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# Sequential sodium–proton exchange in thrombin-induced human platelets

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**Thrombin stimulation of human platelets initiates a membrane depolarization attributable to a $Na^+$ influx into, and an alkalinization of, the cytoplasm, both of which follow a similar rapid time scale and thrombin-dose dependence. These responses precede secretion of the contents of the dense granules (serotonin) and, after 1 minute, of lysosomes ($\beta$-glucuronidase). We have evaluated these parameters in the presence of $^2H_2O$ in order to determine if the $Na^+$ influx and $H^+$ efflux are sequential or simultaneous. NMR evidence indicates that $^2H_2O$ equilibration in rapid, and virtually complete within the 3 min prestimulation platelet equilibration period. In response to an 0.05 U/ml addition of thrombin, the rate of depolarization is 70–80% slower in $^2H_2O$ than in $H_2O$. The time to reach maximal depolarization is 5 to 10 seconds longer in $^2H_2O$, the extent of depolarization 60% inhibited, and the pH change 85% inhibited. The serotonin secretion is unaltered, while the $\beta$-glucuronidase secretion is 130–180% enhanced. Dimethylamiloride inhibits the $Na^+$ influx and the pH change completely. These results suggest that the $Na^+$ and $H^+$ fluxes across the plasma membrane are interdependent but neither simultaneous nor electroneutral. Furthermore, granule secretion, previously shown by us to be independent of the existent $Na^+$ gradient, depends on the cytoplasmic $K^+$ and $H^+$ concentrations.**

All living cells, including the human platelet, maintain a voltage gradient across their membrane, the inside being negative with respect to the exterior milieu. The potential, generally −100 to −10 mV for circulating blood cells, is attributable in part to their interior's high potassium and low sodium concentrations, maintained by a sodium-potassium ATPase [1]. These cells also maintain a transmembrane pH gradient [2–4].

The platelet membrane resting potential has been reported to be between −46 and −60 mV [2,3,5], decreasing to −15 mV upon stimulation with a saturating concentration (4.5 nM) of thrombin [2]; concomitantly their cytoplasmic pH rises from the resting 7.01 to 7.30 [2.4]. It has previously been shown that both the sodium influx and cytoplasmic alkalinization are inhibited by amiloride [11] ($10^{-3}$ M, passive $Na^+$-channel blocker) and dimethylamiloride ($10^{-4}$ M, $Na^+/H^+$ antiport blocker). The ability to block either the passive transport or the antiport is indistinguishable since both amilorides were used at such high concentrations. These potential and pH changes involve a sodium/proton counter-transport system [6–11]. However it had not, to date, been possible to deduce whether these effects are attri-

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butable to a stimulation-activated $Na^+$-$H^+$ or to a linked but non-stoichiometric $Na^+$ and $H^+$ passive transport across the membrane.

Heavy water ($^2H_2O$), the nonradioactive oxide of deuterium ($^2H$), differs significantly from $H_2O$ in its biological functions. It has been speculated that $^2H_2O$ causes a change in membrane receptor conformation, stimulus affinity, and/or enzymatic activity, which results in decreased ADP and collagen-induced aggregation, clot retraction, and formation of pseudopodia [12]. Calcium uptake in heavy water is also diminished [12,13]. These studies were performed after long incubations with $^2H_2O$, conditions under which extensive alterations of platelet function could be expected.

We have chosen to address the antiport versus linked but non-stoichiometric passive transport problem by performing platelet activations in $^2H_2O$ after short incubations ($< 3$ min). In such a system, corrected to achieve an apparent $pH_i = 7.4$ (since $p^2H = pH + 0.4 = 7.0$) [14], the kinetics of both sodium influx, as measured by the membrane potential and proton efflux, as measured by cytoplasmic pH, should remain linked, i.e. if a slower alkalinization occurs, the membrane potential change, fully attributable to the $Na^+$ flux [15], should be similarly slowed. In contrast to the antiport, no such tight coupling is required by a linked but non-stoichiometric transport system.

