Intracellular pH (pHi) of human platelets was measured with the fluorescent dye 2',7'-bis(carboxyethyl)5,6-carboxyfluorescein under various conditions. Stimulation by thrombin at 23 °C caused a biphasic change in pHi (initial pHi, 7.09); a rapid fall of 0.01–0.04 units (correlated with the rise of [Ca²⁺]), measured with quin2) followed after 10–15 s by a sustained rise of 0.1–0.15 units pHi. The fall of pHi, and [Ca²⁺], mobilization was reduced by early (5 s) addition of hirudin, but the later elevated pHi was not reversed by hirudin added after 30 s, although this strips thrombin from receptors and rapidly returns [Ca²⁺], to basal levels. In Na⁺-free medium, or in presence of the Na⁺/H⁺ antiport inhibitors, 5-(N,N-dimethyl)amiloride (DMA) or 5-(N-ethyl-N-isopropyl)amiloride (EIPA), thrombin caused a greater fall of pHi (0.22–0.26 units) that was sustained. DMA or EIPA could also reverse the alkalinization response to thrombin. Ca²⁺ ionophores (ionomycin, A23187) decreased plateau pHi; DMA and EIPA enhanced the fall of pHi (0.14–0.33 units). Cytoplasmic acidification produced by nigericin (K⁺/H⁺ ionophore) was followed by return towards normal that was abolished by Na⁺/H⁺ antiport inhibitors. The phorbol diester phorbol 12-myristate 13-acetate had little effect on pHi, but increased the rate of recovery 2–3-fold after cytoplasmic acidification by nigericin, ionomycin, or sodium propionate. These results indicate that 1) elevation of [Ca²⁺], by thrombin enhances pH⁺ production, but the subsequent alkalinization is independent of receptor occupancy or elevated [Ca²⁺], and 2) stimulation of the Na⁺/H⁺ antiport by thrombin probably involves some mechanism apart from regulation by pH⁺ and protein kinase C.

Among the many events that occur in stimulated cells, there is almost always a significant shift in intracellular pH (pHi) that, in nearly all cases that have been studied, results in cytoplasmic alkalinization (1, 2). The expression of some cellular responses after stimulation have been attributed to this pH shift. In neutrophils, stimulus-induced alkaline shifts in pH, have been proposed to modulate chemotaxis, aggregation, phagocytosis, secretion and the generation of superoxide radicals (3–7). Also, an early event in mitogenic growth stimulation of mammalian fibroblasts is intracellular alkalinization (8) which may be necessary for initiation of DNA synthesis (9). The direction of the pH shift in stimulated cells depends on the balance between the rate of metabolic proton generation, the intracellular buffering capacity, and the transport of protons out of the cell. In many cells stimulation produces a burst of metabolic activity that results in net ATP consumption and other reactions that generate protons (2). The fact that stimulation actually often causes net alkalinization indicates that cells have developed efficient, rapidly activated mechanisms that neutralize or expel protons. One such mechanism is the Na⁺/H⁺ exchanger (antipporter, countertransporter) that rapidly ejects protons from the cytoplasm in exchange for extracellular Na⁺ ions which enter down their concentration gradient.

In all cells that have been examined for the presence of the Na⁺/H⁺ exchanger, its existence has been established (10); however, the mechanisms for regulation and activation of the Na⁺/H⁺ exchanger have not been completely elucidated. Presently it is known that Na⁺/H⁺ exchange is dependent on extracellular Na⁺, is sensitive to pH, and can be inhibited by amiloride and certain of its analogs (10). Direct activation of the Na⁺/H⁺ exchanger, as opposed to thermodynamic regulation that is dependent on the steepness and directions of the Na⁺ and proton gradients, has been suggested to be due to the phosphorylation by protein kinase C (8), Ca²⁺/calmodulin (11), or by protons acting allosterically at a site different from the transport site (7, 12).

In platelets the mechanisms for regulation of pH, and the role played by Na⁺/H⁺ exchange during activation of this cell are not well understood. The most direct studies of intracellular pH in platelets were made by Horne et al. (13) and Simons et al. (14) who used uptake of 9-aminoacridine, and pH-sensitive fluorescent probes trapped inside the cytoplasm to show that stimulation by thrombin caused a rapid alkalinization.

1 The abbreviations used are: pHi, intracellular pH; BCECF, 2',7'-bis(carboxyethyl)5,6-carboxyfluorescein; BCECF-AM, 2',7'-bis(carboxyethyl)5,6-carboxyfluorescein acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate; DMA, 3-amino-5-dimethylamino-6-chloro-N-(diaminomethyl)pyrazinecarboxamine hydrochloride; EIPA, 3-amino-5-ethylisopropylamino-6-chloro-N-(diaminomethyl)pyrazinecarboxamine hydrochloride; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; protein kinase C, Ca²⁺/phospholipid-dependent enzyme; PGD₂, prostaglandin D₂.
ization response with no recovery towards resting pH. However, Rotman and Heldman (15) detected no change in pH, using the intracellular probe azidofluorescein diacetate. Subsequently, it was shown that uptake of a permeant acid led to Na+ influx and platelet swelling that was inhibited by amiloride (16), and the initial phase of proton ejection caused by thrombin or A23187 (measured with an extracellular glass electrode) was inhibited by amiloride or lack of extracellular Na+ (17, 18). These latter studies point to the presence of the Na+/H+ exchanger in platelets, but do not establish it because measurements of pH2 were lacking and possible nonspecific effects of amiloride.

