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Na^+/H^+ exchange and aggregation of human platelets activated by ADP: the exchange is not required for aggregation

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Isolated human blood platelets, loaded with the pH-sensitive fluorescence dye 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein show cytoplasmic alkalinization upon stimulation with thrombin but acidification with ADP stimulation. In both cases a Na^+/H^+ exchange is activated. This can be revealed by sensitivity of the induced pH changes to amiloride and to 5-*N*-(3-aminophenyl)amiloride (APA), known inhibitors of the Na^+/H^+ exchanger, and by a dependence on sodium in the external medium. ADP-induced platelet aggregation is not affected by omission of sodium from the external medium. Furthermore, aggregation is barely inhibited (less than 10%) by amiloride or APA at concentrations up to 50 μM while the K_i values in affecting the Na^+/H^+ exchange are 5.9 and 1.6 μM for amiloride and APA, respectively. Platelet aggregation is inhibited by amiloride or APA at concentrations higher than 50 μM, but this inhibition is apparently due to a secondary effect of the agents. It is concluded that platelet aggregation induced by ADP is not dependent on activation of Na^+/H^+ exchange.

Introduction

Na^+/H^+ exchange is present in human blood platelets. This exchange system appears to play a significant role in platelet functions, as illustrated by many studies. (a) The exchanger is considerably activated when the cytoplasm is acidified [1,2]. This behavior apparently reflects its role in intracellular pH (pH_i) homeostasis [3] and is probably determined by a pH_i-sensitive allosteric activator or 'modifier' site [4,5]. (b) Activation of Na^+/H^+ exchange is observed in platelets stimulated by thrombin [3,6–9], epinephrine [10,11], Ca^{2+} ionophore A23187 [12] as well as by a phorbol ester and a diacylglycerol [13,8]. Recently it has been noted that Na^+/H^+ exchange is activated in osmotically swollen platelets [14]. Suppression of Na^+/H^+ exchange either by removal of extracellular Na^+ or by application of amiloride is reported to inhibit shape change, secretion of granule contents and aggregation [6,12,15,16]. (c) Activation of Na^+/H^+ exchange is reported to function in Ca^{2+} mobilization in platelets: both an increase in intracellular pH_i and an increase in intracellular inositol 1,4,5-trisphosphate (IP_3) are simultaneously required for Ca^{2+} mobilization [9,17]. (d) The Na^+/H^+ exchange regulates receptor-mediated phospholipase A_2 activation in platelets [18]. Furthermore, Na^+/H^+ exchange appears to selectively modulate arachidonic acid mobilization in response to 'weak agonists' (ADP, epinephrine and thrombin

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Abbreviations: APA, 5-*N*-(3-aminophenyl)amiloride. BCECF, 2,′7′-bis(carboxyethyl)-5,6-carboxyfluorescein; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid.

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at low concentration) which subsequently effects vigorous aggregation and dense granule secretion [19]. (e) The activity of the exchange is correlated with the clinical manifestations of hypertension [20].

The objective of the present study is to examine the relationship between activation of platelets by ADP and the Na^+/H^+ exchange. Specifically, our question is: does ADP lead to activation of Na^+/H^+ exchange in platelets, as observed for other inducers? No direct evidence for the activation of platelet Na^+/H^+ exchange by ADP has thus far been presented, but such activation could account for the observation that stimulation of platelets by ADP results in an uptake of Na^+ ions [21,22]. If Na^+/H^+ exchange is activated our subsequent question is: does this exchange play a role in platelet aggregation induced by ADP?

Part of this study has been presented in an abstract form [23].

Materials and Methods

Reagents

Propionic acid, monensin, albumin (bovine, essentially fatty acid-free, from fraction V albumin), nigericin and ADP were from Sigma. 2′,7′-Bis-carboxyethyl)-5,6-carboxyfluorescein (BCECF) acetoxymethyl ester was from Molecular Probes. Amiloride was from Merck, Sharp and Dohme, 5-*N*-(3-aminophenyl)amiloride (APA) [24] was a gift from Dr. D. Cassel. Fibrinogen (human, 97% clottable) was from Cutter, Berkeley, CA. Heparin (Thromboliquine) was from Organon, Oss, Holland. Bovine thrombin from Parke-Davis, Detroit, MI was used in this study. The same results were obtained with highly purified human thrombin (99.5%, from Dr. J.W. Fenton II).

