Influence of Cytoplasmic pH on the Aggregation and Ca\(^{2+}\) Mobilization in Rabbit Platelets

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The aggregability of rabbit platelets was studied under various cytoplasmic pHs (pHi). Nigericin, a K\(^{+}/H\(^{+}\) ionophore, which can induce a decrease in pHi, at 2–10 μM in 2 min incubation reduced both platelet aggregation and an increase in cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) stimulated with thrombin or U46619. The reduced aggregability recovered 10 min after incubation with nigericin in parallel with an increase in pHi. In contrast, when pHi was increased by simultaneous addition of NH\(_{4}\)Cl, methylamine or monensin, aggregation in response to a low concentration of thrombin, U46619, arachidonic acid or A23187 was enhanced significantly. The enhancing effect of NH\(_{4}\)Cl was lowered by prolonged incubation with NH\(_{4}\)Cl, by which the increased pHi was improved concomitantly. Indomethacin, an inhibitor of cyclooxygenase, failed to inhibit the enhancement of aggregation by NH\(_{4}\)Cl under stimulation with U46619. In addition, treatment with NH\(_{4}\)Cl enhanced an increase in [Ca\(^{2+}\)]\(_{i}\) in response to U46619 in a concentration-dependent manner, although the treatment by NH\(_{4}\)Cl alone did not affect [Ca\(^{2+}\)]\(_{i}\). When pHi was artificially altered during the ranges of 6.6–7.4 by treatment with nigericin in K\(^{+}\)-rich medium, aggregation by low concentrations of thrombin was dependent on the pHi. These data indicate that pHi is an important factor for platelet activation including intracellular Ca\(^{2+}\) mobilization and aggregation.

**Key words** aggregation; cytoplasmic pH; platelet; Ca\(^{2+}\) mobilization.

Platelet has a physiological role for hemostasis through its aggregation, and the aggregability is influenced by the factors of cell number, medicines and diseases. Although cytoplasmic pH (pHi) in resting platelets is maintained at about 7.1, it shifts to alkaline pH when platelets are activated by agonists such as thrombin.\(^{1}\) The increase in pHi has been demonstrated to be based on activation of Na\(^{+}/H\(^{+}\) exchanger, which is sensitive to amiloride derivatives\(^{1}\) and is regulated by protein kinase C, Ca\(^{2+}\), mitogen-activated protein kinase\(^{5,6}\) and the lipid mediators arachidonic acid and 1,2-diacylglycerol.\(^{5,6}\) It has been reported that activation of Na\(^{+}/H\(^{+}\) exchanger is essential for platelet activation by epinephrine or ADP,\(^{7,8}\) and for Ca\(^{2+}\) mobilization by thrombin,\(^{9}\) whereas there are several reports contradicting the role of the exchanger for platelet activation.\(^{10,10}\) Although the significance of Na\(^{+}/H\(^{+}\) exchange on platelet activation is open to consideration, pHi is generally known to affect cell functions. We previously reported that the enhancement of arachidonic acid liberation by protein kinase C is partially mediated by cytoplasmic alkalization.\(^{11}\) In this work, to characterize the relation between pHi, and platelet activation, we studied the effect of change in pHi on agonist-stimulated Ca\(^{2+}\) mobilization and aggregation of platelets using these agents: nigericin to decrease pHi, and NH\(_{4}\)Cl, methylamine and monensin to increase pHi.

**MATERIALS AND METHODS**

**Materials** Nigericin, monensin, arachidonic acid and A23187 were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Nigericin and monensin were dissolved in ethanol and methanol at a concentration 300-fold higher than the final concentration, respectively. NH\(_{4}\)Cl and methylamine were obtained from Wako Pure Chemical Industries (Osaka, Japan). U46619 (9,11-dideoxy-9α,11α-methanoepoxy-prostaglandin F\(_{2α}\)) was obtained from Cayman Chemical (Ann Arbor, MI, U.S.A.). Thrombin (bovine plasma) was from Mochida Pharmaceutical (Tokyo, Japan). Fura2 pentacetoxymethyl ester (fura-2-AM) and 2',7'-bis(carboxyethyl)5,6-carboxyfluorescein tetraacetoxymethyl ester (BCECF-AM) were from Dohjin Chemical (Kumamoto, Japan). Other reagents were obtained from commercial sources.

