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# Mobilization of intracellular $Ca^{2+}$ modulates activation of $Na^+/H^+$ exchange in thrombin-stimulated platelets[1]

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**KEY WORDS** calcium; sodium-hydrogen antiporter; nigericin; egtazic acid; ionomycin; thrombin; blood platelets

**AIM:** To study the relationship between intracellular calcium translocation and activation of $Na^+/H^+$ exchange in thrombin-stimulated platelets. **METHODS:** Intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$) and pH ($pH_i$) were measured by a dual wavelength fluorophotometer with Fura-2 and pH-sensitive probe BCECF. **RESULTS:** Thrombin 0.1 $IU \cdot L^{-1}$ elicited an increase in platelet $[Ca^{2+}]_i$ and $pH_i$, the maximal increase in $[Ca^{2+}]_i$ occurred earlier than the rise in $pH_i$. In $Na^+$-free buffers, the $Na^+/H^+$ exchange was markedly suppressed without affecting the elevation of $[Ca^{2+}]_i$; while intracellular acidification with nigericin 1 $mg \cdot L^{-1}$ inhibited the increment of $[Ca^{2+}]_i$. Blockade of $Ca^{2+}$-influx with egtazic acid (EGTA) did not affect cytosolic alkalinization. Depletion of intracellular $Ca^{2+}$ store with ionomycin in the presence of EGTA, no increment in $pH_i$ was observed, the basal value of $pH_i$ was even more acidic, this response of $pH_i$ to thrombin was rehabilitated after refilling of intracellular $Ca^{2+}$ store with extracellular $Ca^{2+}$ 1 $mmol \cdot L^{-1}$. **CONCLUSION:** Intracellular $Ca^{2+}$ mobilization modulated activation of $Na^+/H^+$ exchange, which required an effective increment of $[Ca^{2+}]_i$.

$Na^+/H^+$ exchange plays an important role in the processes that initiate specific platelet functions[1]. Intracellular pH ($pH_i$) acts as a synergistic messenger generating a metabolic context through which the actions of other effectors are integrated[2,3]. Inhibition of $Na^+/H^+$ exchange might affect a variety of additional steps in platelet signal transduction. A key event in platelet activation is the $Ca^{2+}$ translocation. There exists a controversy about the relationship between $[Ca^{2+}]_i$ elevation and activation of $Na^+/H^+$ exchange, some authors proposed that $Na^+/H^+$ exchange triggered the mobilization of $Ca^{2+}$ [4], while others demonstrated the change in $pH_i$ did not modify much $Ca^{2+}$ mobilization[5]. The present study was designed to investigate whether emptying or refilling the internal $Ca^{2+}$ stores affected the activation of $Na^+/H^+$ exchange or changing the $pH_i$ affected modulation of intracellular calcium levels, and to obtain further insights into the interaction of $Na^+/H^+$ exchange and $Ca^{2+}$ mobilization in platelet signal transduction.

## MATERIALS AND METHODS

**Drugs and reigents** 2′, 7′-Biscarboxyethyl-5, 6-carboxyfluorescein acetoxymethyl ester (BCECF/AM) and Fura-2 acetoxymethyl ester (Fura 2-AM) were purchased from Molecular Probes Co. Thrombin, nigericin, indometacin, HEPES, Triton-X-100, bovine serum albumin (BSA), ionomycin, and egtazic acid (EGTA) were purchased from Sigma.

**Preparation of platelet suspension** Washed rabbit platelets were prepared[6]. The platelets ($2 \times 10^{11} \cdot L^{-1}$) was labeled with either Fura 2-AM 1 $\mu mol \cdot L^{-1}$ or BCECF/AM 2 $\mu mol \cdot L^{-1}$, and resuspended in HEPES-buffered saline (HBS) (NaCl 145, KCl 5, $MgCl_2$ 1, $NaH_2PO_4$ 0.5, glucose 10, HEPES 10 $mmol \cdot L^{-1}$, and BSA 0.1 %, pH 7.4, apyrase 1 mg $\cdot L^{-1}$, indometacin 0.1 $\mu mol \cdot L^{-1}$) or $Na^+$-free buffers (choline choride 140, KCl 5, $MgCl_2$ 1, glucose 10, HEPES 10 $mmol \cdot L^{-1}$, BSA 0.1 %, pH 7.4, adjusted by KOH).

**Measurement of $[Ca^{2+}]_i$ and $pH_i$** Fluorescence was monitored by a spectrofluorimeter (RF-5000, Shimadzu, Japan). The $[Ca^{2+}]_i$ was measured[6]. For the assay of $pH_i$, $\lambda_{ex}$ was set at 440 and 505 nm, $\lambda_{em}$ at 526 nm, the basal and stimulated $pH_i$ were estimated by the fluorescence ratio of 505/440[7].

