Intracellular calcium mobilization and activation of the Na⁺/H⁺ exchanger in platelets

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The aim of the present study was to evaluate the regulatory relationship between the cytosolic free calcium concentration ($[Ca^{2+}]_i$) and cytosolic pH (pH_i). $[Ca^{2+}]_i$ and pH_i were measured using the fluorescent dyes fura-2 and BCECF [2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein] respectively. In a medium with 1 mmol/l extracellular calcium, thrombin (2.5 units/ml) induced an increment in $[Ca^{2+}]_i$ of 638 ± 31 nmol/l ($n = 5$) and an intracellular alkalinization of 0.14 ± 0.01 pH units ($n = 8$). Both responses were dependent on the concentration of thrombin, displaying a sigmoidal dose-response pattern. The intracellular alkalinization was dependent upon extracellular Na⁺ and was amiloride-sensitive, indicating that it was mediated by activation of the Na⁺/H⁺ exchanger. When extracellular calcium was chelated with EGTA prior to the addition of thrombin, the intracellular alkalinization was not affected (0.15 ± 0.02 at 2.5 units/ml thrombin, $n = 8$). Under these circumstances, the $[Ca^{2+}]_i$ increment represents mobilization from internal stores, reaching 157 ± 42 nmol/l at 2.5 units/ml thrombin. When plate-

lets were preloaded with the intracellular calcium chelator MAPTAM (1,2-bis-5-methylaminophenoxyethane-*NNN'*-tetra-acetoxymethyl acetate) to block the increase in $[Ca^{2+}]_i$ induced by thrombin, no increment in pH_i was observed. Moreover, MAPTAM-loaded calcium-depleted platelets had a basal pH_i that was more acidic than in the presence of 1 mmol/l extracellular calcium (6.93 ± 0.09 versus 7.14 ± 0.01 , $n = 26$, $P < 0.001$). Ionomycin induced an elevation of $[Ca^{2+}]_i$ that was accompanied by a concomitant increase in pH_i , which was Na⁺-dependent and amiloride-sensitive. $[Ca^{2+}]_i$ and pH_i increases induced by ionomycin were both dependent on the concentration of ionomycin. In conclusion, an increase in $[Ca^{2+}]_i$ is necessary for the agonist-induced activation of the Na⁺/H⁺ exchanger in platelets. Non-agonist-induced increases in $[Ca^{2+}]_i$ seems to prompt activation of the exchanger. In addition, Ca²⁺-depleted platelets have a more acidic basal pH_i , indicating that the basal level of $[Ca^{2+}]_i$ is also important for maintaining the basal pH_i .

INTRODUCTION

A number of cell activators (e.g. hormones, neurotransmitters and growth factors) are able to stimulate phospholipase C, resulting in the formation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol (Zavoico et al., 1986; King and Rittenhouse, 1989). The intracellular increases in these mediators are associated with activation of protein kinase C (PKC) and elevation of the cytosolic free calcium concentration ($[Ca^{2+}]_i$) and of the intracellular pH (pH_i). Most of the metabolic changes involving $[Ca^{2+}]_i$ shifts are known to be related to alterations in pH_i . It has been proposed that pH_i acts as a synergistic messenger generating a metabolic context through which the actions of other effectors are integrated (Busa and Nuccitelli, 1984). Several reports have confirmed that the intracellular alkalinization caused by certain stimuli (hormones and growth factors) is due to the activation of the amiloride-sensitive Na⁺/H⁺ exchanger (Grinstein et al., 1985a,b; Grinstein and Rothstein, 1986; Zavoico et al., 1986; Siffert et al., 1987), mainly through the action of PKC, a Ca²⁺-dependent enzyme (Moolenaar et al., 1984; Mahnensmith and Aronson, 1985). The exchanger can also be stimulated by osmotic shrinking or by a decrease in pH_i (Grinstein et al., 1985a,b; Grinstein and Rothstein, 1986; Mahnensmith and Aronson, 1985). Nevertheless, there is controversy about the relationship between $[Ca^{2+}]_i$ elevations and cytoplasmic alkalinization. While some authors have reported that activation of the Na⁺/H⁺

