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Thrombin-induced platelet aggregation is affected by external Na^+ independently of the Na^+/H^+ exchange

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Thrombin affects blood platelets by activation of Na^+/H^+ exchange and induction of aggregation, but the relationship between these effects is under debate. The present study attempts to clarify whether the activation of the exchanger activity is required for platelet aggregation. In apparent support of such a requirement, thrombin-induced aggregation is higher in Na^+ medium than in *N*-methylglucamine$^+$ medium and is inhibited by sphingosine, an inhibitor of protein kinase C known to regulate the Na^+/H^+ exchanger. However, the inhibition of aggregation by sphingosine occurs in both Na^+-containing and Na^+-free media, the aggregation is identical in Na^+ and K^+-containing media, and is not inhibited by 5-*N*-(3-aminophenyl)amiloride, at a concentration 10-fold higher than its K_i for platelet Na^+/H^+ exchange. Furthermore, at low concentration (0.005 U/ml) thrombin induces aggregation but does not activate the exchange. It is concluded that the activation of Na^+/H^+ exchange is not required for thrombin-induced platelet aggregation and that the apparent augmentation of aggregation by Na^+ is due to an inhibitory effect of *N*-methylglucamine$^+$.

Platelet; Thrombin; Aggregation; Na^+/H^+ exchange

1. INTRODUCTION

The plasma membrane Na^+/H^+ exchange system, an electroneutral transport system, is present in human blood platelets [1]. Like in other unstimulated nonepithelial cell types, the platelet exchanger appears to be nearly quiescent when the cytoplasmic pH is in the physiological range, but becomes increasingly active as pH_i is reduced [2]. It is well established that stimulation of platelets by thrombin is accompanied by activation of the Na^+/H^+ exchanger, resulting in cytoplasmic alkalinization [3–7]. However, there has recently been debate in the literature as to whether activation of the Na^+/H^+ exchanger is a prerequisite for Ca^{2+} mobilization and/or platelet secretion leading to aggregation [3–14]. We have addressed this disagreement in view of an observation that external Na^+ potentiates thrombin-induced platelet aggregation. It became of interest to examine whether the role of external Na^+ is related to the operation of Na^+/H^+ exchange.

The present investigation demonstrates that neither extracellular Na^+ nor Na^+/H^+ activity is essential for thrombin-induced platelet aggregation. An apparent potentiation of aggregation by the presence of Na^+ in the medium does occur but is independent of the Na^+/H^+ exchange.

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2. MATERIALS AND METHODS

2.1. *Reagents*

Albumin (bovine, essentially fatty acid-free, from fraction V albumin), nigericin and probenecid were from Sigma. 2′,7′-Biscarboxyethyl-5,6-carboxyfluorescein (BCECF) acetoxymethyl ester was obtained from Molecular Probes. Amiloride was from Merck, Sharp and Dohme. 5-*N*-(3-Aminophenyl) amiloride (APA) [15] was a gift from Dr D. Cassel. Bovine thrombin from Parke-Davis, was used in this study.

2.2. *Solutions*

Acid-citrate-dextrose solution was composed of 65 mM citric acid, 11 mM glucose and 85 mM trisodium citrate. The standard Na^+ medium contained (in mM): 140 NaCl, 5 KCl, 0.42 NaH_2PO_4, 10 glucose and 20 Hepes (pH 7.35). K^+, Li^+ and *N*-

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methylglutamine$^+$ (NMG^+) media were prepared by iso-osmotic replacement of NaCl by KCl, LiCl and NMG-HCl, respectively. *N*-Methylglucamine·HCl was prepared by titrating *N*-methyl-D-glucamine to neutrality with HCl. For the measurements of aggregation and pH changes the media were supplemented with 1 mM $CaCl_2$. The osmolarity of all media was adjusted to 290 mosM with distilled water or the major salt. Stock solutions of nigericin (1 mM) and BCECF acetoxymethyl ester (1 mg/ml) were prepared in ethanol and dimethyl sulfoxide, respectively. Determination of Na^+ in the media were performed with a Varian Spectra AA 30 atomic absorption spectrophotometer.