Solutions

Acid-citrate-dextrose solution (buffer 1) was composed of 65 mM citric acid, 11 mM glucose and 85 mM trisodium citrate. The standard NaCl medium contained (in mM): 140 NaCl, 5 KCl, 0.42 NaH_2PO_4, 1 $MgCl_2$, 10 glucose and 20 Hepes (pH 7.35). KCl- and *N*-methylglucamine · HCl media were prepared by isoosmotic replacement of NaCl by the indicated salts. *N*-Methylglucamine · HCl was prepared by titrating *N*-methyl-D-glucamine to neutrality with HCl. Sodium propionate solution contained (in mM): 140 sodium propionate, 1 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 20 Hepes (pH 6.5). 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 in dimethylsulfoxide, respectively. Determination of sodium in the media were performed with a Corning Flame photometer.

Preparation of platelet suspension

Venous blood was drawn from healthy volunteers, aged 25–58 years, who had been without medication the previous 10 days. The blood was anticoagulated with buffer 1 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. Heparin (50 U/ml) was added and the platelets were 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 cm × 0.76 cm). The solution used to equilibrate the column and to elute the platelets was the NaCl medium or, where specified, the *N*-methylglucamine medium described above, but modified as follows: $MgCl_2$ was omitted, albumin (1 mg/ml) and heparin (50 U/ml) were added and the pH was adjusted to 6.8.

Determination of cytoplasmic pH changes

Changes in cytoplasmic pH were determined essentially as described earlier [2,25]. Fluorescence was measured in a FP-770 Jasco spectrofluorometer with wavelength setting 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 NaCl medium, unless otherwise noted, fortified with 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mg/ml albumin. The measurements were performed at 22–23° C in a plastic cuvette using $4–6 \cdot 10^7$ platelets/ml and a total volume of 1.8 ml with continuous stirring. Fibrinogen (200 μg/ml) was also added when

ADP was used as an inducer, unless stated otherwise. Calibration of the pH versus fluorescence was performed according to Thomas et al. [26], in the standard medium free of sodium but containing 100 mM KCl and 40 mM *N*-methylglucamine with 1.6 μM nigericin, and 200 μM amiloride, using Mops as titrant. The leak of BCECF [25] in the first hour, during which the experiments were done, amounted to 15%. The pH_i of untreated gel-filtered platelets was 7.32 (S.E. = 0.02, $n = 8$), similarly to an earlier report [2]. The data are presented as changes in pH.

Determination of Na^+/H^+ exchange by cell sizing

The activity of the Na^+/H^+ exchanger was measured indirectly as changes in platelet volume in acid-loaded cells, using sodium propionate, essentially as described previously [1,2]. Volume measurements were conducted by electronic sizing, using a Coulter counter model ZM with a Channelyzer 256. Orifice diameter was 70 μm. An aliquot of the platelet suspension was diluted to 120000 platelets per ml of the medium. The mean cell volume was calculated from the volume distribution curves using polystyrene latex beads for calibration. Measurements were conducted at 22–23°C in triplicates. Volume determinations for each platelet sample were taken at 10–30 s intervals. The Na^+/H^+ exchange rates were calculated from the initial linear rate of swelling in sodium propionate medium. The small passive swelling component (5 to 10% increment over isotonic value) due to penetration of propionic acid was determined in sodium propionate medium containing 200 μM amiloride to prevent Na^+/H^+ exchange. This value was subtracted from all the determinations.

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 (platelet-rich plasma or gel-filtered platelets) and the media used were as specified in Results. 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 results 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.

