

# Thrombin-induced platelet aggregation is affected by external $\text{Na}^+$ independently of the $\text{Na}^+/\text{H}^+$ exchange

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Thrombin affects blood platelets by activation of  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+$  medium than in *N*-methylglucamine $^+$  medium and is inhibited by sphingosine, an inhibitor of protein kinase C known to regulate the  $\text{Na}^+/\text{H}^+$  exchanger. However, the inhibition of aggregation by sphingosine occurs in both  $\text{Na}^+$ -containing and  $\text{Na}^+$ -free media, the aggregation is identical in  $\text{Na}^+$  and  $\text{K}^+$ -containing media, and is not inhibited by 5-*N*-(3-aminophenyl)amiloride, at a concentration 10-fold higher than its  $K_i$  for platelet  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+/\text{H}^+$  exchange is not required for thrombin-induced platelet aggregation and that the apparent augmentation of aggregation by  $\text{Na}^+$  is due to an inhibitory effect of *N*-methylglucamine $^+$ .

Platelet; Thrombin; Aggregation;  $\text{Na}^+/\text{H}^+$  exchange

## 1. INTRODUCTION

The plasma membrane  $\text{Na}^+/\text{H}^+$  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  $\text{pH}_i$  is reduced [2]. It is well established that stimulation of platelets by thrombin is accompanied by activation of the  $\text{Na}^+/\text{H}^+$  exchanger, resulting in cytoplasmic alkalinization [3–7]. However, there has recently been debate in the literature as to whether activation of the  $\text{Na}^+/\text{H}^+$  exchanger is a prerequisite for  $\text{Ca}^{2+}$  mobilization and/or platelet secretion leading to aggregation [3–14]. We have addressed this disagreement in view of an observation that external  $\text{Na}^+$  potentiates thrombin-induced platelet aggregation. It became of interest to ex-

amine whether the role of external  $\text{Na}^+$  is related to the operation of  $\text{Na}^+/\text{H}^+$  exchange.

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

## 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  $\text{Na}^+$  medium contained (in mM): 140 NaCl, 5 KCl, 0.42  $\text{NaH}_2\text{PO}_4$ , 10 glucose and 20 Hepes (pH 7.35).  $\text{K}^+$ ,  $\text{Li}^+$  and *N*-

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methylglutamine<sup>+</sup> (NMG<sup>+</sup>) 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<sub>2</sub>. The osmolality 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<sup>+</sup> 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 ( $0.7 \mu\text{g/ml} = 0.87 \mu\text{M}$ , final) for 30 min at 22–23°C. The platelets were then gel-filtered through a Sepharose 2B column ( $6 \times 0.76 \text{ cm}$ ). The solution used to equilibrate the column and to elute the platelets was the Na<sup>+</sup> medium or, where specified, the NMG<sup>+</sup> 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<sup>+</sup> 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<sub>i</sub> changes in BCECF-loaded platelets was composed of the Na<sup>+</sup> 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<sup>+</sup> 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<sub>i</sub> 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<sup>+</sup>-free medium. The presence of Na<sup>+</sup> 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<sup>+</sup>/H<sup>+</sup> 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<sup>+</sup>/H<sup>+</sup> exchange in platelets [7], APA does not affect the aggregation in Na<sup>+</sup> 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<sup>+</sup>/H<sup>+</sup> 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<sup>+</sup>.

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<sup>+</sup>/H<sup>+</sup> exchange has been reported [10] to regulate phospholipase A<sub>2</sub> 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<sup>+</sup>/H<sup>+</sup> 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

