Lowering pH in Blood Platelets Dissociates Myosin Phosphorylation from Shape Change and Myosin Association with the Cytoskeleton

Vivianne T. Nachmias, Ken-ichi Yoshida, and Major Clay Glennon

Department of Anatomy, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Abstract. Platelet shape change induced by ADP is relatively independent of external pH over the range 6–7. If the chloride ion in the buffer is replaced by weak acids, however, shape change is rapidly and reversibly inhibited as a function of lowered pH (92% at pH 6.0). This inhibition is correlated with lowered internal pH caused by the weak acids, as measured by the 5,5-dimethyloxazolidine 2,4-dione technique. Shape change was 50% inhibited at internal pH 6.4 when 50 mM NaCl was replaced by propionate (PR). When platelets were stimulated with ADP 10–20 s after addition of PR to a final pH of 6 (PR6), both myosin light chain (MLC) phosphorylation and myosin and actin association with the cytoskeleton were reduced in correlation with the inhibition of shape change. But when ADP was added 30 s after PR6, the MLC phosphorylation was essentially the same in PR or in chloride, although shape change and myosin and actin association with the cytoskeleton remained inhibited. This was shown to be due mainly to endogenous phosphorylation of MLC. On return to neutral pH, platelets in PR immediately changed shape and myosin and actin became associated with the cytoskeleton. Two-dimensional tryptic peptides of MLC showed two major spots after PR6 treatment, indicating that both the MLC kinase site and the protein kinase C sites were phosphorylated.

The results show that increased internal pH is not required for shape change, although it may affect the rate. In PR6, as after phorbol esters, MLC phosphorylation can be uncoupled from shape change. The association of myosin and actin with the cytoskeleton is closely correlated with shape change, suggesting that shape change requires the active interaction of these contractile proteins.

MANY cells form ruffled membranes and change shape during locomotion or morphogenesis or when stimulated with agonists. Often these changes are asynchronous or slow, making them difficult to study. Mammalian blood platelets circulate as resting suspensions of anucleate disks, which rapidly and synchronously change shape when stimulated with agonists. The change includes formation of filopodia and lamellipodia, which are similar to fine ruffled membranes (Born, 1970; Daniel et al., 1984; Zucker and Nachmias, 1985). The response can be conveniently followed by measuring absorbance of stirred samples in correlation with microscopy. These characteristics make platelets favorable objects for study of the mechanisms underlying shape change.

Recently it has been shown that when platelets are treated with the agonists ADP or thrombin, phosphorylation of myosin regulatory light chain occurs rapidly (Daniel et al., 1981; Fox and Phillips, 1982), and shape change occurs in correlation with the phosphorylation (Daniel et al., 1984). When platelets are chilled, shape change occurs much more slowly, but again it is in correlation with the phosphorylation of the regulatory light chain (Nachmias et al., 1985). Shape change and phosphorylation both return to control levels when chilled platelets are rewarmed.

These correlations strongly suggest that myosin light chain phosphorylation is necessary for platelet shape change, and, since such phosphorylation activates myosin in its interaction with actin (Adelstein and Conti, 1975), imply that shape change is, at least in part, caused by myosin–actin interaction. Since myosin light chain phosphorylation requires calcium and calmodulin in vitro (Dabrowska and Hartshorne, 1978; Hathaway and Adelstein, 1979), these results agree with other evidence that there is a rise in free calcium when platelets are activated. However, it is not clear whether a rise in free calcium leading to the activation of myosin is sufficient to induce platelet shape change. Other internal changes occur after activation (see Zucker and Nachmias, 1985 for review). Of special interest is the rise of 0.3 pH units after activation with thrombin. Proton release, which increases when platelets are stimulated by thrombin, is inhibited by amiloride, which inhibits sodium–proton exchange (Siffert et al., 1984). Amiloride or the removal of external sodium strongly decreases the aggregation induced by ADP, epinephrine, and low doses of thrombin, or by the ionophore

A 23187 (Connolly and Limbird, 1983; Horne and Simons, 1978; Siffert et al., 1984). It is not clear whether the change in internal pH (pH_i)[1] is essential for shape change. Previous work with platelet cytoskeletons (Gonnella and Nachmias, 1981) showed that transformations of short filaments and amorphous material into bundles of actin filaments could be induced by incubation in media containing 0.1–1 mM free calcium only if the pH were also increased from 6.8 to 7.6. These results suggested that cytoplasmic pH in the intact platelet may modify the shape change response, and that shape change might be inhibited if the pH_i of the platelet were decreased.

In this paper we compared the effects of lowering external pH or pH_i on the rate of shape change. We used weak acids to alter pH_i. Our results show that the rate of platelet shape change is reversibly sensitive to pH_i, but 50% inhibition requires a pH_i of 6.4. Unexpectedly, our results also show that myosin phosphorylation can be uncoupled from platelet shape change.

Materials and Methods

Platelet Preparation and Measurement of Shape Change

Blood was drawn by free flow through a 19-gauge butterfly from healthy human volunteers under informed consent into 1/10 vol of 80 mM trisodium citrate and 70 mM citric acid. The citrated blood was centrifuged (280 *g* for 10 min) to obtain platelet-rich plasma (PRP), pH 6.6–6.8. Shape change measurements were performed after Born's method (1970) in 1.5 ml samples in a siliconized cuvette at 37°C (light path, 1 cm) in a spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). The PRP was constantly stirred with a specially designed magnetic stirrer. For tests at different pHs, platelets were diluted with solutions of propionate (PR) or chloride (CL) at appropriate pHs immediately before starting the measurements. Optical density of stirred suspensions (10^8 platelets per ml) was recorded at 609 nm using maximum sensitivity (50 mV = 0.122 A) with the chart speed at 5 or 15 s per inch. ADP, 10 μM final concentration, was added through a light tight port above the cuvette in a volume 1/100 of the final. Rates of shape change were estimated from the slope of the initial, linear portion of the increase in absorbance. Extent of shape change was estimated from the final height of the absorbance record and from the diminution of the oscillatory signal. Samples were examined by phase microscopy and scanning EM for morphological changes after agonist addition.