Results

ADP- and thrombin-induced changes in platelet pH

pH changes in the cytoplasm of human platelets can be followed by recording the fluorescent signal of platelets loaded with the pH-sensitive dye BCECF [2,3,9]. We have used this technique to investigate the effect of ADP and thrombin on cytoplasmic pH of human platelets. Traces A and C (solid line) of Fig. 1 illustrate in two platelet samples that the addition of 10 μM ADP caused an immediate decrease in fluorescence showing that the cytoplasm became more acidic, rather

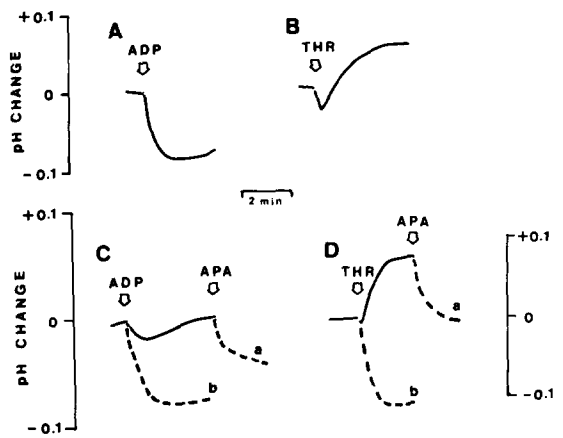

Fig. 1. ADP- and thrombin-induced changes in platelet pH. Examples are given of the changes in cytoplasmic pH by recording the fluorescence signal from BCECF-loaded platelets. The pH changes are induced by 10 μM ADP or 0.1 U/ml of thrombin added to the platelet suspensions at the time indicated by the arrows. The dashed lines in C and D show the effect of APA. In the traces marked a, APA (20 μM) was added after the pH changes inititated by thei nducers had levelled off; in traces marked b, APA (20 μM) was added to the platelet suspension one minute prior to the inducer. The ordinates give the changes in intraplatelet pH, a negative value indicating an acidification.

than the alkalinization expected on stimulation of Na^+/H^+ exchange. This acidification reached its maximal value in about one minute. In the following minute the pH became stable at the low value or started to return towards the original value. This finding is in apparent contrast to the pH changes induced by thrombin as described by Zavoico et al. [3] and Siffert and Akkerman [9] who have reported that thrombin induces an alkalinization due to an activation of the Na^+/H^+ exchange. The absence of ADP-induced alkalinization found in our experiments might be due to a loss of Na^+/H^+ exchange activity during the preparation of the platelets or because ADP, in contrast to other inducers of aggregation, does not activate the Na^+/H^+ exchanger. To test the first possibility the effect of thrombin on pH_i changes was studied in the same platelet preparations as used for the ADP experiments. Fig. 1 B and D (solid line) show that as noted in previous reports [3,9] thrombin-stimulated platelets responded by cytoplasmic alkalinization. As illustrated, this alkalinization either started immediatley or it followed an initial acidification, which was of variable extent and duration.

The described pH changes were obtained in nominally bicarbonate free solutions. The addition of 10 mM bicarbonate to the platelet suspensions 30 minutes before ADP or thrombin, to ensure equilibration of HCO_3^-/CO_2, did not alter the pattern of the described pH changes. In some of the experiments, however, the presence of bicarbonate dampened the changes in pH about 20% (not shown).

ADP-induced acidification in the presence of inhibitors of Na^+/H^+ exchange

To examine the possibility that activation of the exchanger by ADP was masked by the initial acidification, we followed ADP-induced changes in pH_i in the presence of inhibitors of the Na^+/H^+ exchange mechanism. Amiloride and aminophenylamiloride (APA), a more potent derivative, were used for this purpose. These compounds alone did not cause noticeable changes in pH_i. Trace Cb in Fig. 1 (dashed line) shows that when APA was added to the platelet suspension one minute before ADP the decrease in pH_i was substantially greater. When APA was added to the platelet suspension after the ADP-induced decline in pH_i had levelled off (trace Ca), a further, immediate acidification was initiated. These findings indicate that ADP activates the Na^+/H^+ exchanger but that its expression is masked by the initial acidification process and thus a net alkalinization is not observed, as is the case with thrombin. Fig. 1 D (dashed lines) show the effect of APA in thrombin-stimulated platelets. APA prevented the alkalinization when added prior to thrombin (trace b) and it caused a drop in pH_i when added to the medium after the thrombin-induced alkalinization had levelled off (trace a). The activation of the Na^+/H^+ exchange appears to be greater with thrombin than with ADP, so that net alkalinization is observed.

