric acid, 1.7 mM dextrose). The PRP was diluted 1:1 with acid-citrate-Tyrode's salt solution (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4; pH adjusted to 6.7 with stock acid-citrate-dextrose) prior to centrifugation at 500 × *g* for 15 min at 20°C. Following resuspension in the acid-citrate-Tyrode's salt solution buffer, the platelets were filtered on a Sepharose CL-4B-200 column. The column was equilibrated, and the platelets were eluted with column buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$; pH 7.4). Platelets eluted from the column were diluted in column buffer to a final concentration of 100,000 to 200,000 platelets per μl.

Aggregation studies. Human fibrinogen for reconstituting the aggregation response of the GFP was obtained from Sigma [fraction I, "essentially plasmin(ogen) free"], and plasmin(ogen) was further removed by two sequential batch adsorptions with lysine-Sepharose (6). The lysine-Sepharose-treated fibrinogen was added to the GFP at a final concentration of 200 μg/ml. Aggregation responses were evaluated in silanized glass cuvettes with 0.5 ml of platelet sample at 37°C in a dual-channel aggregometer (Peyton Scientific, Inc., Buffalo, N.Y.). The instrument was calibrated to measure turbidity as percent transmission with platelet-poor plasma = 100% and PRP = 10%. The response to 10 μM ATP was used to assess platelet function prior to the use of each platelet preparation. The aggregation response was defined in these experiments as the maximum slope of the change in turbidity. The delay from the addition of agonist to the initiation of the slope response (latency) was also used to characterize the response. Tyrode's solution or saline was used to dilute all reagents and agonists. Inhibitors were incubated with the platelets for 3 to 5 min prior to the addition of agonist.

Spectrofluorometry. Changes in platelet intracellular calcium concentration were measured with an SLM 8000 spectrofluorometer (Aminco, Urbana, Ill.). PRP supplemented with 0.4 U of apyrase and 0.15 U of hirudin per ml was incubated with 2 μM indo-1/AM (Molecular Probes) at 37°C for 45 min prior to gel filtration. Measurements were made on a 2-ml sample of stirred GFP (100,000 per μl) in a 1-cm quartz cuvette excited at 340 nm, and emissions at 398 and 480 nm were measured simultaneously to give a ratio value indicative of free intracellular calcium. Measurements were made over 120 s, with datum points taken every 0.2 s. Agonists were added at 10 s after the establishment of a baseline, while inhibitors were incubated with the platelets for 3 to 5 min prior to the addition of agonists. Calcium values were derived from the ratios and calibration measurements, using ionomycin (Calbiochem)- and EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid]-treated samples, according to the method of Grynkiewicz et al. (10).

[^{14}C]5-HT release studies. PRP were suspended in acid-citrate-dextrose and incubated with 1 μM 5-hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate ([^{14}C]5-HT) (55 mCi/mmol; CFA.170; Amersham) for 15 min at 37°C. The PRP was then centrifuged at 400 × *g* for 13 min, and the platelet pellet was resuspended in Tyrode's saline–acid-citrate-dextrose buffer (pH 6.9). The labelled platelets were washed by gel filtration (Sepharose CL-4B) and supplemented with imiprimine HCl (RBI, Natick, Mass.) to a final concentration of 2 mM. Radiolabelled platelets were used at a concentration of 135,000 dpm per tube (0.5 ml) and assayed in triplicate for agonist-evoked [^{14}C]5-HT release. Platelet suspensions were exposed to blocking antibodies and/or fibrinogen for 5 min prior to addition of PT or agonist. The platelets were exposed to PT or other agonists for 2 min with rotary mixing at 22°C. Control suspensions to measure basal release without addition of PT or agonist were treated in an identical manner. After 2 min, the platelets were immediately centrifuged at 8,000 × *g* for 5 min. The supernatants were removed, added to 10 ml of Universal Cocktail (ICN, Irvine, Calif.) scintillation fluid, and counted in a Beckman LS9800 scintillation counter to measure the amount of 5-HT released. For measurement of total platelet [^{14}C]5-HT, pellets of the control samples were solubilized in 100 μl of 1% sodium dodecyl sulfate (SDS) and counted in Universal Cocktail.

Platelet membrane proteins. GFP or platelets from PRP in 10 mM HEPES–10 mM EDTA–1 μg of leupeptin per ml–1 mM phenylmethylsulfonyl fluoride were vortexed and then quick-frozen in an ethanol-dry ice bath. Samples were thawed and centrifuged at 200 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 1 h. The platelet membranes were resuspended in 50 mM Tris (pH 8)–150 mM NaCl–10 mM EDTA–1 μg of leupeptin per ml–1 mM phenylmethylsulfonyl fluoride–2 mM *N*-ethylmaleimide–1% Triton X-100. After incubation overnight at 4°C, solubilized membrane proteins were isolated from insoluble components by centrifugation at 100,000 × *g* for 45 min.

