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Effect of epinephrine on the regulation of Na^+/H^+ exchange in human platelets

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In the present study, we investigated whether stimulation of platelets by epinephrine affects the Na^+/H^+ exchanger, an antiport that regulates the cytosolic pH (pH_i). Epinephrine alone failed to modulate Na^+/H^+ exchange, as reflected by a constant fluorescence of the pH_i indicator BCECF. In contrast, epinephrine accelerated Na^+/H^+ exchange upon stimulation with a threshold concentration of platelet activating factor (PAF). The extra Na^+/H^+ exchange was not caused by a better binding of PAF to platelets and occurred also in the presence of indomethacin, excluding a role for cyclooxygenase products. Epinephrine failed to mobilize Ca_i^{2+} (measured by fura-2 fluorescence) and did not activate protein kinase C ([^{32}P]pleckstrin) or phospholipase C ([^{32}P]phosphatidic acid). In combination with PAF, epinephrine left the PAF-induced mobilization of Ca_i^{2+} and accumulation of [^{32}P]phosphatidic acid unchanged, but induced a 1.3-fold increase in the phosphorylation of pleckstrin. These data indicate that epinephrine enhances Na^+/H^+ exchange via a direct effect of α_{2A}-adrenergic receptors on protein kinase C.

Introduction

The Na^+/H^+ exchanger mediates the efflux of intracellular protons in exchange for extracellular sodium ions and plays an important role in the regulation of the cytosolic pH (pH_i). In human platelets, α-thrombin, ADP and platelet-activating factor (PAF) activate the Na^+/H^+ exchanger via a phospholipase C (PLC)-dependent mechanism. PLC cleaves phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol [1]. IP_3 induces release of Ca^{2+} from the dense tubular system [2] and diacylglycerol promotes the insertion of protein kinase C (PKC) into the lipid bilayer [3,4]. The affinity of PKC for calcium and phospholipid increases [5] and PKC is activated. Both cytosolic Ca^{2+} ions and PKC control the activation of the Na^+/H^+ exchanger during platelet stimulation [6,7]. Depletion of Ca_i^{2+} leads to a decrease in the agonist-mediated activation of the antiporter and an acidic shift in the basal pH_i [6]. On the other hand, activation of PKC enhances Na^+/H^+ exchange through an alkaline shift in the pH_i set-point, resulting in faster recovery from cytosolic acidification [8]. In addition, GTP-binding proteins may control the Na^+/H^+ exchanger, since fluoride, an activator of these proteins, blocks the α-thrombin-induced proton efflux [9].

Epinephrine is a unique platelet agonist. Initially, epinephrine was thought to activate platelets only in a synergistic action with trace amounts of other agonists. However, recent evidence indicates that epinephrine activates platelets [10], resulting in the exposure of fibrinogen binding sites, fibrinogen binding and aggregation [11,12]. In contrast to α-thrombin, ADP and PAF, epinephrine fails to activate PLC [11] and PKC [13] and does not mobilize Ca^{2+}, at least as detected by fura-2 fluorescence [14]. This suggests that epinephrine utilizes pathways for platelet activation that are different from those initiated by other agonists.

Epinephrine binds to α_{2A}-adrenergic receptors and inhibits adenylyl cyclase and cyclic AMP formation via an inhibitory GTP-binding protein, G_i [15–17]. Epinephrine also activates phospholipase A_2, resulting in liberation of arachidonic acid and formation of prostaglandin endoperoxides and thromboxane A_2

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Abbreviations: pH_i, cytosolic pH; PAF, platelet-activating factor; IP_3, inositol 1,4,5-trisphosphate; PLC, phospholipase C; PLA_2, phospholipase A_2; PKC, protein kinase C; BCECF-AM, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein acethoxymethyl ester; fura-2-AM, fura-2 acethoxymethyl ester.

