Different pathways for control of Na^+/H^+ exchange via activation of the thrombin receptor

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The aim of the present study was to clarify the control of Na^+/H^+ exchange in platelets activated via the thrombin receptor. When human BCECF-loaded platelets were stimulated with the thrombin-receptor-activating peptide (TRAP; amino acid sequence SFLLRN), which activates the receptor independently of proteolysis, the cytosolic pH (pH_i) rose from 7.13 ± 0.04 ($n = 6$) to 7.27 ± 0.04 ($n = 5$), followed by a rapid decrease to resting values. Trypsin, which cleaves the receptor, induced a rapid and irreversible rise in pH_i to 7.31 ± 0.06 ($n = 5$). γ-Thrombin, which cleaves the receptor but is unable to bind to the hirudin-like domain, induced a slow and irreversible rise in pH_i to 7.31 ± 0.04 ($n = 14$). α-Thrombin, which cleaves the receptor and binds to its hirudin-like domain, induced a rapid and irreversible rise in pH_i to 7.31 ± 0.04 ($n = 22$). Changes in pH_i induced by TRAP, trypsin, γ- and α-thrombin were accompanied by similar changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and ^{32}P-pleckstrin, a substrate of protein kinase C (PKC). The separate chelation of $Ca^{2+}{}_i$ (30 μM BAPTA-AM) or inhibition of PKC (1 μM staurosporine) induced about 50% inhibition of the pH_i responses triggered by TRAP, trypsin, γ- and α-thrombin, but the combination induced complete inhibition. Thus the different types of activation of the thrombin receptor control Na^+/H^+ exchange via the same mechanism. Binding of thrombin to the hirudin-like domain accelerates exchange activation, whereas proteolysis of the receptor is essential for a sustained increase in pH_i.

INTRODUCTION

Like many other cells, platelets contain a Na^+/H^+ exchanger which removes cytosolic protons in exchange for extracellular Na^+ ions and regulates cell volume and cytosolic pH (pH_i) [1,2]. The exchanger responds to an increase in the cytosolic H^+ concentration, $[H^+]_i$, by a faster exchange activity [3]. In addition, Na^+/H^+ exchange accelerates during platelet activation, thereby raising the pH_i. Early studies showed alkalinization upon stimulation with α-thrombin, platelet-activating factor and ADP, but also with phorbol esters, suggesting that activation of protein kinase C (PKC) was responsible for the enhanced exchange activity [4–10]. Indeed, recent findings by Livne et al. [11] show concurrent activation of Na^+/H^+ exchange and phosphorylation of a 105–110 kDa protein that was precipitated with an antibody against the C-terminus of the cloned human Na^+/H^+ exchanger-1 (NHE-1) from human lymphocytes. The cloned exchanger contains several serine residues at the C-terminus that are phosphorylated upon stimulation by α-thrombin, providing a basis for exchange regulation [12,13].

This concept predicts that staurosporine, an inhibitor of PKC, decreases the activation of the exchanger during platelet stimulation. Phosphorylation of the 110 kDa protein was indeed absent from staurosporine-treated platelets [11], but the exchange activity at 0.1 unit/ml α-thrombin remained undisturbed [14]. Thus α-thrombin activates the NHE also via a PKC-independent mechanism, possibly by stimulating a Ca^{2+}/calmodulin-dependent process, as has been demonstrated for several other cell types, including fibroblasts and smooth-muscle cells [15–17].

Recently a thrombin receptor was cloned from mRNA obtained from HEL and DAMI cells, which are megakaryocyte-like cell lines [18]. Thrombin cleaves this receptor at Arg^{41}, thereby creating a new N-terminus that acts as a 'tethered ligand'. Synthetic peptides homologous to this new N-terminus stimulate several cell types, including hamster fibroblasts [19], gastric smooth-muscle cells [20] and platelets [21,22]. In addition to the cleavage site, the thrombin receptor contains a hirudin-like domain at the extracellular N-terminal extension that functions as a binding site for α-thrombin [22].

The aim of the present study was to gain more insight in the regulation of the Na^+/H^+ exchanger in platelets activated via the thrombin receptor. The receptor was activated by the thrombin-receptor-activating peptide TRAP, with trypsin, with γ-thrombin and with α-thrombin. TRAP (SFLLRN) is homologous to the new N-terminus of the thrombin receptor that is liberated after cleavage of the receptor and activates the receptor independently of proteolysis [18]. Trypsin and γ-thrombin, an autoproteolysis product of α-thrombin, activate the receptor by proteolysis without binding to the hirudin-like domain on the receptor [18,23,24]. α-Thrombin also activates the receptor by proteolysis, but in addition binds to its hirudin-like domain. The data show that each of these properties contributes to the control of Na^+/H^+ exchange during platelet stimulation by α-thrombin.