exchanger after agonist stimuli is a prerequisite for Ca²⁺ mobilization in human platelets (Siffert and Akkerman, 1987), others have demonstrated that agonist-induced activation of the Na⁺/H⁺ exchanger can occur independently of Ca²⁺ mobilization in murine fibroblasts (Ives and Daniel, 1987) and in human platelets (Simpson and Rink, 1987; Zavoico et al., 1986; Zavoico and Cragoe, 1988; Sage et al., 1990). Experiments in which the $[Ca^{2+}]_i$ and pH_i are monitored simultaneously have demonstrated that, after stimulation by agonists, the increase in $[Ca^{2+}]_i$ occurs before cytoplasmic alkalinization (Zavoico and Cragoe, 1988; Sage et al., 1990; Rink and Sage, 1990). Accordingly, Ca²⁺ mobilization has been reported to modulate activation of the Na⁺/H⁺ exchanger in response to certain stimuli in vascular smooth muscle cells (Mitsuhashi and Ives, 1988). Moreover, several reports have pointed out that agonist-induced intracellular Ca²⁺ mobilization stimulates the Na⁺/H⁺ exchanger in human fibroblasts (Muldoon et al., 1985; Hendey et al., 1989).

The present study was designed to examine the relationships between agonist- and non-agonist-induced intracellular Ca²⁺ mobilization and activation of the Na⁺/H⁺ exchanger. We used increasing doses of human thrombin to evaluate the agonist-induced responses, and the calcium ionophore ionomycin was used to produce the non-agonist-induced increases in $[Ca^{2+}]_i$. It is concluded that agonist-induced activation of the Na⁺/H⁺ exchanger requires intracellular Ca²⁺ mobilization. A non-agonist-induced rise in $[Ca^{2+}]_i$ is able to stimulate the Na⁺/H⁺

Abbreviations used: PKC, protein kinase C; $[Ca^{2+}]_i$, cytosolic free Ca²⁺ concentration; pH_i , cytosolic pH; BCECF, 2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein; MAPTAM, 1,2-bis-5-methylaminophenoxyethane-*NNN'*-tetra-acetoxymethyl acetate; DMSO, dimethyl sulphoxide; EIPA, 3-amino-5-ethylisopropylamino-6-chloro-*N*-(diaminoethylene)pyrazinecarboxamine hydrochloride; PRP, platelet-rich plasma; HBS, Hepes-buffered saline.

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Figure 1 pH-dependence of BCECF fluorescence in intact platelets (○) and in solution (●)

Cells were loaded with BCECF as described in the Experimental section and suspended in high- K^+ Hepes, pH 6. After nigericin ($2 \mu\text{g/ml}$) was added, the pH was increased stepwise to approx. 8 and the fluorescence was recorded once the signal had stabilized (○). Afterwards, the platelets were lysed with digitonin ($50 \mu\text{mol/l}$), the pH was changed stepwise back to approx. 6 and the fluorescence of BCECF in solution was recorded (●). The experiment illustrated is representative of 21 similar determinations.

exchanger. Increments of $[\text{Ca}^{2+}]_i$ and pH_i appear to display a sigmoidal relationship. However, low doses of ionomycin, which induce small increases in $[\text{Ca}^{2+}]_i$, appear to be insufficient to activate the Na^+/H^+ exchanger.

EXPERIMENTAL

Materials

Human thrombin (Sigma, St. Louis, MO, U.S.A.) was resuspended in distilled water and stored at -20°C until use. Fura-2 acetoxymethylester (fura-2/AM), 2',7'-bis-(carboxyethyl)-5,6-carboxyfluorescein tetra-acetoxymethylester (BCECF/AM) and 1,2-bis-5-methylaminophenoxyethane-*NNN*'-tetra-acetoxymethyl acetate (MAPTAM) were from Calbiochem (La Jolla, CA, U.S.A.), prepared in dimethyl sulphoxide (DMSO) and stored at -20°C until use. EIPA [3-amino-5-ethylisopropyl-amino-6-chloro-*N*-(diaminoethylene)pyrazinecarboxamine hydrochloride] was provided by Merck Sharp & Dohme, Spain. Nigericin, ionomycin, EGTA, digitonin and choline chloride

were from Sigma. All other chemicals were of analytical grade, purchased from Merck (Darmstadt, Germany).