[18,19]. Banga et al. [18] measured pH_i with 9-aminoacridine and observed an increase in pH_i after stimulation with epinephrine [18]. Surprisingly, pretreatment with ethylisopropylamiloride, an inhibitor of Na^+/H^+ exchange, not only prevented the increase in pH_i, but also blocked the formation of prostaglandin endoperoxides/thromboxane A_2 and the subsequent accumulation of IP_3. These findings made these authors propose, that the α_{2A}-adrenergic receptor is coupled to the Na^+/H^+ exchanger and that its activation precedes the activation of phospholipase A_2 and PLC. In the present study we investigated the coupling between the α_{2A}-adrenergic receptor and Na^+/H^+ exchanger in more detail.

Materials and Methods

Materials

2,7-Biscarboxyethyl-5(6)-carboxyfluorescein acethoxymethyl ester (BCECF-AM) and PAF were obtained from Calbiochem (San Diego, CA, USA). Epinephrine, indomethacin and nigericin were from Sigma (St. Louis, MO, USA). Fura-2 acetoxymethyl ester (fura-2-AM) was purchased from Boehringer-Mannheim (Mannheim, Germany), [^{32}P]orthophosphate (spec. act. 314 TBq/mmol) from Dupont (New England Nuclear) and [^{3}H]PAF from Amersham International. Sepharose 2B was obtained from Pharmacia-LKB (Uppsala, Sweden). Ethylisopropylamiloride was provided by Dr. W. Siffert. All other chemicals were of analytical grade.

Methods

Platelet isolation. Freshly-drawn venous blood from healthy volunteers (with informed consent) was collected into tri-sodium citrate (0.1 vol. of 130 mM). The donors claimed not to have taken any medication during the previous ten days. Platelets were isolated by either gel filtration (measurement of pH_i) or centrifugation (measurement of PKC activity).

Measurement of pH_i. Citrated blood was centrifuged ($200 \times g$, 10 min, 22°C) and the platelet-rich plasma was collected and acidified to pH 6.5 with 1/6 vol. of ACD (2.5 g tri-sodium citrate, 1.5 g citric acid, 2.0 g D-glucose in 100 ml water). The platelets were isolated by centrifugation ($700 \times g$, 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 $MgSO_4$, 10 mM Hepes (pH 6.5)), containing 0.1% glucose (w/v) and 0.2% gelatin (w/v). The cells were incubated with BCECF-AM (3 μM) in the absence or presence of indomethacin (30 μM) for 30 min at 37°C, 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. Aliquots of 50 μl were added to 1450 μl of prewarmed, gelatin-free Hepes-Tyrode buffer (pH 7.2 37°C), resulting in a final platelet count of $(1.5–2) \cdot 10^5/\mu$l. Fluorescence was determined on a Hitachi F-3000 spectrofluorometer at 37°C, using wavelengths of 495 and 530 nm for excitation and emission, respectively. The suspension was gently stirred (100 rpm). Calibration was carried out by diluting the BCECF-loaded platelets in a high potassium 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. [20].

Measurement of cytosolic calcium. Platelet-rich plasma was incubated with fura-2-AM (3 μM) and indomethacin (30 μM) for 45 min at 37°C. The platelets were washed by centrifugation (15 min, $700 \times g$ at room temperature); 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 in Measurement of intracellular pH. 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 $(1.5–2) \cdot 10^5/\mu$l. Fluorescence was measured at 37°C with exitation at 345 nm and emission at 495 nm on a Hitachi F-3000 spectrofluorometer in gently stirred suspensions (100 rpm).

Measurement of PKC activity. Platelets were labelled with 3.7 MBq carrier-free [^{32}P]orthophosphate/ml acidified platelet-rich plasma (pH 6.5) in the presence of indomethacin (30 μM) for 1 h at 37°C. The platelets were isolated by centrifugation ($700 \times g$, 15 min, 22°C) and resuspended in Hepes-Tyrode buffer (pH 7.2), resulting in a final platelet count of $(1.5–2) \cdot 10^5/\mu$l. Labelled platelets were stimulated with different agonists at 37°C and samples were collected at the times indicated in Results, transferred into 0.5 vol. of 3× concentrated Laemmli sample buffer and heated for 2 min at 100°C prior to electrophoresis. Proteins were separated by electrophoresis through a 11% polyacrylamide gel, according to Laemmli [21]. Gels were stained with Coomassie brillant 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 dried gels and heated for 2 h at 80°C in 30% 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 (data not shown). Data are expressed as percentage of pleckstrin phosphorylation in unstimulated platelets.