In this report we have employed the pH-sensitive fluorescent probe BCECF to continuously monitor pH in platelets. Our objective has been to examine in more detail the shifts in pH, caused by thrombin, PGD2, divalent and monovalent ionophores, and the tumor-promoting phorbol ester PMA, so as to assess the roles of intracellular Ca2+, H+, and protein kinases in the regulation of pH. To evaluate the contribution of the Na+/H+ exchanger to the maintenance of pH, under various conditions, we have employed as transport inhibitors the more potent and selective derivatives of amiloride, 5- (N,N-dimethylamino)-fluoride (DMA) and 5-(N-ethyl-N-isopropyl)amiloride (EIPA).

**EXPERIMENTAL PROCEDURES**

**Materials**—High purity human thrombin (activity exceeds 1000 NIH units/mg) from United States Biochemical Corp. (Cleveland, OH) was reconstituted in 50% glycerol and stored at −20 °C. PMA and bovine serum albumin (BSA) were from Sigma. PMA was dissolved in anhydrous dimethyl sulfoxide (Me2SO) and stored as single-use aliquots at −70 °C. Aliquots were diluted in water and used within 2 h. NIGMA, ionomycin, A23187, and quin2-2-acetoxymethylene (quin2-AM) were from Behring Diagnostics. Digitonin was from Pfaltz & Bauer (Stamford, CT). 2′,7′-Bis(carboxyethyl)-5′,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was from HSC Research Development Corp. (Toronto, Ontario, Canada). BCECF-AM and quin2-AM were prepared in dimethyl sulfoxide and stored at −20 °C. DMA (3-amino-5-dimethylamino-6-chloro-N-(diaminomethylene)-pyrazinecarboxamine hydrochloride) and EIPA (3-amino-5-ethylisopropylamino-6-chloro-N-(diaminomethylene) pyrazinecarboxamine hydrochloride) were synthesized as previously described by Cragoe et al. (19, 20). All other chemicals were of reagent grade obtained from various sources.

**Platelet Preparation**—A concentrated platelet suspension (3 × 109/ml) in buffer A (10 mM Na HEPES, pH 7.15, 135 mM NaCl, 5 mM KCl, and 5.5 mM glucose) was prepared as described previously (21) from platelet concentrates obtained within 24 h of collection from the Connecticut Red Cross Blood Center. BCECF-AM (6 μM) or 10-15 μM quin2-AM (final dimethyl sulfoxide concentration was not more than 0.15%) was added to the platelet suspension and incubated at 37 °C for 30-60 min with gentle agitation in a water bath. The permeable acetoxyamethyl esters of BCECF and quin2 are hydrolyzed by cellular esterases upon entering the cell (22, 23) and the bath. The permeable acetooxymethyl esters of BCECF and quin2 formed in this way are relatively impermeable and become trapped in the cytoplasm. The labeled platelet suspension was diluted with buffer A containing 0.2 mg/ml BSA and centrifuged at 1000 × g for 10 min. The platelet pellet was resuspended in buffer B (buffer A plus 1 mM CaCl2, 1 mM MgCl2, and 0.2 mg/ml BSA) to a concentration of 1.3 × 109 platelets/ml and kept at room temperature until use. For each experiment 1 ml of the concentrated suspension was diluted to 106 cells/ml in 12 ml of buffer B or Na+-free, high K+ buffer (10 mM K+ HEPES, pH 7.15, 140 mM KCl, 5.5 mM glucose, 1 mM CaCl2, 1 mM MgCl2, and 0.2 mg/ml BSA). Since little quin2 leaks out of the cells, quin2-labeled platelets were generally diluted one in 100 into the assay buffer. However, BCECF is not as well retained by cells (22, 23), and a centrifugation step was used just prior to measurements of pH, to remove extracellular BCECF. This procedure was also used to remove extracellular Na+ by replacing buffer B with Na+-free buffer. The concentrated platelet suspension (1 ml) was centrifuged for 5 min at 1000 × g in an Eppendorf microfuge in a 2-ml screw cap microfuge tube (Sarstedt, Princeton, NJ). The supernatant was discarded and the platelet pellet was resuspended with 1 ml of assay buffer and transferred to the fluorometer cuvette containing 12 ml of assay buffer.