## Methods

*Materials.* Crude bovine topical thrombin was obtained from Parke-Davis, 5-*N*,*N*-dimethylamiloride was synthesized as previously described [16] and deuterium oxide was purchased from Isotopes™, a division of KOR, Inc. The fluorescent probes 3,3′-dipropylthiodicarbocyanine (cyanine probe), 6-carboxyfluorescein diacetate (fluorescein probe) and 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (carboxyethylfluorescein probe) were supplied by Molecular Probes. Sepharose 2B and Sephadex C-50 were obtained from Pharmacia. Tritium and $^{14}C$-labeled serotonin were obtained from New England Nuclear. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), imipramine, the sodium salt of phenolphthalein mono-β-glucuronic acid and valinomycin were purchased from Sigma Chemical Co. All Other chemicals are of reagent grade.

*Preparation of thrombin.* α-Thrombin was prepared from crude bovine topical thrombin by ion exchange chromatography according to the method of Lundblad et al. [17].

*Platelet preparation.* Platelets were prepared from blood donated by normal volunteers and mixed with 0.38% sodium citrate as an anticoagulant. All procedures were performed using plastic or siliconized glassware. The blood was centrifuged at $126 \times g$ for 10 min at room temperature. The platelets were isolated from the platelet-rich plasma as described previously [18] using Hepes buffer pH 7.4 (137 mM NaCl, 3.8 mM Hepes, 5.6 mM D-glucose, 3.8 monobasic sodium phosphate, 2.7 mM KCl, 1 mM $MgCl_2$ and 0.15 U/ml apyrase) prepared with $H_2O$ or $^2H_2O$ ($p^2H = pH + 0.4$). Platelet concentration was determined turbidometrically on a Zeiss spectrophotometer at 436 nm and evaluated by means of a calibration curve prepared from Coulter Counter measurements.

*Membrane potential studies.* Membrane potential changes were measured by changes in the fluorescence of 3,3′-dipropylthiodicarbocyanine (cyanine probe) (1.5 μM) loaded platelets as a suspension of $30 \cdot 10^6$ platelets per ml in Hepes buffer. The fluorescence was monitored continuously ($\lambda_{ex} = 620$ nm, $\lambda_{em} = 670$ nm) during stimulation by thrombin (0.005–0.05 U/ml) as previously described [3,11,18–20]. The ability of these platelets to respond to thrombin with a change in transmembrane potential, in cytoplasmic pH, and with secretion of serotonin and β-glucuronidase, was monitored in platelets diluted in normal Hepes or in Hepes prepared with $^2H_2O$. Platelets were diluted in $^2H_2O$ immediately prior to functional studies (final $^2H_2O$ concentration = 90%).

Studies were performed in the presence and absence of three perturbants, 5 μM ouabain, a sodium-potassium ATPase inhibitor, $10^{-4}$ M dimethylamiloride or 5 μM valinomycin.

The resting potential of platelets diluted in deuterium oxide was determined by the addition of valinomycin to platelets preincubated with the cyanine probe and diluted in various high potassium and high sodium deuterium oxide Hepes

buffers. The resting potential was calculated as previously described [18].

*Cytoplasmic pH measurements.* Washed platelets, pH 7.0, were incubated for 12 min at 37°C with either 5 μM 6-carboxyfluorescein diacetate or 1 μM 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (final concentration). Stock solutions of probes were prepared in dimethylsulfoxide (DMSO), and the final DMSO concentration was < 0.1%. After the incubation, 3 mM ethylenediaminetetraacetic acid (EDTA) was added and the platelets were centrifuged for 10 min, 25°C, in a RC 2B Sorvall centrifuge, SS 35 rotor at 5000 rpm. The supernatant was discarded and the pellet resuspended in 0.2 ml Hepes buffer (pH 7.4) containing 3 mM EDTA. The resuspended pellet was then diluted immediately before use to a final concentration of $30 \cdot 10^6$ platelets per ml with normal Hepes buffer or Hepes prepared with deuterium oxide; the pH measurements were then performed.