pH Measurement

The pH of the PRP diluted with different buffers was measured at the start and end of each experiment. Without buffer, loss of CO_2 caused an increased pH of several tenths of a unit. Imidazole and Pipes at 50 mM were not very effective in stabilizing external pH and higher concentrations of imidazole increased platelet pH_i. Sodium phosphate at 50 mM gave excellent stabilization. For measurements of volume and pH_i, PRP was diluted 1:1 with the diluent. We found that platelet volumes increased after dilution in weak acids. Such increases are not surprising, considering the widespread occurrence of sodium–proton antiporters, which are activated by decreased pH_i, which leads to sodium uptake followed by water uptake (Dr. Sergio Grinstein, University of Toronto, personal communication). Therefore, we added amiloride, which inhibits sodium–proton exchange, at final concentrations of 0.5–1 mM. Tritiated water was added to diluted PRP to a concentration of 6.5 μCi/ml. This mixture was then divided into two aliquots and [^{14}C]sucrose was added to one for determination of total pellet volume and external volume. To the other [^{14}C]dimethyloxazolidinedione (DMO) was added for determination of total pellet volume and pH. Both labeled compounds were also added at 6.5 μCi/ml. The PRP was incubated at room temperature for 10 min to allow full equilibration. It was then divided into 200-μL aliquots, which were layered onto silicone oil and spun for 2 min in a microfuge (model B; Beckman Instruments, Inc., Berkeley, CA). Samples of 100 μL were mixed with 2.5 ml of Aquasol-2 (New England Nuclear, Boston, MA). Remaining supernatant and oil were aspirated and the pellets dissolved in half-saturated ammonium chloride then in Aquasol. Double-label counting was carried out in a scintillation spectrometer (SL-4000; Intertechnique, Plaisir, France) with appropriate windows to 0.7% maximum error. Platelet volumes were estimated from samples containing labeled water and sucrose by subtraction of the included supernatant volume from the total pellet volume; platelet pH_i was estimated from samples containing labeled water and DMO. Preliminary experiments were run to determine platelet pH as a function of DMO concentration. The final experiments were run with DMO at 10^{-5} M. No difference was observed in equilibrium values from 2 to 10 min. Duplicate measurements were run for each point. The data were analyzed with a Visicalc program according to the equation:

$$pH_i = pKa_i + \log_{10}\left\{\frac{[T_i]}{[T_e]}\left(10^{pHe-pKa_e} + 1\right) - 1\right\}$$

where $[T_i]$ is concentration of DMO in platelet volume; $[T_e]$ is concentration of DMO in external volume; pKa_i is pK of DMO in the platelet cytoplasm; pKa_e is pK of DMO in the external solution.

The pKa for DMO was taken to be 6.2 for both external and internal solutions (Boron and Roos, 1976). The platelet count was determined in a hemocytometer and recorded as the mean of two readings; agreement was within 10%. The diluents used were either 100 mM M NaCl, 50 mM sodium phosphate, 5 mM KCl, 10 mM EGTA, or with NaCl replaced with 100 mM PR plus 1 mM amiloride (also added to some NaCl samples). The pHs of these solutions were adjusted so that a 1:1 mixture with PRP would yield the appropriate final pH. The pHs of the diluted PRP differed by only 0.02 between the start and end of each experiment.

Phosphorylation Studies

For [^{32}P]PO4 studies, PRP was concentrated 20-fold on a 40% BSA step gradient, incubated with carrier-free phosphate (1 mCi/ml; New England Nuclear) for 1 h at 37°C, then gel filtered through Sepharose 2B. The peak was collected, immediately added to either CL buffer or PR buffer, incubated with stirring in small plastic tubes for varying amounts of time, and then challenged with 10 μM ADP. The lighting was arranged so that loss of swirling, indicative of loss of the discoid shape of platelets, could be easily observed. The response of these platelets was similar to that seen when shape changes were measured in the spectrophotometer. For SDS gels, an equal volume of hot Laemmli sample buffer was added 15 s after ADP, the mixture boiled for 2 min, and then frozen before analysis by gel electrophoresis (Laemmli, 1970). Controls were untreated platelets collected at the start and end of each experiment.

Gel Analysis

The design for urea gels was the same except that the platelets were collected and washed by the method of Zucker and Masiello (1983). All platelets used showed a marked swirling effect on rotation of the tubes. The response to agonists was terminated with an equal volume of ice-cold 1 M perchloric acid. The precipitates were kept on ice for 10 min, collected at 12,000 *g* for 2 min, the perchloric acid carefully removed, and the pellets dissolved in urea sample buffer. Additional Tris was added to bring the samples to alkaline pH. The method was that of Daniel et al. (1981).

Preparation of Thiophosphorylated Actomyosin

Platelet actomyosin was prepared by the method of Adelstein and Conti (1975). It was thiophosphorylated with adenosine-5′-O-(3-thiotriphosphate) by shaking for 2 h at 30°C in the presence of 10 μM calmodulin, 0.1 mM calcium CL, and 100 μg/ml leupeptin. In early trials myosin light chain kinase was added (generous gift of Dr. Robert S. Adelstein, National Institutes of Health, Bethesda, MD), but it was found that endogenous kinase was sufficient, and in later runs this was omitted. The actomyosin was at least 90% phosphorylated after the incubation and after storage in glycerol at −20°C for 10 d by analysis on alkaline urea gels. For unphosphorylated samples, incubation was in the absence of calcium, calmodulin, or kinase at 30°C for 2 h.

