Thrombin stimulates Na^+-H^+ exchange across the human platelet plasma membrane

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Received 7 May 1984

We have investigated the release of protons from thrombin-stimulated platelets. Addition of thrombin to suspensions of washed platelets resulted in fast liberation of H^+. In the presence of 0.1 mM amiloride, a potent inhibitor of the Na^+/H^+ transport system, the amount of protons liberated was decreased by about 50%, and was further reduced to about 15% by 1 mM amiloride. Similar inhibition of H^+ release was observed after Na^+ in the incubating medium had been replaced by choline. We conclude that one of the earliest events in thrombin-stimulated platelets consists of the activation of an Na^+/H^+ countertransport, which leads to an increase in intracellular pH.

Human platelet *Thrombin* *Platelet activation* *Na^+/H^+ countertransport* *Amiloride*

1. INTRODUCTION

Stimulation of human platelets by thrombin leads to a rapid acidification of their environment [1,2]. Whereas part of this acidification is due to increased lactate formation in stimulated platelets, the major part of proton liberation cannot be accounted for by increased glycolytic activity and has, therefore, descriptively been addressed as a 'lactate-unrelated burst of proton liberation' [2]. Recently, authors in [3] demonstrated rapid alkalinization of the platelet interior, by about 0.3 pH units, following activation by thrombin. The diuretic drug amiloride, which is known to specifically inhibit Na^+/H^+ exchange systems [4], inhibits thrombin-stimulated platelet aggregation and release of granular contents [5]. Inhibitory effects on platelet aggregation, similar to those with amiloride, have been observed after removal of extraplatelet Na^+ [6]. These reports led us to investigate whether stimulation of platelets by thrombin might activate an Na^+/H^+ countertransport system.

2. EXPERIMENTAL

Human platelet-rich plasma (PRP) was obtained from fresh, citrated blood by centrifugation (150 × *g* for 12 min) and adjusted to pH 6.5 by adding 0.1 N HCl. Platelets were pelleted by centrifugation of PRP (1500 × *g* for 20 min) and resuspended in buffer (136 mM NaCl, 2.7 mM KCl, 0.18 mM KH_2PO_4, 5 mM glucose, 0.2 mM EDTA, pH 6.5) and washed twice in this medium. The pH of the platelet suspension was finally adjusted to pH 7.4 with 0.01 M KOH, and the platelet count to $5-8 \times 10^{11}$/l.

In a reaction vessel (volume, 0.7 ml; thermostatted at 37°C) platelets were stimulated with bovine thrombin (Sigma, München) and changes in pH in the suspending buffer were monitored with an electrode (Ingold, Frankfurt/Main) while stirring the buffer. Using the buffer capacity of the incubation buffer [77 μmol·(l·pH)$^{-1}$], the recorded pH changes allowed us to calculate the total amount of H^+ released from thrombin-stimulated platelets.

In some experiments, amiloride (Merck, Sharp & Dohme, München) was added to the suspension

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00145793/84/$3.00

and 30 min were allowed for equilibration of platelets with this drug. In other experiments, platelets were suspended in buffer in which NaCl had been replaced by equimolar concentrations of choline chloride.

3. RESULTS AND DISCUSSION

Fig.1 illustrates the release of protons from unactivated (control) and thrombin-stimulated human platelets as the increase in total H^+ concentration in the suspending buffer, $\Delta[H^+]$.

In the absence of thrombin, there is a spontaneous H^+ release from platelets, which probably reflects lactic acid formation in the resting platelet [2]. Addition of thrombin (2 units/ml, arrow) results, after a lag of about 5 s, in a distinct increase in proton liberation, the kinetics of which may be described by (at least) two phases: a fast phase, which decays after about 40 s, and a slower phase, which is still faster than the control release. These kinetics are in qualitative agreement with [2] where the slow phase was related to increased lactic acid formation of thrombin-stimulated platelets. The early, fast phase could, however, not be attributed to changes in lactic acid formation [2]. The remainder of the discussion will be concerned with this early, fast phase.

The total proton release of the early phase, $\Delta[H^+]_{P\rightarrow B}$, was obtained by extrapolating the slow phase after thrombin stimulation to zero time (see fig.1). In the experiment of fig.1, $\Delta[H^+]_{P\rightarrow B}$ amounted to 8.75 μmol/l buffer. Using a mean value for platelet volume of 8×10^{-15} l [7], and a platelet concentration of 5.6×10^{11}/l, this proton release amounts to $\mu[H^+]_{P\rightarrow B}$ = 2.0 mmol/(l platelet) in this experiment. The average value obtained in 6 determinations was 1.9 mmol/(l platelet) (SE = 0.2). Using an average value for pH changes in platelet upon thrombin stimulation [3] of ΔpH = 0.15, an intracellular total buffer value of $\beta = \Delta[H^+]_{P\rightarrow B}/\Delta pH$ = 13 mmol·(l·pH)$^{-1}$ may be estimated. This buffer value would reflect non-bicarbonate buffering since HCO_3^- was nearly absent in our preparation.