Measurement of phosphatidic acid. Changes in phosphatidic acid were measured according to a modified procedure described by Jolles et al. [22] for rat brain

cells. In short, platelets were labelled with [^{32}P]orthophosphate in the presence of indomethacin and isolated by centrifugation as described in Measurement of PKC activity. The platelets were stimulated with epinephrine (10 μM) and/or PAF (20 nM). The reactions were terminated at different time-points by adding 2 ml chloroform/methanol/13 M HCl (100:50:1, by volume; 0°C). The lipids were extracted as described by Bligh and Dyer [23]. Phospholipids were separated by high-performance thin-layer chromatography and the radioactivity was determined by autoradiography on Kodak Royal X-Omat film. The spots were scraped off and counted for ^{32}P by liquid scintillation counting. The ^{32}P content of phosphatidic acid is expressed as a percentage of that of unstimulated platelets.

Binding of [^{3}H]PAF. Samples of gel-filtered platelets were incubated in duplicate with different concentrations of [^{3}H]PAF (22°C) with and without a 200-fold molar excess of unlabelled PAF to measure the specific binding of PAF, as described previously [24]. Binding studies were performed at 22°C, since at 37°C [^{3}H]PAF is rapidly taken up and metabolized, thus making it difficult to assess receptor-[^{3}H]PAF interactions [24]. After incubation, the samples were placed on 20% sucrose in Tyrode (w/v) and centrifuged (12000 × g, 2 min, 22°C). Platelet pellets were lysed in 1% Triton X-100 and the radioactivity was measured by standard methods.

Presentation of data. Data are expressed as means ± S.D. (n = 3–8). Statistical significances were determined by Student's t-tests, and were considered significant at $P < 0.05$.

Results

Effect of epinephrine on the Na^+/H^+ exchanger

Fig. 1a shows the changes in pH_i measured in BCECF-loaded platelets stimulated with epinephrine