Some variability in the ADP response is evident from the two examples given in Fig. 1. Table I summarizes the results obtained with ADP in 11 paired experiments with and without APA. In all experiments ADP initiated a cytoplasmic acidification which averaged 0.05 pH units after 1 minute. The presence of APA caused a further decline in pH in all assays averaging about 0.03 pH units, (termed ΔpH). Both the ADP-induced acidification and the increment in acidification that is APA-inhibitable are statistically significant ($P < 0.005$). APA was used in the following concentrations: 4.4, 10, 20 and 44 μM. Because of a rather big interindividual scatter in the ADP-induced acidification in platelets from different donors the data do not allow a calculation of a

TABLE I

INTRAPLATELET pH CHANGES INDUCED BY 10 μM ADP IN THE ABSENCE AND PRESENCE OF APA IN THE MEDIUM

Negative values indicate an acidification of the platelet cytoplasm.

APA (μM)	ADP-induced pH change (mean $\pm$ S.E., $n = 11$)	
	1 min	2 min
0	-0.054 ± 0.011 [a]	-0.051 ± 0.010 [a]
4–44	-0.080 ± 0.014	-0.093 ± 0.016
ΔpH	0.026 ± 0.004 [b]	0.042 ± 0.016 [b]

[a] $P < 0.005$, compared to pH values prior to the addition of ADP.

[b] $P < 0.005$.

dose-dependent relation between the APA concentration and ΔpH. Amiloride at 200 μM caused a similar extended drop in pH_i ($P < 0.005$).

Additional indication that ADP induces a Na^+/H^+ exchange was apparent in experiments conducted in *N*-methylglucamine medium. Under such conditions, ADP caused a two to three times greater acidification than that observed in sodium containing medium with APA (results not shown).

Na^+/H^+ exchange and platelet aggregation

Siffert et al. [27] observed that amiloride inhibits ADP-induced aggregation of platelet-rich plasma. The results shown in Fig. 2 appear to support this observation, as evaluated by several characteristics of aggregation measurement: rate (slope of the aggregation curve), extent (total change in light transmission within 4 minutes) and size of aggregates (magnitude of the oscillations). The shape change, however, was not affected by the amiloride treatment.

It is unlikely, however, that the sensitivity of aggregation to amiloride, as presented in Fig. 2, indicates the involvement of Na^+/H^+ exchange in aggregation for several reasons. Fig. 3 shows that

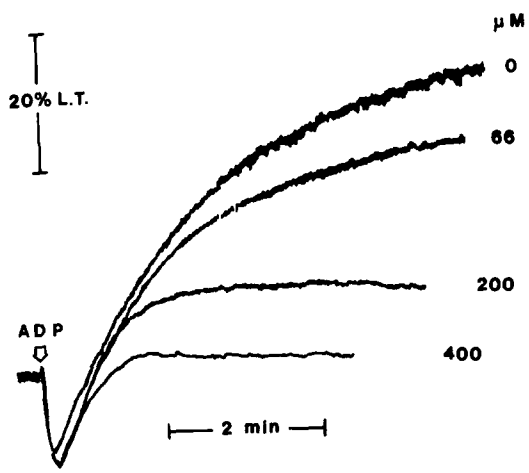

Fig. 2. Effect of amiloride on ADP-induced aggregation. Platelets ($2.2 \cdot 10^7$/ml) suspended in sodium media were incubated with amiloride (the concentration is indicated at the right side of each trace) for 5 min at 37 °C before the addition of 10 μM ADP. The ordinate is the change in light transmission (LT) as defined in Materials and Methods. The experiment shown was one of three performed with platelet-rich plasma. Similar results were also obtained with gel-filtered platelets.