**Fluorescence Measurements**—Fluorescence was measured with a Perkin-Elmer MPF-2A spectrophotometer with the sample contained in a 15-ml quartz cuvette. The excitation wavelength was 500 nm with a 5-nm slit for BCECF and 393 nm with a 6-9-nm slit for quin2. The emission wavelength was 517 nm with a 9-12-nm slit for BCECF and 492 nm with a 9-12-nm slit for quin2. A magnetic stirrer was mounted in the cuvette chamber for continual stirring of the platelet suspension. All additions were made by syringe directly into the cuvette through small holes in the top of the cuvette chamber. Mixing times were determined to be about 1 s by injecting rhodamine into buffer and observing the rise in fluorescence. All experiments were performed at room temperature (22-24 °C).

**Calculation of pH, and [Ca2+]i**—The fluorescence signal of BCECF was calibrated to pH at the end of every experiment as described by Rink et al. (23). The pH was calibrated by adding 1 μM digitonin to release the BCECF into the medium and the fluorescence signal was recorded at known values of pH measured with a combination glass/reference electrode (Radiometer) inserted directly in the cuvette. The estimated pH, calculated from this calibration was corrected for the 0.5-nm difference in the excitation maximum of intracellular and extracellular BCECF by the method of Rink et al. (23) and Sacks et al. (24). Platelets were resuspended in Na+-free, high K+ buffer and 1-2 μg/ml nigericin was added to collapse the proton and K+ gradients which sets pH, equal to extracellular pH (pHo). The platelets were then lysed with digitonin releasing BCECF and causing the fluorescence signal to increase with no change in pHo. In several experiments this response was used to determine the extracellular pH, values estimated by the calibration procedure to obtain the true pH. In all cases the corrected pH, is given in the text. [Ca2+]i was calculated from quin2 fluorescence as described previously (21, 22).

**Protein Phosphorylation**—A concentrated platelet suspension was prepared as described above and incubated for 45 min at 37 °C with 0.2 μc/ml of ortho[32P]phosphoric acid (carrier-free, New England Nuclear). Buffer A containing 0.2 mM EGTA was added and the suspension was centrifuged for 10 min at 750 × g. The platelet pellet was resuspended in buffer A containing 1 mM MgCl2 and 40 μM CaCl2 to a concentration of 5 × 108 cells/ml and then diluted to 2.5 × 106 cells/ml in the experiments. Reactions were stopped with 2.5% diethothreitol, 2% sodium dodecyl sulfate (SDS), 12.5% glycerol, and a trace of bromophenol blue and then placed in a boiling water bath for 5 min. The samples were electrophoresed essentially as described by Sacks et al. (23) on 5-15% polyacrylamide gradient gels containing SDS. The gels were stained with Coomassie Brilliant Blue R, destained, and the phosphorylated proteins located by autoradiography.

**RESULTS**

**Thrombin-stimulated Shifts in Platelet pH**—The pH, of stirred, unstimulated platelets from nine different donors labeled with BCECF in Na+-free HEPES buffer (pH = 7.15) at 21-23 °C was 7.09 (±0.12 S.D.; n = 18). The addition of 1-2 units/ml thrombin caused a biphasic shift in pH, (Fig. 1a). Initially there was a brief fall of 0.01-0.04 pH units that began in about 4-6 s and was maximal in 10-25 s. This was followed by a rapid alkaline shift that rose above resting pH, in less than 30 s and stabilized by 2-3 min at a level greater than resting pH, (Fig. 1a). The pH, 3 min after thrombin was maximally increased by 0.1-0.15 pH units above resting pH. The alkalinization response was always observed, however, the initial acidification was not observed in 3 out of 18 experiments.

**Platelet Stimulation in Na+-free Media**—If the alkalization response to thrombin is mediated by the Na+/H+ exchanger it should be dependent on an inwardly directed Na+ concentration gradient that would be abolished in platelets resuspended and stimulated in Na+-free buffer. To test this hypothesis platelets were prepared and stored in normal Na+ buffers and then the Na+ was replaced with K+ as described under “Methods.” With this procedure the platelets were in Na+-free, high K+ buffer for less than 5 min before stimulation.
with thrombin. Under these conditions 1 unit/ml thrombin caused a rapid and sustained fall of pH$_i$ by as much as 0.26 pH units (±0.06 S.D.) (Fig. 1, b), demonstrating conclusively that the stimulus-induced rise of pH$_i$ was absolutely Na$^+$-dependent.