ATPase activity was determined after reprecipitating the thiophosphory-

1. *Abbreviations used in this paper*: CL, chloride; DMO, 5,5-dimethyloxazolidine 2,4-dione; pH_i, internal pH; PR, propionate; PR6, PR at pH6; PRP, platelet-rich plasma.

lated actomyosin and washing it twice with 0.05 M KCl, 2 mM $MgCl_2$, 50 mM Hepes, pH 6.8. To compare the activity of the actomyosin in CL versus PR, the preparation was resuspended in 0.2 M KCl, 5 mM Hepes, 0.5 mM $MgCl_2$, 0.5 mM EGTA at pH 7.0, divided into aliquots in Eppendorf tubes, and 9/10 vol of 55 mM NaCl or sodium PR in 50 mM Hepes and 4.8 mM $MgCl_2$, pH 6 or 7, was added to each sample. Then 20 μL 0.1 M ATP (2 mM final) was pipetted into the cap of each tube on a rotator and the reaction begun by starting the rotator. The reaction was stopped with 8% final TCA and samples were placed on ice. After centrifugation, an aliquot of the supernatant was taken and assayed for inorganic phosphate. The pellets were assayed for protein after ethanol extraction.

Measurement of Mitochondrial Areas

Mitochondrial areas were measured with a planimeter (Numonics Corp., Lansdale, PA) on micrographs of sectioned platelets in which the contours were sharp and the double membrane and cristae clearly visible.

Platelet Cytoskeletons

Platelet cytoskeletons from 50 ml of blood were washed by the method of Zucker and Masiello (1983) but without albumin. For each point, 1.0 ml of washed platelet suspension was mixed with 1.0 ml of buffer containing either 100 mM NaCl, 5 mM KCl, 50 mM sodium phosphate, 20 mM EGTA, or PR replacing the CL buffer. To inhibit swelling, 0.2 mM of the amiloride analogue L591605 was added. Each buffer was prepared so that the 1:1 mixture gave the pH indicated. After 15 s, 10 μM ADP was added and incubated for 15 s at 37°C. For realkalinization, NaOH was added to give the final pH values seen in Fig. 8. Platelets were lysed with 1/5 vol of 5% Triton X-100, 0.5 mg/ml leupeptin, and 5 mM PMSF. The final pH of all lysates was brought to 7.0. Cytoskeletons were collected on ice for 5–10 min, sedimented at 5,000 *g* for 10 min at 4°C, washed with 2.0 ml of 1:1 mixture of the modified Tyrode's solution, and CL at pH 7, dissolved in 0.1 ml of Laemmli buffer, boiled for 2 min, and subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel.

Tryptic Peptides

These were performed as in Naka et al. (1983). Myosin light chain 1 was identified by standards and autoradiography, cut from the gel, and washed exhaustively in 25% isopropanol and 10% methanol. Slices were treated with 25 μg trypsin in ammonium bicarbonate (pH 8.4) overnight at 37°C. After repeated lyophilization to remove bicarbonate, the residue was dissolved in acetic acid/formic acid/water (15:5:80) and spotted onto a silica gel thin-layer plate. Electrophoresis was at 950 V for 90 min. After drying, chromatography was performed in a buffer of *n*-butyl alcohol/pyridine/acetic acid/water (32.2:25:5:20) in an ascending direction. Autoradiography was performed with Kodak X-Omat R film at −70°C.

Sodium PR was from Sigma Chemical Co. (St. Louis, MO). The urea was ultrapure (Schwarz-Mann Biotech, Cleveland, OH). All other chemicals were of reagent grade. Adenosine-5'-*O*-(3-thiotriphosphate) and calmodulin were from Boehringer Mannheim Biochemicals (Indianapolis, IN). $[^3H]H_2O$ was from New England Nuclear, and the [^{14}C]sucrose and inorganic [^{32}P]phosphate (carrier-free) and 2-[^{14}C]DMO were all purchased from ICN Pharmaceuticals, Inc. (Irvine, CA).

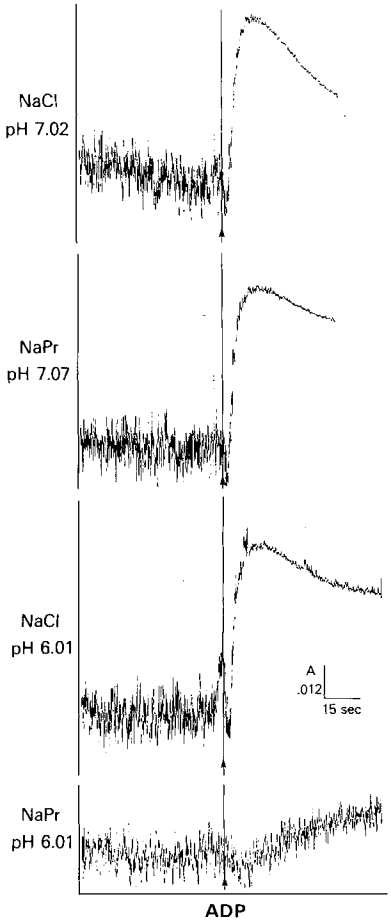

Figure 1. The effect of pH_e on platelet shape change in PR or CL. Platelets in PRP were diluted 1:1 with buffer containing 100 mM NaCl, 50 mM sodium phosphate, 5 mM KCl, 10 mM EGTA, 1 mM amiloride, or with NaCl replaced by sodium PR. The pH of the diluent was adjusted before mixing with platelets to yield the final pH shown at the left of each curve. Platelets were stirred at 37°C as described. The oscillatory tracing is indicative of the discoid shape of platelets, while the increased absorbance and narrowing of the oscillations accompanies the change in shape to irregular spheres with filopodia. At the arrow, 10 μM (final) ADP is added. Note that there are only slight differences in the curves when platelets are diluted with sodium CL to pH 7 or 6, or when dilution is with sodium PR at pH 7, but that there is a marked inhibition of rate of shape change if platelets are diluted into PR at a final pH of 6.