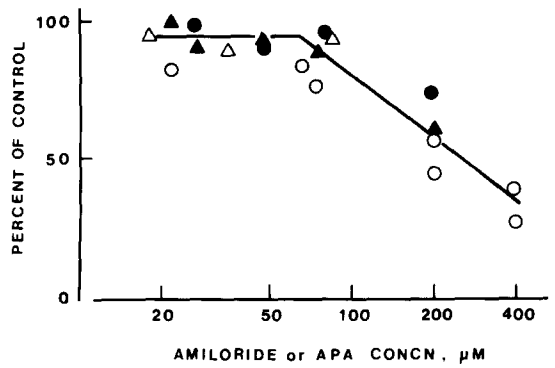

Fig. 3. ADP-induced aggregation in the presence of inhibitors of Na^+/H^+ exchange. Aggregation, induced by 10 μM ADP, was determined by the change in light transmission 4 min after the addition of ADP. The control values (without added inhibitor) were taken as 100%. The results were compiled from four platelet preparations from three donors. Three preparations were obtained as described in Materials and Methods. In one preparation the platelet-rich plasma was obtained from blood anticoagulated with 3.8% (w/v) trisodium citrate with a volume ratio of blood/anticoagulant of 9:1. All experiments were performed in sodium medium and the number of platelets was: platelet-rich plasma $2.2 \cdot 10^7$/ml, gel-filtered platelets $7 \cdot 10^7$/ml. Symbols used: Amiloride/platelet-rich plasma, ○; amiloride/gel-filtered platelets, ●; APA/platelet-rich plasma, △; APA/gel-filtered platelets, ▲.

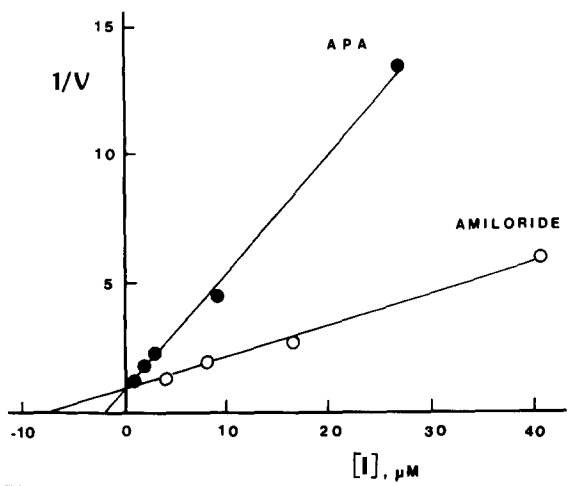

Fig. 4. Inhibitory effect of amiloride and APA on Na^+/H^+ exchange. Dixon plot showing the inhibitory effect of amiloride and APA on the Na^+/H^+ exchange measured by the cell sizing technique [2,44]. V is calculated as the slope of the initial linear rate of swelling in sodium propionate medium (pH 6.5). [I] is the concentration of the inhibitors. Lines were fitted by least squares method and the K_i for amiloride and APA was in this experiment determined to be 5.8 μM and 1.8 μM, respectively, with a correlation coefficient for both lines of 0.99. Representative of three similar experiments performed with platelet-rich plasma.

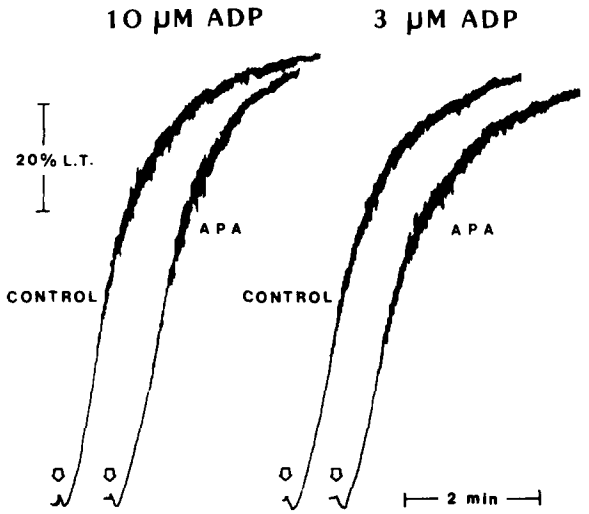

Fig. 5. Effect of APA on ADP-induced aggregation. Gel-filtered platelets ($7 \cdot 10^7$/ml) were incubated at 37° C with or without 20 μM APA for 5 min prior to the addition of ADP (arrow). Ordinate as in Fig. 2.