MATERIALS AND METHODS

Materials

TRAP (amino acids SFLLRN) was prepared by standard solid-phase procedures. γ-Thrombin was a gift from John W. Fenton II, New York State Department of Health, Albany, NY, U.S.A. α-Thrombin, nigericin and propionic acid were obtained from Sigma (St. Louis, MO, U.S.A.). 1-(Isoquinolinylsulphonyl)-2-

Abbreviations used: TRAP, thrombin-receptor-activating peptide; AM, acetoxymethyl ester; PRP, platelet-rich plasma; BCECF, 2′,7′-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein; BAPTA, bis-(o-aminophenoxy)ethane-*NNN′N′*-tetra-acetic acid; H7, 1-(isoquinolinylsulphonyl)-2-methylpiperazine; pH_i, cytosolic pH; $Ca^{2+}{}_i$, cytosolic free Ca^{2+} ions; NHE, Na^+/H^+ exchanger; PKC, protein kinase C; EIPA, ethylisopropylamiloride.

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methylpiperazine (H7) and K252A were from Biomol Research Laboratories (Plymouth Meeting, PA, U.S.A.), and calphostin C was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA, U.S.A.). Fura-2 AM (AM, acetoxymethyl ester), BCECF-AM, trypsin and staurosporine were purchased from Boehringer (Mannhein, Germany), and [^{32}P]P$_i$ (sp. radioactivity 314 TBq/mmol) from New England Nuclear (Boston, MA, U.S.A.). Sepharose 2B was obtained from Pharmacia–LKB (Uppsala, Sweden), and BAPTA-AM from Molecular Probes (Junction City, OR, U.S.A.). Ethylisopropylamiloride (EIPA) was a gift from Dr. W. Siffert, Max-Planck Institut für Biophysik, Frankfurt, Germany. All other chemicals were of analytical grade.

Blood collection

Freshly drawn venous blood from healthy volunteers (with informed consent) was collected into trisodium citrate (0.1 vol. of 130 mM). The donors claimed not to have taken any medication during the previous 10 days.

Measurement of pH$_i$

Citrated blood was centrifuged (200 g, 10 min, 22 °C), and the platelet-rich plasma (PRP) was collected and acidified to pH 6.5 with $\frac{1}{6}$ vol. of ACD (2.5 g of trisodium citrate, 1.5 g of citric acid, 2.0 g of D-glucose in 100 ml of water). The platelets were isolated by centrifugation (700 g, 15 min, 22 °C) and resuspended in 1 ml of Hepes–Tyrode buffer (145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 20 μM $CaCl_2$, 10 mM Hepes, pH 6.5), containing 0.1 % (w/v) glucose and 0.2 % (w/v) gelatin. The cells were incubated for 30 min at 37 °C with BCECF-AM (3 μM) in the absence or presence of BAPTA-AM (30 μM), to chelate intracellular Ca^{2+}, and subsequently washed on a Sepharose 2B column, equilibrated in Hepes–Tyrode buffer (pH 6.5). The platelets were stored in the dark at room temperature until use. Samples (50 μl) were added to 1450 μl of pre-warmed gelatin-free Hepes–Tyrode buffer (pH 7.2; 37 °C), resulting in a final platelet count of (150–200) × $10^3/\mu$l. Fluorescence was determined on a Hitachi F-3000 spectrofluorimeter at 37 °C, using wavelengths of 495 and 530 nm for excitation and emission respectively. The suspension was gently stirred (100 rev./min). Calibration was carried out after diluting the BCECF-loaded platelets in a high-K^+ buffer (120 mM KCl, 30 mM NaCl, 1 mM $MgSO_4$, 5 mM glucose) in the presence of nigericin (2 μM), as described by Horne et al. [4]. In all experiments platelets were stimulated with 15 μM TRAP, 20 μg/ml trypsin, 30 nM γ-thrombin or 4.5 nM α-thrombin. These concentrations induced maximal activation of the Na^+/H^+ exchanger.

Measurement of cytosolic Ca^{2+} ions (Ca_i^{2+})

PRP was incubated with fura-2 AM (3 μM) in the absence or presence of BAPTA-AM (30 μM) for 45 min at 37 °C. The platelets were washed by centrifugation (15 min, 700 g, 22 °C) and the pellet was resuspended in 1 ml of Hepes–Tyrode buffer (pH 6.5) and washed a second time by gel-filtration through Sepharose 2B, as described under 'Measurement of pH$_i$'. The cells were stored in the dark at room temperature until use. Samples (50 μl) of this suspension were diluted into 1450 μl of prewarmed gelatin-free Hepes–Tyrode buffer (pH 7.2, 37 °C), resulting in a final platelet count of (150–200) × $10^3/\mu$l. The suspension was gently stirred (100 rev./min) during the measurement. Fluorescence was measured with excitation at 345 nm and emission at 495 nm.

