Deficient Collagen-Induced Activation in the Newborn Platelet

SARA J. ISRAELS, MICHELE DANIELS, AND EILEEN M. McMILLAN

Manitoba Institute of Cell Biology and the Department of Pediatrics, University of Manitoba, Winnipeg, Manitoba, Canada

ABSTRACT. We have investigated the impaired secretion response of neonatal platelets. We compared the response of washed neonatal and adult platelets to thrombin and collagen, and to specific activators of calcium flux (inositol trisphosphate) and protein kinase C activation (oleoyl-acetyl glycerol). Neonatal platelets show no impairment of aggregation, secretion of $[^{14}C]$serotonin or phosphorylation of specific intracellular proteins in response to thrombin, inositol trisphosphate, or oleoyl-acetyl glycerol. However, neonatal platelets have a markedly decreased response to collagen. To further evaluate this deficient response, we examined specific aspects of the collagen activation pathway. Collagen-platelet interaction as measured by adhesion of platelets to collagen-coated dishes showed no difference in adhesion of neonatal platelets compared to adult controls (20.1 ± 11.6 versus 18.6 ± 9.3%). The presence of GPIa/IIa, a Mg^{2+}-dependent collagen receptor, was evaluated by flow cytometric analysis of binding of fluorescein-tagged monoclonal antibody, 6F1 (directed against GPIa/IIa). There was no difference either in the percent of platelets that bound antibody (80 versus 81%) or in the mean fluorescence intensity of the adult and neonatal samples. Phosphoinositide hydrolysis was decreased in neonatal platelets in response to collagen but normal in response to thrombin. Neonatal platelets released more arachidonic acid than adult platelets in response to thrombin (29.5 ± 3.2 versus 19.6 ± 1.8%) but less than adult platelets in response to 10 μg/mL collagen (3.2 ± 1.1 versus 9.3 ± 3.0%). Thromboxane B_2 production was also decreased in response to collagen (52.7 ± 12.6 versus 101.4 ± 18.7 ng/mL). These results suggest that the deficient collagen response in neonatal platelets may lie in transduction of the collagen signal to phospholipases A_2 and C. (*Pediatr Res* 27: 337–343, 1990)

Abbreviations

AA, arachidonic acid
IP_3, inositol-1,4,5-trisphosphate
OAG, oleoyl-acetyl glycerol
PIP_2, phosphatidylinositol-4,5-bisphosphate
MLC, myosin light chain
47 K, 47000 dalton protein
TxA_2, thromboxane A_2
TxB_2, thromboxane B_2
HBSS, Hanks' balanced salt solution

The transient physiologic alterations in the neonatal hemostatic system have been of interest for many years, and of major

clinical importance to those caring for sick and premature infants. The differences in clotting factor activity and fibrinolytic enzymes between cord blood and that of adult controls are well established (1, 2). The abnormalities of neonatal platelet function are less completely understood. In the mid-1970s, differences in the *in vitro* responses of cord and newborn platelets compared to those from older subjects were identified (3–6). These abnormalities include a decreased aggregation response to certain agonists including ADP, epinephrine and collagen (3, 4), and decreased secretion of dense granule contents (5, 6). Neonatal platelets were shown to have decreased endogenous stores of dense granule contents, ADP, and serotonin, although Whaun demonstrated that their uptake and storage of radiolabeled serotonin was as effective as that of adult platelets (6). In addition, electron microscopy has not revealed a difference in the number or structure of these granules in neonatal platelets (7).

Granule secretion is necessary for irreversible aggregation of platelets as it potentiates the primary aggregatory response produced by an external stimulus. Platelets with either absent granule contents (8) or a defect in the release mechanism (9, 10) show abnormal responses *in vitro* and are the cause of clinical bleeding syndromes. Therefore, we began our investigations of neonatal platelet function by reexamining the secretion response. As in previous studies, we observed decreased secretion, which was most pronounced in response to collagen. We proceeded to study specific aspects of the activation pathway leading to secretion, including those uniquely involved in the response to collagen, to identify the defect in the neonatal platelet response to collagen.

MATERIALS AND METHODS

Blood collection and preparation. Cord blood was collected from the umbilical vein, immediately after clamping of the cord. Samples were obtained from elective cesarean births in which both neonate and mother were in good health and the mother was not taking medication. Cord blood was drawn into plastic syringes containing citrate/citric acid/dextrose anticoagulant. Adult blood was obtained by venipuncture into the same anticoagulant from adult volunteers with no history of bleeding diathesis or drug ingestion. In each case, platelet rich plasma was prepared by centrifugation of the citrated whole blood at 200 × g for 20 min at 20°C. Platelet counts were done using a Coulter counter model ZB1 (Coulter Electronics, Hialeah, FL). Platelets were then washed (11) and resuspended in buffer appropriate for the particular study.

