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Inhibition of Na^+/H^+ exchange reduces Ca^{2+} mobilization without affecting the initial cleavage of phosphatidylinositol 4,5-bisphosphate in thrombin-stimulated platelets

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Stimulation of human platelets increases cytoplasmic pH (pH_i) via activation of Na^+/H^+ exchange. We have determined the effect of inhibiting Na^+/H^+ exchange on (i) thrombin-induced Ca^{2+} mobilization and (ii) turnover of ^{32}P-labelled phospholipids. Blocking Na^+/H^+ exchange by removal of extracellular Na^+ or by ethylisopropylamiloride (EIPA) inhibited Ca^{2+} mobilization induced by 0.2 U/ml thrombin, whereas increasing pH_i by NH_4Cl enhanced the thrombin-induced increase in cytosolic free Ca^{2+}. The effect of EIPA was bypassed after increasing pH_i by moneasin. The thrombin-induced cleavage of phosphatidylinositol 4,5-bisphosphate (PIP_2) was unaffected by treatments that blocked Na^+/H^+ exchange or increased pH_i. It is concluded that activation of Na^+/H^+ exchange is a prerequisite for Ca^{2+} mobilization in human platelets but not for the stimulus-induced hydrolysis of PIP_2.

Na^+/H^+ exchange; Amiloride; Phosphoinositide; Quin 2; Ca^{2+} mobilization; Platelet activation

1. INTRODUCTION

The stimulation of platelets by thrombin results in a rapid phosphodiesteratic cleavage of PIP_2 [1]. This reaction which is activated by phospholipase C leads to the formation of two important second messengers: IP_3, which mobilizes Ca^{2+} from intracellular, non-mitochondrial pools [2,3], and 1,2-diacylglycerol which activates protein kinase C [4]. Another early event following stimulation of platelets consists of activation of a Na^+/H^+ antiport [5] with subsequent cytoplasmic alkalinization [6]. Although it has been demonstrated that blocking Na^+/H^+ exchange in platelets by either removal of extracellular Na^+ or by amiloride also inhibits the production of arachidonic acid, secretion of granule contents, and aggregation [7–9], the molecular basis of how changes in cytoplasmic pH (pH_i) can modulate platelet activation has remained obscure. This study aimed at investigating whether Na^+/H^+ exchange might affect platelet activation via a possible effect on the metabolism phosphoinositide turnover and Ca^{2+} mobilization.

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Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; Ca_o^{2+}, external Ca^{2+}; Na_o^+, external Na^+; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; PA, phosphatidic acid; EIPA, ethylisopropylamiloride

2. EXPERIMENTAL

2.1. *Preparation of platelets and determination of* $[Ca^{2+}]_i$

Freshly drawn citrated blood was centrifuged at 200 × *g* for 15 min to obtain platelet-rich plasma (PRP). The PRP was then incubated for 20 min with 20 μM quin2 acetoxymethyl ester (Sigma) at room temperature. After addition of 0.1 μM forskolin (Calbiochem, final conc.) the quin2-loaded cells were centrifuged at 700 × *g* for 20 min. The

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pellet was resuspended into 1 ml Hepes buffer (140 mM choline chloride, 5 mM KCl, 1 mM $MgSO_4$, 10 mM Hepes, 5 mM glucose, pH 7.5), and 0.1 μM forskolin and 10 μM EGTA (final conc.) were added. Finally, the suspension was passed through a Sepharose 2B column (Pharmacia) pre-equilibrated with the same buffer (without forskolin) in order to remove extracellular quin2 as well as extracellular Na^+ (Na_o^+). Subsequently, cell suspensions were diluted in Hepes buffer (37°C) containing either 140 mM Na^+ or 140 mM choline. 1 mM $CaCl_2$ or 2 mM EGTA was added as required and the cells were pre-equilibrated in a cuvette at 37°C for 5 min before addition of 0.2 U/ml thrombin (bovine; Sigma). The quin2 fluorescence was measured in a Perkin Elmer MPF-3 spectrofluorometer, the experimental details for calibration of the quin2 signals being identical to those described in the literature [10,11].

2.2. *Determination of ^{32}P-labelled phospholipids*

PRP was incubated with ^{32}P (0.1 mCi/ml) for 1 h at 37°C. Thereafter, gel-filtered platelets were prepared as described above. Aliquots (1 ml) of platelets were incubated at 37°C and stimulated with 0.2 U/ml thrombin (bovine; Sigma). The reaction was stopped at various time intervals after stimulation by addition of 4 ml cold (0°C) chloroform/methanol/13 M HCl (100:50:1, v/v). The phospholipids were extracted and subsequently separated by high-performance thin layer chromatography as described by Jolles et al. [12]. Labelled lipids were visualized by overnight radioautography, scraped from the plates and counted for radioactivity according to standard procedures.