Preparation of cells

Blood was obtained from volunteer donors who had not taken any aspirin-containing medications in the previous 10 days. Venous blood (40 ml) was drawn into 3.15% sodium citrate (9:1, v/v) and immediately centrifuged at $200 g$ for 10 min at 20°C to obtain the platelet-rich plasma (PRP). EGTA (0.5 mmol/l) was added to prevent platelet activation and the PRP was centrifuged at $1000 g$ for 20 min at 20°C . The pellet was resuspended in Hepes-buffered saline (HBS) consisting of (in mmol/l): NaCl 145, KCl 5, MgCl_2 1, glucose 6, Hepes 10 and BSA 0.2 mg/ml, pH 7.4, at 37°C . The platelet suspension was incubated with either $2 \mu\text{mol/l}$ Fura-2/AM or $0.75 \mu\text{mol/l}$ BCECF/AM for 35 min at 37°C in a shaking water-bath. The labelled platelets were centrifuged at $1000 g$ for 20 min to remove the extracellular dye and the pellet was resuspended in HBS. The platelet count was adjusted to $0.5 \times 10^8/\text{ml}$ with Technikon H-1 System Coulter counter. CaCl_2 was added to 1 mmol/l to replenish intracellular Ca^{2+} stores. After an equilibration period of 10 min at 37°C , 2 ml of the platelet suspension was placed in a 3 ml quartz cuvette for fluorescence measurements.

Fluorescence measurements

Fluorescence measurements were carried out using an Hitachi F-2000 spectrofluorimeter (Hitachi, Tokyo, Japan). The sample was contained in a 3 ml quartz cuvette placed in a cuvette chamber thermostatted at 37°C . The platelet suspension was continually stirred at low speed throughout the individual fluorescence readings with a magnetic stirrer. All additions were made directly into the cuvette through a small hole in the top of the cuvette chamber using microsample syringes. The fluorescence signal was obtained once every second, with alternate excitation wavelengths of 340 and 380 nm for fura-2, and 500 and 440 nm for BCECF. $[\text{Ca}^{2+}]_i$ and pH_i were estimated by the ratio of values at the excitation wavelengths (340/380 and 500/440 respectively). The emission wavelength was 510 nm for fura-2 and 530 nm for BCECF. The autofluorescence of the unlabelled cell suspension with the vehicle DMSO was recorded before and after lysis with digitonin and subtracted from the total fluorescence; this was always $< 15\%$ and $< 6\%$ of the fluorescence signal for fura-2 and BCECF readings respectively. Cells were stimulated with

Table 1 Requirement of mobilization of intracellular Ca^{2+} for thrombin-induced intracellular alkalization

Cells were loaded with either fura-2/AM or BCECF/AM, and $[\text{Ca}^{2+}]_i$ and pH_i were calculated as described in the Experimental section. The increases in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$; nmol/l) and pH_i (ΔpH_i ; pH units) were calculated by subtracting the baseline from the maximal response or from the response at 300 s respectively. Values are means $\pm$ S.E.M. from different experiments using platelets from different donors, as indicated by the numbers in parentheses. Chelation of extracellular Ca^{2+} with EGTA did not significantly affect the pH_i response to thrombin. However, addition of the intracellular calcium chelator MAPTAM blocked completely the intracellular alkalization in response to thrombin.

Thrombin (units/ml)	$[\text{Ca}^{2+}]_0$ (1 mmol/l)		EGTA (1.2 mmol/l)		MAPTAM (40 $\mu\text{mol/l}$)
	$\Delta[\text{Ca}^{2+}]_i$	ΔpH_i	$\Delta[\text{Ca}^{2+}]_i$	ΔpH_i	ΔpH_i
0.025	29 ± 15 (4)	-0.03 ± 0.003 (3)	7 ± 2 (3)	-0.01 ± 0.005 (3)	—
0.05	81 ± 18 (5)	0.02 ± 0.01 (7)	19 ± 9 (3)	0.01 ± 0.01 (8)	-0.02 ± 0.01 (5)
0.1	140 ± 16 (5)	0.08 ± 0.02 (5)	61 ± 22 (3)	0.04 ± 0.01 (5)	-0.02 ± 0.02 (5)
0.5	377 ± 29 (5)	0.13 ± 0.003 (6)	118 ± 32 (3)	0.11 ± 0.02 (7)	-0.02 ± 0.01 (5)
1.0	510 ± 12 (5)	0.14 ± 0.01 (8)	126 ± 19 (3)	0.14 ± 0.01 (8)	-0.01 ± 0.01 (5)
2.5	638 ± 31 (5)	0.14 ± 0.01 (8)	157 ± 42 (3)	0.15 ± 0.02 (8)	-0.003 ± 0.004 (6)

increasing doses of thrombin (0.025, 0.05, 0.1, 0.5, 1 and 2.5 units/ml), with each dose being applied to aliquots of the same platelet suspension. Ionomycin dissolved in DMSO was applied at 60, 120, 250, 500 and 1000 nmol/l to aliquots of the same platelet suspension.