Secretion of H^+ from stimulated platelets was further measured at various concentrations of amiloride, ranging from 10^{-5} to 10^{-3} mol/l. This compound has been shown to block Na^+ transport systems in a variety of cells and tissues [4]. In fig.2, the early, fast release of protons from thrombin-stimulated platelets, expressed as percentage of control (no amiloride) is plotted against inhibitor concentration. There exists a dose-dependent inhibition of proton release, 50% of the total inhibitory effect being obtained with 10^{-4} M, and 85% with 10^{-3} M amiloride. This suggests that H^+ release is linked to Na^+ transport, as might be expected if Na^+/H^+ counter-transport provided the fast H^+ release.

To further test this hypothesis, we have repeated the experiments with platelets suspended in buffer in which most of the extracellular Na^+ was re-

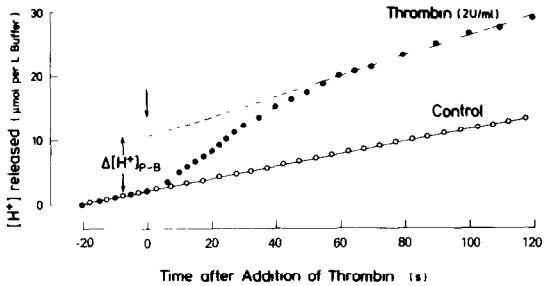

Fig.1. Release of protons from human platelets as a function of time. (○) Control. (●) Thrombin activation at time zero (arrow). Platelet concentration, 5.6×10^{11}/l. Temperature, 37°C. Proton release from platelets to buffer during the early, fast phase, $\Delta[H^+]_{P\rightarrow B}$, obtained by base-line extrapolation at $t = 0$.

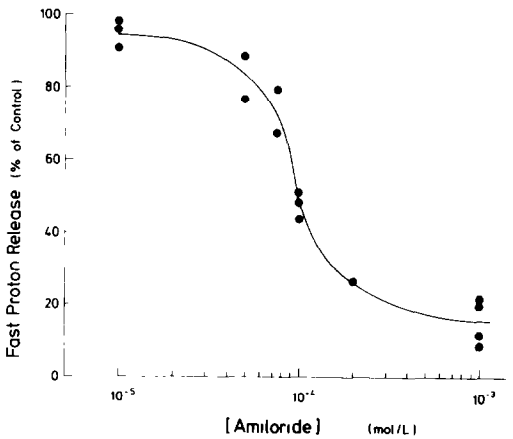

Fig.2. Effect of amiloride on the early phase of proton-release from thrombin-stimulated platelets. Ordinate, amount of H^+ released in the early phase, $\Delta[H^+]_{P\rightarrow B}$ as % of control (absence of amiloride); abscissa, concentration of amiloride (logarithmic scale).

Table 1

Effect of replacing Na^+ on the early, fast release of protons from thrombin-stimulated platelets

$[Na^+]_o$ (mmol/l)	$\Delta[H^+]_{P \rightarrow B}$		N
	mmol/l platelet	% of control	
136	1.8 ± 0.3	100	4
23	1.1 ± 0.2	59	4
6	0.4 ± 0.13	22	4

Mean values ± SE; N, number of measurements

placed by choline. Table 1 summarizes the results. The early, fast release of protons from thrombin-activated platelets is largely reduced when extracellular Na^+ concentration, $[Na^+]_o$, is lowered. With both amiloride and Na^+-depletion, the slow, late phase of H^+ release was retarded but not suppressed.

Both results, inhibition of the early H^+ release by amiloride or by decreased extracellular Na^+, suggest that one of the earliest steps following thrombin stimulation of platelets consists of the activation of Na^+/H^+ exchange across the platelet plasma membrane with subsequent alkalinization of intracellular pH.

Recent reports from the literature suggest that other platelet-activating substances may exert their action via similar mechanisms. It has, thus, been shown that incubation of platelets with ADP, which constitutes a physiologically important platelet-aggregating substance, stimulates net uptake of Na^+ into the activated cells [8,9]. Furthermore, inhibition of the Na^+ transport system by amiloride leads to a marked reduction of ADP-induced platelet shape change and cytoskeletal assembly [10]. The physiological significance of this process, however, is still poorly understood.

ACKNOWLEDGEMENTS

We would like to thank Mrs D. Schüler-Diettrich for her skilful technical assistance. We are indebted to Merck, Sharp & Dohme Co., München, for a generous supply of amiloride.

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