Effects of DMA and EIPA on Platelet pH$_i$ and Response to Thrombin—To further evaluate the role of Na$^+$/H$^+$ exchange in thrombin-stimulated shifts of pH$_i$, the Na$^+$/H$^+$ exchanger was inhibited by DMA or EIPA. These amiloride analogs are much more potent and specific in their inhibition of the Na$^+$/

H$^+$ exchanger than is amiloride itself. At concentrations that inhibit Na$^+$/H$^+$ exchange, the selectivity of amiloride is poor since it has been shown to also inhibit protein kinase C (26), protein synthesis (27), Na$^+$/K$^+$-ATPase (28), and Na$^+$/Ca$^{2+}$ exchange (29, 30). The derivatives EIPA and DMA are, respectively, 140 and 23 times more potent than amiloride in inhibiting the Na$^+$/H$^+$ exchanger (31), and Besterman et al. (26) showed that these analogs strongly inhibited Na$^+$/H$^+$ exchange in neutrophils with no significant effect on protein kinase C. In platelets, a M$_r$-47,000 protein is rapidly phosphorylated by protein kinase C following agonist-induced activation (32). The phosphorylation of this protein induced by 1.0 or 0.1 Unit/ml thrombin was not inhibited by preincubating platelets with 50 μM EIPA or 100 μM DMA; this was in contrast to amiloride which caused a small, but detectable (~15–20%), inhibition (not shown). Therefore, only EIPA and DMA were used in subsequent experiments to selectively inactivate the Na$^+$/H$^+$ exchanger.

The normal alkalinization response elicited by thrombin could be completely reversed by the Na$^+$/H$^+$ exchange inhibitors (Fig. 1, c and d). When platelets were preincubated with 100 μM DMA or 50 μM EIPA for 1 min and then stimulated with 1 Unit/ml thrombin, there occurred a rapid acidification, stabilizing at 0.22 (± 0.05 S.D., n = 3) pH units with DMA and 0.22 (± 0.03 S.D., n = 3) pH units with EIPA below resting pH$_i$ after 5–6 min. By themselves, 100 μM DMA and 50 μM EIPA added to unstimulated platelets initiated a slow fall of pH$_i$ that leveled off in about 6 min (Fig. 2A,a and 2B,a). This effect was slower and of lesser magnitude than the acidification caused by thrombin in the presence of the inhibitors (Fig. 2A,b and 2B,b and c) and was not blocked by PGD$_2$ plus theophylline (not shown), indicating that the Na$^+$/

H$^+$ exchanger is involved in maintaining the steady-state level of pH$_i$ even in unstimulated platelets.

To determine if the thrombin-induced alkalinization, once initiated, could be reversed, we added DMA or EIPA after stimulation by thrombin. When 50 μM EIPA was added 30 s or 6 min after 2 Unit/ml thrombin a rapid (within 5 s) acidification ensued (Fig. 2A,b and c). Note that the rate of fall of pH$_i$ due to EIPA was much greater in the platelets stimulated by thrombin, compared to unstimulated platelets. Such effects were elicited when DMA or EIPA were added as late as 10–12 min after stimulation by thrombin, however, the drop in pH$_i$ became slower and less extensive the greater the time post-thrombin that the inhibitors were added. These results show that the great increase of proton-generating reactions induced by thrombin persist for some minutes and then slowly dissipate with time and that the maintenance of the elevated steady-state pH$_i$ requires the activity of the Na$^+$/

H$^+$ exchanger.

Fig. 3A shows how pretreatment with increasing concentrations of DMA effects the response to thrombin. The initial fall of pH$_i$ caused by thrombin was accentuated at low concentrations of DMA (1–20 μM), and the alkaline rebound to higher pH$_i$ became slower and less in magnitude. At 50–100 μM DMA, only a sustained fall of pH$_i$ occurred (Fig. 3A). Thus, progressive blockade of the proton transporter abolishes the ability of the platelet to cope with the increased rate of H$^+$ generation caused by thrombin, and pH$_i$ falls as protons are trapped within the cell. DMA produced maximum inhibition of proton transport within 15 s (Fig. 3B), which is consistent with evidence that these drugs act on an exposed extracellular site on the transporter. Essentially the same results were obtained with EIPA which was 4–5 times more potent than DMA (Fig. 3C), as found in other systems (31, 33).

Inhibition of pH$_i$, Responses by PGD$_2$—Many platelet responses to thrombin, ranging from calcium mobilization and phosphoinositide hydrolysis to secretion and aggregation,

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are inhibited by agents that increase cyclic AMP. We, therefore, treated platelets with PGD₂ plus theophylline to increase cyclic AMP levels, and then stimulated them with thrombin in the presence or absence of DMA or EIPA. It may be seen in Fig. 4 that PGD₂ completely inhibited the alkalinizing response seen in normal platelets (Fig. 4, a and b) and inhibited the fall in pH, that occurs in platelets exposed to the Na⁺/H⁺ exchange inhibitors by 67% (Fig. 4, c-e). Under similar conditions calcium mobilization (38) and phosphatidylinositol 4,5-bisphosphate hydrolysis were inhibited by about 80%. As mentioned above, the decrease in pH, caused by the transport inhibitors in unstimulated platelets was unaffected by PGD₂.

\[ \text{Ca}^{2+} \text{ Ionophore-stimulated Shifts in pH.} \]—The rise in intracellular Ca²⁺ is an early response to thrombin and could conceivably play an important role in the reactions generating protons and perhaps also more directly in the activation of the Na⁺/H⁺-exchanger. The ionophores ionomycin and A23187 can also be used to elevate intracellular Ca²⁺ and thereby to probe the role of calcium in the regulation of pH. Ionomycin and A23187 had similar effects on platelet pH, which differed significantly from the response to thrombin.