Immunoprecipitation of platelet glycoproteins and ligand blotting. Platelet glycoproteins (~80 μg per sample) were immunoprecipitated with protein A-Sepharose CL-4B coupled with antibodies (1:10 dilution) against platelet glycoproteins purchased from AMACS, Inc. (Westbrook, Maine) (monoclonal antibodies [MAbs] against GPIb and GPIIb are both of the immunoglobulin G subclass 1 isotype) or DAKO (Carpinteria, Calif.). Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on a 7.5% acrylamide slab gel and transferred onto polyvinylidene difluoride membranes (Millipore), which were then incubated overnight at 4°C in TBS (50 mM Tris [pH 7.5], 200 mM NaCl) with 1% bovine serum albumin. For ligand blotting, 10 μg of PT was added to the polyvinylidene difluoride membrane, and the membrane was incubated for 1 h at room temperature. After the membrane was washed in TBS, a mouse MAb (6DX3) against PT was

TABLE 1. Concentration dependence of platelet aggregation response to holotoxin, B oligomer, and 9K/129G mutant[a]

Agonist (μg/ml)	Slope	Delay (s)
PT133		
1.0	2.5	20
2.5	14.5	30
5.0	21.0	15
10.0	25.5	20
20.0	29.0	15
10.0 + GPIb Ab[b]	1.5	45
B oligomer		
1.0	2.0	30
2.5	9.5	35
5.0	18.0	20
10.0	39.0	15
10.0 + GPIb Ab	4.5	25
9K/129G		
1.0	1.5	45
2.5	11.0	25
5.0	22.0	15
10.0	17.5	15
10.0 + GPIb Ab	8.0	30

[a] Data are from a representative experiment.
[b] 10 μg/ml. Ab, antibody.

added (1:1,000) for 1 h. The membrane was then incubated with goat anti-mouse horseradish peroxidase-conjugated antibody (Jackson ImmunoResearch, West Grove, Pa.) for 1 h, washed, and developed in 25 ml of TBS–0.1 ml of 3% hydrogen peroxide–5 ml of a 3-mg/ml solution of chloronaphthol. The reaction was stopped by rinsing with water. The PT MAb 6DX3 was a kind gift from Elizabeth Leininger and James Kenimer of the Laboratory of Allergy and Immunochemistry, Division of Bacterial Products, CBER, Food and Drug Administration.

RESULTS

Platelet aggregation and increases in intracellular calcium concentrations ($[Ca^{2+}]_i$) in response to the B oligomer of PT. To identify a PT binding site on platelets, we first examined the platelet response to the B oligomer of PT by characterizing aggregation elicited by holotoxin, purified B oligomer, and a PT mutant. As shown in Table 1 and Fig. 1, the aggregation response to each of these is irreversible and comparable to that of ADP. In contrast to the strict dependence of ADP-evoked aggregation on supplementation of the GFP with fibrinogen, PT-induced aggregation was variably affected by, but did not require, fibrinogen supplementation (data not shown).

Activation of human platelets by PT, as assessed by changes in the intracellular calcium concentration, was measured on GFP suspensions loaded with indo-1/AM (Fig. 2A and B). The B-oligomer effect of PT was concentration dependent, with 10 μg (80 nM) of holotoxin per ml causing an increase in intracellular calcium ranging from 0.4 to 0.8 μM. Purified B oligomer and 9K/129G (Fig. 2B) produced similar increases in the intracellular calcium concentration. Rosoff et al. (23) reported a 50% effective concentration of 2 nM PT for increases in $[Ca^{2+}]_i$ in lymphocytes. Our results indicate that B-oligomer responses in platelets require a higher concentration of toxin than is necessary in Jurkat cells and are comparable to the concentrations found by Banga et al. to be required in human platelets (1). In the absence of platelet stirring, there was no response to PT, although stirring alone did not evoke any increase in $[Ca^{2+}]_i$ (data not shown).