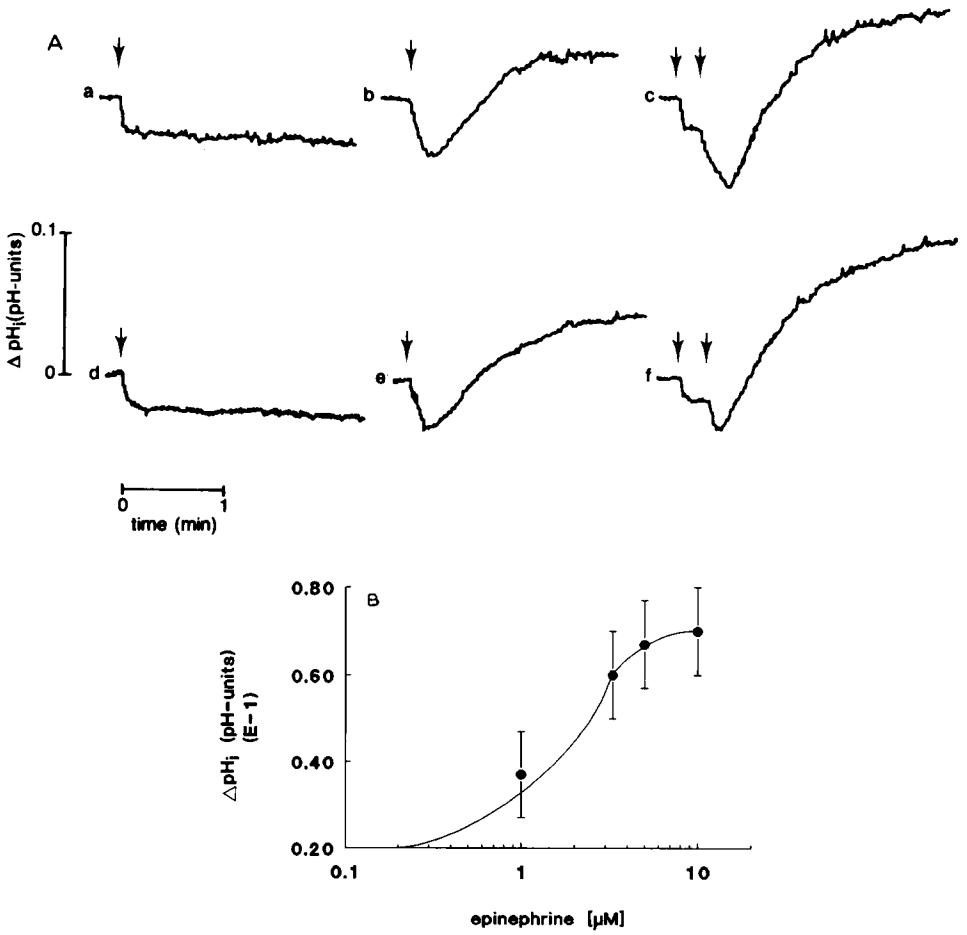

Fig. 1. Effect of epinephrine and PAF on Na^+/H^+ exchange. (A) Platelets were loaded with BCECF-AM (3 μM) in the absence (a–c) or presence (d–f) indomethacin (30 μM). The platelets were stimulated at 37°C with epinephrine (10 μM; curves a,d), PAF (20 nM; b,e) or PAF added 15 s after the addition of epinephrine (c,f). The arrows indicate the additions of agonists. The figure shows fluorescence tracings obtained from one experiment and are representative for three other experiments. Fig. 1B shows the effect of different concentrations of epinephrine on activation of the Na^+/H^+ exchanger by PAF (20 nM). Platelets were loaded with BCECF-AM (3 μM) in the presence of indomethacin (30 μM), preincubated (15 s) with different concentrations of epinephrine (1–10 μM) and stimulated with PAF (20 nM).

and PAF. Resting platelets had a pH_i of 7.13 ± 0.06 ($n = 6$). Addition of epinephrine (10 μM) resulted in a small decrease in BCECF-fluorescence due to dilution of the suspension but no further changes in fluorescence were observed (Fig. 1A,a). Thus, epinephrine failed to change pH_i as measured with BCECF-fluorescence.

We next investigated whether epinephrine affected the regulation of the Na^+/H^+ exchanger by a second agonist. Platelets were stimulated with PAF, a potent activator of the Na^+/H^+ exchanger [25]. The concentration of PAF was lowered to a level that minimally affected BCECF-fluorescence, thereby making the control of pH_i optimally responsive to possible modulation by epinephrine. PAF (20 nM) induced a fall in BCECF fluorescence, that was followed by a slow recovery until pH_i stabilized at levels slightly above the resting pH_i (Fig. 1A,b; Table I). When epinephrine was added 15 s before PAF, the rate of alkalinization was almost two-fold faster than in the absence of epinephrine (Fig. 1A,c). Also, the final pH_i was higher than in the absence of epinephrine and stabilized at 7.20 ± 0.02. To demonstrate that the observed alkalinization was due to an increased Na^+/H^+ exchange, platelets were pretreated with ethylisopropylamiloride. Addition of PAF (20 nM) after a 1-min preincubation with ethylisopropylamiloride (25 μM) resulted in a slight acidification, that was more than 2-fold higher in the presence of epinephrine (10 μM; Table I). These data demonstrate that epinephrine affects the control of the Na^+/H^+ antiporter, although this is only apparent in the presence of a second agonist.

A first step in PAF-induced exchange control is the binding to its receptors on the plasma membrane. In the absence of epinephrine 223 ± 63 molecules [^{3}H] PAF bound specifically per platelet ($n = 5$) at 22°C. In the presence of epinephrine (10 μM) this number was 216 ± 56 molecules/platelet ($P > 0.05$). Also, the binding affinity for [^{3}H]PAF was not affected by epinephrine and was 0.27 ± 0.04 nM in the absence of epinephrine and 0.27 ± 0.08 nM in its presence, ($n = 8$, $P > 0.05$) [26].