Measurement of PKC activity

Platelets were labelled with 3.7 MBq of carrier-free [^{32}P]P$_i$/ml of acidified PRP (pH 6.5), with or without BAPTA-AM (30 μM) for 1 h at 37 °C. Platelets were isolated by centrifugation (700 g, 15 min, 22 °C) and resuspended in Hepes–Tyrode buffer (pH 7.2), resulting in a final platelet count of (150–200) × $10^3/\mu$l. Labelled platelets were stimulated at 37 °C and samples were collected at the times indicated in the Results section, transferred into 0.5 vol. of 3-times-concentrated Laemmli sample buffer and heated for 2 min at 100 °C before electrophoresis. Proteins were separated by electrophoresis through an 11 %-polyacrylamide gel, as described by Laemmli [25]. Gels were stained with Coomassie Brilliant Blue, and the distribution of radioactivity was determined by autoradiography of dried gels on Kodak Royal X-Omat film. For determination of the radioactivity of pleckstrin, the specific area was cut out of the gels and heated for 2 h at 80 °C in 30 % (v/v) H_2O_2. The radioactivity was determined by liquid-scintillation counting. In control experiments no difference in phosphorylation patterns could be detected between centrifuged platelets and gel-filtered platelets (G. van Willigen, unpublished work). Data are expressed as percentage of pleckstrin phosphorylation in unstimulated platelets.

Presentation of data

Data are expressed as means ± S.D. Statistical significance was determined by Student's t test and considered significant at $P < 0.05$. All experiments were performed with 3–5 different platelet preparations.

RESULTS

Activation of Na^+/H^+ exchange by TRAP, trypsin, γ-thrombin and α-thrombin

Figure 1 shows representative fluorescence tracings of BCECF-loaded platelets. The resting pH$_i$ was 7.13 ± 0.04 (n = 6). Addition of TRAP (Figure 1a; Table 1) resulted in a rapid increase in BCECF fluorescence, equivalent to an increase in pH$_i$ of about 0.14 pH units. Maximum fluorescence was reached about 30 s after addition of TRAP. Thereafter the pH$_i$ rapidly decreased and stabilized at the resting pH$_i$. When trypsin was added

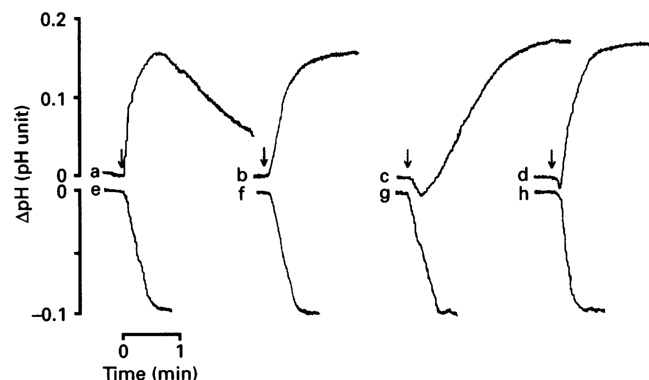

Figure 1 Activation of the Na^+/H^+ exchanger via the thrombin receptor

Gel-filtered BCECF-loaded platelets were stimulated at 37 °C with TRAP (15 μM; **a, e**), trypsin (20 μg/ml; **b, f**), γ-thrombin (30 nM; **c, g**) or α-thrombin (4.5 nM; **d, h**) in the absence (**a–d**) or presence (**e–h**) of EIPA (25 μM). All tracings were obtained within one experiment, but are representative of four similar experiments. The arrows indicate the addition of agonists.

Table 1 Effect of Ca^{2+}_i chelation and inhibition of PKC on thrombin-receptor-induced platelet activation

BCECF- or fura-2-loaded platelets were stimulated at 37 °C with TRAP (15 μM), trypsin (20 μg/ml), γ-thrombin (30 nM) or α-thrombin (4.5 nM). B: where indicated, BAPTA-AM (30 μM)-loaded platelets were used. S: preincubated with staurosporine (1 μM) before addition of agonists. Acidification and alkalinization are expressed as pH units; acidification was determined after a preincubation with EIPA (25 μM) for 1 min. Data are means ± S.D. ($n = 5$–6): *significantly different ($P < 0.05$) from control (TRAP, trypsin, γ-thrombin or α-thrombin without additions).