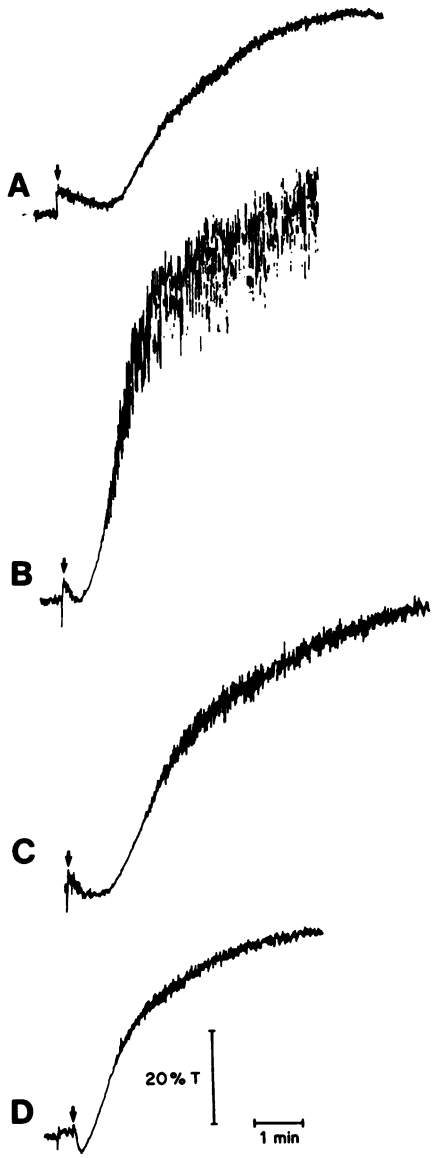

FIG. 1. Platelet aggregation in response to PT. Platelet aggregation experiments were performed as described in Materials and Methods. Agonists added at the arrows were (A) PT at 10 μg/ml, (B) B oligomer at 10 μg/ml, (C) 9K/129G at 10 μg/ml, and (D) ADP at 10 μM. T, transmission.

Inhibition of platelet activation with a MAb against GPIb. The marked effects of stirring on the platelet response to PT B oligomer suggested a role for increased cellular contact and/or shear, possibly similar to platelet adhesion to vWF mediated by GPIb (33). To investigate the possible role of GPIb in B-oligomer-induced activation of platelets, a MAb against GPIb (DAKO antibody) was used. MAb against GPIb at a concentration of 10 μg/ml inhibited both platelet aggregation (Fig. 3; Table 1) and changes in the intracellular calcium concentration (Fig. 4A) elicited by PT. In contrast to the inhibitory effects of the MAb to GPIb, MAbs against GPIIbIIIa (Fig. 4B) and GPIIb (data not shown) did not block the PT B-oligomer effects.

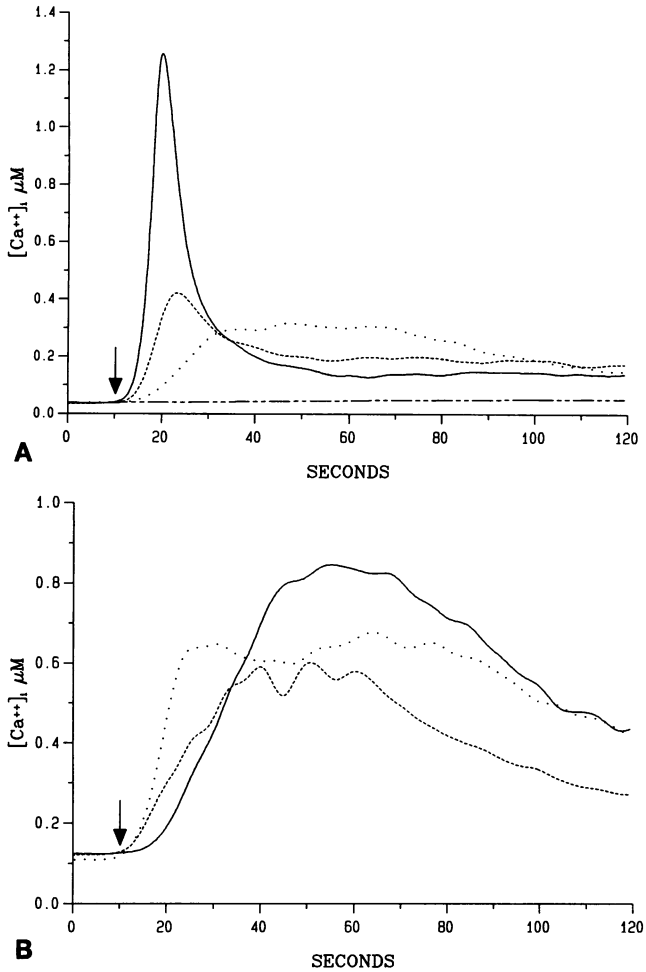

FIG. 2. Platelet $[Ca^{2+}]_i$ responses to PT. (A) Platelets were incubated with PT at 1 (— - - —), 5 (· · ·), 10 (– – –), and 20 (——) μg/ml, and increases in $[Ca^{2+}]_i$ were measured as described in Materials and Methods. (B) Increases in $[Ca_{2+}]_i$ in response to 10 μg of PT (——), B oligomer (· · ·), or 9K/129G (– – –) per ml. Arrows indicate the addition of PT.