**Preparation of Platelets** Fresh rabbit blood anti-coagulated with 0.1 volumes of 1% EDTA was centrifuged at 230×g for 10 min at room temperature to obtain platelet-rich plasma. The platelets separated from this plasma were washed twice as described previously.\(^{12}\) Finally, the platelet suspension was adjusted to 5×10⁶ cells/ml in modified Tyrode-Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5.6 mM glucose, 2.9 mM NaH₂PO₄, 3.8 mM Hepes, pH 7.35) containing 0.35% bovine serum albumin. For measurement of pHi and cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), washed platelets were incubated with 3 μM BCECF-AM and 2 μM fura2-AM at 37°C for 30 min, respectively, and then washed.

**Platelet Aggregation** Platelet aggregation was measured as the change in light transmission after 5 min stimulation at 37°C in the presence of 1 mM CaCl₂, with an aggregometer (NKK Hema Tracer 1; Niko Bioscience, Tokyo).

**Measurement of pHi** and [Ca\(^{2+}\)]\(_{i}\). The pHi and [Ca\(^{2+}\)]\(_{i}\) were determined with a fluorescent dye, BCECF or fura2. BCECF- or fura2-loaded platelets were diluted to 1.5×10⁶ cells/ml with the buffer and then analyzed. The fluorescence from the suspension was continuously monitored with a spectrofluorometer (F-2000; Hitachi) with excitation at 500 nm and emission at 530 nm, and then pHi was calculated by the method of Thomas et al.\(^{13}\) The measurement of [Ca\(^{2+}\)]\(_{i}\) in platelets with fura2 was carried out essentially as described previously.\(^{14}\) The fluorescence from the suspension was continuously monitored with excitation at 340 nm and 380 nm and emission at 500 nm, and then [Ca\(^{2+}\)]\(_{i}\) was calculated.

**Modification of pHi** Washed or fura2-loaded platelets

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were suspended with KCl-MOPS (3-morpholinopropanesulfonic acid) solution (130 mM KCl, 10 mM NaCl, 1 mM MgSO₄, 10 mM MOPS), which was adjusted to various pHs (6.6–7.4), and then incubated with 3 μM nigericin at 37°C for 5 min.

RESULTS AND DISCUSSION

Inhibitory Effect of Nigericin on Platelet Aggregation
Nigericin is a monovalent ionophore which alters Na⁺, K⁺ and proton gradients across cell membranes. In the physiological Na⁺-rich medium, nigericin is known to cause the efflux of cellular K⁺ and the concomitant influx of both Na⁺ and protons, thereby causing intracellular acidification. In fact, addition of nigericin to BCECF-loaded platelets in Tyrode-Hepes buffer (pH 7.35) induced a rapid fall in pHᵢ and the subsequent restoration to initial pHᵢ (the inset in Fig. 2).

The effect of nigericin was dose-dependent, and the maximum drop of pHᵢ were 0.09 at 2 μM, 0.14 at 5 μM and 0.19 at 10 μM. Under these conditions, platelet aggregation upon stimulation was examined. As shown in Fig. 1A, incubation for 2 min with nigericin elicited reduction of platelet aggregation stimulated by thrombin, U46619 or arachidonic acid in a concentration-dependent manner. Nigericin at 10 μM produced a 49% reduction in thrombin-stimulated aggregation. The reduction was strengthened with an increase in nigericin-treated time up to 5 min, while the reduced aggregability was recovered 10 min after incubation with nigericin in parallel with restoration of pHᵢ (Fig. 2).

Study of effect of nigericin on an increase in [Ca²⁺], which is an important step for platelet activation, showed that nigericin induced dose-dependent inhibition of the response to thrombin or U46619 in the presence of Ca²⁺ (Fig. 1B), the inhibition being in agreement with that of the aggregation. The inhibitory effect of nigericin was also observed in the absence of Ca²⁺ (data not shown). These data indicate that nigericin exerts its inhibitory effects on platelet aggregation and an increase in [Ca²⁺] in response to agonists, probably due to the induction of intracellular acidification.