Results

Platelets in plasma diluted with NaCl were compared with identical samples diluted with the sodium salts of the weak acids pyruvate, lactate, acetate, or the nonmetabolized DMO. Within 30–60 s after dilution, the shape change response was recorded by measuring absorbance in stirred samples in response to 10 μM ADP, a concentration of this agonist that gives a maximal response. At neutral pH, there was little difference between the different diluents. When the external pH was dropped below 7, the shape change response was almost unchanged in CL, but was markedly inhibited in weak acids. The weak acids caused platelet swelling, documented by volume measurements and by electron micrographs (data not shown). This swelling was prevented by 1 mM amiloride.

Hemptinne et al. (1983) studied the effects of weak acids on the pH of muscle cytoplasm, and reported very useful properties of PR. It has a relatively high pK of 4.67 and was found to acidify rapidly. It was therefore chosen for systematic study. Fig. 1 shows the platelet shape change response measured within 30 s after dilution into CL or PR at pH 7 and 6. The shape change curve is affected very little by lowering the external pH to 6 in CL, but is strongly inhibited when platelets in PR are brought to pH 6 (PR6). Note that shape change gives rise to two different optical effects, a rise in mean absorbance and a diminution of the oscillations in absorbance, which are due to stirring of the discoid platelets. Both are inhibited in PR6. Fig. 2 shows that the inhibited response is immediately reversed on alkalinization without further addition of agonist. These changes in optical proper-

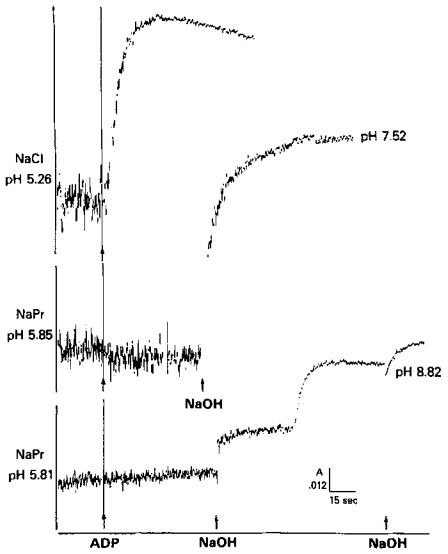

Figure 2. Reversal of the effect of low pH_e on shape change in PRP diluted with NaCl or Na PR in imidazole buffer. The inhibition of shape change in PR at pH 6.02 is immediately reversed upon realkalinization with small aliquots of NaOH without further addition of agonist. Note that the extent of shape change increases as the pH is increased (*lower curve*).

ties were correlated by phase microscopy, which showed retention of discoid shape in inhibited platelets and the appearance of irregular spheres bearing many filopodia in reversed samples.

Similar results were observed with all of the weak acids mentioned above. The threshold for inhibition with acetate at pH 6.5 was about 22 mM. Inhibition of shape change was observed as soon as it could be measured after dilution of the platelets (~15 s).

These results suggested that we had inhibited shape change by decreasing pH_i, since weak acids of different degrees of hydrophobicity, including both metabolized and unmetabolized forms, gave similar results. To test this, we measured pH_i using the labeled DMO method (Boron and Roos, 1976; Gillies and Deamer, 1979; Deutsch et al., 1979). Platelet volumes were measured in the same experiments. The marker for external volume in both cases was ^{14}C-labeled sucrose.

The dependence of pH_i on external pH is shown in Fig. 3. In CL, platelets buffer pH_i well over the range of 6–7 with a slope of 0.33. If only points below 6.7 are included, the slope of pH_i in chloride is 0.16. However, in PR the pH_i almost follows external pH; the slope is 0.81. In CL, the points taken with or without amiloride lie on the same line. In PR, only a few points were measured in the absence of amiloride; these gave a slightly higher pH_i than in its presence (Fig. 3). To determine whether our pH_i measurements were significantly affected by the volume of the mitochondria, the area of the cytoplasm occupied by mitochondria was estimated from electron micrographs. Mitochondria occupied an area of 2.09% (mean), with a range from 0.23 to 3.9.

We tested whether inhibition of shape change could be due to swelling, by measuring platelet volumes in the presence and absence of amiloride in PR. Platelet volume in PRP was relatively constant over a 40-min period. In one experiment we obtained a mean volume of 8.94 ± 0.25 μm^3 over this period. Platelets diluted with CL swelled transiently by 5%, returning to control levels by 40 min. In 43 measurements from nine experiments and four donors, in which the pH ranged from 5.8 to 7.0, the mean volume was 7.04, with a range from 5.55 to 10.20 μm^3. In PR without amiloride, the volume of a platelet averaged 1.4–2.8 μm^3 greater than in CL, with greater increases at lower pHs. Amiloride at 0.5 mM prevented an initial downward drift of absorbance in the shape change record and decreased the volume difference to 0.94–1.75 μm^3. Amiloride at 1 mM completely eliminated the volume difference between platelets in CL or PR. Samples diluted into PR6 buffer with either 0.5 or 1 mM amiloride showed the same amount of inhibition of shape change at low pH.

The rate of shape change was relatively independent of external pH in CL buffer (Fig. 4, left) but highly dependent on pH_i (Fig. 4, right), with 100% inhibition at pH_i of 6, and 50% inhibition at pH 6.4. Similar results were obtained with acetate, lactate, pyruvate, and DMO at 23–65 mM.