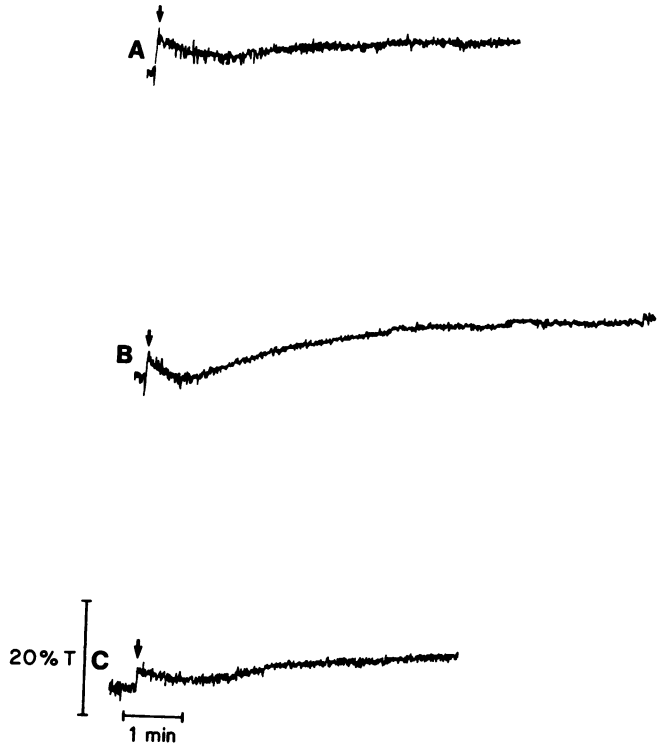

FIG. 3. Inhibition of PT-induced platelet aggregation. Platelet aggregation was measured in response to 10 μg of (A) PT, (B) B oligomer, or (C) 9K/129G per ml after a 3- to 5-min incubation with a MAb against GPIb at 10 μg/ml. T, transmission.

Release of [^{14}C]5-HT by PT. Dense granule release occurs during the physiologic response of platelets to agonists and can be monitored quantitatively by release of [^{14}C]5-HT from previously loaded platelets. PT evoked serotonin release that was 60% of the maximum response to thrombin (1 U/ml) and more than fourfold greater than the response to 10 μM ADP (Fig. 5). With the same platelet preparation, 10 μM ADP and 80 nM PT elicited comparable aggregation responses. The reduced response to ADP as measured by serotonin release is likely due to the conditions (lower temperature and less vigorous rotary mixing) in the release assays. The MAb against GPIb decreased the PT B-oligomer response by about 66% but had essentially no effect on the thrombin response (Fig. 5), suggesting that the PT response is mediated, at least in part, by mechanisms blocked at the GPIb site.

Ligand blotting of platelet membrane proteins with PT. To determine if PT was binding to GPIb, a ligand blotting technique used by Brennan et al. (2) to identify a PT receptor on CHO cells was used. PT bound to several platelet membrane proteins (Fig. 6A); therefore, the solubilized membrane proteins were immunoprecipitated with the MAb against GPIb (AMACS, Inc., antibody) to determine if PT bound to GPIb. As illustrated in Fig. 6B, when immunoprecipitated GPIb was examined under nonreducing conditions by SDS-PAGE, binding of PT to GPIb was observed. In contrast, when examined under reducing conditions, PT did not bind to GPIb. Both subunits of GPIb may be necessary for PT recognition of GPIb because PT did not bind to either subunit independently (Fig. 6B and data not shown). Alternatively, reduction may change the conformation of the GPIb chain to which PT binds. As shown in Fig. 6C, PT also binds to GPIIb. Nevertheless, a MAb against GPIIb does not affect PT-induced platelet responses, suggesting that at least some of the PT interactions on platelet surfaces are not involved in the responses measured. PT binding to GPIb by ligand blotting was not inhibited by the presence of the MAb against GPIb (1:1,000) or by vWF (10 μg of vWF plus 15 mg of ristocetin). Binding of PT to GPIIb was also not inhibited by an antibody against the receptor.