Typical effects of ionomycin, shown in Fig. 5, varied from a rapid and sustained fall of pH, (Fig. 5, A and C) which occurred in two stages (Fig. 5C), or less frequently to an initial fall followed by a return towards normal, or just slightly above the initial pH (Fig. 5, B). The decline of pH, ranged from 0.02 to 0.15 units, and in no experiments did pH, increase to the extent seen with thrombin. The responses to ionophores were unaffected by treatment of platelets with aspirin and apyrase which, respectively, abolish the indirect, receptor-mediated, effects that are attributable to formation of thromboxane A₂ and secretion of ADP that are stimulated by the ionophores. When the Na⁺/H⁺ exchanger was inhibited by 100 µM DMA or 50 µM EIPA, ionomycin caused an even greater acidification that eventually leveled off and stabilized (Fig. 5, A and B) at a pH of from 0.14 to 0.33 pH units below the resting level. The addition of inhibitor after the ionophore also elicited a large and rapid fall of pH, (Fig. 5C), indicating that the Na⁺/H⁺ transporter was partially compensating for increased intracellular proton generation caused by the ionophore.

\[ \text{Ca}^{2+} \text{ Mobilization and pH.} \]—Because a rise of [Ca²⁺], by itself appeared to be sufficient to produce significant changes of pH, we investigated the temporal relationship between changes in pH, and [Ca²⁺], after thrombin stimulation. One of the earliest measurable events in thrombin-stimulated platelets is an increase in [Ca²⁺]. In quin2-labeled platelets at 23 °C, thrombin produced a rise in [Ca²⁺], that was detectable in about 3 s and which reached a maximum of over 1 µM in 8–12 s. Thereafter, [Ca²⁺], spontaneously declined to its original basal level of 50–100 µM over a period of 10–15 min (Fig. 6A). The earliest change in pH, consisting of the brief acidification response, appeared to occur at nearly the same time as the initial rise in [Ca²⁺], (Fig. 6A,b), and the much greater fall in pH, that occurred in the presence of DMA or EIPA began immediately during the most rapid phase of Ca²⁺ mobilization (Fig. 6A,c), suggesting a possible [Ca²⁺]-dependency for proton-generating reactions. With respect to the alkalinizing effect of the exchange transporter the role of

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**Fig. 3. Effects of thrombin on pH in platelets treated with DMA or EIPA.** A: time course of thrombin-induced changes of pH in platelets treated with concentrations of DMA ranging from 0.1 to 100 µM; 1 unit/ml thrombin (T) added 1 min after DMA. B: response to thrombin added 15 s after 100 µM DMA. C: dose-response relationship for effects of EIPA and DMA on thrombin-induced changes in pH. Platelets were preincubated with DMA (●) or EIPA (○) for 1 min before stimulation with 1 unit/ml thrombin. The shift in pH, relative to resting pH, measured 5 min later is plotted as a function of inhibitor concentrations.

**Fig. 4. Inhibition of thrombin-induced changes in pH by PGD₂.** a, control pH response to 1 unit/ml thrombin (T); b, response to thrombin after pretreatment of platelets with 1 µM PGD₂ plus 1 mM theophylline for 2 min; c–e, responses in platelets treated with 50 µM EIPA; c, effect of EIPA on pH in unstimulated platelets (± PGD₂/theophylline); d, platelets treated with PGD₂/theophylline and EIPA and then stimulated with 1 unit/ml thrombin (T); e, effect of thrombin on pH in EIPA-treated platelets in the absence of PGD₂/theophylline.

**Fig. 5. Effects of ionomycin on platelet pH in the presence and absence of Na⁺/H⁺ exchange inhibitors.** A: a, 1 µM ionomycin added alone or b, 2 min after 100 µM DMA. B: left, response to 1 µM ionomycin; right, response to ionomycin added 1 min after 50 µM EIPA. C: response to ionomycin, followed 5 min later by 100 µM DMA.
calcium is more dubious. The rise of $[\text{Ca}^{2+}]$, preceded the increase of pH, and was completed well before the new steady-state levels of pH were attained (Fig. 6A,a and b). Furthermore, the rise in pH, was stable, whereas $[\text{Ca}^{2+}]$, declined steadily for 10–15 min (Fig. 6A,a). The temporal relationship between $[\text{Ca}^{2+}]$, and the rise of pH, was also assessed by using excess hirudin to rapidly strip thrombin from its receptors. When hirudin was added 5 s after thrombin (Fig. 6B,b), $[\text{Ca}^{2+}]$, mobilization was attenuated (Fig. 6B,e) and the rise of pH, was largely abolished. However, when hirudin was added after 30 s (Fig. 6B,a), there was no attenuation or reversal of the increase of pH, despite the fact that $[\text{Ca}^{2+}]$, rapidly declined to its resting level (Fig. 6B,d). Thus, the sustained elevation of pH, caused by thrombin, which is due to stimulation of the proton transporter, does not require elevated $[\text{Ca}^{2+}]$.