**Protocol** In the presence of $Ca^{2+}$ 1 $mmol \cdot L^{-1}$, the effects of thrombin 0.1 $IU \cdot L^{-1}$ on $[Ca^{2+}]_i$ and $pH_i$ were observed respectively in HBS and $Na^+$-free buffers. Nigericin 1

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*mg · $L^{-1}$ was used to induce intracellular acidification.* The effects of inhibiting $Ca^{2+}$ entry with EGTA 1 mmol · $L^{-1}$ on $[Ca^{2+}]_i$ and $pH_i$ were also estimated. To deplete the intracellular $Ca^{2+}$ stores, the labeled platelets were incubated with ionomycin 1 μmol · $L^{-1}$ in the presence of EGTA 1 mmol · $L^{-1}$ at 37 ℃ for 20 min, then washed once, and resuspended in HBS. The effects of emptying $Ca^{2+}$ stores and refilling by readdition of extracellular $Ca^{2+}$ 1 mmol · $L^{-1}$ on $pH_i$ were assessed.

**Statistical analysis** Data were expressed as $\bar{x} \pm s$ and assessed by ANOVA and *t*-test.

## RESULTS

**Thrombin-stimulated shifts in $pH_i$ and $[Ca^{2+}]_i$** In the presence of $Ca^{2+}$ 1 mmol · $L^{-1}$, the basal level of $[Ca^{2+}]_i$ was 107 ± 21 nmol · $L^{-1}$, the resting $pH_i$ was 7.42 ± 0.03 (*n* = 10). Thrombin elicited an increase of 362 ± 26 nmol · $L^{-1}$ in $[Ca^{2+}]_i$ within 10 – 20 s (Fig 1A) and a maximal rise of 0.16 ± 0.03 pH in $pH_i$ which was stabilized after 3 – 5 min (Tab 1, Fig 2A). Addition of EGTA prior to thrombin inhibited the $[Ca^{2+}]_i$ increments (Tab 1), but the cytosolic alkalinization was not much influenced (Tab 1).

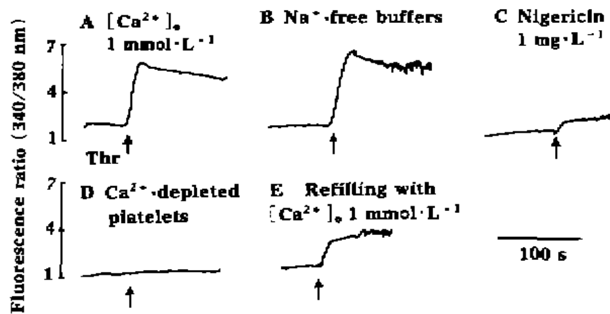


**Fig 1. Fura fluorescence in response to thrombin (Thr 0.1 IU · $L^{-1}$; arrow) with different treatments.**

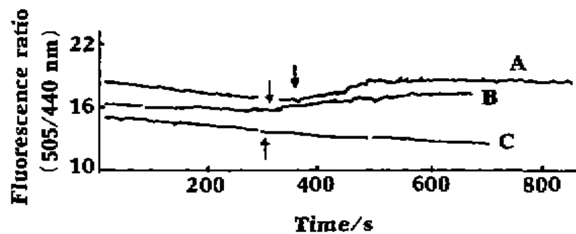


**Fig 2. Effects of depleting or refilling the intracellular $Ca^{2+}$ stores on $pH_i$. Trace A: control; B: refilling with $Ca^{2+}$ in $Ca^{2+}$-depleted platelets; C: $Ca^{2+}$-depleted platelets in $Ca^{2+}$-free HBS (Thrombin ↑).**

***Tab 1. Thrombin-evoked shifts in $[Ca^{2+}]_i$ and $pH_i$. (number in parenthesis). $\bar{x} \pm s$. ${}^aP > 0.05$, ${}^cP < 0.01$ vs control.***

| | $\Delta[Ca^{2+}]_i$ (nmol · $L^{-1}$) | $\Delta pH_i$ |
|---|---|---|
| Control ($[Ca^{2+}]_o$ 1 mmol · $L^{-1}$) | 362 ± 26 (6) | 0.16 ± 0.03 (7) |
| EGTA (1 mmol · $L^{-1}$) | 274 ± 8[c] (4) | 0.15 ± 0.02[a] (4) |
| $Na^+$-free buffers | 365 ± 50[a] (5) | −0.11 ± 0.01[c] (4) |
| Nigericin (1 mg · $L^{-1}$) | 145 ± 37[c] (4) | - |
| Depletion of $Ca^{2+}$ with ionomycin | 37 ± 14[c] (8) | −0.019 ± 0.008[c] (4) |
| Refilling with $[Ca^{2+}]_o$ 1 mmol · $L^{-1}$ | 403 ± 26[a] (4) | 0.13 ± 0.017[a] (4) |