2.3. *Modulation of Na^+/H^+ exchange and cytoplasmic pH*

In some experiments Na^+/H^+ exchange was inhibited by isotonic replacement of NaCl by choline chloride. In other experiments Na^+/H^+ exchange was blocked by amiloride (obtained as a gift from MSD Sharp & Dohme, Munich, FRG) or by EIPA which has a higher affinity towards the Na^+/H^+ exchanger than amiloride [13].

Cytoplasmic alkalinization was induced by addition of 10 mM NH_4Cl to the platelet suspensions or by use of the Na^+/H^+ ionophore monensin (Sigma; 1 mM stock in methanol). The effect of the latter two manipulations on pH_i was monitored in platelets isolated and suspended in NaCl-Hepes buffer, pH 7.5, as described above and loaded with the fluorescent intracellular pH indicator biscarboxyethylcarboxyfluorescein (BCECF; HSC Research Development Corporation, Toronto, Canada) as described by Rink et al. [14]. Addition of 10 mM NH_4Cl to the extracellular medium induced a rapid increase in pH_i from 7.15 to about 7.7 and 10 μM monensin raised pH_i to the extracellular value of about pH 7.5 (not shown).

3. RESULTS

The effects of inhibiting Na^+/H^+ exchange or increasing pH_i on Ca^{2+} mobilization are summarized in table 1. Thrombin raised $[Ca^{2+}]_i$ at 140 mM Na_o^+ in both the absence and presence of Ca_o^{2+}. Replacement of Na_o^+ by choline led to a reduction of Ca^{2+} mobilization by 82 and 85% in the absence or presence of Ca_o^{2+}, respectively. A substantial inhibition of the thrombin-induced in-

Table 1

Effects of blocking Na^+/H^+ exchange or cytoplasmic alkalinization on the thrombin-induced rise in cytosolic free calcium in human platelets

	$[Ca^{2+}]_i$ (nM) at 0 Ca_o^{2+} (2 mM EGTA)	$[Ca^{2+}]_i$ (nM) at 1 mM Ca_o^{2+}
140 mM Na_o^+	100 ± 15 (8)	786 ± 212 (9)
140 mM choline	18 ± 6 (7)	114 ± 13 (11)
140 mM Na_o^+ + 60 μM EIPA	21 ± 8 (8)	312 ± 55 (7)
140 mM Na_o^+ + 60 μM EIPA + 10 μM monensin	84 ± 15 (9)	701 ± 112 (10)
140 mM Na_o^+ + 10 mM NH_4Cl	212 ± 60 (15)	N.D.

The change in the concentration of cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, upon stimulation by 0.2 U/ml thrombin was measured in human platelets loaded with the fluorescent dye quin2. For experimental details see section 2. Values represent means (± SD); the number of experiments, performed in at least 3 different preparations, is given in parentheses. N.D., not determined

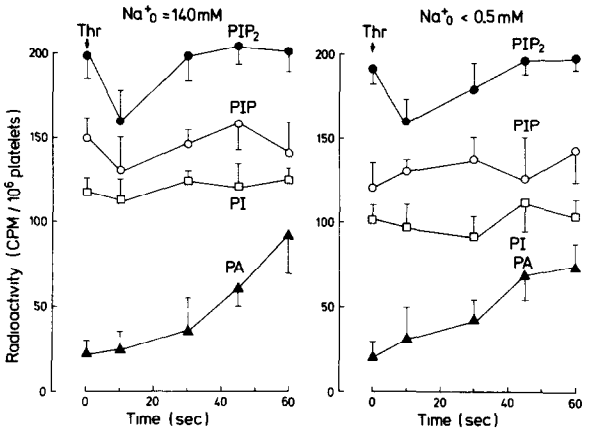

Fig.1. Effect of Na^+ removal on the turnover of ^{32}P-labelled phospholipids in thrombin-stimulated platelets. Left panel: control, presence of 140 mM NaCl; right panel: NaCl replaced by choline chloride. Displayed is the radioactivity in the separated [^{32}P]phospholipids at various times after addition of thrombin (0.2 U/ml). The symbols represent means (±) obtained in 6 different preparations. The radioactivity of the different phospholipid fractions was normalized to the average values of the corresponding metabolites before stimulation. Data were compared at 10, 30, 45, and 60 s for PIP_2, and at 45 and 60 s for the other phospholipids by Student's *t*-test. Removal of Na^+ did not affect the stimulus-induced changes in phospholipids ($p > 0.05$). (●) PIP_2, phosphatidylinositol 4,5-bisphosphate; (○) PIP, phosphatidylinositol 4-phosphate; (□) PI, phosphatidylinositol; (▲) PA, phosphatidate.