Preparation of collagen. Acid soluble collagen was prepared by homogenizing calf skin collagen (type I) with 6% acetic acid. The supernatant and gelatinous precipitate were diluted and centrifuged at 1000 × g for 20 min (12). Before use as monomeric collagen the preparation was dialyzed against 0.05% acetic acid.

Aggregation studies. Aggregation was studied using a Payton dual channel aggregometer (Payton Associates, Scarborough,

Ontario) at 37°C under stirring conditions. Platelets were suspended in a Krebs-Ringer's-HEPES buffer (120 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.2 mM MgCl$_2$, 10 mM HEPES, 15 mM dextrose, 0.2% albumin) pH 7.4 (13), for the study of all agonists except IP$_3$. For agonists other than IP$_3$, 1 mM CaCl$_2$ was added to each sample 2 min before the addition of agonist. The agonists used were 0.2 U/mL thrombin, 1.0 μg/mL, and 10 μg/mL bovine collagen, 100 μg/mL OAG, and the calcium ionophore, A23187 (4.0 μM). The platelet response to IP$_3$ was monitored as previously described (14). In brief, platelets were resuspended in a high potassium buffer with 40 μM EGTA to chelate any external calcium and permeabilized using saponin (12 μg/mL) to permit entry of IP$_3$ (5–10 μM) into the cell. Maximum aggregation was recorded at 5 min after addition of the agonist.

Secretion of serotonin from platelet-dense granules. Platelets labeled with 1.0 μCi/mL of [^{14}C]serotonin were studied by the method of Jerushalmy and Zucker (15). Platelet samples were stimulated in the aggregometer with agonists, as described above, and allowed to aggregate for 2 min before the response was halted with an equal volume of 0.1% glutaraldehyde. These samples were centrifuged at 2000 × g for 10 min to pellet the platelets. Percent release was calculated by comparing radioactivity in the supernatants of stimulated samples to that of unstimulated controls.

Protein phosphorylation studies. Phosphorylation of MLC and 47K were studied as previously described (16). In brief, 1.0 mCi/mL [^{32}P]orthophosphoric acid was included in the final volume of washed platelets. The platelet suspensions were incubated for 1 h at 37°C, then washed and resuspended at a final concentration of 3 × 10^8/mL. Samples of labeled platelets were stimulated with agonists, as above. Aliquots were removed prior to and at 20 and 60 s after the agonist, added immediately to denaturing solution containing 2% mercaptoethanol and held at 100°C for 3 min. Proteins were separated on a 5–18% gradient polyacrylamide-SDS slab gel. Phosphorylated bands were identified by autoradiogram then cut from the dried gel and solubilized in 80% Protosol before counting using a Beckman LS 7800 liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA).

Flow cytometry analysis of GP Ia/IIa. Washed platelets were resuspended in NH$_4$Cl-TBS (NaCl 119 mM, Tris 30 mM, NH$_4$Cl 500 mM, pH 7.4) and then fixed with an equal volume of 2% paraformaldehyde in 0.2 M cacodylate for 1 h. The fixed platelets were incubated with monoclonal antibody 6F1 (a gift of Dr. B. S. Coller, Stony Brook, NY) directed against GP Ia/IIa as described elsewhere (17), for 2 h at 25°C. Binding was visualized using a biotinylated goat anti-mouse second antibody (HyClone Laboratories, Logan, VT) and fluorescein-avidin (Dimension Lab Inc., Mississauga, Ontario). After each incubation, the platelets were washed three times in TBS/0.1% BSA. The 300-μL aliquots at a final platelet concentration of 4 × 10^9/L were used for analysis. Platelet-associated fluorescence was quantified with an EPICS V flow cytometer (Coulter) equipped with an Argon-Ion laser (400 mW, 488 nm). Fluorescence was detected at 525 nm. Platelets were analyzed at a rate of 300/s and were identified by forward angle and orthogonal light scatter. Single parameter, 255-channel, log integral green fluorescent histograms were obtained, and the negative and positive fluorescent populations were resolved. For each sample, mean fluorescence intensity was obtained by dividing total fluorescence recorded by the number of platelets analyzed (2 × 10^4 platelets) (18).

Platelet adhesion assay. Platelet adhesion to collagen-coated polystyrene plates was performed by the method of Santoro (19). Washed platelets were resuspended in Tris-NaCl (NaCl 140 mM, Tris 50 mM, glucose 5 mM, 0.5% BSA, pH 7.4) before incubation with Na^{51}CrO$_4$ (100 μCi/mL) for 1 h at 37°C. Platelets were washed and resuspended in Tris-NaCl, containing 2 mM MgCl$_2$ at a final concentration of 2.5 × 10^9/L. The 1-mL platelet suspension was added to polystyrene dishes (35-mm diameter, Falcon Labware, Oxnard, CA) previously coated with 25 μg/mL

monomeric collagen. Collagen denatured by heating to 100°C for 10 min was used as control. After incubating for 30 min at 25°C, the platelets were removed by aspiration and the plates washed three times. Adherent platelets were removed by incubating the dish twice with 1 mL of 2% SDS for 30 min. The extracts were aspirated and pooled for determination of radioactivity by scintillation counting. The radioactivity in each sample was compared with that in 1 mL of the original platelet suspension to determine the percent of platelets adhering to the collagen.