Thrombin (0.005–0.05 U/ml) was added to an aliquot of platelets and the fluorescence at a constant emission wavelength (6-carboxyfluorescein: $\lambda = 518$ nm, carboxyethylfluorescein probe: $\lambda = 530$ nm) was recorded continuously. The excitation wavelengths for 6-carboxyfluorescein were changed from 464 nm, the isobestic point for pH induced changes, to 494 nm, the wavelength of maximal pH sensitivity [18] at intervals of 30 s. The excitation wavelengths for carboxyethylfluorescein probe were 500 and 450 nm, respectively. An aliquot of cells was taken immediately before and 30–60 s after thrombin stimulation and centrifuged at $12000 \times g$. The fluorescence of the supernatant was measured at each excitation wavelength and subtracted from that of the cell suspension to correct for the presence of extracellular dye. The ratio of fluorescence ($F_{500}/F_{450}$ or $F_{492}/F_{464}$ for the carboxyethylfluorescein probe and 6-carboxyfluorescein, respectively) before and after thrombin stimulation was determined. Intracellular pH was determined from a calibration curve prepared using the same ratio procedure with nigericin and potassium Hepes, pH 6.6–7.6 [18,22]. The relative pH change was determined by measuring the initial slope of the thrombin-induced fluorescence change (cm/min) and dividing by $F_{450}$ or $F_{464}$ for each probe.

*Serotonin secretion.* Dense granule secretion was measured by a previously described [15] variation of the method of Jerushalmy and Zucker [20].

*β-Glucuronidase secretion.* β-Glucuronidase was measured, as previously described [15] as a marker for lysosomal granule secretion from thrombin-induced platelets diluted in normal Hepes and Hepes prepared with deuterium oxide.

*Ammonium chloride studies.* Various amounts of ammonium chloride (1 M stock) were added to platelets in order to change the intracellular pH, which was measured immediately. A thrombin

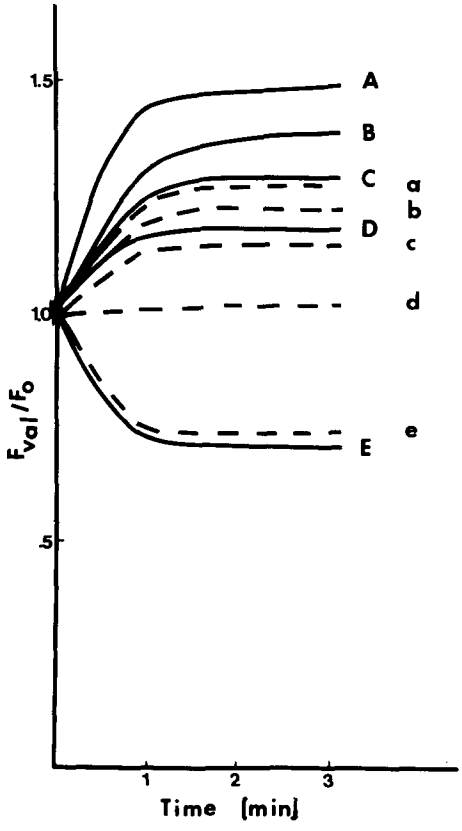


Fig. 1. Changes in fluorescence resulting from exposure of platelets to valinomycin. Relative changes in fluorescence of diS-$C_3(5)$ equilibrated platelets from exposure to 5 μM valinomycin. Fluorescence is expressed as a ratio of observed fluorescence ($F_{val}$) to the fluorescence before the addition of valinomycin ($F_0$). The platelets were suspended in various buffers in which $K^+$ replaces $Na^+$, prepared in $H_2O$/Hepes (———) and $^2H_2O$/Hepes (— — —). Extracellular [$K^+$] concentrations are labeled as A, B, C, D, and E for $H_2O$ and a, b, c, d, and e for $^2H_2O$, corresponding to 2.7, 32.4, 62.3, 92.2 and 122 mM, respectively.

dose curve, measuring membrane depolarization and granule secretion after addition of ammonium chloride to $pH_i = 7.3$, was performed.