DISCUSSION

The B-oligomer effects of PT, including the toxin's ability to activate platelets and lymphocytes, are important to the assessment of new acellular vaccines of which PT is a major component (7, 18, 20). The mechanisms and binding sites involved in B-oligomer-induced lymphocyte activation are beginning to be understood. Rosoff and Mohan (22), in a model based on their studies of lymphocyte activation, proposed that a 43-kDa PT binding protein requires the presence of the CD3-TCR complex because the PT receptor signals the

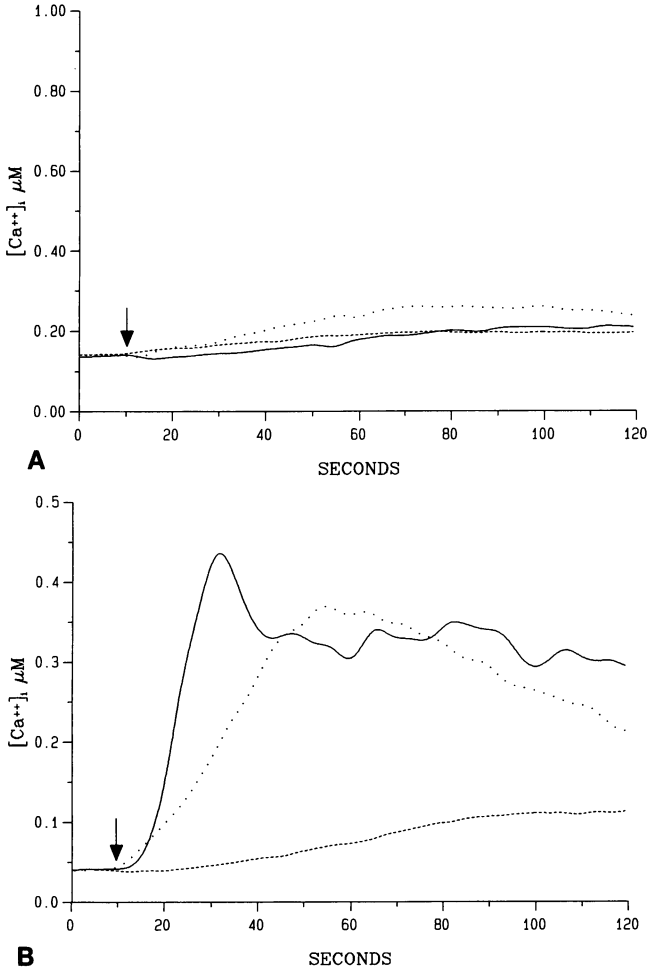

FIG. 4. Inhibition of platelet $[Ca^{2+}]_i$ responses to PT. (A) Increases in $[Ca^{2+}]_i$ elicited by 10 μg of PT (——), B oligomer (– – –), and 9K/129G (· · ·) per ml were inhibited by a 10-μg/ml portion of MAb against GPIb. (B) Increases in $[Ca^{2+}]_i$ elicited by 10 μg of PT per ml were not inhibited by a 10-μg/ml amount of a MAb against GPIIbIIIa (· · ·). The solid line represents a typical PT response (10 μg/ml), and the dashed line shows inhibition of the PT response by a MAb against GPIb, as in panel A. The MAbs were added 3 to 5 min before the addition of PT (arrows).

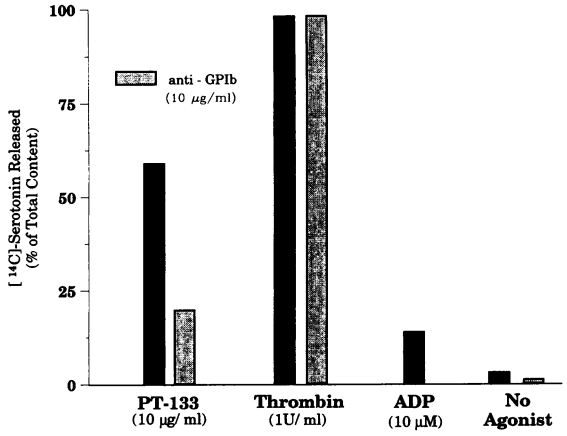

FIG. 5. [^{14}C]5-HT release studies. Platelets were loaded with [^{14}C]5-HT and assayed for agonist response as described in Materials and Methods. Maximal responses to thrombin were 98% of total extractable intracellular [^{14}C]5-HT. Each response was measured in triplicate. The standard errors of the mean ranged from 0.04 to 1.2% of the total [^{14}C]5-HT content and were <6% of the released [^{14}C]5-HT. Addition of blocking antibodies alone did not affect the low levels of basal release. Cells were incubated with blocking MAbs (10 μg/ml) for 5 min prior to a 2-min exposure to PT or thrombin.