ADP-induced aggregation was affected similarly by amiloride and APA, when either platelet-rich plasma or gel-filtered platelets were tested. Furthermore, the effective concentrations of these Na^+/H^+ exchange inhibitors required to inhibit aggregation were found to be above 50 μM. Fig. 4 shows that much lower concentrations were required to inhibit the Na^+/H^+ exchange rate of these platelets and that the two inhibitors clearly differed in potency: The K_i values determined from a Dixon plot [28] were 5.9 ± 0.2 (S.D.) and 1.6 ± 0.2 (S.D.) μM for amiloride and APA, respectively, as tested by the cell sizing technique ($n = 3$).

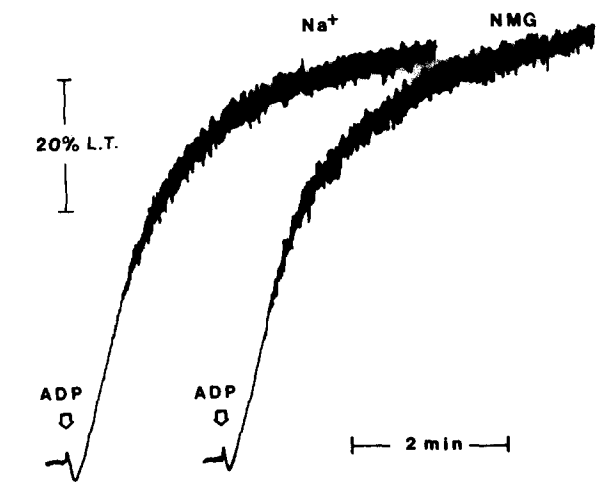

Fig. 6. ADP-induced aggregation in sodium- and *N*-methylglucamine media (NMG). Platelets were gel-filtered in *N*-methylglucamine medium and assayed at $7.3 \cdot 10^7$/ml in either sodium (106 mM) or *N*-methylglucamine medium ($[Na]_o = 0.3$ mM). 10 μM ADP was added (arrows). Ordinate as described in Fig. 2. Similar results were obtained in three additional experiments.

Fig. 5 shows that APA in the concentration of 20 μM, that is over 10 times the K_i value for inhibition of Na^+/H^+ exchange, did not change either the rate or the extent of aggregation nor the size of the aggregates when 10, 3 or 1 μM ADP was added (trace with 1 μM ADP not shown).

If Na^+/H^+ exchange plays a role in platelet aggregation, the aggregation should be dependent on external sodium. To test this possibility platelets were gel-filtered in *N*-methylglucamine medium and then assayed for aggregation in either sodium- or *N*-methylglucamine-containing media. Fig. 6 shows that a similar aggregation pattern was obtained in sodium medium ($[Na]_o = 106$ mM) and in *N*-methylglucamine medium ($[Na]_0 = 0.3$ mM).

Discussion

A major finding of this study is that ADP activates Na^+/H^+ exchange in human platelets but the exchange activated by ADP is not expressed by a net cytoplasmic alkalinization due to an overriding intracellular acidification. The activation of the exchange by ADP is revealed by the inhibitory effect of amiloride and its more potent analog APA and by the dependence of pH_i changes on external sodium.

The pH_i changes in platelets following the stimulation by thrombin and ADP seem to be different: thrombin induces an acidification that is brief and transient followed by a sustained alkalinization (Fig. 1B) as already reported [3]. In contrast, ADP induces primarily a sustained acidification (Fig. 1A), but a tendency for a reversal towards the initial pH_i was also observed (Fig. 1C). Biphasic pH_i changes have been observed in other cell systems, for example in vascular smooth muscle cells in which an activation of the Na^+/H^+

exchange by angiotensin II is responsible for the alkalinization phase [29,30].