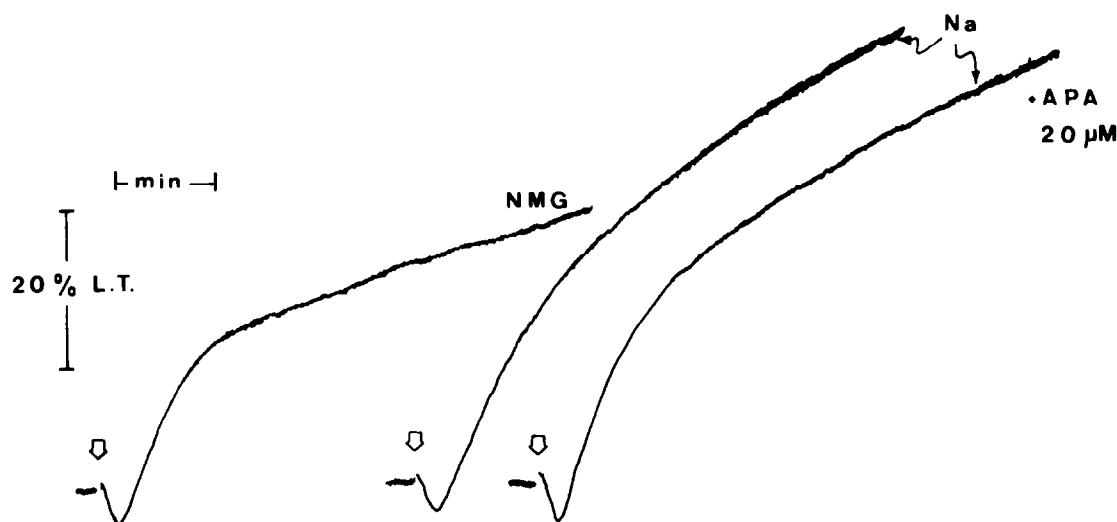


Fig.1. Effect of  $\text{Na}^+$  and APA on thrombin-induced aggregation. Platelets ( $2.2 \times 10^7/\text{ml}$ ) gel-filtered in  $\text{NMG}^+$  medium and suspended in  $\text{NMG}^+$  or  $\text{Na}^+$  media were incubated, where indicated, with  $20 \mu\text{M}$  APA or, for control, with  $0.1 \text{ mM}$   $\text{HCl}$  for  $25 \text{ s}$  at  $37^\circ\text{C}$  before the addition of  $0.01 \text{ U/ml}$  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  $2.2 \text{ U/ml}$ , were used.

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

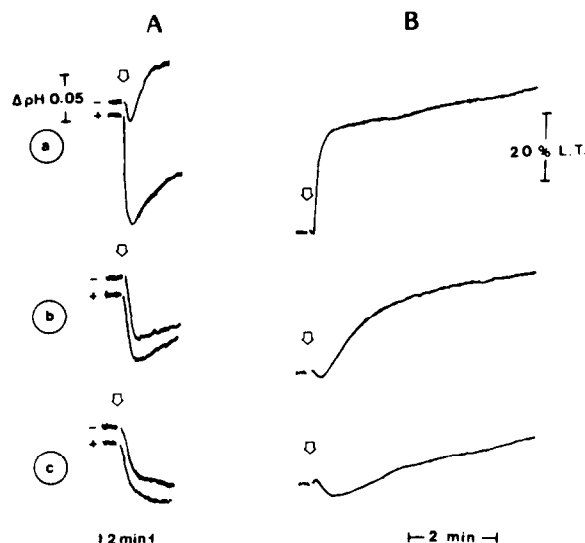


Fig.2. A comparison between changes in platelet  $\text{pH}_i$  and aggregation at different thrombin concentrations. (A) Changes in cytoplasmic  $\text{pH}$  recorded by the fluorescence signal from BCECF-loaded platelets in the absence (—) and presence (+) of  $20 \mu\text{M}$  APA. (B) Aggregation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

Table 1

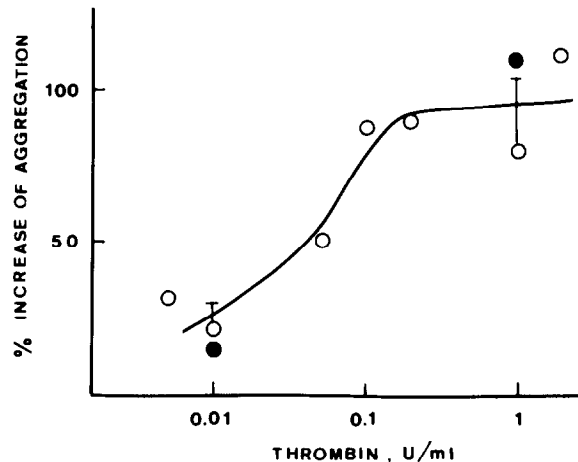
Effect of sphingosine on platelet aggregation

Aggregation medium	Inhibition (%) by sphingosine	
	$2 \mu\text{M}$	$10 \mu\text{M}$
$\text{Na}^+$	$31 \pm 6$	$100 \pm 0$
$\text{NMG}^+$	$11 \pm 7^a$	$100 \pm 0$