T cell by activating a TCR-associated phospholipase C. The LPS p73 receptor on lymphocytes also binds PT (14), yet whether this receptor is involved in B-oligomer effects is unknown. Binding sites have also been addressed by Lobet et al. (15), using site-directed mutagenesis to investigate amino acid residues in the B oligomer which are involved in these activities. They found that Asn-105 in S_2 and Lys-105 in S_3 are residues in the B oligomer needed to cause lymphocyte mitogenicity.

The ability of PT to activate platelets, however, is less well understood. Banga et al. (1) demonstrated that the ADP-ribosyltransferase activity of PT was not necessary for PT to cause platelet aggregation and phospholipase C activation. In this earlier work, PT catalytic activity was abolished by modifying the toxin with *N*-ethylmaleimide, but the question of whether PT was binding to a specific receptor was not addressed. The use of chemically modified PT has previously led to the conclusion that the histamine-sensitizing effects of PT did not require ADP-ribosyltransferase activity (19). Later, however, it was shown that this was not the case (18), and therefore, analysis of the aggregation response with the B oligomer and the PT mutant 9K/129G was necessary to be

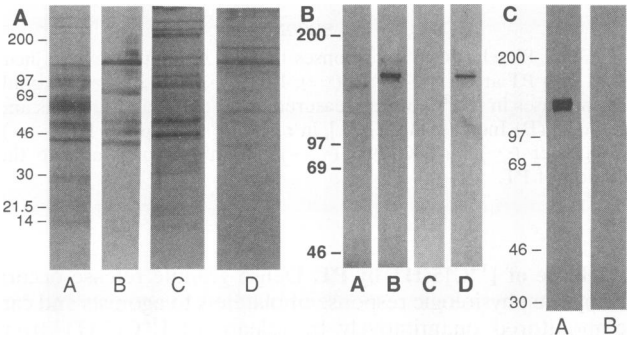

FIG. 6. Ligand blotting studies. (A) PT binding to platelet membrane proteins. PT binding to membrane proteins under both reducing conditions (lane A, Coomassie blue-stained platelet membrane proteins; lane B, platelet membrane proteins ligand blotted with PT and then probed with mouse polyclonal ascites) and nonreducing SDS-PAGE conditions (lane C, Coomassie blue-stained platelet membrane proteins; lane D, platelet membrane proteins ligand blotted with PT and then probed with mouse polyclonal ascites). (B) PT binding to GPIb. Lane A is immunoprecipitated GPIb separated under reducing conditions. Lane B is immunoprecipitated GPIb separated under nonreducing conditions. Both lanes were probed with a MAb against GPIb. Lane C is immunoprecipitated GPIb separated under reducing conditions, ligand blotted with PT, and then probed with 6DX3. Lane D is immunoprecipitated GPIb separated under nonreducing conditions, ligand blotted with PT, and then probed with 6DX3. (C) PT binding to GPIIb. Lane A is immunoprecipitated GPIIb separated under reduced conditions. Lane B is immunoprecipitated GPIIb separated under reduced conditions, ligand blotted with PT, and then probed with 6DX3. In all panels, molecular weight (10^3) markers are shown on the left.

certain that PT catalytic activity does not contribute to platelet activation.

Our data show that increases in platelet intracellular calcium, aggregation, and granule release initiated by PT are inhibited by a MAb against GPIb. GPIb is the major sialoglycoprotein on the platelet surface, with most of its carbohydrate found in the glycocalicin portion of the α chain of the glycoprotein. An estimated 60% of the weight of GPIb is attributed to its carbohydrate content, with most being O-linked residues (13). Sialic acid residues have been shown to be important for PT binding to CHO cells (2), suggesting the sialic acid residues on GPIb may be involved in PT binding to the glycoprotein. We show by ligand blotting that only after nonreducing SDS-PAGE does PT bind to GPIb. Because GPIbα and Ibβ chains are connected by a disulfide bond, this suggests that both chains of GPIb are necessary for PT to interact with GPIb.

Platelet adhesion studies suggest that GPIb is linked to a signal transduction pathway stimulating protein kinase C, which may, in turn, activate GPIIbIIIa, the platelet glycoprotein receptor for fibrinogen (25). Additionally, binding of vWF to GPIb under high shear conditions leads to an influx of calcium. This response is not initiated, however, if a recombinant fragment of vWF containing only the GPIb binding site is used (12). The data of Ikeda et al. suggest that occupancy of a single receptor is not sufficient and that the GPIIbIIIa binding site of vWF may be important for the calcium influx in response to vWF (12). While PT does bind to GPIb and its effects are inhibited by a MAb against GPIb, the possibility that there are additional receptors for PT has not been eliminated. The [^{14}C]5-HT release studies suggest that PT interactions with a GPIb site alone do not account for B-oligomer-induced platelet activation. One hypothesis for the mechanism by which PT activates platelets is that its binding to GPIb leads to the activation of GPIIbIIIa, associated with further stimulatory events and aggregation.