## Results

Platelet studies were performed in normal Hepes or in Hepes prepared with deuterium oxide. The relative initial resting potential, evaluated by the $[K^+]_{out}$ concentration at which valinomycin induces no change in the cyanine probe fluorescence, is nearly identical (Figs. 1 and 2) in $H_2O$/Hepes and $^2H_2O$/Hepes at physiologic sodium concentrations (2.7 mM $K^+$). The $K^+$ concentration of 2.7 mM for $H_2O$ and $^2H_2O$ corresponds to approximate resting potentials of $-50$ and $-40$ mV for $H_2O$ and $^2H_2O$, respectively. The linear dependence of the platelet potential on log[$K^+$] is slightly steeper in $H_2O$ than in $^2H_2O$.

A typical tracing of the thrombin-induced change in the cyanine probe fluorescence for platelets diluted in $^2H_2O$/Hepes and in $H_2O$/Hepes is shown in Fig. 3. The relative changes at

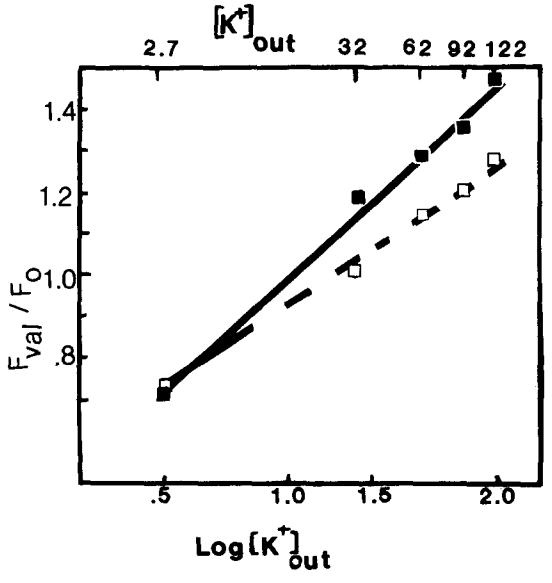


Fig. 2. Observed fluorescence changes versus log [$K^+$] of valinomycin-treated platelets. Relative change in diS-$C_3$(5) fluorescence upon addition of 5 $\mu$M valinomycin: conditions as described in Fig. 1. Potassium concentrations are 2.3, 32.4, 62.3, 92.2 and 122.2 mM, respectively, for $H_2O$/Hepes (■) and $^2H_2O$/Hepes (□).

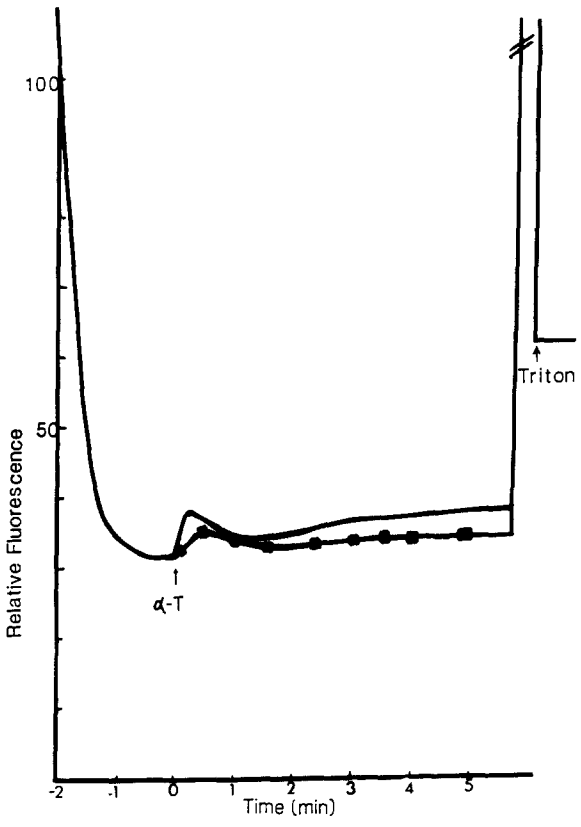


Fig. 3. Fluorimetric analysis of platelets depolarization to thrombin. Superimposed tracings of the response of diS-$C_3$(5) equilibrated platelets in $H_2O$/Hepes (———) or $^2H_2O$/Hepes to 0.05 U/ml thrombin.