Enhancing Effects of NH₄Cl, Methylamine and Monensin on Platelet Aggregation

We next examined the effects of 3 drugs which induce cytoplasmic alkalization on platelet aggregation and Ca²⁺ mobilization. It is reported that addition of NH₄Cl or methylamine to cell suspension leads to a rapid rise in pHᵢ by entry of NH₃ or methylamine into cytoplasm and by their protonations. Actually, when BCECF-loaded platelets were treated with 5 mM NH₄Cl, the pHᵢ rapidly increased by 0.17±0.04 (mean±S.E., n=3) and was subsequently restored. Under the conditions used, the effect of intracellular alkalization on platelet activation was examined upon stimulation.

As shown in Fig. 3, the addition of NH₄Cl (1—10 mM) significantly enhanced both aggregation and an increase in [Ca²⁺], when stimulated by a low concentration of U46619 (0.2 μM), a concentration which induces only slight aggregation. However, prolonged incubation with NH₄Cl before addition of U46619 attenuated the enhancement of platelet aggregation, and the increased pHᵢ just after addition of NH₄Cl lowered to the resting level (Fig. 4). NH₄Cl also enhanced ATP secretion induced by U46619 (0.27 nmol/10⁶ cells without NH₄Cl and 2.31 nmol/10⁶ cells with NH₄Cl). It may be possible that phospholipase A₂ is implicated in the NH₄Cl-induced platelet activation, since its activity is known to increase under alkaline conditions. However, we have reported that stimulation of platelets with U46619 does not induce a significant activation of the enzyme. Furthermore, we observed in the present work that even when the platelets were pretreated with 10 μM indomethacin, no effect was found on NH₄Cl-induced hyperaggregability (data not shown). These results suggest that neither phospholipase A₂ nor cyclooxygenase is involved in the enhancement of aggregation by NH₄Cl. NH₄Cl by itself failed to change the level of [Ca²⁺]; therefore, cytoplasmic alkalization alone is necessary but is not enough to induce enhancement of Ca²⁺ mo-
Fig. 3. Effect of NH$_4$Cl on Platelet Aggregation (A) and an Increase in [Ca$^{2+}$$]_i$. (B) Induced by U46619
Washed (A) or fura2-loaded (B) platelets were treated at 37°C with various concentrations of NH$_4$Cl, and simultaneously stimulated with 0.2 μM U46619 in the presence of 1 mM CaCl$_2$. (A) The results are expressed as percentage of maximum aggregation induced by high concentration of U46619 (1 μM) in the absence of NH$_4$Cl, and are the means of 2 experiments. (B) The change in fura$^{2+}$ fluorescence was measured, and the traces are representative of 2 experiments.

Fig. 4. Time Course of Enhancement by NH$_4$Cl Treatment on Platelet Aggregation
Washed platelets were incubated with 10 mM NH$_4$Cl (●) or buffer (○) at 37°C for various times and then stimulated with 0.2 μM U46619 in the presence of 1 mM CaCl$_2$. The results are expressed as percentage of maximum aggregation induced by high concentration of U46619 (1 μM) in the absence of NH$_4$Cl, and are the means of 2 experiments. The inset shows effect of 10 mM NH$_4$Cl (added at arrow) on pH$_i$ in BCECF-loaded platelets.

Table 1. Effects of Various Agents on Platelet Aggregation

<table>
<thead>
<tr>
<th>Agonist</th>
<th>None</th>
<th>NH$_4$Cl</th>
<th>Methyamine</th>
<th>Monensin</th>
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<tr>
<td>U46619</td>
<td>4.8</td>
<td>81.6</td>
<td>77.6</td>
<td>68.8</td>
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<tr>
<td>Thrombin</td>
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<td>37.3</td>
<td>18.7</td>
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<tr>
<td>Arachidonic acid</td>
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<td>73.1</td>
<td>52.3</td>
<td>41.5</td>
</tr>
<tr>
<td>A23187</td>
<td>10.3</td>
<td>42.9</td>
<td>34.6</td>
<td>37.2</td>
</tr>
</tbody>
</table>