Assay of inositol phosphates. Washed platelets were resuspended in HBSS containing [^{3}H]inositol (80 μCi/mL) and incubated at 37°C for 2 h. The platelets were washed, resuspended in HBSS containing 10 mM LiCl and incubated for 1 h before aggregation studies. One min after addition of agonists the reaction was terminated by transferring the platelet suspensions to tubes containing chloroform/methanol/HCl (100:200:2, vol/vol/vol). Water was added to the tubes before centrifugation and the upper phase was removed and diluted. The inositol phosphates in the upper phase were separated on Dowex-1 anion exchange columns by differential elution (20) of inositol phosphate, inositol bisphosphate, and inositol trisphosphate. Radioisotope measurements of these fractions were determined by scintillation counting using a Beckman LS 7800 liquid scintillation counter.

AA release and TxB$_2$ formation. Washed platelets were resuspended in Krebs-Ringers-HEPES buffer containing [^{14}C]-AA (50 μCi/mL) and incubated for 2 h. The platelets were then washed and resuspended before aggregation studies. Aliquots of platelets were removed before and 2 min after the addition of agonist and added immediately to ethyl acetate, agitated, centrifuged, and the organic phase collected. Two further extractions were performed with ethyl acetate, the last after addition of 10 μL of 1 M HCl. The solvent was evaporated under N$_2$, unlabeled AA, and TxB$_2$ were added as standards, and the extracts were resuspended in ethyl acetate. These were applied to Baker Si-250 TLC plates (J. T. Baker Co., Phillipsburg, NJ) that were activated at 100°C for 60 min (21). Lipid migration took place in a solvent system of chloroform:methanol:acetic acid:water (90:8:1:0.8, vol/vol/vol/vol) (22). The lipids were localized by brief exposure to iodine vapor and their mobility compared to the standards on the same plate. Radioactivity was measured using a System 200 imaging scanner (Bioscan Inc., Washington, DC) coupled to an IBM-PC-XT. The radioactivity appearing in the AA and TxB$_2$ "peaks" were compared pre- and poststimulation to determine the percent of AA released from the membrane phospholipids and subsequently converted to TxB$_2$.

TxB$_2$ was quantitated by ELISA using specific rabbit antibody to TxB$_2$ as previously described (14, 23). Briefly, after aggregation of washed platelets, the suspension was centrifuged and the supernatant removed and diluted in PBS-BSA. Aliquots plus anti-TxB$_2$ were added to wells of microtiter plates, previously coated with TxB$_2$-BSA conjugate, and allowed to react for 18 h. The plates were washed and goat anti-rabbit IgG-alkaline phosphatase conjugate was added and after incubation, the plates were washed again before the addition of phosphatase substrate. The absorbance at 405 nm was read for each well using a microtiter plate reader (Titertek Multiscan, Mississauga, Ontario).

Statistical analysis. Student's *t* test was used to compare the mean values of the results obtained for adult and neonatal platelets for each study.

RESULTS

Platelet aggregation. Aggregation was compared in 16 cord blood/adult pairs. All cord blood samples described were from elective term cesarean births, but studies of cord blood from full-term vaginal births did not show significant differences from our results. Aggregation responses in neonatal platelets and adult

platelets varied depending on the agonist used. Responses to thrombin and IP3 were similar in the two groups (Fig. 1A). The response of neonatal platelets to OAG was moderately reduced compared to adult platelets. The collagen response in neonatal platelets was markedly reduced compared to adult controls both at 1.0 and at 10.0 μg/mL.

Serotonin secretion from dense granules. The release of [¹⁴C] serotonin was studied in 10 cord blood/adult pairs (Fig. 1B). Secretion induced by thrombin, OAG, and IP3 was not different in neonatal and adult platelets. However, the response to collagen was markedly diminished in the neonatal platelets.

Phosphorylation of platelet proteins. The phosphorylation of MLC and 47K is shown in Figure 2 A and B. For these studies, the calcium ionophore, A23187, was used instead of IP3 to stimulate calcium flux so that all samples could be studied in intact (nonpermeabilized) platelets. Phosphorylation of MLC and 47K in unstimulated platelets was not different in the two groups. Baseline phosphorylation of MLC was 146.4 ± 74.8 and 161.2 ± 78.0 cpm/4 × 10⁸ platelets for adult and neonatal platelets, respectively. Baseline phosphorylation of 47K was 432.0 ± 124.0 cpm/4 × 10⁸ platelets for adult platelets and 381.7 ± 116.1 cpm/4 × 10⁸ platelets for neonatal platelets. Phosphorylation of MLC and 47K in response to thrombin, OAG, and A23187 was similar in neonatal and adult platelets. In contrast, in neonatal platelets, phosphorylation of both MLC and 47K was minimal in response to 10.0 μg/mL collagen.

