Effects of Local Anesthetics on Human Platelets: Filopodial Suppression and Endogenous Proteolysis

By V. T. Nachmias, J. S. Sullender, and J. R. Fallon

Agents that affect platelet shape may be useful in understanding the mechanism of shape change; for this reason the effects of local anesthetics are worthy of further study. Local anesthetics cause platelets to retract filopodia. At short time intervals (up to about 30 min) and low concentrations of the drugs, the filopodia are reextended when the platelets are gel filtered with eluant free of anesthetic. At longer time intervals (1–2 hr) or higher drug concentrations, the retraction becomes irreversible. When the polypeptide composition of the total platelet lysate was examined on SDS gels, proteolysis of two high molecular weight bands was seen when the suppression became irreversible. These polypeptides, estimated as 250,000 and 230,000 daltons, were major components of a precipitate that formed when platelets were lysed at low ionic strength and were also enriched in a "cytoskeletal" preparation made by lysing platelets attached to glass beads and analyzing the adherent residue. Electron micrographs of platelets lysed on surfaces showed an intermeshed network of filaments to be a major component of the residue. The results suggest that the proteins comprised of these bands may be part of the cytoskeletal system and that their integrity may be necessary for the platelet to reextend filopodia following suppression.

PLATELETS are frequently obtained in plasma by drawing blood through a needle into sodium citrate anticoagulant and centrifuging out the red and white cells. In such preparations 50%–80% of the platelet population is found to have formed long thin extensions called microspikes,[1] which are similar to filopodia formed by many tissue cells. We reported that the local anesthetic lidocaine caused the retraction of these microspikes or filopodia together with sphering of the cells.[1] We found that at short time intervals (up to about 30 min) lidocaine suppression of filopodia was completely reversible when the platelets were then gel filtered free of anesthetic using Tyrode's buffer as eluant. The effect of the anesthetic was correlated with a striking change in the appearance of the cytoskeleton seen after rapid lysis of whole platelets followed by negative staining on electron microscope grids. In untreated platelets a microtubule coil and nets and bundles of thin filaments were observed; in treated platelets both coil and thin filaments were absent and replaced by a matrix that appeared largely granular but also contained short (0.05 μm) disconnected fragments of thin filaments.

We subsequently observed that tetracaine, especially when dissolved in low concentrations of dimethylsulfoxide (DMSO) (noted by J.S.S.) is a more effective suppressant of filopodia at much lower concentrations than lidocaine. We report

---

From the Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Submitted February 2, 1978; accepted August 20, 1978.

Supported by NIH Grant HL-15835 to the Pennsylvania Muscle Institute and Grant T01-GM-00281 to the Dept. of Anatomy, University of Pennsylvania.

Address reprint requests to Dr. V. T. Nachmias, Dept. of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pa. 19104.

here the results obtained when we examined the polypeptides of platelets treated with either lidocaine or tetracaine. Using SDS gel analysis, we found a correlation between internal proteolysis of two high molecular weight polypeptides and the reversibility of the drug effect. While one or two other polypeptides were also affected, the proteolysis of the two high molecular weight bands was the most striking and consistently observed change.

MATERIALS AND METHODS

Preparation of platelets. Blood was drawn from normal human volunteers into plastic syringes using 0.1 vol 3.8% sodium citrate as anticoagulant, immediately mixed and centrifuged at about 200 *g* for 10 min at 37°C to remove red cells and leukocytes, to yield platelet-rich plasma (PRP) and kept covered at 37°C until used within a few hours. All procedures were carried out in plastic containers. When platelets free from plasma were needed, they were separated by rapid gel filtration through Sepharose 2B at room temperature using calcium-free Tyrode's solution (pH 7.4) as eluant (see below for composition). The platelets were examined after fixation with 2% formaldehyde-dichromate, 2.5% glutaraldehyde, or 2% formaldehyde and found to consist of 98% discoid forms; however, it is noteworthy that 50%–80% of those platelets in plasma contained one or more filopodia emerging from the disc. Platelets fixed directly after gel filtration in Tyrode's solution showed discoid forms but 95% had filopodia. No differences between the fixatives were observed. All filopodia counts were done using a Zeiss phase-contrast microscope under oil immersion. Counts were made independently by two observers.