Responses to Nigericin—Nigericin is a monovalent carboxylic ionophore that is commonly used to alter the Na+, K+, and proton gradients across cell membranes. Nigericin has a 100-fold greater affinity for K+ over Na+, but also transports protons enabling manipulation of pH (34). Both the Na+ and K+ gradients across the platelet plasma membrane are collapsed by nigericin without stimulation of platelet aggregation or secretion (35). In the presence of a normal outwardly directed K+ gradient nigericin causes the efflux of cellular K+ in exchange for influx of both Na+ and protons, thereby causing intracellular acidification (34). As expected, nigericin added to unstimulated platelets caused a dramatic, rapid, dose-dependent fall in pH, followed by recovery towards resting pH, that began almost immediately after maximal acidification was achieved. Addition of 2 μg/ml nigericin decreased pH, by 0.47 units within 30 s, which was followed by 50% recovery in about 5 min (Fig. 7A). The rate of the ensuing recovery phase was also dose-dependent, so that recovery was faster when the initial acidification was greatest. In the experiment shown in Fig. 7A the recovery measured 3 min after the maximal acidic pH, shift was 67, 35, and 12% complete with 2, 1, and 0.2 μg/ml nigericin, respectively: at 1 μg/ml nigericin the recovery rate (increase of pH,) was 0.023 pH units/min, compared to 0.062 pH units/min at 2 μg/ml nigericin. In the presence of Na+/H+ exchange inhibitors the nigericin-induced fall of pH, was enhanced and recovery was blocked. A typical example presented in Fig. 7A shows that 1 μg/ml nigericin by itself caused pH, to fall 0.38 units in 45 s followed by alkalization, whereas in the presence of 50 μM EIPA pH, dropped 0.58 units in 30 s and to a maximum of 0.73 units in 3 min with no ensuing recovery. Thus, the ionophore-mediated influx of H+ was opposed by an activated Na+/H+ exchanger.

Functional Consequences of pH,—One of our interests has
been to determine the functional consequences of changes in platelet pH, especially with regard to those early receptor- mediated activation processes, such as Ca2+ mobilization. Because the fluorescence of the Na+/H+ exchange inhibitors interferes with measurements of quin2 fluorescence we were not able to test responses to thrombin under those conditions. However, the use of nigericin afforded the opportunity to explore the effect of reduced pH on [Ca2+]i. Added by itself, nigericin produced either no effect or in several experiments caused a small (30–100 nM), transient rise of [Ca2+]i (Fig. 7,B and C) that was not accompanied by secretion or aggregation (not shown). Under these conditions, the subsequent addition of a maximally effective concentration of thrombin elicited a small (0.5–2 nM), sustained decrease of Ca2+-quin2 fluorescence observed at low levels of Ca2+ mobilization may be due in part, but not entirely, to the approximately 18% decrease of Ca2+ in quin2 fluorescence observed at low levels (e.g. 100 nM) of ambient Ca2+ when pH is lowered about 0.3 units (22).

**Effects of PMA on Platelet pH.—** Because the activity of the Na+/H+ exchanger may be regulated by protein kinase C (8) it was of interest to study the effects of the phorbol ester PMA, which activates protein kinase C (32), on pH. In unstimulated platelets, 50–100 ng/ml PMA had virtually no effect on pH, (Fig. 8,a and b). In two experiments, there was no change in pH, after 10 min of incubation while in a third experiment there was a slight acidification of about 0.01 units after 1 min, followed by a slow alkalization that continued for 7–8 min and leveled off 0.01 units above resting pH, (Fig. 8,a). These results suggest that PMA has little or no effect on the activity of the Na+/H+ exchanger in unstimulated platelets. However, if the cytoplasm was acidified by the addition of ionomycin, nigericin, or Na+ propionate (16), the presence of PMA enhanced the rate at which pH returned towards resting pH, by 2–3 fold (Fig. 8,d–f). In contrast, thrombin caused an initial rise of pH, in nigericin-treated platelets that was about 7 times greater than the effect of PMA (Fig. 8,g).

**DISCUSSION**

Stimulation of human platelets by thrombin causes dramatic changes in their cellular biochemistry that results in shape change, aggregation, degranulation, and mobilization of arachidonic acid. These responses occur concurrently with a sustained alkaline shift in pH. The mechanism of this change in pH, and its relationship to the other platelet responses is not well understood. Previous studies have suggested the presence of the Na+/H+ exchanger in platelets, based on the inhibition by amiloride of the early phase of H+ ejection (measured extracellularly) caused by thrombin or A23187 (17, 18) and the Na+-dependent cellular swelling caused by influx of a permeant acid (16). However, direct measurements of pH under these conditions were not made, and amiloride has less than ideal pharmacological selectivity. We have, therefore, employed amiloride analogs of much greater potency and selectivity and an intracellular fluorescent probe to measure pH.