Flow cytometric analysis of GP Ia/IIa. 6F1 binding was studied in seven neonatal/adult pairs (Fig. 3 A and B). The mean fluorescence intensity for neonatal samples was not different for adult samples (mean channel, 72, for both). Paired samples, studied on the same day, had similar fluorescence intensities, although this value varied between experiments (Fig. 3B). Of the particles in the platelet of neonatal and adult samples, 80% and

Fig. 1. *A*, percent maximum aggregation of washed platelet suspensions in response to thrombin 0.2 U/mL (*THR*), collagen 10 μg/mL (*COLL 10*), collagen 1.0 μg/mL (*COLL 1*), OAG 100 μg/mL, and IP3 10 μM. Each *bar* represents the mean ± 1 SD of 16 experiments. *B*, percent secretion of [¹⁴C]serotonin from washed platelet suspensions in response to thrombin 0.2 U/mL (*THR*), collagen 10 μg/mL (*COLL 10*), collagen 1.0 μg/mL (*COLL 1*), OAG 100 μg/mL, and IP3 10 μM. Each *bar* represents the mean ± 1 SD of 10 experiments.

81%, respectively, gave a positive reaction with 6F1. This is similar to the 83% positivity reported by Coller *et al.* (17) for 6F1 binding.

Adhesion of platelets to collagen. Adhesion of platelets to dishes coated with 25 μg/mL of monomeric collagen was determined in the presence of 2 mM Mg²⁺ for 10 neonate/adult pairs. There was no significant difference in percent adhesion between the neonatal and adult platelets using this assay (Table 1).

Formation of inositol phosphates. Stimulation of platelets with thrombin and collagen resulted in formation of all species of inositol phosphate. In Figure 4, the increase in total inositol phosphates are shown. Neonatal and adult platelet production of inositol phosphates in response to 0.2 U/mL of thrombin were not significantly different. However, significant differences between adult and neonatal platelets were observed when collagen was used as the agonist. Using 100 μg/mL collagen, there was no further increase in inositol phosphates produced by adult platelets compared to the response to 10 μg/mL. The neonatal platelets did produce inositol phosphates at 100 μg/mL of collagen, but the response was still significantly less than that of the adult control. The relative production of inositol mono-, bis-, and trisphosphate formed were not different in adult and neonatal platelets.

AA release and Tx formation. Release of [¹⁴C]-AA from platelet phospholipids is shown in Figure 5. In response to thrombin, neonatal platelets released more AA (29.5 ± 3.2%) than adult platelets (19.6 ± 1.8%). However, in response to collagen, neonatal platelets released significantly less AA.

Identification of labeled TxB2 separated by thin-layer chromatography allowed determination of the percent of released AA that was subsequently converted to TxB2. The conversion of AA to TxB2 was less in the neonatal platelets regardless of whether the stimulus was thrombin or collagen (Table 2). Quantitation of TxB2 production by ELISA, however, showed no difference in the absolute amount of TxB2 produced in response to thrombin. In contrast, neonatal platelets did produce less TxB2 in response to collagen.

DISCUSSION

We have evaluated the collagen-stimulated activation pathway in neonatal platelets. Because previous investigators have identified a defect in dense granule secretion of ADP and serotonin, we began our studies by looking at the secretion and aggregation responses of neonatal platelets to two physiologic agonists, thrombin and collagen. From these initial studies, it became clear that there was a differential response of neonatal platelets to thrombin and collagen. The response to thrombin did not differ from the platelets of adult controls, but the response to collagen even at relatively high concentrations was minimal. Therefore, we investigated aspects of the activation pathway that leads to dense granule secretion to determine whether these are deficient in the neonatal platelet, particularly when collagen is the activation stimulus.