Since previous work had shown that shape change is correlated with phosphorylation of myosin regulatory light chain, it seemed most likely that decreasing pH_i would inhibit this phosphorylation. Surprisingly, the situation was more complicated. We first studied phosphorylation after a 30-s incubation in PR6 followed by an ADP stimulus stopped 20 s later by lysis with Laemmli buffer. Table I shows the results of autoradiography in the platelets labeled with ^{32}P. Scans of gels across the myosin light chain, which was well resolved, showed an increase in endogenous myosin light chain phosphorylation when platelets were suspended in PR6. After the ADP stimulus, although the increment was smaller, the final level of phosphorylation was about the same as in CL at pH 6. This conclusion was borne out by separating the phosphorylated myosin light chain on alkaline urea gels (Fig. 5). Again, there was endogenous phosphorylation in PR6 (cf. lanes *3* and *2*) while after ADP the level of phosphorylated myosin light chain was the same or slightly increased over that in CL (cf. lanes *5* and *4*).

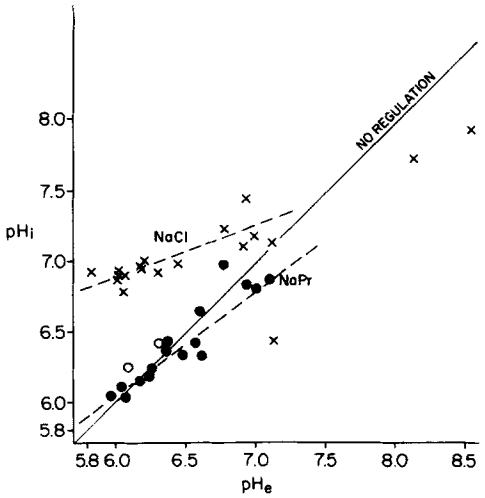

Figure 3. The pH_i of platelets diluted as in Fig. 1 to different external pH values was estimated by the DMO method as described, allowing 10 min for equilibration. The continuous line is the line of no regulation. The points below pH 7.5 were estimated for sodium CL and gave a regression line of slope 0.31; in PR the slope was 0.85. If only points below pH 6.7 were included, the respective slopes were 0.16 and 0.81.

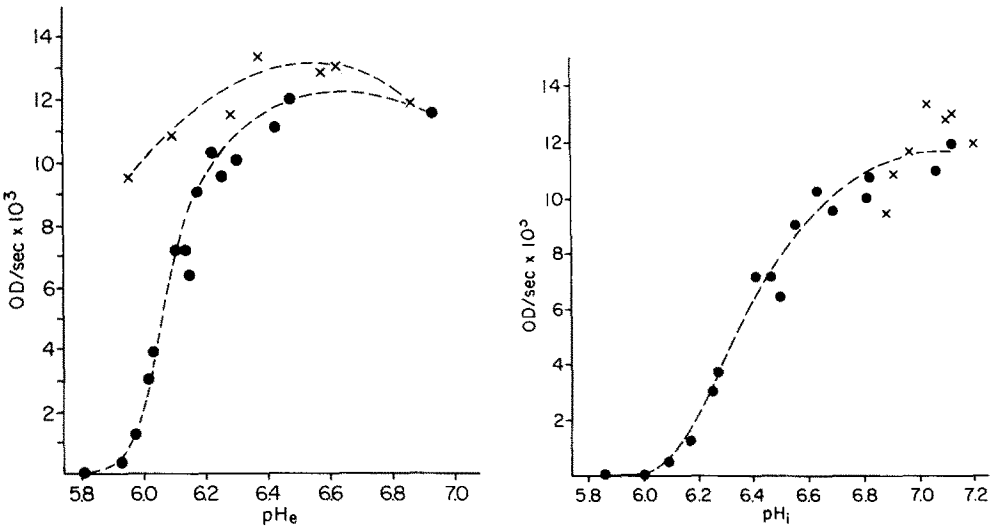

Figure 4. (*Left*) The rate of platelet shape change as a function of external pH was estimated from the initial, linear portion of the slope under the conditions of Fig. 1. (x) Points in sodium CL; (•) points in PR. (*Right*) The rate of platelet shape change, estimated as at left, as a function of the pH_i measured as in Fig. 3. Note that while at left the CL and PR curves are separate, here they are described by a single smooth curve.

Although myosin was phosphorylated in PR6, the acid condition and PR within the platelet might inhibit actin-activated ATPase, but experiments with fully phosphorylated actomyosin showed that the ATPase measured under low ionic strength conditions was nearly the same in PR6 as in CL at pH 7 or PR at pH 7. The rates ranged from 83 to 125% of the CL rates at pH 7.

On the other hand, incubation in PR6 had a striking effect on the composition of the platelet cytoskeleton, as shown in Fig. 6. Cytoskeletons made from control platelets in CL or PR6 (lane *1*) contained only traces of myosin heavy chain and small amounts of actin. Cytoskeletons prepared 15 s after ADP stimulation in CL at pH 6 or 7 or in PR at pH 7 contained larger amounts of both myosin and actin (lanes *4*, *7*, and *5*). This increase in myosin and actin was almost completely inhibited when platelets were stimulated with ADP in PR6 (lanes *2* and *6*) and the resulting cytoskeletons resembled the unstimulated controls. These results were not due to a difference in the buffer composition or pH at the time of lysis, since control experiments in which both pH and salt were varied showed that these did not alter the myosin content of the cytoskeleton. To see whether an increase in myosin and actin would correlate with rapid shape change after realkalinization, we studied the cytoskeletons of controls with those of PR6 platelets that were first treated with ADP and then brought to final pHs of 7 and above. Under these conditions the shape change response recovers, as shown in Fig. 2. Cytoskeletons from these reversed platelets now contained some myosin heavy chain and increased actin (Fig. 7). Although shape change occurred rapidly after realkalinization, the amount of myosin heavy chain in the cytoskeletons was less than that in samples that had never been exposed to PR6; cf lanes *7–9* in Fig. 7 with lanes *3–5*.