In unstimulated human platelets the mean pH, of 7.09 ± 0.12 S.D., measured with BCECF at 21–23 °C and pHr = 7.15, initially fell in response to thrombin by 0.01 ±0.01 pH units. This response was of rapid onset (4–6 s lag time) and brief duration (5–20 s) and was immediately followed by a rapid and sustained alkalization which increased pH, by as much as 0.15 unit above resting levels. Horne et al. (13) and Simons et al. (14) used the fluorescent probe 6-carboxyfluorescein to detect a sustained increase of 0.30 units pH, due to thrombin at 37 °C, but no initial acidification was observed. We believe that the initial fall of pH, was driven out of our experiments because the lower temperature permitted a finer resolution of the rapidly occurring events that cannot be adequately separated at 37 °C. A similar acidification preceding the rise in pH, was noted earlier in stimulated neutrophils (3, 5) and is a sign of the onset of early H+-generating reactions, initiated by receptor activation, that are rapidly compensated by increased H+ extrusion.

The extrusion of protons that are generated following cellular stimulation is believed in many cases to be mediated by the Na+/H+ exchanger (1, 2). The energy required for this exchange is derived from the inwardly directed Na+ concentration gradient. Our results confirm the presence of the Na+/ H+ exchanger in platelets and establish its involvement in regulating pH, in thrombin-activated platelets. By direct measurement of pH, under conditions in which Na+/H+ exchange was rendered inoperable by Na+-free media, or by the amiloride analogs DMA and EIPA, we were able to demonstrate that the normal alkalization response was completely abolished. Indeed, under these conditions thrombin induced a rapid and substantial acidification that was sustained when Na+ lack or transport inhibitor concentrations were sufficient. The involvement of the Na+/H+ exchanger in the maintenance of the sustained post-stimulation steady-state alkalinization was also demonstrated by the dramatic acidification caused by adding DMA or EIPA after thrombin stimulation had already raised pH. The effectiveness of the Na+/H+ exchange is derived from the inwardly directed Na+ concentration gradient.
The ensuing alkalinization is long lasting. Nevertheless, the protons that are generated intracellularly act as a direct response to the extent of the original acidification. Furthermore, that Ca++-dependent reactions (e.g. increased myosin ATPase activity) contribute to H+ production. Treatment of platelets with PGD2 plus theophylline, which raises cyclic AMP levels and partially inhibits thrombin-induced hydrolysis of phosphoinositides and the mobilization of Ca++ (36), also inhibited the acidification response. This result is consistent with the view that the fall in pH caused by thrombin is substantially attributable to mobilization of Ca++. Although receptor-linked reactions apart from calcium mobilization may increase H+ production, the ionophores do so under conditions that prevent receptor-activation and phosphoinositide hydrolysis (37) (i.e. in aspirin-treated platelets with ADP scavengers). In this case the fall in pH is more clearly attributable directly to Ca++-mediated events and is partly offset by the Na+/H+ exchanger since pH decreases to a greater extent in the presence of DMA or EIPA.

The stimulation of the Na+/H+ exchanger following platelet activation by thrombin, which is responsible for the increase in pH, that offsets the increased proton production, could occur by several mechanisms. One possibility is that the protons that are generated intracellularly act as a direct stimulus to the transmembrane ion exchanger. This mechanism probably accounts for the response to nigericin, since the recovery of pH, following the initial drop caused by the ionophore, increased at a rate that was proportional to the extent of the original acidification. Furthermore, that increased rate of recovery was clearly attributable to the ion exchanger since it was abolished by DMA and EIPA. The situation in thrombin-stimulated platelets is quite different. A mechanism driven by low cytoplasmic pH, could not be sustained since the initial acidification is transient, whereas the ensuing alkalinization is long lasting. Nevertheless, the sustained increase of pH is clearly due to the Na+/H+ exchanger since it is rapidly reversed by DMA and EIPA.

Another possible mechanism for regulation of pH may be mediated by protein kinase C. Unlike some other cell types, the pH of unstimulated platelets was usually not significantly elevated by the phorbol ester PMA, despite the fact that activation of protein kinase C was maximal as determined by measurement of protein phosphorylation. However, PMA did stimulate Na+/H+ exchange if the platelets were first treated with ionomycin, nigericin, or Na+ propionate to acidify the cytoplasm. The role of protein kinase C may be to increase the sensitivity of the exchange transporter to H+ or to increase the turnover rate of the exchanger. Thus, in the platelet, protein kinase C may play a permissive or modulatory role in regulating pH, but it does not appear to account for the much greater and more rapid alkalinizing response to thrombin. One caveat in this regard is the possibility that physiological activation of protein kinase C by endogenous diglyceride may be a more effective mechanism for activation of the Na+/H+ exchanger than treatment with PMA.