**Effects of nigericin and $Na^+$-free buffer on $[Ca^{2+}]_i$** In $Na^+$-free buffers, the intracellular alkalinization was markedly inhibited, while the rise of $[Ca^{2+}]_i$ evoked by thrombin was unaffected (Tab 1, Fig 1B). Pretreatment with nigericin caused an intracellular acidification with $pH_i$ stabilizing about 0.2 pH below resting $pH_i$, the increase of $[Ca^{2+}]_i$ was partially inhibited (Tab 1, Fig 1C).

**Effects of depleting or refilling of intracellular $Ca^{2+}$ stores on $pH_i$** In ionomycin-treated platelets, no elevation of $[Ca^{2+}]_i$ induced by thrombin was seen (Fig 1D), confirming that depletion of the intracellular $Ca^{2+}$ store had been achieved. If exposure of the same platelets in $Ca^{2+}$-containing buffers, the $[Ca^{2+}]_i$ response to thrombin was restored (Fig 1E), readdition of extracellular $Ca^{2+}$ was apparently sufficient to refill the internal stores. No intracellular alkalinization was seen in ionomycin-treatment platelets (Fig 2C), the rise in $pH_i$ was also rehabilitated in $Ca^{2+}$-containing buffers (Tab 1, Fig 2B).

## DISCUSSION

The present study showed that platelet activation was associated with a rapid elevation in $[Ca^{2+}]_i$ and a gradual intracellular alkalinization. The former is

mediated by both $Ca^{2+}$ influx and $Ca^{2+}$ mobilization from internal stores, the latter is attributable to activation of $Na^+/H^+$ exchange. It was found that the maximal increase in $[Ca^{2+}]_i$ occurred earlier than the rise in $pH_i$, this potentiation may reflect accumulation of cycling $Ca^{2+}$ upstream of the activation of $Na^+/H^+$ exchange, raised $Ca^{2+}$ triggers a proton efflux which shows the characteristics of $Na^+/H^+$ exchange. Inhibition of $Na^+/H^+$ exchange did not reduce the rise of $[Ca^{2+}]_i$ and lead to a complete blockade of $Ca^{2+}$ translocation, this might result from the existence of multiple pathways for thrombin-induced $Ca^{2+}$ increment, eg, receptor-mediated $Ca^{2+}$ entry or high concentration agonist produce sufficient inositol-1,4,5-trisphosphate ($IP_3$) to make calcium mobilization in a $pH_i$ independent manner. Under the more acidic cytosolic condition, the $Ca^{2+}$ increment was partially inhibited. According to previous report, $IP_3$-mediated release of stored $Ca^{2+}$ is $pH_i$ sensitive and increases with increasing $pH_i$ in the relevant range of 7.0 – 7.4[9], the alkalinization that results from receptor-accelerated $Na^+/H^+$ exchange may increase $[Ca^{2+}]_i$, thereby enhancing calcium dependent events. So we speculated that increasing $Na^+/H^+$ exchange activity might be useful in preventing an intracellular acidification which could inhibit the $[Ca^{2+}]_i$ increase.

This study also showed that inhibition of $Ca^{2+}$ entry did not affect $Na^+/H^+$ exchange, whereas depletion of intracellular $Ca^{2+}$ stores abolished the increase in $pH_i$ and showed a lower basal $pH_i$. The effect of depletion of internal $Ca^{2+}$ stores was more complete than that directly exposure of the cells in $Ca^{2+}$-free buffers and did not result in platelet aggregation by ionomycin treatment[10,11]. Readdition of extracellular $Ca^{2+}$ restored the thrombin response on $[Ca^{2+}]_i$ and $pH_i$, confirming earlier reports that $Ca^{2+}$ was taken up into the intracellular stores and was released into the cytoplasm upon agonist stimulation[12]. Refilling occurs without a detectable elevation of $[Ca^{2+}]_i$, suggested that it might occur by a 'capacitative' route which have been demonstrated to exist in platelets[13]. Our results indicated that mobilization of intracellular $Ca^{2+}$ was necessary for activation of $Na^+/H^+$ exchange and played a role in platelet $pH_i$ regulation.

In summary, our finding suggested that mobilization of intracellular $Ca^{2+}$ modulated activation of $Na^+/H^+$ exchange, which required an effective increase in $[Ca^{2+}]_i$.