Calculation of $[\text{Ca}^{2+}]_i$

The relationship between the fluorescence ratio at 340/380 and $[\text{Ca}^{2+}]_i$ was obtained using the equation:

$$[\text{Ca}^{2+}]_i = K_d (R - R_{\min.}) / (R_{\max.} - R) \times F_{\min.} / F_{\max.}$$

(Grynkiewicz et al., 1985), where $R_{\max.}$ is the fluorescence ratio at saturating Ca^{2+} , $R_{\min.}$ is the ratio at zero Ca^{2+} , and $F_{\min.}$ and $F_{\max.}$ are the fluorescence readings at 380 nm at zero and saturating Ca^{2+} respectively. K_d is the effective dissociation constant of fura-2 in appropriate conditions and was taken to be 224 nmol/l, according to Grynkiewicz et al. (1985). Calibration was performed by the following procedure. After each single experiment, cells were lysed with digitonin (50 $\mu\text{mol/l}$) in the presence of 1 mmol/l CaCl_2 to obtain $R_{\max.}$ and $F_{\max.}$. Thereafter, to obtain $R_{\min.}$ and $F_{\min.}$, EGTA (6 mmol/l) was added and the pH of the cuvette was adjusted to 8.3 with 20 mmol/l Tris base.

Calculation of pH_i

The fluorescence signal of BCECF was calibrated to the pH at the end of each experiment, as described by Rink et al. (1982). Briefly, platelets were lysed with digitonin (50 $\mu\text{mol/l}$) and the fluorescence signal was recorded at known values of pH from 6 to 8, simultaneously monitored using a combined microelectrode (Hamilton; Bonaduz AG) inserted directly into the cuvette. The pH_i estimated from this calibration was corrected for the shift in the excitation maximum of intracellular and extracellular BCECF according to the method of Rink et al. (1982). Platelets were resuspended in high- K^+ Hepes, which contained (in mmol/l): KCl 120, NaCl 20, glucose 6, MgCl_2 1 and Hepes 10 (pH 6 at 37 °C), plus 2 $\mu\text{g/ml}$ nigericin, allowing the external pH to equal the pH_i . The external pH was increased stepwise from pH 6 to pH 8, and the fluorescence signal was recorded at each step. The cells were then lysed and the pH was decreased stepwise back to pH 6. The difference between the intracellular and the post-lysis calibration curves showed that the signal in the latter underestimated the pH_i by 0.13 ± 0.02 pH units ($n = 21$) (Figure 1). The values presented in this work have been corrected appropriately, according to these data.

Manipulation of the Na^+/H^+ exchanger

Two methods were employed for inhibition of the Na^+/H^+ exchanger. First, the sodium of the Hepes buffer was iso-osmotically replaced by choline chloride. Secondly, the amiloride derivative specific exchanger blocker EIPA was used at 50 $\mu\text{mol/l}$ (Vigne et al., 1984).

Intracellular Ca^{2+} clamp

At 5 min after the addition of BCECF, the intracellular calcium chelator MAPTAM (40 $\mu\text{mol/l}$) was added to the loading medium and incubated for 30 min at 37 °C. Afterwards, cells were washed as described above. Before addition of the agonist, EGTA (1.2 mmol/l) was added to the platelet suspension.

Statistical and mathematical analyses

Data are presented as means $\pm$ S.E.M. Statistical significances were determined by Student's *t* test, and *P* values > 0.05 were considered not significant. Dose-response curves were adjusted using an InPlot graphical program (Graph-PAD Software Inc.).