2.3. *Preparation of platelet suspension*

Venous blood was drawn from healthy volunteers, aged 25–53 years, who had been without medication the previous 10 days. The blood was anticoagulated with acid-citrate-dextrose solution at a volume ratio of blood:anticoagulant of 6:1. Platelet-rich plasma was obtained by centrifugation at $120 \times g$ for 10 min and had a pH of 6.5. For the spectrofluorimetric measurements of pH changes the platelets were first loaded with the probe BCECF by incubation of platelet-rich plasma with the parent acetoxymethyl ester (0.7 μg/ml = 0.87 μM, final) for 30 min at 22–23°C. The platelets were then gel-filtered through a Sepharose 2B column (6 × 0.76 cm). The solution used to equilibrate the column and to elute the platelets was the Na^+ medium or, where specified, the NMG^+ medium described above, but modified as follows: albumin (1 mg/ml) was added and the pH was adjusted to 6.8. To diminish the leakage of the fluorescent probe BCECF-loaded gel-filtered platelets were diluted 1:1 (v/v) with a solution of 0.25 mM probenecid in Na^+ medium (pH 6.8).

2.4. *Acetylsalicylic acid treatment*

Platelet-rich plasma was incubated for 30 min at 23°C with 1 mg/ml acetylsalicylic acid and then gel-filtered.

2.5. *Assay of aggregation*

Platelet aggregation was followed by recording light transmission through a stirred platelet suspension in an aggregometer (Chronolog) cuvette at 37°C in a final volume of 0.45 ml. The platelet preparation (BCECF-loaded or unloaded gel-filtered platelets) and the media used were as specified in section 3. Aggregation was measured as the percentage change in light transmission through the platelet suspension with 0% defined as the lowest point of the curve after addition of the aggregating agent and 100% defined as maximal transmission through a clear solution.

Because of the decay of the platelet functions with time all the experiments reported here were performed within the first hour after the preparation of the platelets. When a comparison of an inducer or an inhibitor of platelet activity was investigated, time-paired experiments were performed for the same reason.

2.6. *Determination of cytoplasmic pH changes*

Changes in cytoplasmic pH were determined essentially as described [1,16]. Fluorescence was measured in a Jasco FP-770 spectrofluorometer with wavelength settings of 495 and 525 nm for excitation and emission, respectively, using 5 and 10 nm slits, respectively. The assay mixture for measurements of pH_i changes in BCECF-loaded platelets was composed of the Na^+ medium. The measurements were performed at 37°C in a plastic cuvette using $2–3 \times 10^7$ platelets/ml (an equal concentration to that used for the concomitant aggregation assays) and a total volume of 1.8 ml with continuous stirring. Calibration of the pH vs fluorescence was performed according to Thomas et al. [17], in the standard medium free of Na^+ but containing 100 mM KCl and 40 mM NMG with 5.4 μM nigericin, and 200 μM amiloride, using Mops as titrant. The leak of BCECF [16] in the first hour, during which the experiments were performed, amounted to 20%. The pH_i of untreated gel-filtered platelets was 7.35 (SE = 0.04, $n = 4$), similar to earlier reports [1,7]. The data are presented as changes in pH.

3. RESULTS

Fig.1 shows that appreciable thrombin-induced platelet aggregation occurs in an Na^+-free medium. The presence of Na^+ in the medium increases the extent of aggregation but does not influence its rate. APA [5-*N*-(3-aminophenyl)-amiloride] is known to be a more potent inhibitor of Na^+/H^+ exchange in platelets than amiloride [7]. At 20 μM, a concentration that is 10-fold higher than its K_i for the inhibition of the Na^+/H^+ exchange in platelets [7], APA does not affect the aggregation in Na^+ medium. The same pattern of results was obtained for acetylsalicylic acid-treated platelets (not shown).

Shingosine is a specific inhibitor of protein kinase C [18]. Among its various effects the enzyme is known to control the Na^+/H^+ exchange system [6,19,20]. As shown in table 1, sphingosine effectively decreases the extent of thrombin-induced platelet aggregation, in both the presence and absence of extracellular Na^+.