Banga et al. [18] proposed that epinephrine activates the Na^+/H^+ exchanger prior to activation of phospholipase A_2 and formation of cyclooxygenase products. Fig. 1A,a, d and f illustrate the changes in pH_i by epinephrine and PAF in platelets in which cyclooxygenase was inhibited with indomethacin. As in untreated platelets, epinephrine failed to affect pH_i (Fig. 1A,d) and also the changes induced by PAF (Fig. 1A,e) and the combination of PAF and epinephrine (Fig. 1A,f) were similar to those seen in untreated platelets. Thus, in agreement with earlier observations based on 9-aminoacridine [18], epinephrine's effect on the Na^+/H^+ antiporter is not dependent on the formation of prostaglandin endoperoxides and thromboxane A_2.

Fig. 1B shows that concentrations of epinephrine lower than 10 μM also enhanced PAF-induced activation of the exchanger. The IC_{50} for epinephrine was between 1–3 μM.

Possible role of Ca^{2+} mobilization

To determine, whether epinephrine enhanced the PAF-induced Na^+/H^+ exchange by facilitating the mobilization of Ca^{2+}-ions, platelets were incubated with indomethacin and loaded with the fluorescent dye fura-2-AM (3 μM). EGTA (1 mM) was added immediately before addition of the agonists to prevent influx of extracellular Ca^{2+}-ions. The concentration of Ca_i^{2+} in resting platelets was 69 ± 22 nM and not affected by 10 μM epinephrine (Fig. 2a). In contrast, even the low concentration of PAF (20 nM) induced a rapid rise of $[Ca^{2+}]_i$ to 125 ± 33 nM (Fig. 2b). When epinephrine was added 15 s before PAF, no change in PAF-induced Ca^{2+}-mobilization was observed ($P < 0.05$; $n = 4$; Fig. 2c). Since PAF induced more Na^+/H^+ exchange in the presence of epinephrine than alone (Fig. 1Ab,c), whereas the mobilization of Ca^{2+} ions was the same,

TABLE I

Modulation of PAF-induced Na^+/H^+ exchange by epinephrine

Changes in pH_i are expressed in pH units. The resting pH_i was 7.13 ± 0.06 ($n = 6$). Changes in acidification and alkalinization when compared with resting pH_i. Concentrations of ethylisopropylamiloride (EIPA), epinephrine (EPI) and PAF were 25 μM, 10 μM and 20 nM, respectively. Epinephrine was added 15 s before PAF.

Agonist	Acidification	Alkalinization	Δ pH/min
Untreated			
PAF	0.05 ± 0.02	0.03 ± 0.01	0.7 ± 0.2
EPI/PAF	0.04 ± 0.01	0.07 ± 0.02 *	1.3 ± 0.1 *
EIPA PAF	0.04 ± 0.02	0	0
EIPA EPI/PAF	0.10 ± 0.02	0	0
Indomethacin-treated			
PAF	0.04 ± 0.02	0.04 ± 0.02	0.8 ± 0.2
EPI/PAF	0.02 ± 0.01	0.11 ± 0.01 *	1.2 ± 0.1 *

* $P < 0.05$, compared with addition of PAF alone.

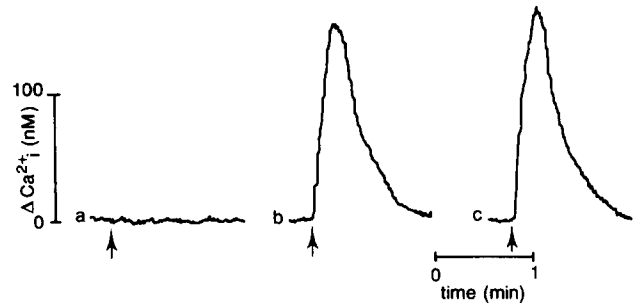

Fig. 2. Role of Ca^{2+}-mobilization. Platelets were loaded with fura-2-AM (3 μM) in the presence of indomethacin (30 μM) for 45 min at 37°C. 1 min before the addition of agonists EGTA (1 mM) was added to chelate extracellular Ca^{2+}-ions. Platelets were stimulated at 37°C with epinephrine (10 μM, curve a), PAF (20 nM, b) or PAF added 15 s after the addition of epinephrine (c). The arrows indicate the additions of agonists. The figure shows fluorescence tracings obtained from one experiment that are representative for three other experiments.