Scanning electron microscopy. Platelets in plasma or buffer under control or experimental conditions were fixed for 30 min at room temperature with 5 vol glutaraldehyde (2% final) in 0.05 *M* imidazole buffer pH 6.7. The fixed platelets were then allowed to adhere to clean carbon-coated coverslips for 10 min. Fixative was washed away with distilled water. To dehydrate the cells, the coverslip was flooded with increasing concentrations of ethanol from 70%–100% for 5 min at each concentration followed by three changes of 100% ethanol and then three changes of dry reagent-grade acetone. Critical-point drying was carried out in a DCP-1 (Denton Vacuum) dryer, and the cells were coated with gold-palladium 60-40 and mounted on graphite stubs for the Philips PSEM 500 scanner operated at 25 kV using a 20° angle. For light (Nomarski) photomicrographs, cells were fixed in 2% formaldehyde in 0.2 *M* phosphate buffer pH 7.0 and photographed under oil immersion.

SDS gel analysis. For low-salt precipitates made by a slight modification of the method of Lucas et al.,[2] the PRP was spun three additional times to remove any remaining red cell contamination. To one of two equal aliquots, anesthetic was added from 100 m*M* stock to yield either 30 m*M* lidocaine in Tyrode's buffer or 1.5 m*M* tetracaine in 0.75% DMSO, respectively. These concentrations were determined to be the lowest that would give 100% filopodial withdrawal. Both treated and control samples (receiving an equal volume of buffer or DMSO) were then incubated at 37°C for 30 min. Both anesthetic-treated and control platelets were then gel filtered on separate identical columns of Sepharose 2B made from 50 ml modified plastic syringes using medium containing 100 m*M* KCl, 10 mM ethylene glycol-bis-(β-ammoethyl ether)-*N,N'* tetraacetic acid (EGTA), and 7 m*M* sodium phosphate (pH 6.8) as eluant. Experimental samples also contained anesthetic in the eluant. The gel-filtered platelets were collected in plastic tubes and transferred to dialysis tubing for concentration with powdered Ficoll 400. This procedure was necessary for gentle concentration without aggregation. The filtered platelets were concentrated approximately tenfold in 2–2.5 hr. Both samples were then counted in a hemocytometer and the number of cells in the concentrates equalized. Cell counts were always within 10%. At this stage red cell contamination was also determined by direct counting and was less than 0.01%. The platelets were then rapidly lysed by adding 0.2 vol 10% Triton X-100 to each (2% final). The samples were immediately placed on ice, and phenylmethysulfonylfluoride (10^{-4}*M* final) was added to each. After 10 min on ice a precipitate was well developed, and the samples were then spun for 20 min at 27,000 *g* at 5°C. The precipitates and supernatants of both samples were prepared for SDS gel electrophoresis by boiling in sample buffer according to the method of Laemmli.[3] Red cells equal to maximum possible contamination (by direct counts after concentration) were also boiled for gel analysis.

For cytoskeletal preparations 50 ml PRP was used, and EGTA was added to 5 m*M* final concentration. Platelets were gel filtered through 300 ml Sepharose 2B using calcium-free Tyrode's for preequilibration and for eluant. EGTA was added to the gel-filtered platelets to make a final

concentration of 5 m*M*, and contaminating red cells were spun out with a low-speed spin. Aliquots of the filtered platelets were then consecutively spun down in the same tube to form a single pellet. The pellet from the original PRP was then resuspended in 6 ml calcium-free Tyrode's solution containing 5 m*M* EGTA by incubating for 20 min at 37°C with occasional gentle stirring. This suspension was poured onto a 15-cm-diameter Petri dish containing a single layer of prechilled acid-washed glass beads (Thomas, Philadelphia, Pa., 3 mm diameter) and allowed to incubate at room temperature for 20 min. The excess suspension with nonadhering cells was thoroughly withdrawn without agitating the beads and poured over the glass beads in an identical second Petri dish and treated in the same way. Both sets of beads, after complete drainage, were then flooded with 10 ml 1% Triton X-100, which was added to the beads, agitated thoroughly, and then removed as completely as possible using fine pipettes. The beads were then flooded with 6 ml 1% SDS and agitated vigorously again. The SDS solution containing solubilized material enriched for "cytoskeletal" proteins from both dishes was then decanted and concentrated in dialysis tubing against Ficoll 400 at 5°C until the final volume reached 0.3–0.5 ml (about 25-fold). At this point the sample was boiled in preparation for slab gel analysis. A control sample for the total platelet homogenate proteins was obtained from the resuspension step.