Agonist	B	S	Acidification	Alkalinization	$[Ca^{2+}]_i$ (nM)
TRAP	−	−	0.10 ± 0.03	0.14 ± 0.04	323 ± 32
	−	+	0.14 ± 0.02	0.09 ± 0.06	302 ± 41
	+	−	0.05 ± 0.02	0.08 ± 0.03	42 ± 14*
	+	+	0.04 ± 0.03	0.01 ± 0.02*	47 ± 15*
Trypsin	−	−	0.10 ± 0.02	0.12 ± 0.03	390 ± 34
	−	+	0.13 ± 0.01	0.05 ± 0.01*	380 ± 48
	+	−	0.08 ± 0.01	0.04 ± 0.01*	52 ± 16*
	+	+	0.01 ± 0.01*	0.01 ± 0.03*	48 ± 7*
γ-Thrombin	−	−	0.10 ± 0.02	0.18 ± 0.04	170 ± 49
	−	+	0.11 ± 0.02	0.09 ± 0.02*	135 ± 13
	+	−	0.05 ± 0.02*	0.06 ± 0.01*	26 ± 4*
	+	+	0.04 ± 0.02*	0.01 ± 0.03*	37 ± 7*
α-Thrombin	−	−	0.10 ± 0.02	0.18 ± 0.04	196 ± 46
	−	+	0.14 ± 0.04	0.08 ± 0.03*	278 ± 36*
	+	−	0.04 ± 0.02*	0.07 ± 0.01*	48 ± 14*
	+	+	0.05 ± 0.01*	0.02 ± 0.02*	46 ± 4*

(Figure 1b; Table 1) there was a very fast irreversible increase in pH_i, which stabilized at about 0.12 pH unit above the resting pH. Addition of γ-thrombin (Figure 1c) resulted in a slow decrease in pH_i. This transient acidification lasted for 30–40 s and was followed by a slow increase in pH_i to about 0.18 pH unit above the resting pH_i. The alkalinization was irreversible and pH_i remained elevated up to 5 min. When platelets were stimulated with α-thrombin (Figure 1d) a very rapid acidification was seen, which lasted only 5–10 s. Thereafter the pH_i increased rapidly and stabilized at 0.18 pH unit above the resting pH_i.

One of the triggers for Na^+/H^+ exchange is an increase in the cytosolic $[H^+]_i$ [3]. To study proton generation during different types of thrombin-receptor activation, BCECF-loaded platelets were stimulated in the presence of EIPA (25 μM), an inhibitor of the exchanger. Addition of TRAP (Figure 1e) induced a rapid fall in pH_i that stabilized at 0.10 pH unit below the resting pH_i (Table 1). Addition of trypsin induced an equally rapid fall in pH_i to about 0.10 pH unit below resting pH_i (Figure 1f). Stimulation with γ-thrombin (Figure 1g) induced an equally rapid decrease in pH_i and the extent of acidification was not different from the TRAP-induced acidification response. Also, the response to α-thrombin (Figure 1h) was not different from that to TRAP. Thus the differences in pH_i response seen with different activators of the thrombin receptor were not due to differences in proton-generating properties.

Stimulation of $[Ca^{2+}]_i$ via the thrombin receptor

One of the factors that control Na^+/H^+ exchange in human platelets is the concentration of cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) [14]. To determine whether cleavage of the thrombin receptor and binding of thrombin to the hirudin-like domain affected $[Ca^{2+}]_i$ homoeostasis, fura-2-loaded platelets were stimulated with TRAP, trypsin, γ-thrombin or α-thrombin. TRAP induced a rapid increase in $[Ca^{2+}]_i$, which was transient, with a maximum

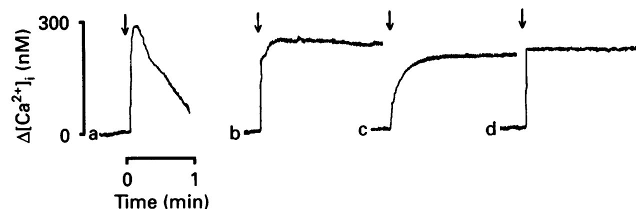

Figure 2 Effect of thrombin-receptor activation of $[Ca^{2+}]_i$

Gel-filtered fura-2-loaded platelets in Hepes–Tyrode buffer containing 20 μM $CaCl_2$ were stimulated at 37 °C with TRAP (15 μM; **a**), trypsin (20 μg/ml; **b**), γ-thrombin (30 nM; **c**) or α-thrombin (4.5 nM; **d**). The tracings were obtained within one experiment, but are representative of three similar experiments. The arrows indicate the addition of agonists.

reached within 10–20 s (Figure 2a; Table 1). Thereafter $[Ca^{2+}]_i$ decreased rapidly to resting levels. In contrast, trypsin induced a rapid, but irreversible, increase in $[Ca^{2+}]_i$ (Figure 2b; Table 1). Addition of γ-thrombin (Figure 2c) induced a slow increase in $[Ca^{2+}]_i$, which thereafter remained high for at least 5 min. When α-thrombin was added (Figure 2d), $[Ca^{2+}]_i$ rapidly increased. This increase was irreversible and remained high up to 5 min. Thus the different activation patterns of the Na^+/H^+ exchanger seen with different activators of the thrombin receptor were paralleled by similar changes in $[Ca^{2+}]_i$.