RESULTS

Thrombin effects on $[\text{Ca}^{2+}]_i$ and pH_i

Under the conditions used, the basal level of platelet $[\text{Ca}^{2+}]_i$ was 45 ± 2 nmol/l ($n = 25$). Thrombin (2.5 units/ml) produced a dose-dependent increment in $[\text{Ca}^{2+}]_i$ in 20–25 s, up to 638 ± 31 nmol/l ($n = 5$) (Table 1; Figure 2a). Blockade of extracellular Ca^{2+} influx by addition of EGTA (1.2 mmol/l) prior to the application of stimulus reduced the $[\text{Ca}^{2+}]_i$ increments at

Figure 2 Representative traces of fura-2 fluorescence at 340 nm in response to thrombin (0.1 unit/ml; arrow) under different conditions: (a) CaCl_2 (1 mmol/l), (b) EGTA (1.2 mmol/l) and (c) MAPTAM (40 $\mu\text{mol/l}$)

A rise in fluorescence, reflecting a rise in $[\text{Ca}^{2+}]_i$ (see the Experimental section), can be observed after addition of thrombin (a). When extracellular Ca^{2+} was chelated with EGTA, the increase in $[\text{Ca}^{2+}]_i$ was reduced (b). When platelets were preloaded with the intracellular calcium chelator MAPTAM, no rise in fluorescence was observed (c). Traces are representative of at least five different experiments done with platelets from different donors.

Figure 3 Representative traces of BCECF fluorescence at 500 nm in response to thrombin (0.1 unit/ml; arrow) under different conditions: (a) CaCl_2 (1 mmol/l), (b) EGTA (1.2 mmol/l) and (c) MAPTAM 40 $\mu\text{mol/l}$

A rise in BCECF fluorescence, reflecting a rise in pH_i (see the Experimental section), can be observed both in CaCl_2 -containing Hepes (a) and in conditions where the extracellular calcium is chelated (b). However, in platelets incubated with MAPTAM, no alkalization, but rather a gradual acidification, was observed (c). Traces are representative of at least five different experiments done with platelets from different donors.

either dose of thrombin ($P < 0.05$) ($n = 5$), representing Ca^{2+} mobilization from internal stores (Table 1, Figure 2b).

The basal pH_i was 7.14 ± 0.01 ($n = 26$). Thrombin produced a dose-dependent intracellular alkalization which was detectable within 1 min, stabilized after 5–7 min and was preceded by an initial and transient acidification of 0.005–0.01 pH unit (Figure 3a). Thrombin-induced intracellular pH changes at 300 s exhibited a sigmoid dose–response pattern, with a maximal increase in pH_i of 0.14 ± 0.01 at 2.5 units/ml thrombin ($n = 8$) (Table 1). When platelets were resuspended in Na^+ -free Hepes or when EIPA (50 $\mu\text{mol/l}$) was added to the platelet suspension prior to the application of thrombin, no intracellular alkalization was observed. Thrombin (1 unit/ml) produced a gradual intracellular acidification at 180 s of 0.05 ± 0.02 pH unit in Na^+ -free Hepes

Table 2 Effects of ionomycin on $[\text{Ca}^{2+}]_i$ and pH_i

Platelets were loaded with either fura-2/AM or BCECF/AM, and $[\text{Ca}^{2+}]_i$ and pH_i were calculated as described in the Experimental section. The increases in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$; nmol/l) and pH_i (ΔpH_i ; pH units) were calculated by subtracting the baseline from the maximal response or from the response at 300 s respectively. Values are means $\pm$ S.E.M. from different experiments using platelets from different donors, as indicated by the numbers in parentheses. Ionomycin produced a sustained rise in pH_i only at doses which raised $[\text{Ca}^{2+}]_i$ to more than 300 nmol/l.

ionomycin (nmol/l)	$\Delta[\text{Ca}^{2+}]_i$ (nmol/l)	ΔpH_i (pH units)
60	89 ± 5 (4)	0.01 ± 0.007 (5)
125	155 ± 21 (4)	0.014 ± 0.012 (5)
250	334 ± 22 (4)	0.07 ± 0.03 (5)
500	681 ± 44 (4)	0.10 ± 0.02 (5)
1000	> 1000 (4)	0.13 ± 0.05 (5)

and of 0.07 ± 0.01 units with EIPA ($n = 4$) (not significant), indicating that thrombin-induced intracellular alkalization was mediated by activation of the Na^+/H^+ exchanger.