varying doses (each point and average of five values) are depicted in Fig. 4, corresponding values in $H_2O$/Hepes being used as controls (= 100%). The rate of depolarization (32.9 ± 13.4%), the rate of alkalinization (12.4 ± 4.7% for 6-carboxyfluorescein-loaded platelets and 22.7 ± 3.0% for carboxyethylfluorescein probe-loaded platelets), the extent of depolarization (43.1 ± 17.3%), and the time to maximal depolarization (5–10 s) induced by 0.05 U/ml thrombin, are all lower in platelets diluted in deuterium oxide than in those diluted in normal Hepes. Furthermore, serotonin secretion is normal in platelets diluted in Hepes prepared with deuterium oxide, but $\beta$-glucuronidase release is significantly enhanced.

To determine if the enhanced $\beta$-glucuronidase secretion was due to the deuterium-induced change in the existing proton gradient in $H_2O$ suspended

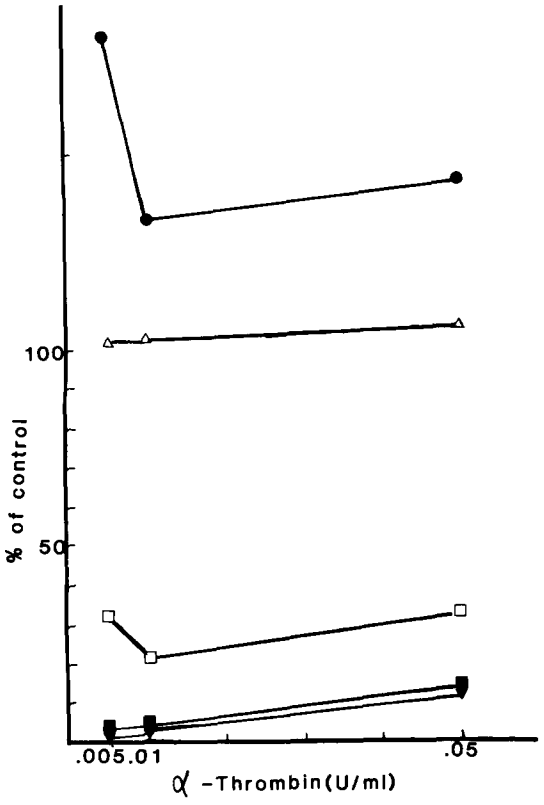


Fig. 4. Relative effect of $^2H_2O$ on the platelet thrombin response. Relative effect of $^2H_2O$ on the initial rate of depolarization (■), the β-glucuronidase secretion (●) and the serotonin secretion (△) by diS-$C_3$(5) equilibrated platelets upon thrombin stimulation. Relative effect of $^2H_2O$ on 6-carboxyfluorescein-loaded (■) and 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF)-loaded (▲) platelets after thrombin stimulation. Thrombin doses were 0.05, 0.01 and 0.05 U/ml.

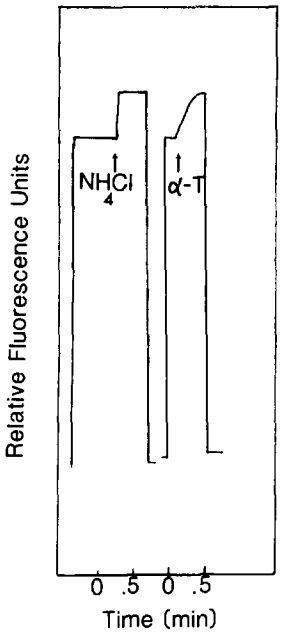


Fig. 5. Alterations in the fluorescence of 6-carboxyfluorescein-loaded platelets upon addition of ammonium chloride. Comparison tracings of 5 mM $NH_4Cl$ treated and 0.05 U/ml α-thrombin (α-T) stimulated 6-carboxyfluorescein-loaded platelets. Fluorescence measurements were taken at $\lambda_{excitation}$ = 492 nm.