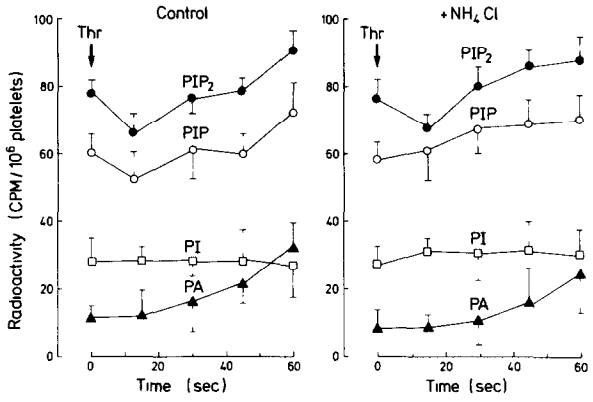

Fig.2. Effect of NH_4Cl on the thrombin-induced turnover of ^{32}P-labelled phospholipids. Left panel: control; right panel: addition of 10 mM NH_4Cl 2 min prior to activation by 0.2 U/ml thrombin. The symbols represent means (± SD) from 4 different experiments. For methods see section 2 and the legend to fig.1. The abbreviations are identical to those of fig.1.

crease in $[Ca^{2+}]_i$ was also observed after inhibition of Na^+/H^+ exchange by EIPA. This latter effect, however, was almost completely reversed when pH_i was artificially increased by the Na^+/H^+ ionophore monensin or by NH_4Cl (not shown). Cytoplasmic alkalinization by addition of NH_4Cl to the platelet suspension (final conc. 10 mM) did not affect $[Ca^{2+}]_i$ in unstimulated cells. In the presence of thrombin, however, the same treatment induced a 2-fold increase in $[Ca^{2+}]_i$. Taken together, these findings suggest an important role of Na^+/H^+ exchange for Ca^{2+} mobilization from internal stores and also demonstrate that Na^+/H^+ exchange may control the influx of Ca^{2+} across the plasma membrane.

In order to investigate whether Na^+/H^+ exchange directly affected Ca^{2+} translocation or acted via mechanisms that were close to receptor activation, we measured the changes in ^{32}P-labelled phosphoinositides which are early events in the sequence of stimulus-response coupling in thrombin-stimulated platelets. At 140 mM Na_o^+, thrombin stimulation led to a fall in the radioactivity of the PIP_2 fraction and a subsequent formation of PA (fig.1). Inhibiting Na^+/H^+ exchange by removal of Na_o^+ had no effect on the fall in PIP_2 and did not significantly reduce PA formation either (fig.1). At 140 mM Na_o^+, amiloride (0.1–1 mM) had no effect on [^{32}P]polyphosphoinositide turnover (not shown). Similarly, exposure of platelets to 10 mM NH_4Cl neither influenced the hydrolytic cleavage of PIP_2 nor significantly altered PA production as compared with untreated controls (fig.2).

4. DISCUSSION

We have presented evidence that the cytoplasmic pH is a crucial factor in the control of stimulus-response coupling in platelets. An increase in pH_i is essential for thrombin-induced Ca^{2+} mobilization. It is unlikely that these observations are due to disturbances of Ca^{2+} homeostasis by removal of Na_o^+ or the presence of EIPA in unstimulated platelets, since (i) none of the experimental conditions shown in table 1 changed the resting levels of $[Ca^{2+}]_i$ (84 ± 11 nM; ± SD, $n = 48$), and (ii) addition of the Ca^{2+} ionophore ionomycin to platelets

suspended in Ca^{2+}-free medium produced identical Ca^{2+} signals at normal and low Na_o^+ or in the presence of EIPA (not shown) which indicates that the total amount of Ca^{2+} releasable from internal stores was unaffected. In addition, it has been demonstrated that the total platelet Ca^{2+} content is independent of Na_o^+ [15]. We could also demonstrate that experimental conditions which either block the thrombin-induced rise in pH_i or induce cytoplasmic alkalinization apparently do not interfere with the stimulus-induced breakdown of PIP_2 (figs 1 and 2). It appears reasonable, therefore, to assume that pH_i plays a role in rather late stages in the sequence that couples receptor occupancy to aggregation and secretion. One possible candidate is the IP_3-mediated Ca^{2+} release from internal storage sites. This idea finds strong support in recent findings by Brass and Joseph [3] who demonstrated that the IP_3-induced Ca^{2+} mobilization from platelet internal membranes can be significantly enhanced by increasing pH_i from 6.9 to 7.4. An alternative possibility is that high pH_i retards the hydrolysis of IP_3. Evidence for this assumption, however, is lacking.

At present it is unknown how pH_i regulates the influx of Ca^{2+} across the plasma membrane. The plasma membrane of platelets lacks voltage-dependent Ca^{2+} channels [16] or a Na^+/Ca^{2+} exchanger [15]. Further work is required to determine how pH_i regulates Ca^{2+} transport across the plasma membrane.

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