<sup>a</sup> Not significantly different from the inhibition by  $2 \mu\text{M}$  sphingosine in  $\text{Na}^+$  medium

Aggregation was induced by  $0.1 \text{ U/ml}$  of thrombin. The data presented are means  $\pm$  SE of two experiments, each in triplicate

Fig.3. Increase of aggregation in  $\text{Na}^+$  medium compared with  $\text{NMG}^+$  medium as a function of thrombin concentration. For each thrombin concentration the change in light transmission (L.T.) after 5 min of aggregation in  $\text{NMG}^+$  medium was referred to as 100% and the augmentation in  $\text{Na}^+$  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  $\text{Na}^+$ , until a plateau of the effect is reached. The same pattern was obtained with acetylsalicylic acid-treated platelets (fig.3, solid circles).  $20 \mu\text{M}$  APA did not inhibit aggregation induced by any of the thrombin concentrations in either  $\text{Na}^+$  or  $\text{NMG}$  media.

Aggregation in  $\text{Na}^+$ ,  $\text{NMG}^+$ ,  $\text{K}^+$  and  $\text{Li}^+$  media was compared in an attempt to elucidate the nature of the difference between the extent of aggregation

in  $\text{Na}^+$  and  $\text{NMG}^+$  media. As shown in fig.4, aggregation activities in  $\text{Na}^+$  and  $\text{K}^+$  media do not differ from each other, both being higher than the

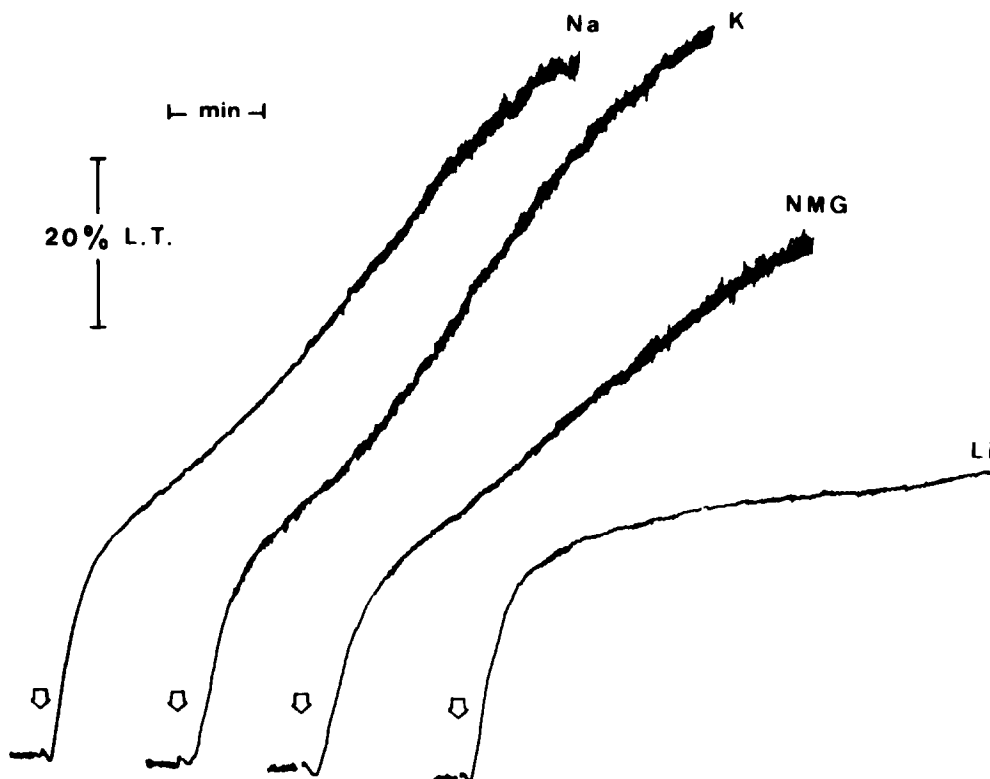


Fig.4. Thrombin-induced platelet aggregation in four different media. Platelets ( $2.0 \times 10^7/\text{ml}$ ), gel-filtered in  $\text{NMG}^+$  medium and suspended (1:9, v/v) in  $\text{Li}^+$ ,  $\text{Na}^+$ ,  $\text{K}^+$  or  $\text{NMG}^+$  media were activated with  $0.1 \text{ U/ml}$  thrombin.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

aggregation in  $\text{NMG}^+$  medium. Interestingly, aggregation in  $\text{Li}^+$  medium was the lowest.