For analysis of total lysates the platelets from 5 ml PRP (approximately 10^9 platelets) were gel filtered as described above, and the platelets from the peak fractions were pelleted. The pellet was rapidly suspended in 100 μl sample buffer[3] mixed with 10 μl 100 m*M* EGTA. The solution was immediately boiled for 2 min and used for slab gel analysis.

Slab gel procedure. Polyacrylamide gel electrophoresis in SDS was performed according to the method of Laemmli[3] on 7.5% acrylamide gels with the following modifications: (1) the procedure was adapted for slab gels, (2) the acrylamide concentration in the stacking gel was increased to 4.75%, and (3) for the gradient gels 7 μ*M* flavin mononucleotide was substituted for ammonium persulfate and the gels were polymerized with fluorescent light for 30 min. Electrophoresis was carried out at a constant current of 20 mA. Gels were stained and fixed in 0.1% Coomassie Brilliant Blue in 50% trichloroacetic acid and destained in 7% acetic acid.

Lidocaine and tetracaine were products of Pfaltz and Bauer. DMSO was obtained from Fisher Scientific. All other chemicals were reagent grade.

The calcium-free Tyrode's solution contained 0.13 *M* NaCl, 2 m*M* KCl, 0.5 m*M* NaH_2PO_4, 10 m*M* Na_2CO_3, and 4 m*M* $MgCl_2$, adjusted to pH 7.4.

RESULTS

Morphology. Tetracaine and lidocaine were incubated with PRP at 37°C. As previously reported[1] 30 m*M* lidocaine caused filopodia to retract in up to 90% of the platelets. Tetracaine was effective at 2–4 m*M* in causing 100% withdrawal. Both drugs caused platelets to become spherical[1] (Fig. 1). However, when tetracaine was used at 1–1.5 m*M* in conjunction with 0.75% DMSO the filopodia were completely retracted but the platelets did not sphere; they assumed a slightly enlarged discoid shape best described as an oblate spheroid. Figure 2A shows the complete filopodial retraction in such a population of platelets as seen by scanning electron microscopy (compare with the control population shown in Fig. 2B).

Both lidocaine- and tetracaine-DMSO-induced retractions were completely reversible at short time intervals when the platelets were gel filtered through Sepharose 2B using calcium-free Tyrode's buffer (see Materials and Methods) as eluant[1] (Table 1). After longer time intervals or increases in concentrations of the drug in the case of tetracaine the effect became irreversible (Table 1).

We had previously found that control platelets lysed on grids with Triton X-100 contained an interconnected filamentous network but that this was replaced in anesthetic-treated platelets by a granular matrix.[1] This suggested that there could be an alteration in the proteins associated with the cytoskeleton. Lucas et al.[2] observed bands corresponding to actin, myosin, and additional polypeptides, including a prominent high molecular weight band in a low-salt precipitate made