Previous data showing PT binding to a 165-kDa protein on CHO cells, either a 43- or a 70-kDa protein on lymphocytes, and a number of receptors on macrophages (32) and our results showing PT binding to GPIb support the conclusion that, unlike other bacterial toxins (8), PT may bind to different surface structures on different cells. Whether some binding sites which mediate the B-oligomer responses could also be used for PT entry and intoxication or the activities are mutually exclusive is not known. One hypothesis is that PT binds to many sites, all capable of leading to toxin entry into the cell, and that in some instances PT interacts with a receptor in such a way that it serendipitously activates a distal signaling pathway. To summarize, the data presented here demonstrate that platelet activation caused by PT is inhibited by a MAb against GPIb. PT binds to GPIb and may initiate platelet activation by triggering the second-messenger system associated with GPIb.

ACKNOWLEDGMENTS

This work was supported by grant RO1-AI-31118 from the National Institutes of Health.

We thank John Humphries for critically reviewing the manuscript.

REFERENCES

1. **Banga, H. S., R. K. Walker, L. K. Winberry, and S. E. Rittenhouse.** 1987. Pertussis toxin can activate human platelets. J. Biol. Chem. **262:**14871–14874.
2. **Brennan, M. J., J. L. David, J. G. Kenimer, and C. R. Manclark.** 1988. Lectin-like binding of pertussis toxin to a 165-kilodalton Chinese hamster ovary cell glycoprotein. J. Biol. Chem. **263:**4895–4899.
3. **Britt, S. G., S. L. Gonias, J. M. Sanders, and S. R. VandenBerg.** 1988. Agonist and antagonist activities of arylpiperazines at human platelet serotonin$_2$ receptor. J. Pharmacol. Exp. Ther. **247:** 965–970.
4. **Burns, D. L., J. G. Kenimer, and C. R. Manclark.** 1987. Role of the A subunit of pertussis toxin in alteration of Chinese hamster ovary cell morphology. Infect. Immun. **55:**24–28.
5. **Clark, C. G., and G. D. Armstrong.** 1990. Lymphocyte receptors for pertussis toxin. Infect. Immun. **58:**3840–3846.
6. **Deutsch, D. G., and E. T. Mertz.** 1970. Plasminogen: purification from human plasma by affinity chromatography. Science **170:** 1095–1096.
7. **Edwards, K. L.** 1993. Acellular pertussis vaccines—a solution to the pertussis problem. J. Infect. Dis. **1:**15–20.
8. **Eidels, L., R. Proia, and D. Hart.** 1983. Membrane receptors for bacterial toxins. Microbiol. Rev. **47:**596–620.
9. **Gray, L. S., K. S. Huber, M. C. Gray, E. L. Hewlett, and V. H. Engelhard.** 1989. Pertussis toxin effects on T lymphocytes are mediated through CD3 and not by pertussis toxin catalyzed modification of a G protein. J. Immunol. **142:**1631–1638.
10. **Grynkiewicz, G., M. Poenie, and R. Y. Tsien.** 1985. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J. Biol. Chem. **260:**3440–3450.
11. **Hewlett, E. L., K. T. Sauer, G. A. Myers, J. L. Cowell, and R. L. Guerrant.** 1983. Induction of a novel morphological response in Chinese hamster ovaries by pertussis toxin. Infect. Immun. **40:** 1198–1203.
12. **Ikeda, Y., M. Handa, T. Kamata, K. Kawano, Y. Kawai, K. Watanabe, K. Kawakami, K. Sakai, M. Fukuyama, I. Itagaki, A. Yoshioka, and Z. M. Ruggeri.** 1993. Transmembrane calcium influx associated with von Willebrand's factor binding to GP Ib in initiation of shear-induced platelet aggregation. Thromb. Haemostasis **69:**496–502.
13. **Kieffer, N., and D. R. Phillips.** 1990. Platelet membrane glycoproteins: functions in cellular interactions. Annu. Rev. Cell Biol. **6:**329–357.
14. **Lei, M. G., and D. C. Morrison.** 1993. Evidence that lipopolysaccharide and pertussis toxin bind to different domains on the same p73 receptor on murine splenocytes. Infect. Immun. **61:**1359–1364.
15. **Lobet, Y., C. Feron, G. Dequesne, E. Simoen, P. Hauser, and C. Locht.** 1993. Site-specific alterations in the B oligomer that affect receptor binding activities and mitogenicity of pertussis toxin. J. Exp. Med. **177:**79–87.
16. **Locht, C., and J. M. Keith.** 1986. Pertussis toxin gene: nucleotide sequence and genetic organization. Science **232:**1258–1264.
17. **MacIntyre, E., P. Tatham, R. Abdul-Gaffar, and D. Linch.** 1988. The effects of pertussis toxin on human T lymphocytes. Immunology **64:**427–432.
18. **Nencioni, L., M. Pizza, D. Volpini, D. T. DeMagistris, F. Giovannoni, and R. Rappouli.** 1991. Properties of the B oligomer of pertussis toxin. Infect. Immun. **59:**4732–4734.
19. **Nogimori, K., M. Tamura, M. Yajima, K. Ito, T. Nakamura, N. Kajikawa, Y. Maruyama, and M. Ui.** 1984. Dual mechanisms involved in development of diverse biological activities of islet-activating protein, pertussis toxin, as revealed by chemical modification of lysine residues in the toxin molecule. Biochim. Biophys. Acta **801:**232–243.
20. **Pizza, M., A. Covacci, A. Bartoloni, M. Perugini, L. Nencioni, M. T. De Magistris, L. Villa, D. Nucci, R. Manetti, M. Bugnoli, F. Giovannoni, R. Olivieri, and R. Rappuoli.** 1989. Mutants of pertussis toxin suitable for vaccine development. Science **246:**497–500.
21. **Rogers, T. S., S. J. Corey, and P. M. Rosoff.** 1990. Identification of a 43-kilodalton human T lymphocyte membrane protein as a receptor for pertussis toxin. J. Immunol. **145:**678–683.
22. **Rosoff, P. M., and C. Mohan.** 1992. Unidirectional heterologous desensitization of the pertussis toxin receptor by the CD3/TCR complex. J. Immunol. **149:**3191–3199.
23. **Rosoff, P. M., R. Walker, and L. Winberry.** 1987. Pertussis toxin triggers rapid second messenger production in human T lymphocytes. J. Immunol. **139:**2419–2423.
24. **Saukkonen, K., W. N. Burnette, V. L. Mar, H. R. Masure, and E. I. Tuomanen.** 1992. Pertussis toxin has eukaryotic-like carbohydrate