Thrombin, at 0.1 U/ml and above, is known to potentiate both the platelet release reaction and aggregation. At lower concentrations the release reaction is activated to a lesser extent [21]. Na^+/H^+ exchange has been reported [10] to regulate phospholipase A_2 activation, which, by itself, controls release of free arachidonic acid, an essential step for the platelet release reaction. pH changes and platelet aggregation at different thrombin concentrations were assayed concomitantly in BCECF-loaded gel-filtered platelets under identical conditions of assay medium contents, platelet concentration and temperature (fig.2). The extent of activation of Na^+/H^+ exchange by thrombin can be appreciated by the impact of APA (lower curve of each pair in fig.2A). The difference between the two traces, rather than

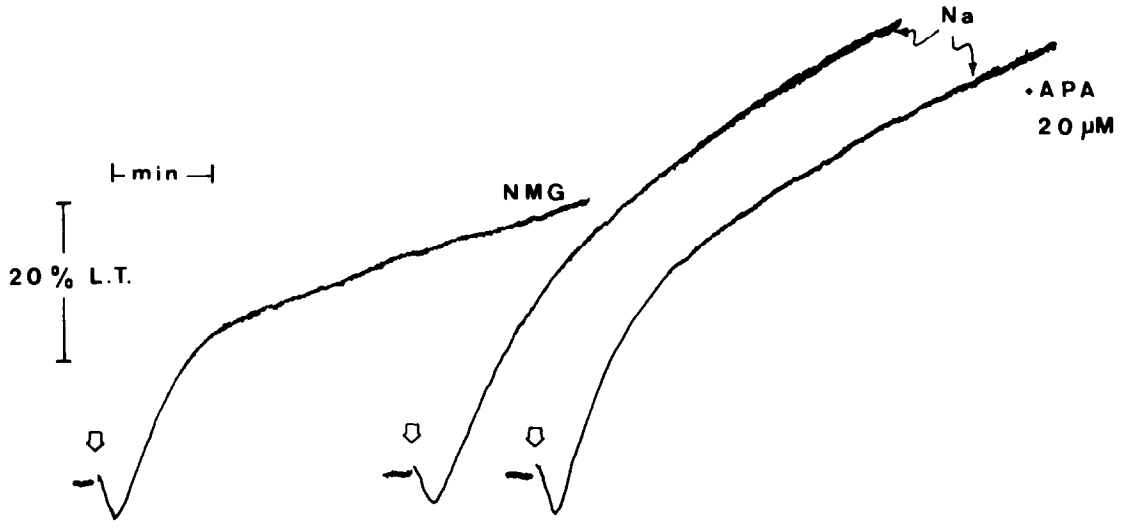

Fig.1. Effect of Na^+ and APA on thrombin-induced aggregation. Platelets (2.2×10^7/ml) gel-filtered in NMG^+ medium and suspended in NMG^+ or Na^+ media were incubated, where indicated, with 20 μM APA or, for control, with 0.1 mM HCl for 25 s at 37°C before the addition of 0.01 U/ml thrombin (arrow). The ordinate is the changes in light transmission (L.T.) as defined in section 2. The experiment shown is one of ten giving the same pattern of results. The same pattern was also obtained when higher thrombin concentrations, up to 2.2 U/ml, were used.

the individual traces, better reflects the impact of thrombin. Fig.2 clearly demonstrates that activation of the Na^+/H^+ exchange system is obvious at 0.1 U/ml thrombin (fig.2A, trace a), negligible at 0.01 U/ml (trace b) and absent at 0.005 U/ml (trace c) as the difference between the traces is nil. On the other hand, an appreciable activity of platelet aggregation is induced by 0.005 U/ml of thrombin (fig.2B, trace c). It is noteworthy that measurable aggregation was still obtained with even lower concentration of thrombin (0.003 U/ml, not shown). Fig.3 demonstrates that