Relationship between $[\text{Ca}^{2+}]_i$ and activation of the Na^+/H^+ exchanger

To investigate the influence of intracellular Ca^{2+} transients in the thrombin-induced intracellular alkalization, the response of the pH_i to thrombin was measured after addition of EGTA (1.2 mmol/l). In the absence of extracellular Ca^{2+} , the final pH_i response to thrombin was not affected (Table 1, Figure 3b). These data suggest that the intracellular alkalization induced by thrombin is not dependent on the influx of extracellular Ca^{2+} . To further investigate the influence of intracellular Ca^{2+} mobilization on the pH_i response, the cell-permeable Ca^{2+} chelator MAPTAM was used to blunt the intracellular Ca^{2+} transient. Under these conditions, no significant intracellular Ca^{2+} increment was observed with either dose of thrombin (Figure 2c). MAPTAM reduced the basal pH_i from 7.14 ± 0.01 to 6.93 ± 0.09 ($n = 26$) ($P < 0.001$). At either dose of thrombin employed, a slight intracellular acidification was observed (Table 1; Figure 3c). These data indicate that the pH_i response to thrombin was dependent upon intracellular Ca^{2+} increments, and that the basal $[\text{Ca}^{2+}]_i$ is important for maintenance of the basal pH_i .

Effects of non-agonist-induced rises in $[\text{Ca}^{2+}]_i$ on pH_i

To examine the effects of non-agonist-induced rises in $[\text{Ca}^{2+}]_i$ on changes in pH_i , platelets were treated with different doses of ionomycin in HBS containing 1 mmol/l CaCl_2 and 0.2 mg/ml BSA, with doses being added to aliquots of the same platelet suspension. The effect of ionomycin on $[\text{Ca}^{2+}]_i$ is shown in Table 2. Ionomycin at low doses (60 and 125 nmol/l), which produced increases in $[\text{Ca}^{2+}]_i$ of 89 ± 5 and 155 ± 47 nmol/l respectively, produced a slight intracellular alkalization. However, ionomycin at 250 and 500 nmol/l, which gave increases in $[\text{Ca}^{2+}]_i$ of 334 ± 45 and 681 ± 44 respectively, produced a brief and small acidification followed by alkalizations of 0.07 ± 0.03 and 0.10 ± 0.02 pH unit respectively at 300 s (Table 2; Figure 4). A higher dose of ionomycin (1 $\mu\text{mol/l}$), which in our hands raised $[\text{Ca}^{2+}]_i$ to $> 1 \mu\text{mol/l}$, produced an alkalization of 0.13 ± 0.05 unit at 300 s ($n = 4$). These data indicate that non-agonist-induced elevations in $[\text{Ca}^{2+}]_i$ of greater than 300 nmol/l can induce intracellular alkalization. The rise in pH_i showed a sigmoidal relationship to the increase in $[\text{Ca}^{2+}]_i$ (Figure 5). The

Figure 4 Responses of pH_i to $[\text{Ca}^{2+}]_i$ increments induced by Ionomycin

Cells were loaded with either fura-2/AM or BCECF/AM and $[\text{Ca}^{2+}]_i$ and pH_i were calculated as described in the Experimental section. Platelets were resuspended in HBS/BSA (0.2 mg/ml), with CaCl_2 added to 1 mmol/l. The increase in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$; nmol/l) and that in pH_i (ΔpH_i ; pH units) were calculated by subtracting the baseline from the maximal response (for $[\text{Ca}^{2+}]_i$) or from the response at 300 s (for pH_i). Ionomycin dissolved in DMSO was added at different doses (60, 125, 250 and 500 nmol/l) to aliquots of the same platelet suspension. Ionomycin produced a sustained rise in pH_i at doses at which the $[\text{Ca}^{2+}]_i$ was increased to more than 300 nmol/l. Values are means $\pm$ S.E.M. from four different experiments with different donors.