Two separable processes contribute synergistically to secretion. These complementary processes, granule centralization and granule membrane fusion are each the end result of two separate pathways that are activated by products of phospholipase C mediated hydrolysis of PIP2. These second messengers are IP3 that releases calcium from intracellular stores (14, 24) and diacylglycerol that activates a calcium and phospholipid-dependent kinase, protein kinase C (25). The increase in cytosolic calcium activates a calcium/calmodulin-dependent MLC kinase that phosphorylates MLC. This promotes interaction of myosin with actin filaments, contraction of the actin-myosin cytoskeleton (26, 27) and centralization of granules. Protein kinase C phosphorylates proteins thought to be associated with the fusion of granular membranes with those of the open canalicular system, the internal membrane channels of the platelet that are in continuity with the plasma membrane (28). Phosphorylation of
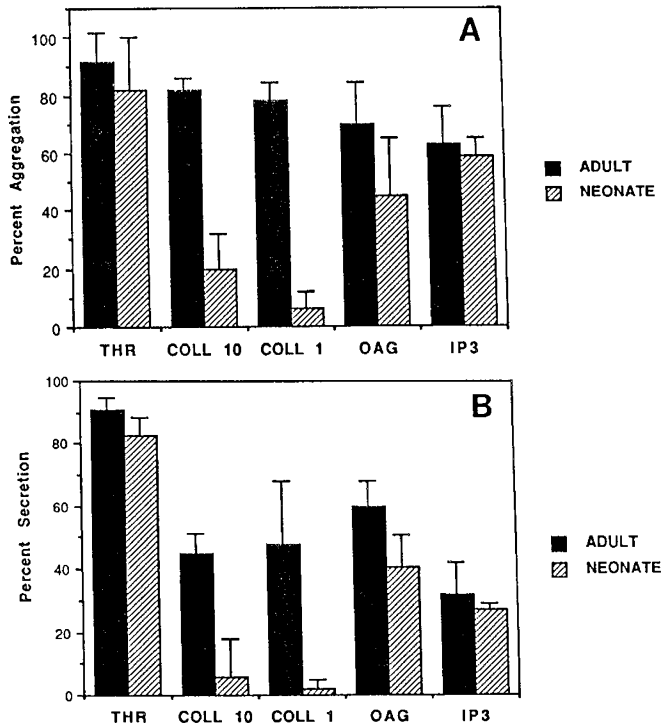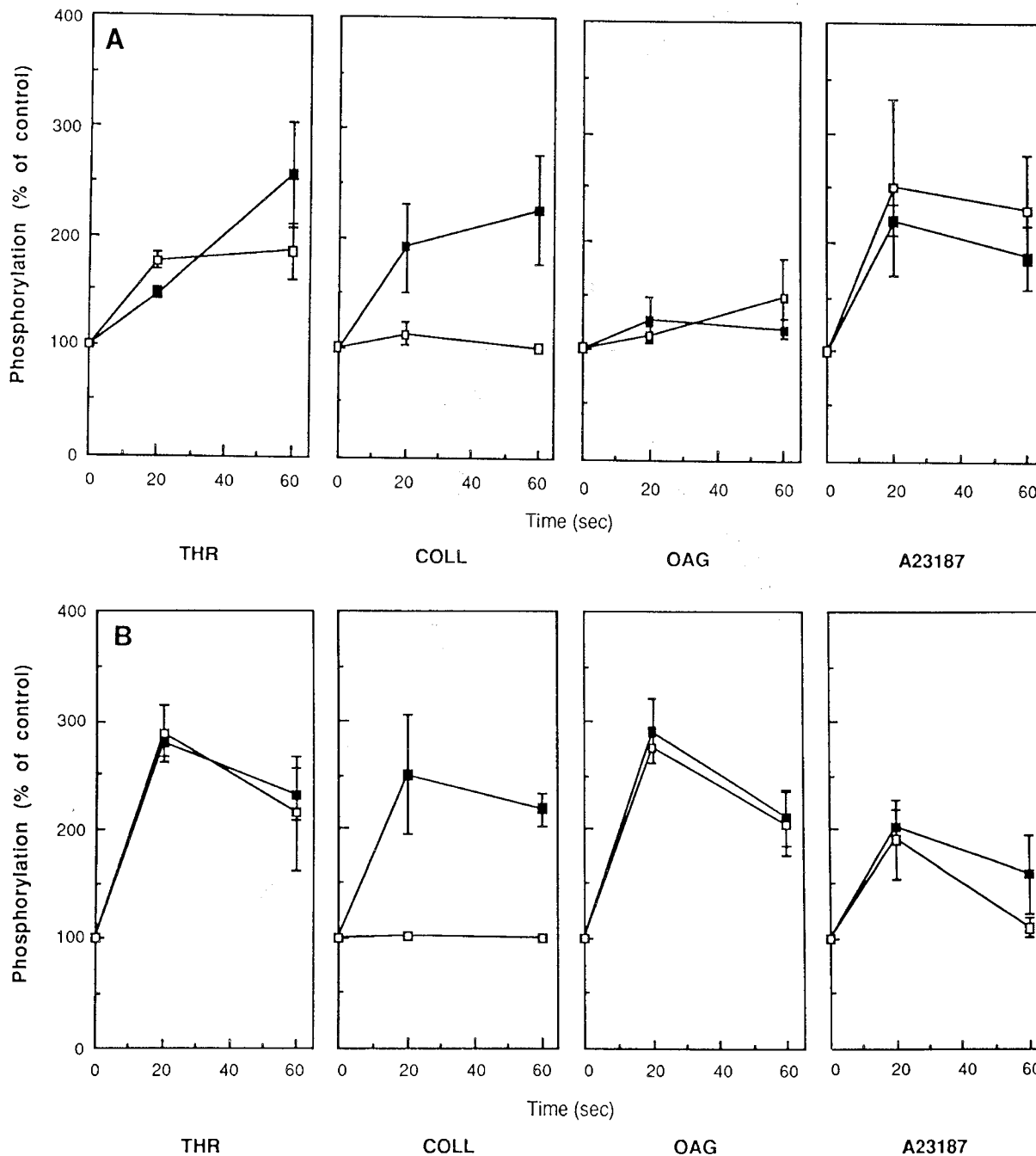
Fig. 2. *A*, phosphorylation of myosin light chain in washed platelets stimulated by thrombin 0.2 U/mL (*THR*), collagen 10 μg/mL (*COLL*), OAG 100 μg/mL, and A23187 4 μM. *Closed symbols* represent adult platelets and *open symbols* represent neonatal platelets. Each *point* is the mean ± 1 SE of four experiments. *B*, phosphorylation of the 47 000 D protein in washed platelets stimulated by thrombin 0.2 U/mL (*THR*), collagen 10 μg/mL (*COLL*), OAG 100 μg/mL, and A23187 4 μM. *Closed symbols* represent adult platelets and *open symbols* represent neonatal platelets. Each *point* is the mean ± 1 SE of four experiments.