cells, we altered $pH_i$. The intracellular pH, ($pH_i$), of the platelets was artificially increased by addition of $NH_4Cl$ to $pH_i = 7.3$, the $pH_i$ achieved after maximal thrombin stimulation [3] as measured by 6-carboxyfluorescein. The cytoplasmic pH rose immediately after addition of 5 mM ammonium chloride (Fig. 5), but the resting potential of these platelets remained unaffected, as measured by their equilibrium cyanine probe fluorescence. Upon subsequent addition of a saturating thrombin does, the rate of depolarization and of serotonin secretion was unaltered (Fig. 6, while the response to subsaturating thrombin doses was partially inhibited. In contrast, β-glucuronidase release by the alkalinized platelets was enhanced at stimulus doses of 0.01 and 0.05 U/ml thrombin. The existence of increased cytoplasmic pH could therefore not itself be inducing the enhanced β-glucurondidase since the pH achieved with $NH_4Cl$ is identical to that found in the cytoplasm after stimulation. Further investigation (Fig. 7) indicates that the enhanced β-glucuronidase may be due to the length of exposure of the platelet to the increased pH.

The effects of three perturbants on platelets diluted in deuterium oxide were also investigated. Dimethylamiloride behaved similarly in both buffers, as did ouabain: amiloride inhibited the initial depolarization completely while ouabain had no effect on the depolarization. Thus the substitution of $^2H_2O$ for $H_2O$ in the platelet buffers did not alter our previously reported [15] findings with respect to these inhibitors of specific functions. Valinomycin counteracted approx. 20% of the deuterium oxide-attributable decrease in thrombin-induced depolarization ($32.9 \pm 13.4\%$ in $^2H_2O$ versus $56.1 \pm 4.6\%$ in $^2H_2O$ plus valinomy-

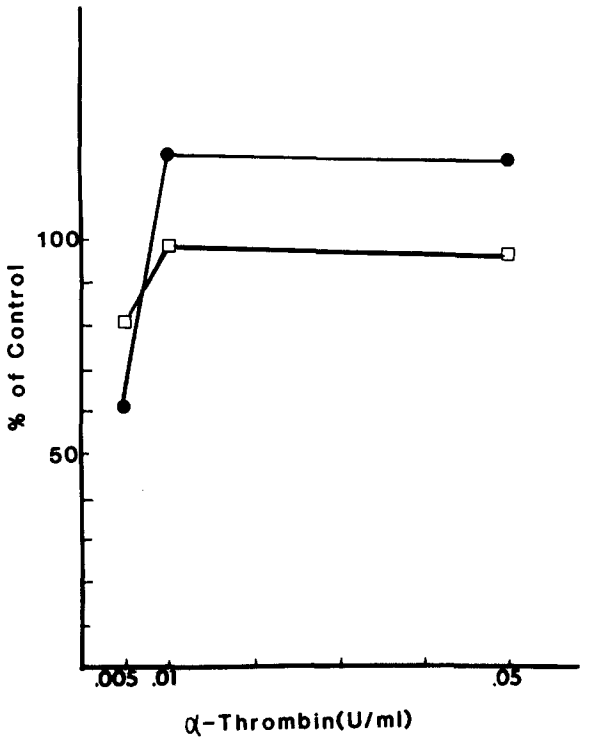


Fig. 6. Relative responses of ammonium chloride treated platelets to thrombin stimulation. Relative response of diS-$C_3(5)$ equilibrated platelets to thrombin after a 30 s pretreatment of platelets with 5 mM ammonium chloride. Doses of $\alpha$-thrombin used were 0.005, 0.01 and 0.05 U/ml. Parameters observed were initial slope (□) and $\beta$-glucuronidase secretion (●). 100% = normal thrombin dose response in the absence of ammonium chloride.