Table 1

Effect of sphingosine on platelet aggregation

Aggregation medium	Inhibition (%) by sphingosine	
	2 μM	10 μM
Na^+	31 ± 6	100 ± 0
NMG^+	11 ± 7[a]	100 ± 0

[a] Not significantly different from the inhibition by 2 μM sphingosine in Na^+ medium

Aggregation was induced by 0.1 U/ml of thrombin. The data presented are means ± SE of two experiments, each in triplicate

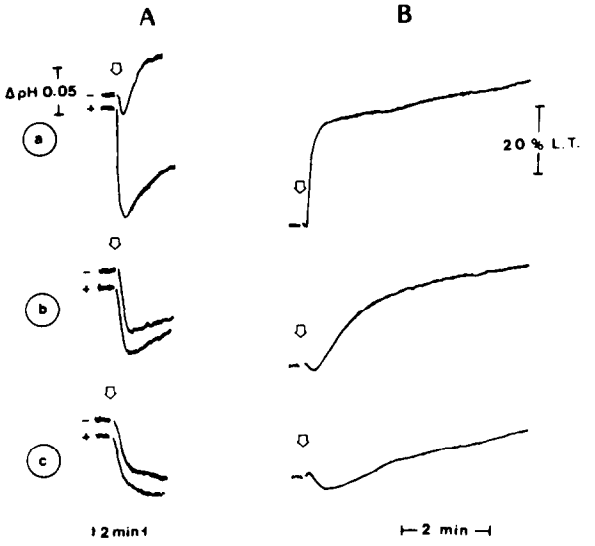

Fig.2. A comparison between changes in platelet pH_i and aggregation at different thrombin concentrations. (A) Changes in cytoplasmic pH recorded by the fluorescence signal from BCECF-loaded platelets in the absence (−) and presence (+) of 20 μM APA. (B) Aggregation traces of changes in light transmission (L.T.). Measurements were conducted concomitantly and under identical conditions. Platelets (2.1×10^7/ml) gel-filtered in Na^+ medium and suspended in Na^+ medium were activated by the following concentrations of thrombin (arrow): (a) 0.1 U/ml, (b) 0.01 U/ml, (c) 0.005 U/ml. A representative experiment out of five showing a similar pattern.

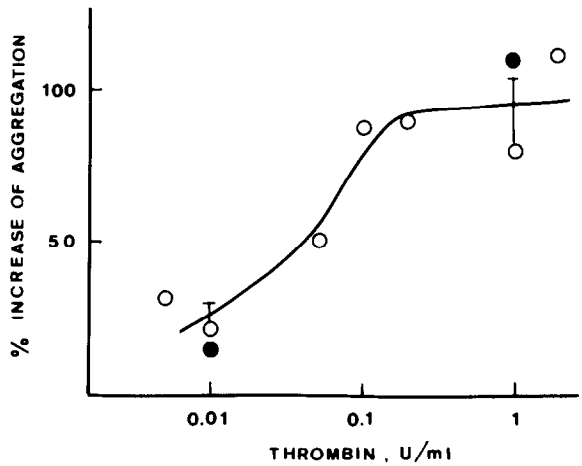

Fig.3. Increase of aggregation in Na^+ medium compared with NMG^+ medium as a function of thrombin concentration. For each thrombin concentration the change in light transmission (L.T.) after 5 min of aggregation in NMG^+ medium was referred to as 100% and the augmentation in Na^+ medium expressed as percent above it. Assay details as in fig.1. Each point is the average of 2–5 experiments. The bars represent SE of five experiments. (○) Untreated platelets, (●) acetylsalicylic acid-treated platelets.

the higher the concentration of thrombin the greater is the extent of potentiation of aggregation exerted by the presence of Na^+, until a plateau of the effect is reached. The same pattern was obtained with acetylsalicylic acid-treated platelets (fig.3, solid circles). 20 μM APA did not inhibit aggregation induced by any of the thrombin concentrations in either Na^+ or NMG media.