Figure 5 Relationship between Ionomycin-induced rises in $[\text{Ca}^{2+}]_i$ and intracellular alkalinization

The conditions were the same as in Figure 4. Values of $[\text{Ca}^{2+}]_i$ and pH_i are means $\pm$ S.E.M. The rise in pH_i induced by Ionomycin alone, by stimulation of the Na^+/H^+ exchanger (see the text), shows a sigmoidal dependence on the increase in $[\text{Ca}^{2+}]_i$. Values are from four different experiments with platelets from different donors.

rise in pH_i evoked by Ionomycin was dependent on the rise in $[\text{Ca}^{2+}]_i$, since it was blunted when platelets were preloaded with the intracellular Ca^{2+} chelator MAPTAM (results not shown). Pretreatment of platelets with EIPA (50 $\mu\text{mol/l}$) for 1 min prior to the addition of 250 nmol/l Ionomycin produced an acidification of 0.17 ± 0.03 pH unit at 180 s ($P < 0.005$). This indicates that the intracellular alkalinization observed after addition of Ionomycin was due to the activation of the Na^+/H^+ exchanger.

DISCUSSION

Treatment of platelets and other cell types with agonists is known to be associated with a rapid elevation in $[\text{Ca}^{2+}]_i$ and a more sustained intracellular alkalinization, and these changes are

known to be related to cell activation (Moolenaar et al., 1984; Siffert et al., 1989). On stimulation with thrombin, the increase in $[\text{Ca}^{2+}]_i$ is mediated by both the influx of Ca^{2+} across the plasma membrane and the release of Ca^{2+} from internal stores (Sage and Rink, 1987; Siffert et al., 1989), since it is not completely blocked by the addition of EGTA (Table 1). In platelets, agonist-induced changes in pH_i usually comprise two phases: an initial and transient acidification of 0.005–0.01 pH unit, followed by a gradual and sustained alkalinization (Zavoico et al., 1986; Zavoico and Cragoe, 1988; Sage et al., 1990). The intracellular alkalinization is mediated by activation of the Na^+/H^+ exchanger, since it is prevented by removal of extracellular Na^+ or pre-incubation with EIPA, a specific inhibitor of the exchanger (Vigne et al., 1984; Zavoico et al., 1986; Siffert et al., 1989).

The present study shows that the thrombin-induced rise in pH_i in human platelets is dependent upon intracellular Ca^{2+} mobilization. The role of intracellular Ca^{2+} mobilization as a second messenger for activation of the Na^+/H^+ exchanger is under discussion. Several investigators have pointed out that the intracellular alkalinization in response to growth factors is independent of changes in $[\text{Ca}^{2+}]_i$ in fibroblasts (Moolenaar et al., 1983, 1984; Ives and Daniel, 1987). Nevertheless, experiments in which $[\text{Ca}^{2+}]_i$ and pH_i were measured simultaneously in platelets demonstrated that the spike of $[\text{Ca}^{2+}]_i$ produced by thrombin occurred before any increase in pH_i (Zavoico and Cragoe, 1988; Sage et al., 1990), suggesting a causal role for intracellular Ca^{2+} . Moreover, the rapid increase in $[\text{Ca}^{2+}]_i$ occurred simultaneously with the initial and transient acidification phase, which can itself be a stimulus for the Na^+/H^+ exchanger (Mahnensmith and Aronson, 1985). The initial decline in pH_i is probably produced by stimulation of the plasma membrane Ca^{2+} -ATPase, which exchanges external H^+ for cytosolic Ca^{2+} (Carafoli and Zurini, 1982) and is known to be present in platelets (Enyedi et al., 1986). These data contrast with our findings of a decline in pH_i in Ca^{2+} -depleted platelets stimulated with thrombin. Therefore, other, perhaps additive, possibilities may explain the agonist-induced initial acidification, such as enhanced H^+ production resulting from increased hydrolysis of ATP and the formation of lactic acid (Akkerman and Holmsen, 1981).

It has been stated more recently that intracellular Ca^{2+} mobilization appears to display a regulatory function in the activation of the Na^+/H^+ exchanger in vascular smooth muscle cells (Mitsuhashi and Ives, 1988), fibroblasts (Owen and Villereal, 1982a,b; Hendey et al., 1989) and platelets (Kimura et al., 1990). Our results are consistent with an intracellular Ca^{2+} requirement for the activation of the Na^+/H^+ exchanger, which is apparent with a wide range of thrombin concentrations (0.05–2.5 units/ml). After chelation of extracellular Ca^{2+} with EGTA, the increase in pH_i was not affected (Table 1; Figure 3b). Although mitochondrial $\text{Ca}^{2+}/\text{H}^+$ exchange cannot be excluded (Gunter and Pfeiffer, 1990), our data suggest that the alkalinization was mediated by the membrane Na^+/H^+ exchanger (see the Results section). Moreover, it is known that mitochondria are not a significant source of releasable Ca^{2+} (Rink and Sage, 1990). When the increase in intracellular Ca^{2+} was blocked with MAPTAM, the alkalinization phase was not observed (Table 1, Figure 3c). Moreover, our results also suggest that the basal $[\text{Ca}^{2+}]_i$ plays an important role in the maintenance of the basal pH_i , as in Ca^{2+} -depleted platelets the basal pH_i was significantly more acidic than in control platelets, and thrombin produced a slight acidification without a recovery phase.