It has also been suggested that the Na+/H+ exchanger in cultured human fibroblasts may also be regulated by Ca++/ calmodulin (11) or by a direct receptor-linked mechanism. Participation of Ca++ in the initial activation of the Na+/H+ exchanger cannot be excluded and is in fact suggested by comparing the time course of the rise of [Ca++]i, with the shifts in pH. The increase of [Ca++]i precedes the increase of pH and could, therefore, be a causative factor. Conditions that inhibit mobilization of calcium such as pretreatment with PGD2 (36), or early (5 s) post-stimulation treatment with hirudin, also inhibit the rise of pH. However, following stimulation by thrombin, pH quickly stabilizes at an elevated level while [Ca++]i, spontaneously returns toward basal levels. Even more striking was the maintenance of elevated pH despite the rapid decline of [Ca++]i, to resting levels caused by addition of hirudin 30 s after thrombin. These experiments clearly indicate that although the exchange transporter is initially activated to a new heightened kinetic state by receptor-mediated events including, perhaps, the hydrolysis of phosphoinositides and the rise of [Ca++]i, the maintenance of that activated state does not require either elevated [Ca++]i, or continued receptor occupancy. This suggests that some receptor-initiated covalent modification of the exchanger occurs rapidly and is not reversible by hirudin after about 30 s. It is important to note that for some minutes after adding thrombin the generation of H+ remains high (although slowly declining), despite which the pH remains fixed at an elevated level. This is clearly demonstrated by the experiments showing sharp drops in pH, when DMA or EIPA were added from 3 to 12 min after thrombin. This behavior attests to the fact that the exchanger remains for some time at a higher state of activity than in unstimulated platelets.

In platelets it is clear that the metabolic stimulation evoked by thrombin imposes a large stress to pH, homeostasis, which is efficiently handled by the Na+/H+ exchange process. The question is, then, for what functions, if any, is the rise in pH; advantage? For the platelet those hypotheses which propose that the alkalinization response is necessary for DNA synthesis and growth stimulation (2) do not apply, but other theories concerning the role of pH, in cytoskeleton assembly (38), aggregation, and secretion (3–7) are certainly pertinent. Some aspects of platelet function are apparently not impaired if the Na+/H+ exchanger is inhibited or if the cytoplasm is acidified prior to stimulation. We previously showed that platelet aggregation was not inhibited by concentrations of nigericin that we now show would cause a substantial cytoplasmic acidification (35). Also, maximal Ca++ mobilization by thrombin is not impaired at the height of the acidification by nigericin. This shows that stimulus-response coupling is not absolutely dependent on either opposing the fall of pH, or on the elevation of pH. Indeed our kinetic studies show that Ca++ mobilization by thrombin precedes the rise of pH, and is, thus, independent of it. However, there is some tendency for Ca++ mobilization by low concentrations of thrombin to be impaired when pH is reduced. This effect corresponds to recent findings of Swaet et al. (39) who showed that arachidonic acid release from platelets that were stimulated by epinephrine, ADP, or very low concentrations of thrombin was inhibited in Na+-free media and by the presence of EIPA and other amiloride analogs. In contrast, arachidonic acid release by moderate (≥0.1 unit/ml) or maximally effective concentrations of thrombin was unaffected. Shape change and aggregation that was independent of arachidonic acid metabolism was unaffected by EIPA (39). The normal increase of pH, caused by thrombin may have other consequences. The activities of some key enzymes, such as phosphofructokinase, Ca++-transport ATPase, and adenylate cy-
In conclusion, we have conclusively demonstrated the involvement of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in the regulation of pH\textsubscript{i} in platelets, and we have identified at least two mechanisms that can stimulate Na\textsuperscript{+}/H\textsuperscript{+} exchange; i.e., intracellular acidification and activation of protein kinase C. However, neither mechanism is sufficient by itself, or in conjunction with the other, to stimulate Na\textsuperscript{+}/H\textsuperscript{+} exchange comparable to thrombin. The greatest rate and extent of rise of pH\textsubscript{i}, representing the most effective stimulation of Na\textsuperscript{+}/H\textsuperscript{+} exchange, was observed only after thrombin stimulation. It could not be duplicated by Ca\textsuperscript{2+} ionophores or by PMA. It is significant to note that thrombin was also the only agent whose mechanism of action was receptor linked, suggesting that there is still an unidentified mechanism for the stimulation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger in platelets, apart from Ca\textsuperscript{2+} or protein kinase C, that may involve some receptor-associated factor, such as GTP-binding proteins (as in neutrophils, Ref. 40) or some kinase activity, such as a tyrosine kinase which is found in high activity in platelets (41).

REFERENCES