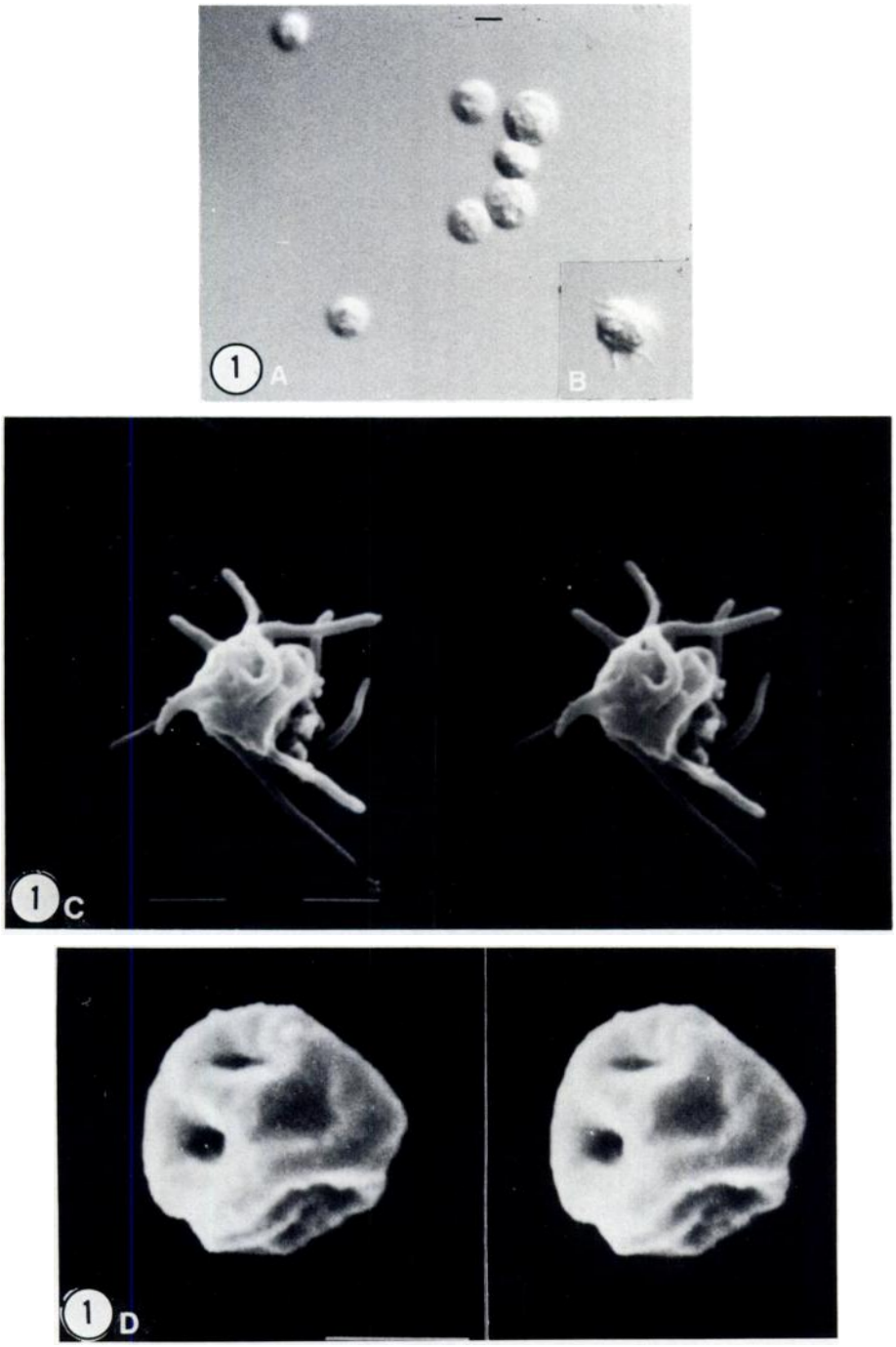

Fig. 1. A and B, platelets fixed in 2% buffered formaldehyde for 10 min before viewing by Normarski optics under oil immersion. × 3600. Bar, 1 μm. (A) Platelets treated with 4 m*M* tetracaine at pH 7.4 for 10 min at 37°C followed by gel filtration in 4 m*M* tetracaine in calcium-free Tyrode's solution. (B) Control platelets incubated in the same way and gel filtered in calcium-free Tyrode's solution. C and D, scanning electron micrographs (stereo pairs) of platelets fixed after gel filtration with 2% glutaraldehyde as described. (C) Control platelet with many filopodia. × 10,000. (D) Platelet treated with 30 m*M* lidocaine for 20 min at 37°C. This example shows more than the usual enlargement of the canalicular system. × 20,000.