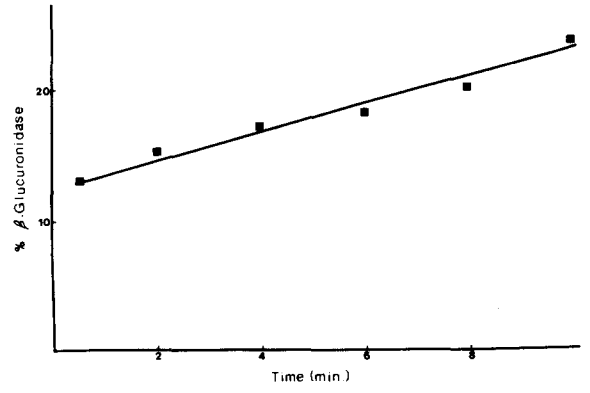


Fig. 7. Time-dependent release of $\beta$-glucuronidase. % $\beta$-Glucuronidase released from lysosomal granules upon stimulation of human platelets by 0.05 U/ml $\alpha$-thrombin as a function of time.

cin) but had no effect on the comparable $H_2O$-based buffers [15].

## Discussion

The sodium and proton fluxes which thrombin induces in human platelets have been demonstrated, but it has not been clear that an actual antiport exists [2]. Such an antiport has been shown to exist in the plasma membrane of several cell types [7–9]. We have therefore extended some previously reported investigations of the effects of heavy water ($^2H_2O$) on platelets [21] and on other cells [10], in order to study this putative antiport.

The effects of lengthy $^2H_2O$ exposure involve membrane receptor conformational changes and binding strength alterations (isotopic effects), as well as altered enzymatic activities of metabolic intermediates due to the increased 'linkage energy' of deuterium bridges (solvent effect). The studies discussed here were designed to investigate the $Na^+/H^+$ exchange and therefore were based on brief exposure to $^2H_2O$. The deuterium ion efflux due to thrombin stimulation should be slower than that of $H_2O$ because of the increased 'linkage energy' of $^2H_2O$ versus $H_2O$ [10,21]. If a stoichiometric antiport exists, then the depolarization should be slowed in parallel with the pH change, since the sodium influx and the proton efflux should exchange through the same channel in a one for one ratio, both being slower in $^2H_2O$. Furthermore, such an antiport should be electrically silent.

Previous platelet activation studies in $^2H_2O$ have reported a significant decrease in ADP and collagen-induced platelet aggregation, while adrenaline-induced aggregation was significantly enhanced [21]. We have found that the membrane potential change due to thrombin-induced stimulation of platelets in $^2H_2O$ was significantly decreased (Fig. 4) and the time to maximal stimulation lengthened (Fig. 3) even if the $^2H_2O$ incubation was brief. This slower response implies a direct link between the sodium influx and proton efflux. The effect of $^2H_2O$ on cytoplasmic alkalinization exceeded the effect on membrane potential, while the rate of pH change in $^2H_2O$ remained similar to that in $H_2O$/Hepes, i.e. on the same time scale. These findings are not com-

patible with a $Na^+/H^+$ antiport, as had been previously suggested [11], but would fit a countertransport mechanism of thrombin-induced sodium/proton exchange. The latter is also compatible with our amiloride studies, which indicate that $pH_i$ changes as well as $Na^+$ influx are affected by amiloride or dimethylamiloride. An electrically non-neutral interconnection must hence be invoked and activation of platelets with thrombin involves activating this linked ion transport system. The transport system for both ions may be the same, or may be different but under tight control.

Granule secretion in $^2H_2O$ was also investigated. Serotonin secretion was unaltered in Hepes buffer prepared with $^2H_2O$ suggesting a lack of proton dependence. Lysosomal granule release in heavy water was significantly enhanced, implicating a proton dependence. This proton dependence was confirmed in platelets with an artificially ($NH_4Cl$) increased cytoplasmic pH, but the $\beta$-glucuronidase time-course studies indicate that the enhancement was due to the length of exposure to the increased cytoplasmic pH and not directly to the altered proton gradients.

Thus thrombin stimulation of human platelets activates a $Na^+/H^+$ ($Na^+/^2H^+$) countertransport leading to a cytoplasmic alkalinization. The latter is enhanced in $^2H_2O$ (versus $H_2O$) based buffers, leading to a higher rate of lysosomal granule release.

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