Thrombin and ADP are known to differ in other actions on platelets. (a) The association between myosin and F-actin does not occur when platelets are stimulated with thrombin [31]. (b) Unlike thrombin, ADP causes very little release of the content of platelet granules in a Ca^{2+}-containing medium [32]. (c) Thrombin increases the turnover of phosphoinositides and stimulates the formation of IP_3, [33–35], but the effect of ADP on the formation of IP_3 is controversial [36–40]. (d) The coupling mechanism for Ca^{2+} entry into platelets induced by ADP and thrombin is different [41,42]. The differences between thrombin and ADP presented in this study further stress the distinction between these two platelet activators.

The acidification could result from several sources. A major source of protons may be associated with glucose 6-phosphate metabolism such as glycolysis and glucose monophosphate shunt. Activation of platelets with ADP is known to be associated with a marked enhancement of energy metabolism [43,44]. Another possible source relates to Ca^{2+}-dependent reactions, such as increased myosin ATPase activity, as suggested for the acidification induced by thrombin [3]. Calcium mobilisation has been reported to be associated with intracellular acidification in cultured vascular smooth muscle cells [45].

While the activation of platelet Na^+/H^+ exchange by ADP has not been directly demonstrated earlier, several studies indicate that such an activation indeed occurs. (a) Epinephrine was shown to activate Na^+/H^+ exchange in platelets [10,11]. Yet, ADP is required for epinephrine-induced platelet activation [46]. (b) Na^+/H^+ exchange appears to be dependent upon the binding of fibrinogen to glycoprotein IIb/IIIa, therefore it was suggested that the operation of this exchange should also be a function of ADP binding [11]. (c) Activation of platelet with ADP is associated with uptake of sodium ions [21,22]. In order to establish the relationship between this uptake and the operation of Na^+/H^+ exchange, it would be of interest to verify whether the uptake is sensitive to amiloride and its potent analogs.

The present study deals with the relationships between Na^+/H^+ exchange and aggregation in platelets activated by ADP. Platelet aggregation has been linked under certain conditions to the operation of Na^+/H^+ exchanger [6,9,15,27,47]. However, several lines of evidence support our conclusion that Na^+/H^+ exchange is not required for ADP-induced platelet aggregation. (a) A differential dependence of aggregation and the exchange on external Na: while the K_m for $[Na]_o$ in Na^+/H^+ exchange is 75 to 90 mM [2], aggregation is not affected by reduction of $[Na]_o$ from 100 mM to 0.3 mM (Fig. 6). (b) A differential sensitivity of the two processes to amiloride and APA: aggregation and Na^+/H^+ exchange are 50% inhibited by APA at vastly different concentrations: 200 and 1.6 μM, respectively. The corresponding values for amiloride are: 200 and 5.9 μM. Siffert et al. [27] concluded that the concentration of amiloride required to inhibit platelet aggregation is compatible with an inhibitory action on the Na^+/H^+ exchange system. Yet they show about 50% inhibition of ADP-induced aggregation by 50 μM amiloride, but this concentration is 8-fold higher than the K_i for the exchange (Fig. 4). Furthermore, while amiloride and APA inhibit aggregation similarly, they differ markedly in inhibition of Na^+/H^+ exchange. It appears that the inhibition of aggregation at relatively high concentrations of amiloride and APA is due to a secondary effect, unrelated to Na^+/H^+ exchange. (c) pH_i does not seem to be critical for platelet aggregation, since the aggregation is not impaired by treatments (nigericin) that cause a substantial cytoplasmic acidification [3,48].

Sweat et al. [9] reported 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 amiloride analogs. Shape change and aggregation that was independent of arachidonic acid metabolism was unaffected by the amiloride analog. Thus it may be concluded that the Na^+/H^+ exchange activity is required for arachidonic acid metabolism and thereby may play a secondary role in release-related aggregation. The present study supports the conclusion that the operation of Na^+/H^+ exchange is not obligatory for primary platelet aggregation induced by ADP.

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