recognition domains. Proc. Natl. Acad. Sci. USA **89:**118–122.
25. **Savage, B., S. J. Shattil, and Z. M. Ruggeri.** 1992. Modulation of platelet function through adhesion receptors. J. Biol. Chem. **267:**11300–11306.
26. **Sommermeyer, H., and K. Resch.** 1990. Pertussis toxin B-subunit-induced Ca^{2+} fluxes in Jurkat human lymphoma cells: the action of long term pre-treatment with cholera and pertussis holotoxins. Cell. Signalling **2:**115–128.
27. **Stewart, S. J., V. Prpic, J. A. Johns, F. S. Powers, S. E. Graber, J. T. Forbes, and J. H. Exton.** 1989. Bacterial toxins affect early events of T-lymphocyte activation. J. Clin. Invest. **83:**234–242.
28. **Strnad, C. F., and R. A. Carchman.** 1987. Human T lymphocyte mitogenesis in response to the B oligomer of pertussis toxin is associated with an early elevation in cytosolic calcium concentrations. FEBS Lett. **225:**16–20.
29. **Tamura, M., K. Nogimori, S. Murai, M. Yajima, K. Ito, T. Katada, M. Ui, and S. Ishii.** 1982. Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry **21:**5516–5522.
30. **Ui, M.** 1984. Islet-activating protein, pertussis toxin: a probe for functions of the inhibitory guanine nucleotide regulatory component of adenylate cyclase. Trends Pharmacol. Sci. **1984:**2377–2379.
31. **VandenBerg, S. R., and S. L. Gonias.** 1989. Covalent complexes of albumin with serotonin, kerranserin, and lysergic acid antagonize the activity of serotonin in human platelets. Life Sci. **44:**1777–1785.
32. **van't Wout, J., W. N. Burnette, V. L. Mar, E. Rozdzinski, S. D. Wright, and E. I. Tuomanen.** 1992. Role of carbohydrate recognition domains of pertussis toxin in adherence of *Bordetella pertussis* to human macrophages. Infect. Immun. **60:**3303–3308.
33. **Weiss, H. J., V. T. Turitto, and H. R. Baumgartner.** 1978. Effect of shear rate on platelet interaction with subendothelium in citrated and native blood. I. Shear rate-dependent decrease of adhesion in von Willebrand's disease and the Bernard-Soulier syndrome. J. Lab. Clin. Med. **92:**750–764.