Stimulation of PKC via the thrombin receptor

In human platelets the Na^+/H^+ exchanger is activated via phosphorylation by PKC [5,7,11]. TRAP induced a rapid 4–5-fold increase in ^{32}P-pleckstrin, a major substrate of PKC in platelets. The maximal phosphorylation was reached within 15 s, and thereafter ^{32}P-pleckstrin returned to control levels (Figure 3). When trypsin was added, the incorporation of ^{32}P into pleckstrin rapidly increased to about 4 times resting values. In contrast with the effect of TRAP, this increase was irreversible. Addition of γ-thrombin resulted in an approx. 5-fold increase in the ^{32}P contents of pleckstrin. This increase was slow and reached a maximum about 60 s after addition of γ-thrombin. Thereafter ^{32}P-pleckstrin remained high. α-Thrombin induced an immediate increase in ^{32}P contents of pleckstrin, which was also irreversible. Taken together, these findings suggest that proteolytic cleavage of the thrombin receptor is required to preserve the increased phosphorylation of pleckstrin. Binding of thrombin to the hirudin-like domain is not essential for activation of PKC, but appears to accelerate this process. The changes in ^{32}P-pleckstrin closely paralleled those seen for the cytosolic pH.

A preincubation with BAPTA-AM before stimulation slowed down the α-thrombin-induced activation of PKC. The maximal amount of ^{32}P-pleckstrin did not change (Figure 3). Because the first 30 s are important for activation of the antiporter, this suggests that at least part of the effect of BAPTA on the Na^+/H^+ exchanger is due to inhibition of PKC.

Role of $[Ca^{2+}]_i$ and PKC in activation of the exchanger

To determine the relative contributions of $[Ca^{2+}]_i$ and phosphorylation in the activation of the exchanger, intracellular Ca^{2+} ions were chelated by loading the cells with BAPTA-AM (30 μM). This concentration of BAPTA completely blocked the increase in Ca^{2+}_i after stimulation with all agonists tested. In a second series of experiments PKC was inhibited by the non-selective PKC inhibitor staurosporine (1 μM), a concentration that was shown to inhibit the phosphorylation of the PKC

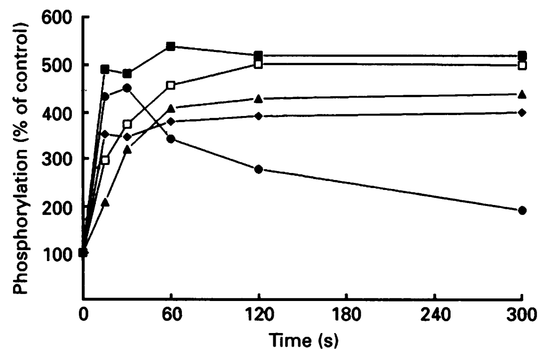

Figure 3 Activation of PKC by TRAP, trypsin, γ- and α-thrombin

Activation of PKC was assessed by determining the incorporation of ^{32}P into pleckstrin, a 47 kDa protein that is one of the major substrates for PKC in platelets. ^{32}P-labelled platelets were stimulated at 37 °C with 15 μM TRAP (●), 20 μg/ml trypsin (◆), 30 nM γ-thrombin (▲) or 4.5 nM α-thrombin (■). The effect of chelation of Ca^{2+}_i on activation of PKC was investigated by preincubating ^{32}P-labelled platelets with BAPTA (30 μM; 45 min, 37 °C). Subsequently these platelets were stimulated with 4.5 nM α-thrombin (□). Data are expressed as percentage of pleckstrin phosphorylation at $t = 0$ and are from one suspension that was representative of three others.

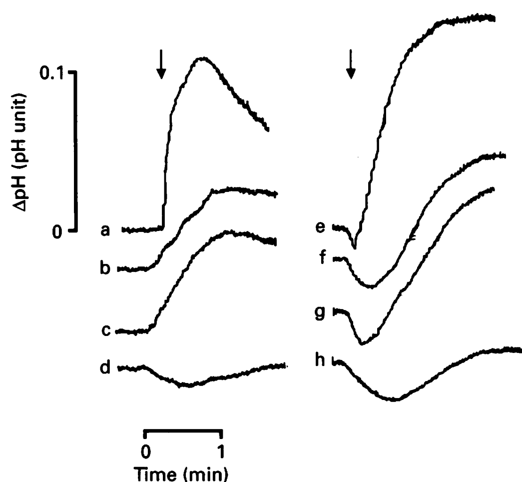

Figure 4 Role of Ca^{2+}_i and PKC in activation of the Na^+/H^+ exchanger

Gel-filtered BCECF-loaded platelets were stimulated at 37 °C with TRAP (15 μM; **a–d**) or α-thrombin (4.5 nM; **e–h**), without (**a, e**) or after a preincubation with BAPTA-AM (30 μM; **b, f**) or staurosporine (1 μM; **c, g**) or both staurosporine and BAPTA-AM (**d, h**). All tracings were from one suspension that was representative of four other experiments. The arrows indicate the addition of agonists.