47K, a specific substrate of protein kinase C, can be used as a marker of enzyme activation. It is these two separate but complementary processes, granule centralization and granule membrane fusion, that together result in granule secretion (28).

Using selective stimuli of either calcium flux (IP_3 and the calcium ionophore A23187) (14) or protein kinase C activation (the synthetic diglyceride, OAG) (29), we examined these two processes separately to identify potential differences in the secretion response of neonatal and adult platelets.

The calcium-dependent processes, as stimulated either by A23187 or by addition of IP_3 to permeabilized platelets, did not differ in neonatal and adult platelets. MLC phosphorylation, in particular, was not deficient in the cord blood platelets. The

protein kinase C-dependent pathways also appeared intact, because OAG-induced secretion and 47K phosphorylation were present. We therefore examined early activation steps that lie upstream from the bifurcation of the calcium-dependent and protein kinase C-dependent pathways, specifically those related to collagen activation.

Collagen binds to specific surface membrane receptors and although there has been some controversy over identification of the specific protein involved, recent evidence supports the role of GP Ia/IIa as a receptor that mediates Mg^{2+}-dependent collagen binding (17, 30). Rare patients with an absent response to collagen have been shown to lack these glycoproteins on their platelet surface (31, 32). Using 6F1, a MAb that recognizes GP
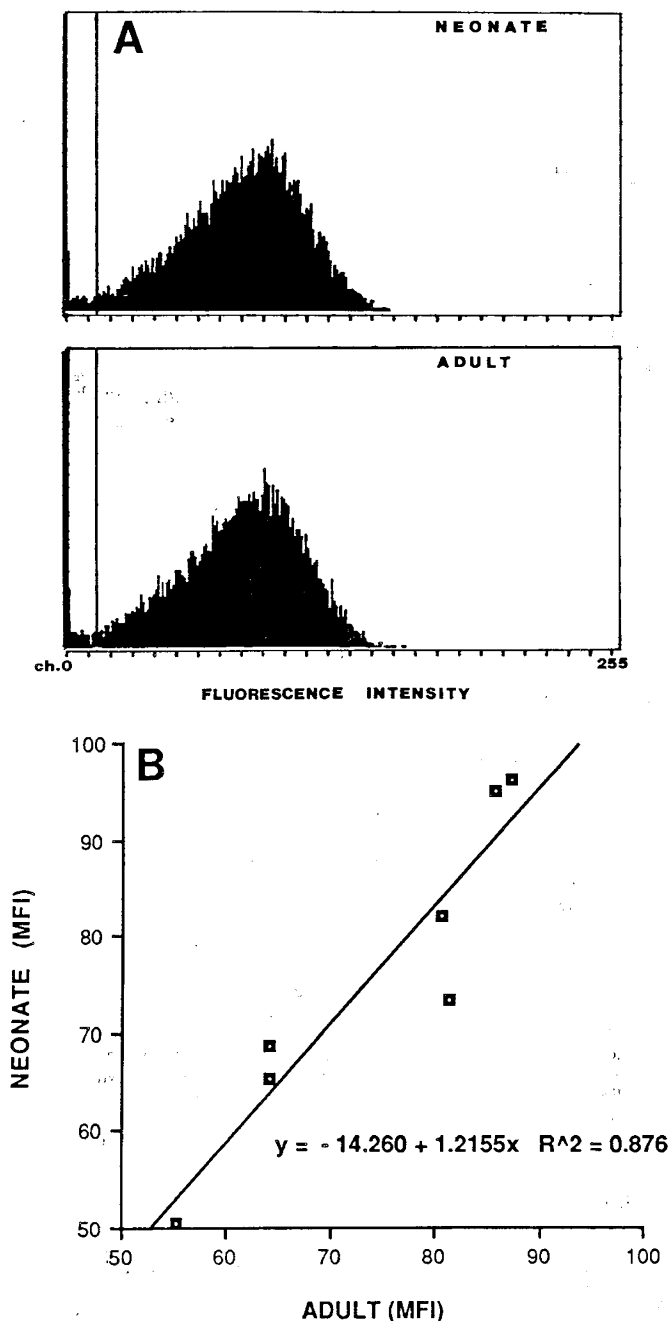
Fig. 3. Flow cytometric analysis of 6F1 binding to adult and neonatal platelets. *A*, representative histograms of fluorescence recorded after binding of 6F1, biotinylated second antibody, and fluorescein-avidin to platelets of a neonate and an adult control. The *vertical line* separates negative and positive fluorescent populations. For each sample, 2×10^4 cells were counted. A three-decade log scale was used. *B*, each point represents the mean fluorescence intensities (*MFI*) recorded for each of seven adult/neonatal pairs after binding of 6F1, biotinylated second antibody, and fluorescein-avidin.