The discrepancy between myosin phosphorylation and myosin association with the cytoskeleton was analyzed further by examining very early time points at pHs from 6 to 7. We found that if washed platelets were stimulated with ADP very rapidly after dilution into PR (10–20 s) myosin

Table I. Phosphorylation of Myosin Light Chain in Intact Platelets Suspended in Buffer with 50 mM CL or 50 mM PR

Experiment	CL	PR	PR/CL
pH 6.1			
Control	1.6	2.1	1.3
ADP	2.5	2.5	1.0
pH 6.0			
Control	0.84	1.14	1.36
ADP	1.12	1.30	1.16

Platelets were concentrated, incubated with [^{32}P]phosphate as described, gel filtered, and diluted 1:1 with CL or PR buffer to the final pH shown. 20 s after the addition of ADP(50 μM in experiment 1 and 25 μM in experiment 2), platelets were lysed with an equal volume of 2% Laemmli sample buffer. Samples were subjected to electrophoresis and autoradiographs were prepared. The results shown are the ratio of the density at the position of the light chain on autoradiographs to loading as determined on the Coomassie Blue-stained gel.

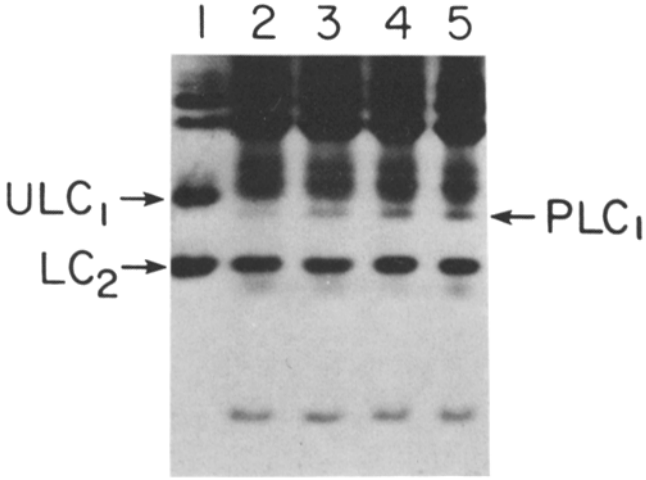

Figure 5. An alkaline urea gel of the perchloric acid precipitate of whole platelets. The washed platelets were diluted 1:1 with CL at pH 6 (lanes *2* and *4*) or PR6 (lanes *3* and *5*). Controls were precipitated with perchloric acid after 15 s. 20 s later, ADP at 20 μM was added to the platelets used for lanes *4* and *5*. Note that lanes *4* and *5* show an increased amount of phosphorylated myosin light chain marked by the position of the arrow (PLC_1). Note that there is also an increase in endogenous phosphorylation in lane *3* as compared with lane *2*. Lane *1* is an actomyosin standard in which the myosin light chain was dephosphorylated (ULC_1 and LC_2 are the positions of the unphosphorylated regulatory light chain and myosin light chain 2, respectively).

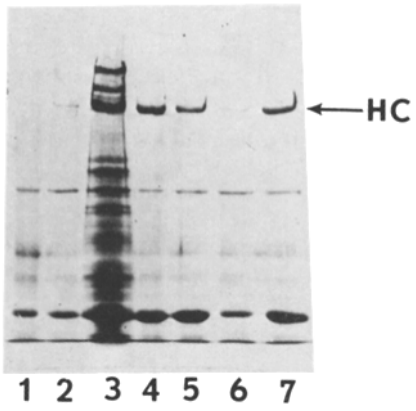

Figure 6. This and the next figure are 7.5% SDS-PAGE gels of cytoskeleton from platelets washed by the method of Dr. M. B. Zucker (see Materials and Methods). The platelets as prepared are discoid and show a good shape change response with ADP. Platelets are mixed at room temperature 1:1 with either CL buffer containing 100 mM sodium chloride, 5 mM KCl, 50 mM sodium phosphate, 10 mM EGTA, or with PR buffer in which the CL is replaced with PR. PR buffer also contained 0.2 mM of the amiloride analogue L591605 to inhibit swelling. Each buffer is prepared so that the 1:1 mixture is pH 6.0 or 7.0, as required. At exactly 15 s after mixing, ADP is added and 15 s later the platelets are lysed by the addition of 1/5 vol of 5% Triton-X 100, 0.5 mg/ml leupeptin, and 5 mM PMSF. The cytoskeletons are collected on ice for 10 min. They are sedimented, washed once with 2.0 ml of 1:1 mixture of the wash solution and CL buffer at pH 6.0, and then dissolved in 0.1 ml of Laemmli buffer for electrophoresis. Control experiments also showed that the composition of the cytoskeletons did not depend on the pH at the time of lysis. Lane *1*, PR control, pH 6.0; lane *2*, PR ADP, pH 6.0; lane *3*, whole platelet lysate; lane *4*, CL ADP, pH 6.0; lane *5*, PR ADP, pH 7.0; lane *6*, PR ADP, pH 6.0; lane *7*, CL ADP, pH 7.0.

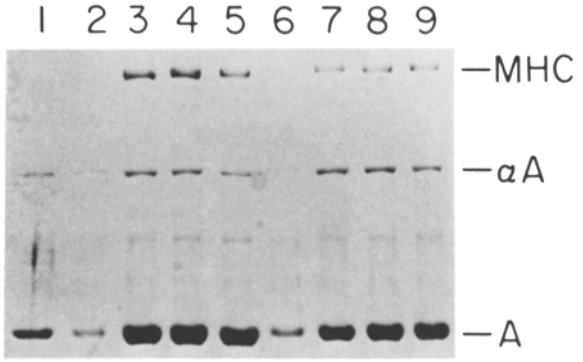

Figure 7. Reversal effect. The cytoskeletons were prepared as described in Fig. 7. In this case, after ADP addition, platelets were treated with an appropriate amount (predetermined) of NaOH for 15 s before lysis. Platelets were diluted 1:1 with PR buffer. Lane *1*, PR control, pH 7.0; lane *2*, PR control, pH 6.0; lane *3*, PR 7.8 ADP; lane *4*, PR 7.4 ADP; lane *5*, PR 7.0 ADP; lane *6*, PR 6 ADP; lanes *7*, *8*, and *9*, PR 6 ADP with pHs reversed to 7.0, 7.4, and 7.8, respectively. To the samples in PR6 buffer we further added NaOH so that the final pH gave the values indicated (lanes *7*, *8*, and *9*). ADP was omitted for controls. After lysis, sufficient NaOH or HCl was added so that the pH of all lysates was 7.0.