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## 内钙动员调控凝血酶诱导的血小板 $Na^+/H^+$ 交换[1]

R973.2

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关键词 钙；$Na^+/H^+$ 交换；尼日利亚菌素；凝血酶
钠 氢 血小板

依他酸；伊屋诺霉素；凝血酶；血小板

**目的**：研究凝血酶诱导的血小板活化中细胞内钙动员和 $Na^+/H^+$ 交换的关系．**方法**：Fura-2 负载测 $[Ca^{2+}]_i$ 和 BCECF 负载测 $pH_i$．**结果**：凝血酶 0.1 $IU \cdot L^{-1}$ 引起 $[Ca^{2+}]_i$ 和 $pH_i$ 增加，$[Ca^{2+}]_i$ 增加先于 $pH_i$ 增加．在无钠溶液中，$Na^+/H^+$ 交换被抑制而 $[Ca^{2+}]_i$ 增加不受影响；用尼日利亚菌素（1 $mg \cdot L^{-1}$）使胞内酸化可抑制 $[Ca^{2+}]_i$ 增加．用依他酸（EGTA）阻断外钙内流，胞浆碱化不受影响．伊屋诺霉素加 EGTA 耗竭胞内钙池时，胞浆碱化效应被取消，且静息 $pH_i$ 更低，加入 1 $mmol \cdot L^{-1}$ 外钙重新充填钙池，胞浆碱化效应又被恢复．**结论**：细胞内钙动员调控 $Na^+/H^+$ 交换，后者需 $[Ca^{2+}]_i$ 增加达一定有效浓度．

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# Blocking effects of phentolamine on L-type calcium current and ATP-sensitive potassium current in guinea pig ventricular myocytes[1]

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**KEY WORDS** phentolamine; prazosin; yohimbine; glyburide; patch-clamp techniques; calcium channels; potassium channels; myocardium

**AIM**: To study the effect of phentolamine on L-type calcium currents ($I_{Ca}$) and ATP-sensitive $K^+$ currents ($I_{K,ATP}$) in ventricular myocytes. **METHODS**: $I_{Ca}$ and $I_{K,ATP}$ were observed using patch clamp techniques in whole-cell recording configuration. **RESULTS**: Phentolamine reduced $I_{Ca}$ of ventricular myocytes in concentration-dependent and voltage-independent manners. Phentolamine 5, 25, and 100 $\mu mol \cdot L^{-1}$ decreased $I_{Ca}$ from $370 \pm 99$ nA to $310 \pm 95$ nA (17 % block, $n = 6$, $P < 0.01$), from $230 \pm 98$ nA to $180 \pm 73$ nA (23 % block, $n = 5$, $P < 0.05$), and from $293 \pm 66$ nA to $206 \pm 44$ nA (30 % block, $n = 5$, $P < 0.01$), respectively, without affecting the current-voltage relationship. Prazosin 100 $\mu mol \cdot L^{-1}$ and yohimbine 100 $\mu mol \cdot L^{-1}$, which were specific blockers of $\alpha_1$ and $\alpha_2$ adrenoceptors respectively, did not show the inhibitory effect on $I_{Ca}$. Phentolamine 100 $\mu mol \cdot L^{-1}$ also inhibited the $I_{K,ATP}$ induced by 2, 4-dinitrophenol (DNP) at 0 mV from $3.2 \pm 0.6$ nA to $0.8 \pm 0.5$ nA (75 % block, $n = 4$, $P < 0.01$). **CONCLUSION**: Phentolamine directly inhibits $I_{Ca}$ and $I_{K,ATP}$ in guinea pig ventricular myocytes.

Phentolamine is a classical nonselective α adrenoceptor blocking agent. The potent anti-arrhythmic effects of phentolamine were shown in cat heart *in vivo*[1] and in rat[2] and guinea pig[3,4] hearts *in vitro*. Phentolamine can block single ATP-sensitive $K^+$ channels in ventricular myocytes of rabbit at the intracellular side of the channel[5]. But, there was no direct evidence that phentolamine can block $K_{ATP}$ channels from the surface of cardiac myocytes and as one of targets of anti-arrhythmic drug, L-type $Ca^{2+}$ channels should be a candidate[6]. Hence we studied the effect of phentolamine on ATP-sensitive $K^+$ currents ($I_{K,ATP}$) and L-type $Ca^{2+}$ currents ($I_{Ca}$) in guinea pig cardiomyocytes.

## MATERIALS AND METHODS

**Cell isolation** Single ventricular myocytes were obtained by enzymatic dissociation[7] from Dunkin Hartley guinea pigs ($235 \pm 15$ g) provided by Experimental Animal Center of Shanxi Medical University. Langendorff hearts were perfused through

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