Washed platelets were treated at 37°C with 5 mM NH$_4$Cl, 5 mM methyamine, 1 mM monensin or buffer vehicle (none) in the presence of 1 mM CaCl$_2$ and simultaneously stimulated with 0.2 μM U46619, 0.005 U/ml thrombin, 10 μM arachidonic acid or 0.1 μM A23187. The results are expressed as percentage of maximum aggregation by high concentration of each agonist (1 μM U46619, 0.1 U/ml thrombin, 100 μM arachidonic acid, 1 μM A23187), and are the means of 2 experiments.

Fig. 5. Thrombin-Induced Platelet Aggregation (A) and an Increase in [Ca$^{2+}$]$_i$ (B) in Various pH$_i$s
Washed (A) or fura2-loaded (B) platelets in KCl-MOPS solution (pH 6.6—7.4) were treated with 3 μM nigericin at 37°C for 5 min in the presence of 1 mM CaCl$_2$, and then stimulated 0.01 (●), 0.02 (▲) or 0.03 (■) U/ml thrombin. (A) The results are expressed as percentage of maximum aggregation obtained with 0.2 U/ml thrombin at pH 7.4. (B) The results are expressed as the increased level of [Ca$^{2+}$]$_i$. The data are the means of 2 experiments.

The Aggregatability in Various pH$_i$s
Although the replacement of Na$^+$ in medium by another ion or N-methyl-

Glucamine was carried out to examine the effect of pH$_i$ on cell responses, the result is reported to be due to the decreased activity of thrombin by the absence of Na$^+$. In the present study, to adjust pH$_i$ to 6.6, 6.8, 7.0 and 7.4, platelets were suspended in K$^+$-rich medium (KCl—MOPS solution) with those of pH$_i$ in the presence of 3 μM nigericin for 5 min. Under these conditions, pH$_i$ is assumed to be adjusted to such pH$_i$s by the ionophoretic action of nigericin. The results in Fig. 5A show that the aggregation induced by low doses of thrombin was apparently suppressed at pH 6.6, while it was enhanced at pH 7.4, in comparison with that at pH 7.0. The effects of various pH$_i$s on an increase in [Ca$^{2+}$]$_i$, in response to thrombin were similar to those on aggregation: the increase was suppressed at pH 6.6 and enhanced at pH 7.4 (Fig. 5B). Thus it seems likely that the effect of pH$_i$ on aggregation results from that on Ca$^{2+}$ mobilization.

The mechanism underlying pH$_i$ dependence on agonist-induced Ca$^{2+}$ mobilization is still not characterized in the present work. It is reported that treatment of platelets with NH$_4$Cl does not influence stimulus-induced hydrolysis of phosphatidylinositol 4,5-bisphosphate as compared with untreated control. Brass and Joseph observed, however, that the increase in pH$_i$, from 6.8 to 7.4, augments inositol 1,4,5-

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trisphosphate-induced Ca\(^{2+}\) release from a dense tubular system.\(^{22}\) Thus, one possibility is that intracellular alkalization may potentiate the biological activity of inositol 1,4,5-trisphosphate, which is formed under stimulation and responsible for Ca\(^{2+}\) mobilization. Since the change of pH\(_i\) is reported to affect thromboxane formation\(^{23}\) and cytoskeletal reorganization,\(^{24}\) these effects, in addition to [Ca\(^{2+}\)], may also be involved in the enhancement of platelet aggregation in response to an increase in pH\(_i\).

It has been shown that platelets from patients with hypertension have an increased pH\(_i\) and a functional hyperreactivity.\(^{25,26}\) Thus, the present results suggest a possibility that functional abnormalities of platelets related to a change in pH\(_i\), such as hyperaggregability, may be implicated in the pathogenesis or development of vascular damage in a disease accompanied by a change in blood pH.

In conclusion, we suggest that an increase in pH\(_i\) of platelets induces pH\(_i\)-dependent acceleration of agonist-induced Ca\(^{2+}\) mobilization, leading to the enhancement of platelet aggregation.

REFERENCES