In order to ascertain the role of an increase in $[\text{Ca}^{2+}]_i$ by itself on changes in pH_i , the effect of the Ca^{2+} ionophore Ionomycin at different doses was evaluated. Only Ionomycin-induced incre-

ments of $[Ca^{2+}]_i$ greater than 300 nmol/l were able to induce a significant and sustained intracellular alkalinization (Table 2, Figure 4), which was preceded by an initial and transient acidification phase in most cases. These data suggest that mobilization of intracellular Ca^{2+} could be sufficient to trigger activation of the Na^+/H^+ exchanger, and are in agreement with studies performed in fibroblasts (Hendey et al., 1989). Ionomycin evokes changes in cell volume which can activate the Na^+/H^+ exchanger (Grinstein and Cohen, 1987). However, the fact that the intracellular alkalinization induced by ionomycin was Ca^{2+} -dependent argues against an osmotic activation of the Na^+/H^+ exchanger, a process reported to be independent of intracellular Ca^{2+} (Grinstein et al., 1985b). An interesting finding was that the ionomycin-induced intracellular alkalinization was dose-dependent, showing sigmoidal relationship with the increase in $[Ca^{2+}]_i$ (Figure 5).

The biochemical pathways that mediate the activation of the Na^+/H^+ exchanger are under study. There is compelling evidence that agonist-induced activation of the Na^+/H^+ exchanger is mediated by stimulation of PKC in a number of cells (Moolenaar et al., 1984; Grinstein et al., 1985a; Grinstein and Rothstein, 1986). However, in vascular smooth muscle cells, mechanisms independent of PKC activation have also been reported (Berk et al., 1987; Huang et al., 1987; Mitsuhashi and Ives, 1988). Activation of the Na^+/H^+ exchanger through Ca^{2+} /calmodulin-dependent processes has also been suggested by some investigators (Owen and Villereal, 1982b; Little et al., 1988). In platelets, the effects of $[Ca^{2+}]_i$ on the activation of the Na^+/H^+ exchanger seems to be more important than PKC stimulation, since inhibition of PKC does not completely block the pH_i response (Kimura et al., 1990). The findings of the present study are in agreement with this hypothesis, in that ionomycin is known not to activate PKC (Fearon and Tashjian, 1985), suggesting that the transient increase in $[Ca^{2+}]_i$ produced by ionomycin and the subsequent intracellular alkalinization occur via a PKC-independent pathway. This would be applicable to the rise in pH_i induced by thrombin (Kimura et al., 1990), but in our hands, the increments in $[Ca^{2+}]_i$ produced by thrombin and ionomycin were not equipotent in promoting activation of the Na^+/H^+ exchanger (Tables 1 and 2), suggesting different pathways for the agonist- and non-agonist-induced rises in pH_i .

It can be concluded that activation of the Na^+/H^+ exchanger requires the presence of an effective increment in $[Ca^{2+}]_i$. A non-agonist-induced rise in $[Ca^{2+}]_i$ also activates the exchanger in a dose-dependent fashion, displaying a sigmoidal relationship between the rise in $[Ca^{2+}]_i$ and the increase in pH_i . Moreover, the data suggest that $[Ca^{2+}]_i$ is important for maintaining the basal pH_i .

We thank Dr. G. Escolar and Dr. J. Monteagudo from the Hemotherapy and Hemostasis Service for their advice. This work was supported by grants from the Fondo de Investigaciones Sanitarias de la SS (FIS 92/0551 and FIS 91/0223) and by grants from the Hospital Clínic i Provincial de Barcelona (1991) and the Direcció General d'Universitats de la Generalitat de Catalunya (Formació d'Investigadors Joves, 1991).

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