phosphorylation was inhibited in parallel with shape change and myosin association with the cytoskeleton (Fig. 8). As the time interval after dilution into PR at low pH was increased, the extent of myosin light chain phosphorylation increased, so that by 30 s it was already nearly constant over the range pH 6–7 (Fig. 8, uppermost curve). When we examined endogenous myosin light chain phosphorylation as a function of time in PR6 buffer, we observed a slow increase up to 10 min from a just discernable effect at 15–20 s.

We performed tryptic peptide analysis on the myosin light chain labeled with [^{32}P]phosphate after 5 min incubation in PR6 and compared it with the results of 0.1 U/ml thrombin for 30 s. We detected two major radioactive spots after treatment with PR with mobilities similar to those reported after stimulation of platelets for 5 min with phorbol esters (Naka et al., 1983). The two-dimensional autoradiograph is shown in Fig. 9.

Discussion

Our measurements show that the rate of platelet shape change is sensitive to pH_i over the range of 7–6, with 50% inhibition at pH 6.4. Platelets also respond to an acid-induced drop in pH by swelling. First, then, how accurate are the pH measurements, and second, is the inhibition of shape change really due to lowered pH, or could it be caused by swelling?

There is only a small effect of DMO concentration on the pH measured. At 50 μM (used here), the pH was only 0.06 pH units less than with 1 mM DMO. If the more alkaline pH is due to DMO binding at 1 mM, there can be very little residual binding at 50 μM. DMO equilibrates rapidly, certainly within 2 min, and most probably across internal membranes. Of these the only ones likely to take up DMO are those of mitochondria, which have mildly alkaline compartments (Deutsch et al., 1981), but mitochondria occupy at most only 2% of the platelet volume. DMO is a weak acid and so should be excluded from acid compartments such as lysosomes and dense granules, making it a better marker than weak bases for a cell such as the platelet which has more

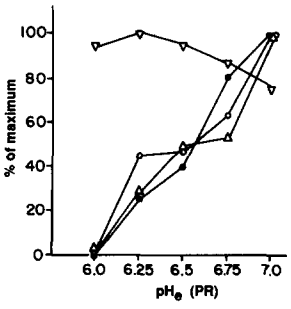

Figure 8. Correlation of myosin light chain phosphorylation with shape change and myosin and actin association with the cytoskeleton as a function of pH after 20 or 30 s incubation in PR6. The platelets were prepared as in Fig. 7 but the time interval after dilution into buffer was strictly monitored before the addition of ADP. Results are expressed as a percent of the maximum, which was pH 7, except for the longer incubation in PR, which gave maximum phosphorylation after ADP at pH 6.25. (●) Shape change at 20 or 30 s; (○) myosin association with the cytoskeleton at 20 or 30 s; (△) myosin light chain phosphorylation at 20 s; (▽) myosin light chain phosphorylation at 30 s. Note that there is a good correlation of all three parameters at 20 s but that by 30 s myosin light chain phosphorylation increases without a change in the inhibition of shape change or myosin association with the cytoskeleton.

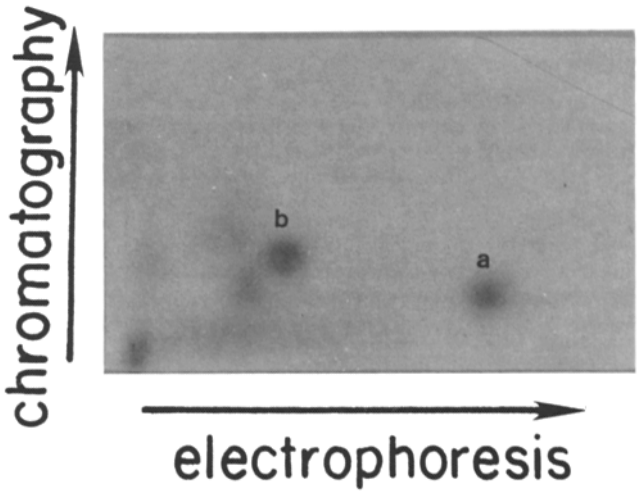

Figure 9. Two-dimensional tryptic peptide map of the myosin regulatory light chain from platelets that had been preincubated with [^{32}P]phosphate as described, washed, and then incubated with PR buffer at pH 6.0 for 5 min. Two major spots are seen, marked *a* and *b*. These are similar in position to the sites phosphorylated by protein kinase C (*a*) and myosin light chain kinase (*b*) as shown by Naka et al. (1983).

granules than mitochondria. Hence we conclude that our measurements of pH_i are good to one-tenth of a pH unit and probably better.

Our results provide a good value for platelet volume. Platelets contain an elaborate system of long indentations of the plasma membrane, the so-called open canalicular system. Previous studies that used iodinated albumin as an external marker (Giles, 1981; Feinberg et al., 1974) found volumes 1–2 μm^3 larger than in our study. Sucrose can penetrate the open canalicular system more completely to yield more accurate values. Interestingly, the mean volume found by Bull and Zucker (1965) using a Coulter counter for platelets at room temperature with citrate as an anticoagulant was 7.1 μm^3, agreeing well with our mean value of 7.04 μm^3.