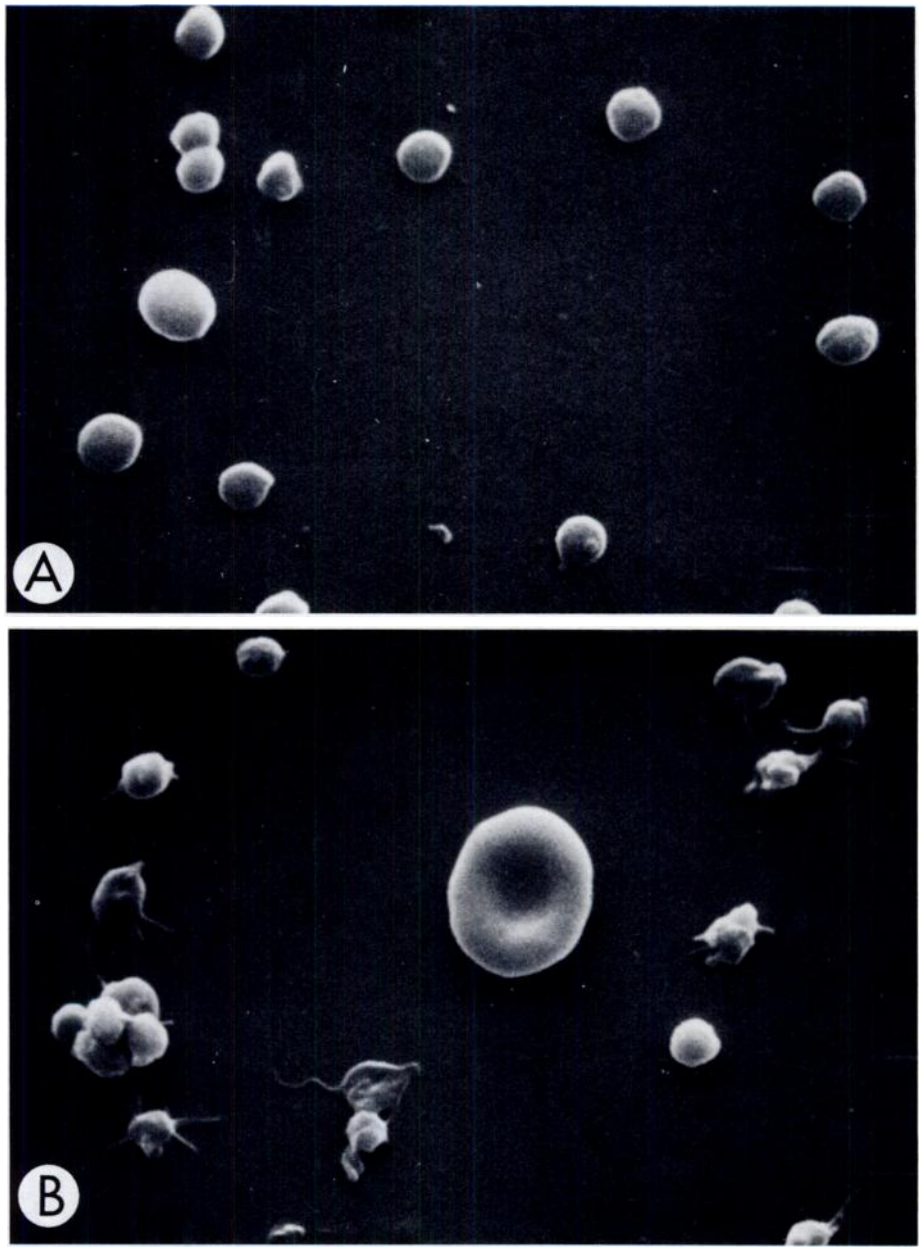

Fig. 2. Scanning electron micrographs of platelets in PRP. × 4000. (A) Platelets incubated with 0.5 m*M* tetracaine in 0.5% DMSO for 10 min. Note complete suppression of filopodia. (B) Platelets incubated with 0.5% DMSO alone for 10 min.

from platelets by direct lysis. Therefore we compared the precipitates from control and treated platelets as described above.

SDS gel analysis. We first wished to be certain that any high molecular weight components seen in platelet precipitates could not be due to red cell contamination. Figure 3 shows (channel 4) that the high molecular weight bands found in the

Table 1. Reversibility of Platelet Morphology After Tetracaine-Induced Filopodia Retraction

Treatment	Percent in Each Form			
	Discs, No Filopodia	Discs With Filopodia	Spheres With Filopodia	Proteolysis of Bands 1 and 2
(1) PRP and buffer	45	55	0	
(1a) After gel filtration	0	100	0	Absent
(2) PRP and 1.5% DMSO, 120 min	50	50	0	
(2a) After gel filtration	0	100	0	Absent
(3) PRP and 1.5 mM tetracaine in 0.75% DMSO, 2 or 30 min	98	2	0	
(3a) After gel filtration, after 2 or 30 min	0	100		Absent
(4) PRP and 3 m*M* tetracaine in 1.5% DMSO, 120 or 180 min	0	0	100	
(4a) After gel filtration, 120 or 180 min	0	0	100	Marked

Platelets in PRP were treated with buffer, tetracaine in DMSO, or with DMSO alone for the times indicated. An aliquot was fixed and counted to determine the percentage of discoid (with or without filopodia) or spherical platelets. The rest of the PRP was then gel filtered at room temperature in calcium-free Tyrode's buffer, and a second sample was fixed and counted directly after collection. The rest of the gel-filtered platelets were spun down and the pellet taken up into sample buffer as described for gel analysis of the total platelet homogenate.