#### 4. DISCUSSION

$\text{Na}^+/\text{H}^+$  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  $\text{A}_2$  activation [10,24],  $\text{Ca}^{2+}$  mobilization [4,5,25], active transport of serotonin [22] and indomethacin-sensitive secretion [26–28]. Thrombin-induced cytoplasmic alkalinization due to  $\text{Na}^+/\text{H}^+$  activation is a well-established phenomenon [3,4,6,10–12,14,29,30]. However, whether  $\text{Na}^+/\text{H}^+$  activation is a prerequisite for  $\text{Ca}^{2+}$  mobilization leading to aggregation remains under debate [3–14]. If  $\text{Na}^+/\text{H}^+$  exchange activity is obligatory for aggregation, then aggregation should not occur in  $\text{Na}^+$ -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  $\text{Ca}^{2+}$  mobilization [4,5,8] by amiloride or its derivatives has been shown. Similar effects were observed when  $\text{Na}^+$  was omitted from the medium [4,5,12,23]. Yet, the data in  $\text{NMG}^+$  medium in the present study, as well as in other work [23], clearly demonstrate that the absence of  $\text{Na}^+$  from the medium only diminishes but does not abolish thrombin-induced platelet aggregation. Moreover, when  $\text{Na}^+$  is replaced by  $\text{K}^+$ , rather than  $\text{NMG}^+$ , 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  $\text{Na}^+/\text{H}^+$  exchange, does not exert any inhibition on thrombin-induced platelet aggregation. If activation of the  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+/\text{H}^+$  antiport activity, is specifically inhibited by sphingosine [31]. If  $\text{Na}^+/\text{H}^+$  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  $\text{Na}^+$  free medium, in which  $\text{Na}^+/\text{H}^+$

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  $\text{Na}^+/\text{H}^+$  exchanger.  $\text{Li}^+$ , but not  $\text{K}^+$ , may substitute for  $\text{Na}^+$  as a substrate for the  $\text{Na}^+/\text{H}^+$  exchange in platelets [1]. Therefore, if the activation of the exchanger is needed for aggregation,  $\text{Li}^+$  medium, but not  $\text{K}^+$  medium, should enable aggregation to occur. However, the results obtained show the inverse pattern, namely, aggregation in  $\text{K}^+$  medium is as high as in  $\text{Na}^+$  medium, while it is much lower in  $\text{Li}^+$  medium.

In summary, the following aspects contradict the proposition that  $\text{Na}^+/\text{H}^+$  exchange activation is a prerequisite for thrombin-induced platelet aggregation: (i) aggregation occurs in  $\text{Na}^+$ -free medium; (ii) aggregation is induced by thrombin concentrations that do not stimulate  $\text{Na}^+/\text{H}^+$  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  $\text{K}^+$  medium but markedly inhibited in  $\text{Li}^+$  medium; and (v) aggregation is inhibited by sphingosine in  $\text{Na}^+$ -free medium similarly to  $\text{Na}^+$  medium. It is thus concluded that, as already shown for ADP [5], the following holds true for thrombin as well: the inducers activate the  $\text{Na}^+/\text{H}^+$  exchange in platelets but the exchange is not required for aggregation.

Intriguingly,  $\text{Na}^+$  appears to affect thrombin-induced platelet aggregation. As shown, aggregation in  $\text{Na}^+$  medium is significantly higher than in  $\text{NMG}^+$  medium. Moreover, the higher the concentration of thrombin, the greater is the increase of aggregation caused by  $\text{Na}^+$ , yet no effect of APA is observed.  $\text{Na}^+$  had been previously implicated as an effector of platelet activities such as serotonin transport [32] and the  $\alpha_2$ -adrenergic receptor system [33,34]. It may therefore be suggested that  $\text{Na}^+$  transport systems, other than the  $\text{Na}^+/\text{H}^+$  exchanger, play a role in thrombin-induced platelet aggregation; the higher the thrombin concentration, the greater is the stimulation of these  $\text{Na}^+$  transport systems. It was of interest to study the nature of the  $\text{Na}^+$  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  $\text{Li}^+$ ,  $\text{Na}^+$  and  $\text{K}^+$  and the organic cation  $\text{NMG}^+$ . Aggregation exhibited the same pattern and

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

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