Aggregation in Na^+, NMG^+, K^+ and Li^+ media was compared in an attempt to elucidate the nature of the difference between the extent of aggregation in Na^+ and NMG^+ media. As shown in fig.4, aggregation activities in Na^+ and K^+ media do not differ from each other, both being higher than the

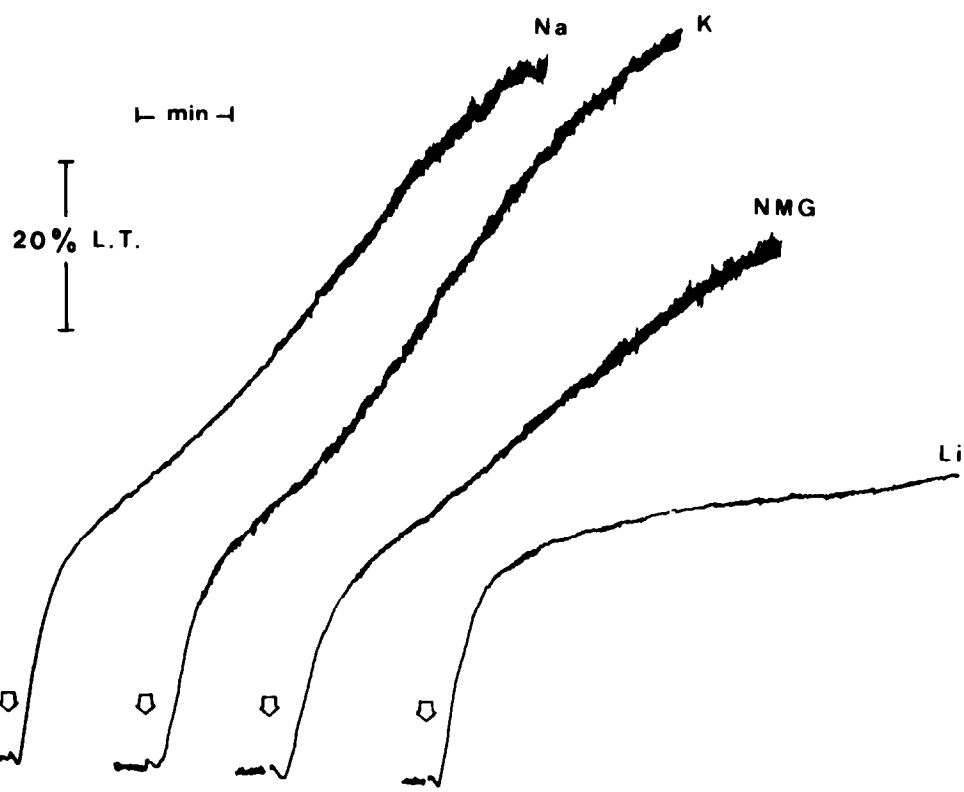

Fig.4. Thrombin-induced platelet aggregation in four different media. Platelets (2.0×10^7/ml), gel-filtered in NMG^+ medium and suspended (1:9, v/v) in Li^+, Na^+, K^+ or NMG^+ media were activated with 0.1 U/ml thrombin. Other assay details as in fig.1. The experiment shown is one of three with the same pattern of results.

aggregation in NMG^+ medium. Interestingly, aggregation in Li^+ medium was the lowest.