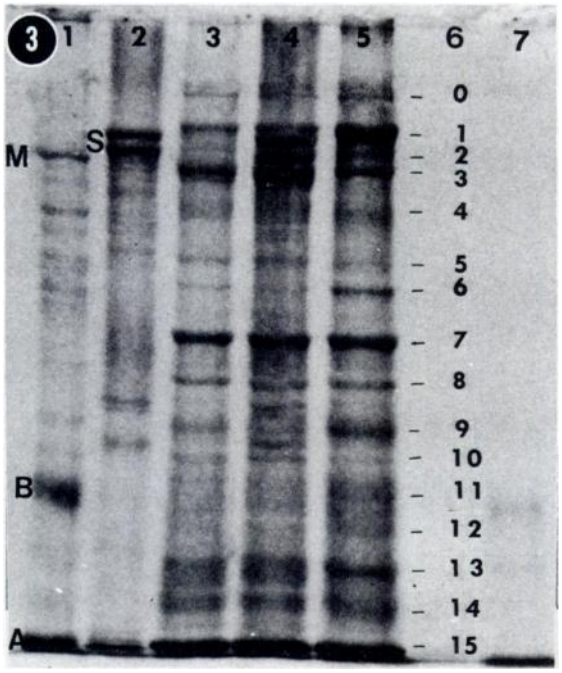

Fig. 3. SDS–7% acrylamide slab gel of low-salt precipitate made as described. Channel 1: standards of chicken skeletal muscle myosin (M), bovine serum albumin (B), and actin (A). Channel 2: red cell ghost indicating spectrin doublet (S); (2) 15 μg. Channel 3: low-salt precipitate from tetracaine-treated platelets. Channel 4: coelectrophoresis of loadings of channels 2 and 3; note that the two high molecular weight bands did not comigrate with spectrin. Channel 5: low-salt precipitate of control platelets; note that the major difference between this and channel 3 is reduction of the high molecular weight band[1] and also of bands 6 and 9, the increase of band 5 in channel 3, and a faint band below myosin. Other slight differences were not present in other runs. Channel 7: maximum possible red cell contamination as determined by direct counts.

low-salt precipitates did not comigrate with the spectrin doublet from red cells. Further evidence that red cell contamination cannot account for the high molecular weight components is shown by channel 7, where red cells equal to the maximum possible contamination were run. As channel 5 shows, we detected a large number of bands (15 detectable on these gels) in the low-salt precipitate. The high molecular weight component consists of two bands (1 and 2) above a band with the mobility of myosin (band 3). The doublet subunit molecular weights of bands 1 and 2 were estimated as approximately 250,000 and 230,000 daltons by comparison with spectrin, taken as 240,000 and 220,000 daltons, respectively. Channel 3 shows also that treatment of platelets with 2 m*M* tetracaine resulted in major reductions in bands 1 and 2. There were increases in a faint band at position 5 and in a band between 13 and 14. There also was some loss of bands 6 and 9. Band 5 had a molecular weight of 135,000 daltons by comparison with standards run on the same gel.

Although this result suggested proteolysis, it was necessary to examine the supernatant to be certain that the doublet had not been solubilized. Figure 4 shows a similar experiment in which both precipitate and supernatant were examined after lidocaine treatment. Analysis of this gel showed that the disappearance of bands 1 and 2 (channels 3 and 4) could not be accounted for by their increased