The swelling response is most likely due to a sodium–proton exchange activated by the decreased pH_i, since it is inhibited by amiloride. The existence of such antiporters has been shown in a number of cell types (Grinstein et al., 1984; Moolenaar et al., 1984; Pouyssegur et al., 1984). The inhibition of shape change in PR6 was as strong in 1 mM amiloride, which prevented swelling completely at pH 6.11, as in 0.5 mM amiloride. We conclude that the inhibition of platelet shape change at low pH_i cannot be due to platelet swelling.

Anderson and Foulks (1978) reported that the small organic anions formate, acetate, butyrate, hexanoate, and octanoate did not alter the pH dependence of the rate of shape change under alkaline conditions, as compared with sodium CL, but produced strong inhibition at pHs below 7. Although they ascribed these results to a surface effect, their data, coupled with the observation that sulfonates (probably less permeable) were much less effective, fit our interpretation that the fundamental variable is pH_i. Independent confirmation of a change in pH comes from a report of Zieve and Solomon (1966) who found that platelets buffered their pH_i poorly when the pCO_2 was increased.

In summary, lowering pH_i inhibits shape change, but a decrease to ~6.4 is necessary for a 50% inhibition. We conclude that a small change in the pH range of 6.8–7.3 would only affect the rate of shape change by ~10%. Our results do not fit well with a recent report from Siffert and Akkerman (1987). They found that lowering sodium to 40 mM and adding ethylisopropyl-amiloride completely inhibited the calcium signal from 0.1 U/ml thrombin and inhibited activation. These treatments should lower pH_i by 0.1–0.2 pH units. They concluded that the sodium–proton antiporter was necessary for activation. Our results suggest that alkalinization is more likely to be an effect of platelet activation than a requirement for the earliest response, i.e., calcium mobilization and shape change. To explain the discrepancy, it would be necessary to measure the calcium signal at the same time as intracellular pH and shape change. We were not able to measure the calcium transient with fura-2 in PR6 due to quenching of the fluorescence.

Myosin phosphorylation is closely correlated with shape change induced by thrombin, ADP, or cold (Daniel et al., 1981, 1984; Nachmias et al., 1985). In the present experiments this correlation was only sustained if the platelets were stimulated within 20 s after being exposed to PR6. The phosphorylation in PR6 appeared to be largely endogenous (Table I) and it increased with time (data not shown). It is known that phosphorylating the myosin regulatory light chains in platelets increases interaction of myosin with actin (Adelstein and Conti, 1975). Therefore, inhibition of shape change might be ascribed to inhibition of actin-activated myosin ATPase either by the lower pH, or by the presence of PR. However, our results indicated only a very weak (maximum 17%) effect of lowered pH and PR on actomyosin ATPase activity. At later time points PR even activated the actomyosin ATPase.

In contrast to these negative effects, the inhibition of shape change correlated well with inhibition of myosin and actin association with the cytoskeleton, even when cytoskeletons were formed at the same final pH. Furthermore, when platelets were treated with ADP in PR6 buffer and then realkalinized, myosin and actin again became associated with the cytoskeleton in parallel with the return of shape change (Fig. 7). This association of actin and myosin with the cytoskeleton would appear to reflect active myosin–actin interaction. An independent inhibition of actin association could be ruled out. If we added excess filamentous actin to cytoskeletons in PR6, we did not cause increased myosin association (data not shown). A similar effect was seen during studies of cold-induced shape change (Nachmias et al., 1985). Thus, a change in actin cannot explain the results.

Our results can be interpreted by assuming that there are two effects of lowering pH_i: a rapid one, which inhibits some aspect of transduction, and a slower one, which leads to phosphorylation of myosin regulatory light chain. Over a much longer time scale (minutes) this leads to shape change. It is known that platelet myosin light chain can be phosphorylated on two sites in vitro, one due to the light chain kinase and one due to protein kinase C (Naka et al., 1983). When the C kinase site is phosphorylated in smooth muscle, it does not stimulate the actin-activated MgATPase and even decreases it over controls when the myosin light chain kinase site is subsequently phosphorylated (Nishikawa et al., 1983;

1984). The results with PR6 are similar to those seen when platelets are stimulated with phorbol esters. These inhibit shape change due to agonists (Yoshida et al., 1986) but cause slow shape change with little association of myosin with the cytoskeleton (Carroll et al., 1982). Phorbol esters cause phosphorylation of both the myosin light chain kinase site and the C kinase site on myosin regulatory light chain (Naka et al., 1983). We found two radioactive spots after tryptic peptide digests of myosin light chain after PR6 incubation (Fig. 9). By relative mobility and comparison with spots found after thrombin, these appear to be similar to the myosin light chain kinase site and the protein kinase C site. They were phosphorylated to an approximately equal extent. In addition, we found strong phosphorylation of the 47-kD band in whole platelets in PR6. This is known to be a good substrate for the C kinase. Our results suggest that PR6 can, like phorbol esters, activate protein kinase C.

A second possibility is that the signaling process is intact, and myosin becomes sufficiently phosphorylated (in part endogenously and in part by the ADP signal) at the myosin light chain kinase site to be activated, but some other step inhibits shape change until the pH is increased. For example, perhaps myosin cannot change conformation from the folded to the unfolded form (Scholey et al., 1980; Trybus and Lowey, 1982) until the pH is reversed. This would imply that although phosphorylated myosin is activated it can only exert active tension in the filamentous form.

In summary, part of the mechanism required for platelet shape change is reversibly inhibited by lowering the pH_i. This mechanism is not the overall level of myosin phosphorylation, nor can it be accounted for by an inhibition in actomyosin ATPase. Thus, myosin phosphorylation can be dissociated from shape change. In some respects, the response of platelets to lowered pH is similar to the response to phorbol esters, which also rapidly inhibit shape change but cause phosphorylation of the regulatory myosin light chain on two sites. On the other hand, our results show that the association of myosin and actin with the cytoskeleton correlates closely with the ability of platelets to change shape. This strengthens the conclusion that active interaction of myosin and actin is necessary for platelet shape change (Daniel et al., 1984).

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