Table 1. *Adhesion of neonatal and adult platelets to collagen*

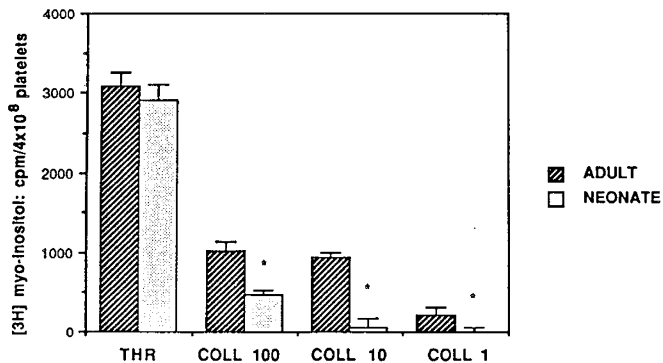| Collagen substrate	% Adhesion	
	Adult	Neonate
Monomeric (25 μg/mL)	18.6 ± 9.3*	20.1 ± 11.6 (NS)
Denatured (25 μg/mL)	6.0 ± 2.1	10.1 ± 3.3 (NS)

* Mean ± 1 SD of 10 experiments.

Fig. 4. Increase in total inositol phosphates in response to thrombin 0.2 U/mL (*THR*), and collagen, 100 μg/mL (*COLL 100*), 10 μg/mL (*COLL 10*), 1 μg/mL (*COLL 1*), over unstimulated controls (3162 ± 68.5 cpm). Each *bar* represents the mean ± SE of six experiments. The *asterisk* identifies a difference between the adult and neonatal means significant at $p < 0.05$.
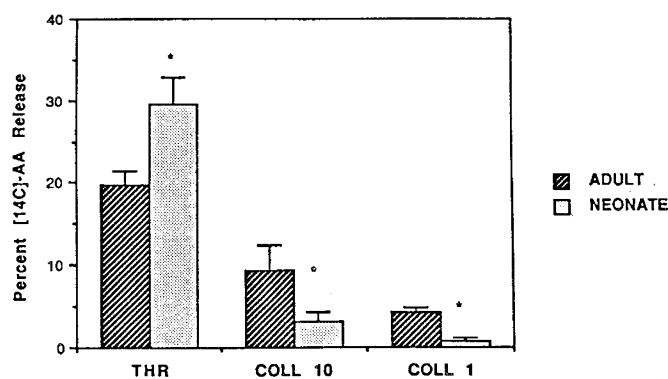

Fig. 5. Percent release of [^{14}C]-AA from platelet membrane phospholipids in adult and neonate in response to thrombin 0.2 U/mL (*THR*) and collagen, 10 μg/mL (*COLL 10*), 1 μg/mL (*COLL 1*). Each *bar* represents the mean ± SE of seven experiments. The *asterisk* identifies a difference between the adult and neonatal means significant at $p < 0.05$.

Ia/IIa, we used flow cytometry to compare the binding of this antibody to adult and neonatal platelets. We did not find differences either in the percent of cells that bound 6F1 or the mean fluorescence intensity, which reflects the relative number of binding sites per cell. This suggests that the Ia/IIa complex is present on neonatal platelets in comparable number to those present on adult platelets. The possibility of decreased numbers of collagen receptors was considered a significant possibility since the number of α-adrenergic receptors on neonatal platelets is markedly decreased, accounting for their minimal response to epinephrine (33). This appears to be an isolated receptor deficiency because the amounts of GP IIb/IIIa and GP Ib have been reported to be normal on the surface of fetal platelets (18).