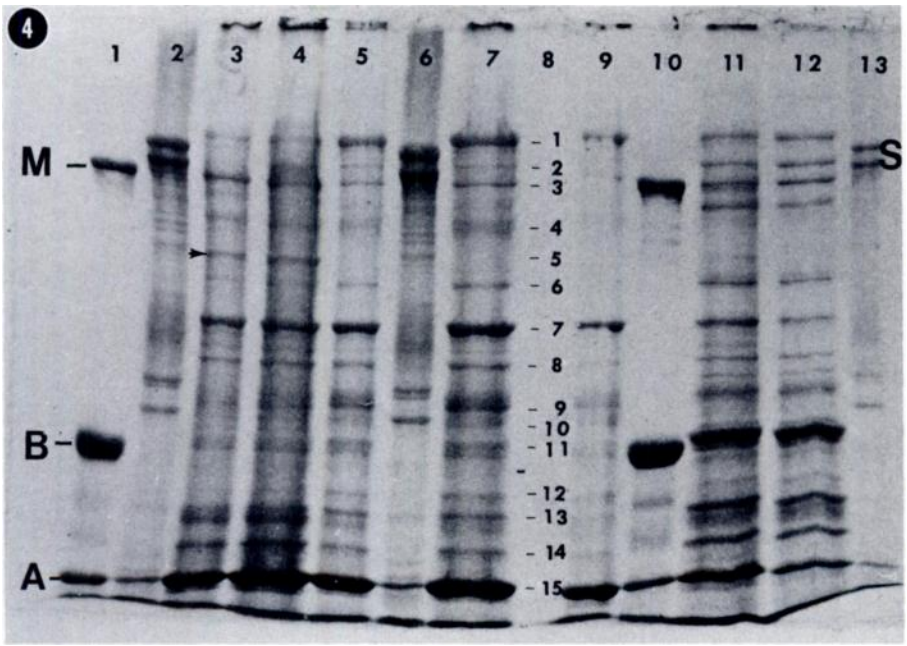

Fig. 4. Prepared as in Fig. 3 but after lidocaine treatment. Channels 1 and 10: standards as in Fig. 3. Channels 2 and 6: red cell ghosts as in Fig. 3. Channels 3 and 4: 10 and 20 μl low-salt precipitate from lidocaine-treated gel-filtered cells; note more striking loss of band 1 and increase of band 5, and a band between 13 and 14, as comapred to channels 5 and 7. Channels 5 and 7: 20 and 50 μl precipitate from control cells. Channel 8: major band numbers. Channel 9: 10 μl control precipitate. Channel 11: supernatant from lidocaine-treated platelets. Channel 12: supernatant from control platelets; note that there was no significant difference in the high molecular weight doublet in the supernatants of control and treated platelets. Channel 13: lower loading of spectrin standard (10 μg ghost) to show difference in mobility from the high molecular weight doublet in platelets.

concentration in the supernatant (compare channels 11 and 12). This result, coupled with the appearance of the 135,000-dalton band more definitely in the precipitate (arrow, Fig. 4), confirmed the supposition of proteolysis. There was also the appearance of a faint band just below the myosin band, especially visible in the supernatant, that may have been a product of the high molecular weight component or could have been due to proteolysis of myosin. There was no obvious reduction in the intensity of the myosin band, however. Again, bands 6 and 9 appeared reduced in the precipitate from treated cells, and a band below 13 was increased.

The concentration step necessary to prepare these precipitates takes several hours. We therefore examined the polypeptides of whole platelets pelleted from the Tyrode's buffer used for gel filtration after the treatments shown in Table 1. This preparative procedure was very rapid (10–15 min). As shown in Table 1, we found that following a 30-min incubation period with tetracaine/DMSO, platelets could still extend filopodia after gel filtration; at this time there was no evidence of internal proteolysis. After a small increase in tetracaine concentration, enough to cause sphering, and a 90-min further incubation (or in platelets treated with 3m*M* tetracaine alone for 120 min), the platelets were no longer able to reextend filopodia and proteolysis was evident. In DMSO alone platelets appeared at least as discoid as controls, and increased numbers of filopodia were seen after gel filtration.

To see if the high molecular weight bands were found associated with platelet actin in another type of preparation, we prepared "cytoskeletal" residues as described. Although these residues contain many polypeptides, our results show that when total lysate and "cytoskeletal components" were compared on gels, there was enrichment of both high molecular weight bands. Figure 5 compares total

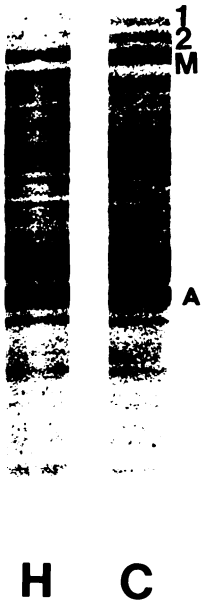

Fig. 5. SDS 5%–20% gradient gels so that actin (A) appeared about halfway down the gel. Photograph shows gel of the platelet homogenate (H) on the left and cytoskeletal preparation (C) on the right. Intensity of the actin bands was equalized. Note that while several other bands were less intense in the cytoskeleton, high molecular weight bands were increased, as was band 3 (myosin).

lysate with "cytoskeleton" on a gel in which the bands with the mobility of actin (A) were roughly equalized. The cytoskeleton was not only enriched in the high molecular weight bands, but also in the band comigrating with myosin, while several other bands were diminished. Other bands enriched with respect to the band comigrating with actin were in the 50,000–60,000-dalton range.