substrate pleckstrin. The findings are summarized in Table 1, and representative BCECF-fluorescence tracings are shown in Figure 4. Addition of TRAP resulted in a transient alkalinization, as shown in Figure 4(a). Chelation of intracellular Ca^{2+} partially inhibited the TRAP-induced activation of the exchanger (Figure 4b). A similar inhibition was found when PKC was inhibited by staurosporine (1 μM; Figure 4c). However, when chelation of Ca^{2+}_i and inhibition of PKC were combined, TRAP induced only a small decrease in pH_i, which was followed by a slow return to resting pH_i without further alkalinization (Figure 4d). Chelation of Ca^{2+}_i and inhibition of PKC affected trypsin- and γ-thrombin-induced pH_i responses (results not shown) and α-thrombin-induced pH_i responses (Figures 4e–4h) in a similar way to that seen with TRAP. Thus both Ca^{2+}_i ions and PKC contribute to the stimulation of the Na^+/H^+ exchanger in platelets stimulated with TRAP, trypsin, γ-thrombin or α-thrombin.

Table 2 Effect of different PKC inhibitors on α-thrombin-induced Na^+/H^+ exchange

BCECF-loaded platelets were stimulated at 37 °C with 4.5 nM α-thrombin. Platelets were preincubated with staurosporine (1 μM; 1 min), H7 (50 μM; 1 min), K252A (10 μM; 1 min) or calphostin C (1 μM; 30 min). Control platelets were preincubated with an equal volume of Hepes/Tyrode buffer, pH 7.2. Data are means ± S.D. ($n = 4$).

Addition	Alkalinization (pH unit)
None (control)	0.17 ± 0.03
Staurosporine	0.08 ± 0.03
H7	0.08 ± 0.04
K252A	0.07 ± 0.02
Calphostin C	0.08 ± 0.03

Table 3 Effect of Ca^{2+}_i chelation and inhibition of PKC on the recovery from a cytosolic acid load

Sodium propionate (40 mM final concn.) was added to BCECF-loaded platelets at 37 °C, which lowered the pH_i by 0.18 ± 0.05 pH unit in all suspensions. Where indicated, BAPTA-AM (30 μM)-loaded platelets were used. Staurosporine (1 μM final concn.) was added 1 min before addition of sodium propionate. Data are means ± S.D. ($n = 5–6$).

BAPTA-AM (30 μM)	Staurosporine (1 μM)	Rate of recovery (pH unit/min)
−	−	0.06 ± 0.02
−	+	0.05 ± 0.01
+	−	0.05 ± 0.01
+	+	0.05 ± 0.01

Because staurosporine is not a selective PKC inhibitor, other PKC inhibitors were tested for their effect on α-thrombin-induced activation of the Na^+/H^+ exchanger. The results are shown in Table 2. The PKC inhibitor H7 (50 μM), as well as K252A (10 μM) and calphostin C (1 μM), blocked the α-thrombin-induced rise in pH_i by about 50 %, which is similar to the inhibition of PKC by staurosporine. This indicates that the effect of staurosporine is probably due to inhibition of PKC.

Control experiments showed the effects of Ca^{2+}_i chelation and inhibition of PKC on proton generation induced by TRAP, trypsin, γ-thrombin and α-thrombin (Table 1). About 50 % less acidification was observed in BAPTA-AM-loaded platelets. In contrast, staurosporine had no effect on proton generation. Since pH_i is an equilibrium between proton extrusion (Na^+/H^+ exchange) and production (ATP hydrolysis), the decreased acidification could also reflect a change in Na^+/H^+ exchange activity. Therefore Na^+/H^+ exchange was activated by an artificial acid load. As shown in Table 3, the recovery from addition of 40 mM propionate, which reflects activation of the exchanger by an increase in [H^+], was not affected by BAPTA-AM. Also, staurosporine did not change the recovery from an acid load. Thus BAPTA-AM and staurosporine left Na^+/H^+ exchange in unstimulated platelets undisturbed.

Effect of staurosporine on activation of the exchanger

In previous studies no effect of staurosporine was found when BCECF-loaded platelets were stimulated with 0.1 unit/ml α-

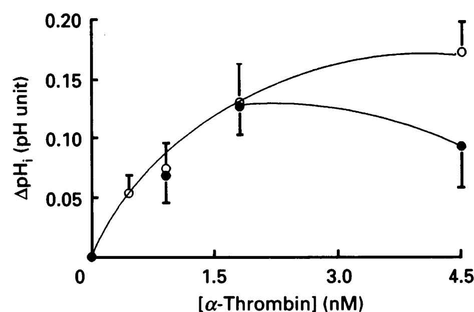

Figure 5 Effect of staurosporine on α-thrombin-induced exchange activation

Gel-filtered BCECF-loaded platelets were stimulated at 37 °C with different concentrations of α-thrombin without (○) or with (●) a 1 min preincubation with 1 μM staurosporine. Data represent means ± S.D. from 15 determinations (4.5 nM α-thrombin; $P < 0.001$).