As we had already demonstrated minimal aggregation and secretion responses to collagen, we also evaluated the function of this collagen receptor using an adhesion assay that was not dependent on activation of the platelet. The adhesion of [^{51}Cr] labeled neonatal platelets to monomeric collagen was not different from adult samples.

In view of these findings, we proceeded to evaluate early steps in the stimulus-response pathway of collagen. The platelets' response to collagen involves both phospholipase C-mediated phosphatidylinositol hydrolysis and phospholipase A₂-mediated release of AA from membrane phospholipids and subsequent formation of TxA₂ (34, 35). Although it is known that both phospholipase C and phospholipase A₂-mediated pathways are involved, it is unclear whether they are activated sequentially or simultaneously. Pollock *et al.* (36) have demonstrated that col-

Table 2. *TxB₂ production in neonatal and adult platelets*

	% AA converted to TxB₂		Quantity TxB₂ produced (ng/mL)	
Agonist	Adult	Neonate	Adult	Neonate
Thrombin 0.2 U/mL	17 ± 3*	11 ± 4	382.5 ± 101.5†	319.0 ± 93.4 (NS)
Collagen 10 μg/mL	15 ± 4	10 ± 2	101.4 ± 18.7	52.7 ± 12.6 ($p < 0.05$)

* Mean ± 1 SD of seven experiments.
† Mean ± 1 SE of eight experiments.

lagen can stimulate AA release without an increase in cytoplasmic calcium, suggesting that the AA/TxA₂ pathway may be the initial activation pathway with subsequent phosphoinositide hydrolysis. Considering this, we evaluated the activity of both phospholipase A₂ and C in neonatal platelets.

Phosphoinositide hydrolysis was measured by labeling platelets with [³H]inositol and stimulating them with thrombin and increasing concentrations of collagen (1-100 μg/mL). In response to thrombin, neonatal platelet phosphoinositide hydrolysis was normal but the response to collagen was markedly decreased although some hydrolysis occurred at the highest collagen concentration (100 μg/mL) used. In contrast, the response of adult platelets to 10 μg/mL collagen *versus* 100 μg/mL collagen did not differ suggesting that a maximal response had been achieved in the adult platelets at the 10 μg/mL concentration. Phospholipase C activity appears to be normal in the neonatal platelet, but activation of this enzyme by collagen is decreased.

Inasmuch as TxA₂ production is an important intermediate in collagen activation and acts as a second messenger to further activate phospholipase C, we investigated the production of TxA₂ in neonatal platelets. Stuart and Allen (37) and Stuart *et al.* (38) have identified some differences in AA release and TxA₂ synthesis between adult and neonatal platelets. In response to maximal stimulation by thrombin, neonatal platelet membranes release more AA than those of the adult. There is also more free AA available in neonatal plasma to provide substrate for TxA₂ synthesis. Although the fraction of AA converted to TxA₂ was less in the neonatal platelet, probably because of decreased activity of cyclooxygenase, the absolute amounts produced were comparable to those in the adult platelet. We found a similar pattern of response to thrombin with increased AA release from the neonatal platelets even in response to the lower dosage of thrombin used in our study (0.2 *versus* 5 U/mL). TxB₂ production measured by ELISA was the same for adult and neonate. In contrast, collagen stimulated less AA release from neonatal platelets than from adult platelets and as a result, less AA is available for conversion to thromboxane. In response to 10 μg/mL of collagen, TxB₂ production was also significantly decreased in neonatal platelets. These studies were done in washed platelets and the difference may not be present if studies are done in neonatal plasma where more free AA may be available for conversion to TxA₂. These results do suggest that collagen activation of phospholipase A₂ is decreased in the neonatal platelet.

It is unclear from these studies whether activation of phospholipase A₂ and C are both deficient in response to collagen or whether the present findings may be the result of a specific deficiency in activation of phospholipase A₂ only, with the decreased activation of phospholipase C due to decreased TxA₂ production. A defect in activation of phospholipase A₂, specifically, would explain the differential response of neonatal platelets to thrombin and collagen, as phospholipase A₂ appears to be more critical in the response to collagen.

Recent studies by Saxena *et al.* (39) have identified a role for intracellular histamine as a second messenger in platelets stimulated with collagen. Their studies have demonstrated that collagen-induced platelet aggregation, secretion, and thromboxane synthesis can be inhibited by inhibition of histamine synthesis or antagonism of histamine binding to its intracellular receptor. This inhibition can be overcome in permeabilized platelets by addition of exogenous histamine suggesting that histamine plays

a role in collagen-stimulated activation at the level of thromboxane generation. These studies identify a mechanism that could contribute to decreased phospholipase A₂ activation in neonatal platelets but also illustrate that the coupling of receptor to enzyme is still not completely understood and there may be other intermediate steps that are responsible for the deficient response to collagen in neonatal platelets.

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