DISCUSSION

We previously showed[1] that platelets exposed to local anesthetics have a remarkably altered cytoskeleton. Similar effects have been observed in tissue culture cells.[4,5] It was proposed from results with red cells[6] that this category of drug affects cell shape because of a differential intercallation into the outer or inner halves of the bilayer with resultant expansion of either layer of the membrane. This may occur, but in platelets clearly the major breakdown of both microfilaments and microtubules is associated with the changes in cell shape. The anesthetics do not seem to affect the cytoskeleton directly, since addition of drugs to lysed platelets on grids for 2 min, enough to cause retraction of filopodia by intact platelets, did not alter the cytoskeleton (ref. 1 and unpublished observations). Platelets can remain essentially discoid or become spherical and still retain the ability to reextend filopodia after gel filtration. There is therefore a separation between filopodial retraction and sphering; they are not necessarily coactivated.

Although proteolysis is not necessary for filopodial retraction during the reversible stage, the irreversible suppression of filopodia does correlate with the proteolysis of bands 1 and 2 and some loss of bands 6 and 9. Phillips and Jakabova reported[7] that four bands (1.1, 1.2, 2.1, and 2.2) with molecular weights similar to those of our bands 1 and 3 (we saw some fine structure between bands 1 and 2, especially in Fig. 4, channel 5) were lysed in platelets homogenated by a calcium-activated protease. One band formed by their protease had a molecular weight of 135,000 daltons, the molecular weight of our band 5. Evidence from model phospholipid membranes suggests that anesthetics compete with calcium for binding sites. An elegant experiment reported by Hauser and Dawson[8] using films of phosphatidylinositol at an air-water interface showed that there was a linear correlation between the displacement of ^{45}Ca from the interface with the increase in surface pressure due to penetration of different amounts of tetracaine into the film from the underlying solution.

Both pieces of evidence suggest that the anesthetic might displace calcium from an intracellular site and that this could lead to the proteolysis we observed in bands 1 and 2. It seems quite unlikely that bands 1 and 2 represent an external protein or glycoprotein, since of those iodinated in intact platelets[9,10] none has the molecular weight of this pair.

What is the relationship between the proteolysis and the inability of platelets to reextend filopodia? At present this relationship represents only circumstantial evidence for a role of the high molecular weight polypeptides in this process or in the structure of the filament bundles shown in our previous paper.[1] However, it is most interesting that Rosenberg et al.[11] reported that a high molecular weight polypeptide estimated as 270,000 daltons (probably equivalent to our band 1) represented an actin-binding protein that caused side-to-side association of microfilaments.

ACKNOWLEDGMENT

We thank Dr. J. W. Sanger for the use of his Normarski optics. Some of these findings were reported at the June 1977 meeting of the Sixth International Congress on Thrombosis and Haemostasis and the November 1977 meeting of the Americal Society for Cell Biology.

REFERENCES

1. Nachmias V, Sullender J, Asch A: Shape and cytoplasmic filaments in control and lidocaine-treated platelets. Blood 50:39–53, 1977

2. Lucas RC, Detwiler TC, Stracher A: The identification and isolation of a high molecular weight (270,000 daltons) actin-binding protein from human platelets. J Cell Biol 70:229A, 1976 (Abstr)

3. Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 227:680–685, 1970

4. Nicolson GI, Smith JR, Poste G: Effects of local anesthetics on cell morphology and membrane associated cytoskeletal organization in BALB/3T3 cells. J Cell Biol 68:393–402, 1976

5. Poste G, Papahadjopoulos D, Nicolson GL: Local anesthetics affect transmembrane cytoskeletal control of mobility and distribution of cell surface receptors. Proc Natl Acad Sci USA 72:4430–4434, 1975

6. Sheetz MP, Singer SJ: Biological membranes as bilayer couples. A molecular mechanism of drug-erythrocyte interactions. Proc Natl Acad Sci USA 71:4457–4461, 1974

7. Phillips DR, Jakabova M: Calcium dependent protease in human platelets. J Biol Chem 252:5602–5605, 1977

8. Hauser H, Dawson RMC: The displacement of calcium ions from phospholipid monolayers by pharmacologically active and other organic bases. Biochem J 109:909–916, 1968

9. Nachman RL, Hubbard A, Ferris B: Iodination of the human platelet membrane. J Biol Chem 248:2928–2936, 1973

10. Philips DR: Effect of trypsin on the exposed polypeptides and glycoproteins in the human platelet membrane. Biochemistry 11:4582–4588, 1972

11. Rosenberg S, Stracher A, Detwiler TC, Lucas RC: Actin-binding protein (ABP) regulates the assembly of platelet microfilaments. Fed Proc 37:1790, 1978 (Abstr)