thrombin [14,26]. In contrast, in the present study approx. 50% inhibition was found when platelets were stimulated with 4.5 nM (Table 1). To address the role of PKC in activation of the exchanger in more detail, BCECF-loaded platelets were stimulated with different concentrations of α-thrombin with and without staurosporine. Figure 5 shows that a preincubation with 1 μM staurosporine partially inhibited the activation of the exchanger at 4.5 nM α-thrombin, but at lower concentrations no inhibition was observed. This explains why staurosporine did not inhibit the exchanger in studies at lower concentrations of α-thrombin [14,26].

DISCUSSION

The results presented in this paper show that activation of the thrombin receptor by TRAP (SFLLRN) resulted in a rapid activation of the Na^+/H^+ exchanger. The activation was transient and was paralleled by an equally rapid and transient increase in $[Ca^{2+}]_i$ and PKC activity. When the thrombin receptor was cleaved, e.g. by addition of trypsin, γ- or α-thrombin, the exchanger was activated irreversibly. These results indicate that receptor cleavage was required to induce permanent activation of the exchanger. Also, the rise in $[Ca^{2+}]_i$ and activation of PKC were irreversible when the thrombin receptor was cleaved. Binding of α-thrombin to the hirudin-like domain made the activation of the Na^+/H^+ exchanger, the increase in $[Ca^{2+}]_i$ and activation of PKC much faster. TRAP, trypsin and γ-thrombin are known not to bind to the hirudin-like domain. Nevertheless, they activated the exchanger, indicating that occupation of the hirudin-like domain is not required for exchange activation. Activation of the Na^+/H^+ exchanger by TRAP was completely inhibited when $Ca^{2+}{}_i$ was chelated and PKC was inhibited. Also, activation of the exchanger by trypsin, γ-thrombin and α-thrombin were prevented under these conditions, indicating that receptor cleavage and binding of thrombin to the hirudin-like domain do not induce additional routes for exchange activation.

Zavoico et al. [7] showed that a 30 s interaction between α-thrombin and platelets was sufficient to induce irreversible activation of the exchanger. When α-thrombin was removed from its receptor by addition of hirudin, the thrombin-induced alkalinization was unaffected. Those authors suggested that cleavage of the thrombin receptor induces a permanent activation of the exchanger, which is in agreement with the present results. Possibly, cleavage of the thrombin receptor results not only in generation of TRAP but also in an irreversible conformational change of the receptor that induces permanent cell activation. In contrast with α-thrombin, TRAP induced a transient alkalinization. Also, other studies showed that cell activation by TRAP is different from activation by α-thrombin. Stimulation of gastric smooth-muscle cells with TRAP resulted in a transient contraction, whereas α-thrombin induced an irreversible contraction [20]. Stimulation of hamster fibroblasts with either TRAP or α-thrombin resulted in [^{3}H]inositol phosphate formation, but TRAP failed to induce mitogenesis, as seen upon stimulation by α-thrombin [27]. These findings were explained by assuming the existence of a second type of thrombin receptor, which induced the onset of mitogenesis. On the basis of our present findings, a different explanation might be that mitogenesis requires proteolysis of the thrombin receptor.

Our data show that γ-thrombin activated the exchanger. γ-Thrombin is an autoproteolysis product of α-thrombin that is cleaved within the anion-binding exosite for fibrin(ogen) recognition and therefore is unable to bind to the hirudin-like domain of the thrombin receptor [28]. In the present study a 6.5-fold higher concentration of γ-thrombin was used in order to obtain an increase in pH_i comparable with that with α-thrombin. Nevertheless, the activation of the exchanger was slower than that after stimulation with α-thrombin. McGowan and Detwiler [23] also found that much more γ-thrombin than α-thrombin had to be used in order to obtain similar platelet responses such as [^{32}P]phosphatidic acid formation and ATP secretion. More recently a mutant thrombin Quick I was described, which was mutated within the anion-binding exosite and did not bind to the hirudin-like domain [29]. The mutated thrombin induced a rise in $[Ca^{2+}]_i$, but this response was slower, and reached only 10–17% of the increase seen upon stimulation with α-thrombin. These findings were explained by assuming that γ-thrombin did not bind to the hirudin-like domain and could therefore not be targeted to its substrate. In addition, when a synthetic peptide, homologous to the hirudin-like domain, attached to α-thrombin, the specificity for artificial substrates changed [30]. Thus binding of α-thrombin to the hirudin-like domain may have two functions: (i) it may act as a binding site to target the protease and (ii) it may enhance thrombin's capability to cleave the receptor.