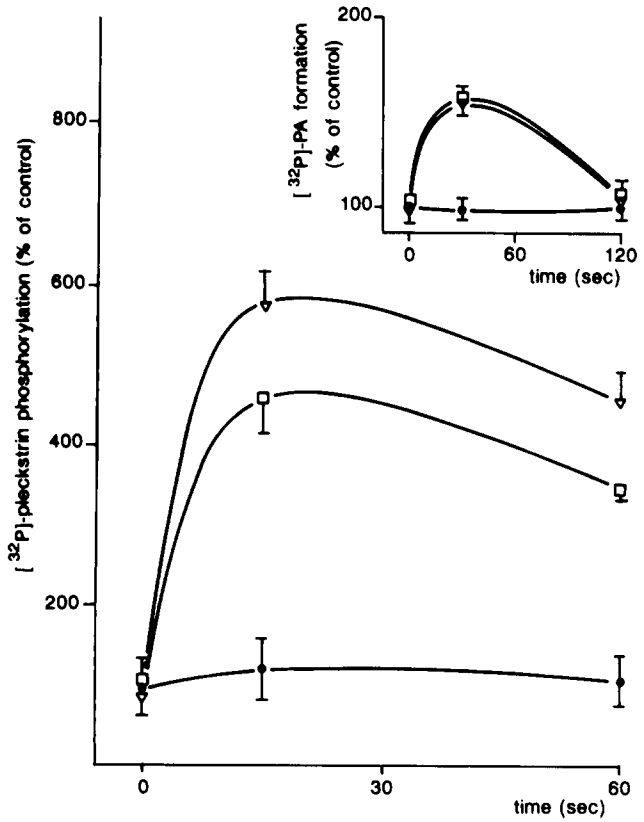

Fig. 3. Role of protein kinase C. Platelets were incubated with indomethacin (30 μM) and [^{32}P]orthophosphate. The platelets were isolated by centrifugation and stimulated with epinephrine (10 μM, •), PAF (20 nM, □) or PAF added 15 s after the addition of epinephrine (▽). The data are expressed as percentage of pleckstrin phosphorylation in unstimulated platelets. Inset: ^{32}P-labelled platelets were stimulated with epinephrine (10 μM, •), PAF (20 nM, □) or a combination of epinephrine and PAF (▽). The amount of [^{32}P]phosphatidic acid radioactivity is expressed as a percentage of that in unstimulated platelets. The data represent means ± S.D. ($n = 5$).

these data indicate that epinephrine does not affect Na^+/H^+ exchange via an enhancement of Ca^{2+} mobilization.

Role of protein kinase C

A second mechanism for the control of Na^+/H^+ exchange in platelets is activation of PKC [8,25]. Fig. 3 illustrates, that the phosphorylation of pleckstrin, which is a major substrate of PKC, remained unchanged during the first minute after addition of epinephrine. Also at later stages (up to 5 min) no signs of PKC activity could be detected (data not shown). In contrast, 20 nM PAF induced a 4-fold increase in pleckstrin phosphorylation. Even more PKC activity was found with the combination of epinephrine and PAF, which resulted in a 6-fold enhancement of pleckstrin phosphorylation at 60 s after addition of PAF ($P = 0.005$; $n = 6$).