4. DISCUSSION

Na^+/H^+ exchange has been suggested to play a dominant role in numerous functions of stimulated human platelets including aggregation [3,22], arachidonic acid mobilization [23], phospholipase A_2 activation [10,24], Ca^{2+} mobilization [4,5,25], active transport of serotonin [22] and indomethacin-sensitive secretion [26–28]. Thrombin-induced cytoplasmic alkalinization due to Na^+/H^+ activation is a well-established phenomenon [3,4,6,10–12,14,29,30]. However, whether Na^+/H^+ activation is a prerequisite for Ca^{2+} mobilization leading to aggregation remains under debate [3–14]. If Na^+/H^+ exchange activity is obligatory for aggregation, then aggregation should not occur in Na^+-free medium and should be inhibited by specific inhibitors of the exchanger. Indeed, inhibition of platelet aggregation [3,7,8,22,23] as well as of Ca^{2+} mobilization [4,5,8] by amiloride or its derivatives has been shown. Similar effects were observed when Na^+ was omitted from the medium [4,5,12,23]. Yet, the data in NMG^+ medium in the present study, as well as in other work [23], clearly demonstrate that the absence of Na^+ from the medium only diminishes but does not abolish thrombin-induced platelet aggregation. Moreover, when Na^+ is replaced by K^+, rather than NMG^+, aggregation is not at all affected. It is also shown that APA, at a concentration over 10-fold higher than its K_i for the Na^+/H^+ exchange, does not exert any inhibition on thrombin-induced platelet aggregation. If activation of the Na^+/H^+ exchange is a crucial step in platelet aggregation, then any thrombin concentration that stimulates aggregation should also potentiate the exchanger. This expectation was also ruled out in the present study.

Protein kinase C, known to be involved in the regulation of Na^+/H^+ antiport activity, is specifically inhibited by sphingosine [31]. If Na^+/H^+ exchange activation is an essential step in the cascade of events leading to aggregation, then aggregation should be inhibited by sphingosine. The present results do show inhibition of aggregation by this inhibitor, but the inhibition is also obtained in Na^+ free medium, in which Na^+/H^+ activity cannot take place. Therefore, these results apparently implicate the involvement of protein kinase C in the mechanism of platelet aggregation, but not via the Na^+/H^+ exchanger. Li^+, but not K^+, may substitute for Na^+ as a substrate for the Na^+/H^+ exchange in platelets [1]. Therefore, if the activation of the exchanger is needed for aggregation, Li^+ medium, but not K^+ medium, should enable aggregation to occur. However, the results obtained show the inverse pattern, namely, aggregation in K^+ medium is as high as in Na^+ medium, while it is much lower in Li^+ medium.

In summary, the following aspects contradict the proposition that Na^+/H^+ exchange activation is a prerequisite for thrombin-induced platelet aggregation: (i) aggregation occurs in Na^+-free medium; (ii) aggregation is induced by thrombin concentrations that do not stimulate Na^+/H^+ exchange; (iii) aggregation is not inhibited by 5-*N*-(3-aminophenyl)amiloride, a potent and specific inhibitor of the exchanger; (iv) aggregation is optimal in K^+ medium but markedly inhibited in Li^+ medium; and (v) aggregation is inhibited by sphingosine in Na^+-free medium similarly to Na^+ medium. It is thus concluded that, as already shown for ADP [5], the following holds true for thrombin as well: the inducers activate the Na^+/H^+ exchange in platelets but the exchange is not required for aggregation.

Intriguingly, Na^+ appears to affect thrombin-induced platelet aggregation. As shown, aggregation in Na^+ medium is significantly higher than in NMG^+ medium. Moreover, the higher the concentration of thrombin, the greater is the increase of aggregation caused by Na^+, yet no effect of APA is observed. Na^+ had been previously implicated as an effector of platelet activities such as serotonin transport [32] and the α_2-adrenergic receptor system [33,34]. It may therefore be suggested that Na^+ transport systems, other than the Na^+/H^+ exchanger, play a role in thrombin-induced platelet aggregation; the higher the thrombin concentration, the greater is the stimulation of these Na^+ transport systems. It was of interest to study the nature of the Na^+ effect on thrombin-induced platelet aggregation. This issue was addressed by the comparison of four cations as the major constituents of the aggregation medium: the alkali series Li^+, Na^+ and K^+ and the organic cation NMG^+. Aggregation exhibited the same pattern and

magnitude in Na^+ and K^+ media indicating that membrane potential does not play a major role in the aggregation pattern observed. The lower aggregation in NMG^+ medium relative to Na^+ and K^+ media suggests that NMG^+ is inhibitory for thrombin-induced platelet aggregation. Thus, the apparent stimulatory effect of Na^+ may be misleading, as it appears higher only relatively to an inhibited state. The inhibitory effect of Li^+ has not been documented previously and requires further elucidation.

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