In many cell types, including fibroblasts [15,17], hepatocytes [31] and smooth-muscle cells [16], Na^+/H^+ exchange activity is regulated by $[Ca^{2+}]_i$. Possibly Ca^{2+} does not affect the exchanger directly, but acts via Ca^{2+}/calmodulin-dependent processes [16]. A purified Ca^{2+}/calmodulin-dependent protein kinase phosphorylated a fusion protein consisting of the C-terminus of the Na^+/H^+ exchanger from rabbit cardiac cells (NHE-1) and β-galactosidase [32]. When $Ca^{2+}{}_i$ was chelated in human platelets, activation of the exchanger by α-thrombin was decreased [14]. Although in the present study similar findings were obtained, we observed in addition that the generation of protons (Table 1) was inhibited by 50% and that activation of PKC (Figure 3) was slower during the first 60 s after α-thrombin stimulation. Chelation of $Ca^{2+}{}_i$ did not change the activity of the exchanger itself, as illustrated by a normal recovery from a cytosolic acidification. The finding that stimulation of PKC was partially inhibited was not very surprising. Platelets contains four subtypes of PKC, of which three are translocated to the membrane upon stimulation with α-thrombin: α, β and ζ [33]. Translocation is a $[Ca^{2+}]_i$-dependent process and is therefore expected to be decreased in BAPTA-AM-loaded cells [33–35].

Early studies by Siffert and Scheid [5] and Zavoico et al. [7] indicated that part of the control of the exchanger in stimulated platelets is mediated by PKC. Activators of PKC increased pH_i

in a process dependent on extracellular Na^+ and sensitive to amiloride and EIPA. The observation that the recovery from an acid load of resting platelets was faster after pretreatment with phorbol 12-myristate 13-acetate, an activator of PKC, made Zavoico et al. [7] and Kimura et al. [14] conclude that phosphorylation increased the affinity of the exchanger for protons. In the present study PKC was inhibited by staurosporine (1 μM). This concentration of staurosporine only partially inhibited thrombin-receptor-induced activation of the exchanger, as has been demonstrated previously [26]. At a lower concentration of α-thrombin, Kimura et al. [14] found no effect of staurosporine treatment, which is in agreement with our present findings. Apparently the relative importance of Ca^{2+} and PKC in control of the exchanger depends on the concentration of α-thrombin. The partial inhibition could not be explained by an incomplete inhibition of PKC, since at this concentration staurosporine completely inhibited the pleckstrin phosphorylation by an excess of α-thrombin (results not shown). Inhibition of PKC did not affect the recovery from a cytosolic acid load and did not inhibit the rise in $[Ca^{2+}]_i$. Also, other PKC inhibitors decreased the α-thrombin-induced alkalinization. Thus activation of the exchanger by the thrombin receptor(s) is mediated, at least in part, via activation of PKC.

Complete inhibition of thrombin-receptor-mediated activation of the exchanger was observed only when $Ca^{2+}{}_i$ was chelated and PKC was inhibited, suggesting that these events independently trigger Na^+/H^+ exchange. Watson and Hambleton [26] showed that staurosporine inhibited phorbol dibutyrate-induced platelet aggregation completely, whereas α-thrombin-induced aggregation was slowed down. Under conditions that prevented an increase in $[Ca^{2+}]_i$, aggregation by α-thrombin was slow and completely inhibited when staurosporine was present. Thus, also platelet responses other than Na^+/H^+-exchange activation depend on both activation of PKC and a rise in $[Ca^{2+}]_i$.

Thrombin-receptor-mediated activation of the Na^+/H^+ exchanger in other cell types may also be mediated by a rise in $[Ca^{2+}]_i$ and activation of PKC. Inhibition of PKC in vascular smooth-muscle cells failed to prevent activation of the exchanger by α-thrombin [36]. When not only PKC was inactivated but also $Ca^{2+}{}_i$ was chelated, no activation of the exchanger occurred. Since an increase in $[Ca^{2+}]_i$ failed to activate the exchanger in these cells, these authors suggested that both activation of PKC and an increase in $[Ca^{2+}]_i$ are required to enhance Na^+/H^+ exchange. In aortic smooth-muscle cells, α-thrombin activated the exchanger under conditions where PKC was inactivated [37]. To prevent activation completely, chelation of $Ca^{2+}{}_i$ was required.

In conclusion, the present findings indicate that activation of the Na^+/H^+ exchanger by α-thrombin occurs via binding of the tethered ligand to the receptor. The binding triggers a transient activation of the exchanger via both a $Ca^{2+}{}_i$- and PKC-dependent pathway. Proteolysis of the receptor changes the transient exchange activation into a permanently activated Na^+/H^+ exchange due to a parallel change in $[Ca^{2+}]_i$ and PKC. Binding to the hirudin-like domain accelerates signal generation and exchange activation, possibly by facilitating the proteolysis of the N-terminal cleavage site of the receptor.

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