The activation of PKC seen in platelets stimulated with α-thrombin, ADP or PAF is known to result from the PLC-mediated formation of diacylglycerol. Since diacylglycerol is rapidly phosphorylated by diacylglycerol-kinase to phosphatidic acid, accumulation of this metabolite is generally taken as a marker for PLC activity in platelets [27]. As shown in the inset of Fig. 3, no increase in [^{32}P]phosphatidic acid was found after addition of epinephrine. In contrast, PAF induced a 1.5-fold increase in [^{32}P]phosphatidic acid within 30 s, followed by a rapid decrease to control levels. Essentially similar patterns were found with the combination of epinephrine and PAF, suggesting that the activation of PLC by PAF was not further enhanced by epinephrine.

Discussion

The results presented in this paper show, that epinephrine fails to raise pH_i, as detected by BCECF-fluorescence. Epinephrine was also unable to increase $[Ca^{2+}]_i$, to activate PKC, or to induce the formation of [^{32}P]phosphatidic acid, which is a reflection of the activation of PLC [27], illustrating that the pathways that control the exchanger during stimulation by other platelet agonists are insufficiently activated by epinephrine. In contrast, in the presence of a threshold concentration of PAF, epinephrine facilitated the activation of the antiporter by PAF leading to a faster and higher alkalinization. This effect was not due to formation of cyclooxygenase products or modulation of the properties of the PAF receptors. Epinephrine had no effect on the PAF-induced mobilization of Ca^{2+}_i-ions or the formation of [^{32}P]phosphatidic acid, but induced a 1.5-fold increase in pleckstrin phosphorylation. Thus, the higher exchange activation by the combination of PAF and epinephrine compared to PAF alone is likely the result of stimulation of PKC activity.

Banga et al. [18] used the pH_i indicator 9-ami-

noacridine and found an increase in pH_i upon addition of epinephrine. Interestingly, the alkalinization depended on the presence of fibrinogen, suggesting that occupation of the glycoprotein IIB/IIIA complex was essential for epinephrine-induced Na^+/H^+ exchange. These findings contrast with the lack of changes in pH_i of BCECF-loaded platelets stimulated with epinephrine and also in the presence of fibrinogen (2 μM) we found no signs of Na^+/H^+ exchange (data not shown). Similar results were obtained by Steen et al. [28], who found only an effect of epinephrine on Na^+/H^+ exchange in the presence of low concentrations of α-thrombin. In addition, we measured the set point for Na^+/H^+ exchange activation, as described by Kimura et al. [6]. BCECF-loaded platelets were acidified by addition of sodium proprionate (20 mM), without or with a 30 s preincubation with epinephrine (10 μM). Also, under these conditions, epinephrine failed to change the pH_i recovery following artificial acidification of the cytosol (data not shown).

Our findings show, that the combination of PAF and epinephrine activates more PKC than PAF alone and that this effect is not caused by a higher increase in cytosolic Ca^{2+} concentration or more activation of the PLC pathway. Saitoh et al. [29] found no activation of PKC by the separate additions of epinephrine and the Ca^{2+}-ionophore A23187, whereas the combination induced a 3-fold increase in [^{32}P]pleckstrin phosphorylation. The A23187-induced increase in $[Ca^{2+}]_i$ and formation of diacylglycerol were not changed by epinephrine, indicating that another mechanism than the PLC pathway was responsible for the increase in PKC activity [29]. In another study [30], epinephrine potentiated the ADP-induced activation of PKC, again without changing the activity of PLC. The fact that this interaction was only seen when the PLC pathway was active led these authors to the conclusion that the interaction between the α_{2A}-adrenergic receptor and PKC depended on an increase in $[Ca^{2+}]_i$ [30]. In a parallel study [31], we found that diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS), an inhibitor of Cl^-/HCO_3^- exchange which stimulates platelets and erythrocytes [32–34], activated PKC via binding to α_{2A}-adrenergic receptors [31,35]. This activation was independent of the PLC pathway, occurred at a stable cytosolic Ca^{2+} content and preceded further steps in platelet activation, such as activation of Na^+/H^+ exchange, binding of fibrinogen to glycoprotein IIB/IIIA and aggregation. Taken together, these data suggest that epinephrine enhances the activation of PKC via a direct effect of the α_{2A}-adrenergic receptor on PKC.

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