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Potentiation by adrenaline of thrombin-induced elevation of pH_i is not essential for synergistic activation of human platelets

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Gel-filtrated human platelets were stimulated with thrombin in the absence and presence of adrenaline. Adrenaline markedly enhanced the thrombin-induced increase in cytoplasmic pH (pH_i) in BCECF-loaded platelets. This rise in pH_i was strongly inhibited by the Na^+/H^+ exchange blocker EIPA. The potentiation by adrenaline of thrombin-induced PLC activation measured as [^{32}P]PA formation and final platelet responses was, however, not blocked by EIPA, even at low concentrations of thrombin. These results indicate that the enhancement by adrenaline of thrombin-induced cytoplasmic alkalinization may be a secondary effect which is not essential for the potentiation by adrenaline of platelet activation by thrombin.

Na^+/H^+ exchange; PLC activation; Aggregation; Secretion

1. INTRODUCTION

The mechanism of adrenaline-mediated human platelet activation has been subject to much debate during the last decade. Especially, it has not been clearly established whether adrenaline is a platelet stimulator per se or only potentiates responses induced by other platelet agonists.

Therefore, the demonstration that adrenaline by itself induces cytoplasmic alkalinization via activation of the Na^+/H^+ antiport attracted great interest, especially since it was shown that this rise in pH_i is parallelled by phospholipase A_2 activation [1,2], thereby constituting a possible pathway for adrenaline-mediated platelet activation.

Recently it was reported that adrenaline reverses thrombin-induced homologous desensitization of human platelets, and this resensitization is not inhibited by the Na^+/H^+-exchange blocker EIPA [3,4]. These results indicated that adrenaline did not affect platelet activation through an effect on pH_i. In the present paper we have investigated whether adrenaline may potentiate thrombin-induced cytoplasmic alkalinization, and determined the importance of this mechanism during potentiation by adrenaline of thrombin-induced platelet activation.

2. MATERIALS AND METHODS

2.1. *Materials*

Stock solutions of bovine thrombin (Hoffman-La Roche), adrenaline (Sigma), acetylsalicylic acid (Sigma), nigericin (Sigma; prepared in DMSO), BCECF-AM (Molecular Probes Inc.) and EIPA (a gift from Dr W. Siffert), both prepared in DMSO, were stored at −20°C. Solutions of CP (Sigma) and CPK (Sigma; type I, rabbit muscle) were freshly prepared in Tyrodes solution. [^{32}P]Orthophosphate was purchased from Amersham (code PBS-11, carrier-free).

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Abbreviations: BCECF, 2,7-biscarboxyethyl-5(6)-carboxyfluorescein; AM, acetooxymethyl ester; CP, creatine phosphate; CPK, creatine phosphokinase; EIPA, ethylisopropylamiloride; PA, phosphatidic acid; pH_i, cytoplasmic pH; P_i, inorganic orthophosphate; PLA_2, phospholipase A_2; PLC, phospholipase C

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2.2. *Platelet isolation, labelling and incubation*

Platelet-rich plasma (PRP) was prepared by differential centrifugation of freshly drawn human venous blood anticoagulated with ACD [5]. For the phosphoinositide experiments, PRP was incubated with [^{32}P]P_i (0.1 mCi/ml) for 60 min at 37°C. In the experiments designed to measure cytoplasmic pH (pH_i), the platelet pellet was resuspended in a Hepes-modified Tyrode's buffer (10 mM Hepes, 0.02% BSA, 5 mM glucose, pH 6.5) and incubated with the fluorescent probe BCECF for approx. 60 min at 37°C. BCECF was added as its tetraacetooxymethyl ester (BCECF-AM; 10 μM final conc.). In all experiments, acetylsalicylic acid (1 mM final conc.) was added to the platelet suspension during the last 15 min of the different preincubation procedures. The platelets were transferred by gelfiltration through Sepharose 2B to a Hepes-modified, nominally HCO_3^- free Tyrode's buffer (10 mM Hepes, 0.2% BSA, 5 mM glucose, pH 7.3) which also contained CP (5 mM)/CPK (10 U/ml). In the phospholipid experiments, the buffer was nominally P_i-free. The platelet concentration was either standardized at 3.5×10^8 cells/ml for phosphoinositide, aggregation and secretion experiments or at 1.0×10^8 cells/ml for pH_i determination. In all experiments, suspensions of platelets were preincubated with DMSO (control; 0.1%) or EIPA (10 μM; 0.1% DMSO) for 10 min at 37°C before the addition of agonist(s). Adrenaline was always added 10 s prior to thrombin.

2.3. *Determination of pH_i*

The fluorescence experiments were performed without stirring at 37°C in a Perkin-Elmer LS 5 luminescence spectrometer. Excitation and emission were at 495 nm and 530 nm, respectively, using bandwidths of 5 nm. Samples of BCECF-loaded platelets were stimulated after a stable fluorescence signal had been established. The pH_i measurements recorded as changes in fluorescence were calibrated according to Horne et al. [6], using a Hepes-buffer with 120 mM KCl, 25 mM NaCl and 2 μM nigericin. BCECF fluorescence was linear with pH in the pH range investigated (6.9–7.4). Saline (0.15 M NaCl) alone did not change the fluorescence signal (not shown). The traces are representative of experiments with platelets from at least 4 different donors.

2.4. *Phospholipid metabolism*

0.5 ml samples were withdrawn from the unstirred suspensions (37°C) 90 s after the addition of agonist, and mixed with 2.0 ml chloroform/methanol/conc. HCl (20:40:1, by vol.; 0°C). The further processing of the samples, including separation by thin-layer chromatography and determination of radioactivity of the phospholipids were performed as described [5]. The results are expressed as a percentage of the saline-treated (control) platelets.

2.5. *Aggregation and dense granule secretion*

Aggregation was recorded as change in optical density of the platelet suspension, using a Payton dual-channel aggregation module [7]. Dense granule secretion was determined after 75 s of incubation as the extracellular appearance of ADP plus ATP, measured by a luciferin-luciferase method [7]. The results are representative of identical experiments from 6 different donors.

3. RESULTS

3.1. *Adrenaline potentiates thrombin-induced elevation of pH_i*

Fig.1 shows typical changes in the fluorescence signal in BCECF-loaded platelets upon stimulation with thrombin and adrenaline. Resting pH_i value was 7.25 ± 0.03 (n = 7; ± SD). The fluorescence signal did not increase by adrenaline alone (10 μM) under the conditions used (fig.1a). Thrombin alone (0.10 U/ml) induced a minimal initial decrease of pH_i (<0.02 pH units) followed by a slow alkalinization to 0.06 pH units (2 min after stimulation) above the initial value (fig.1b). The presence of adrenaline (10 μM) markedly enhanced the thrombin-induced alterations of pH_i, demonstrated by an initial drop in fluorescence (0.05 pH units) followed by a more rapid alkalinization to 0.06 pH units above resting value. Only the rate but not the final extent of the alkalinization was increased by the presence of adrenaline (fig.1c). The increase in pH_i induced by thrombin alone (not shown) and by adrenaline plus thrombin was

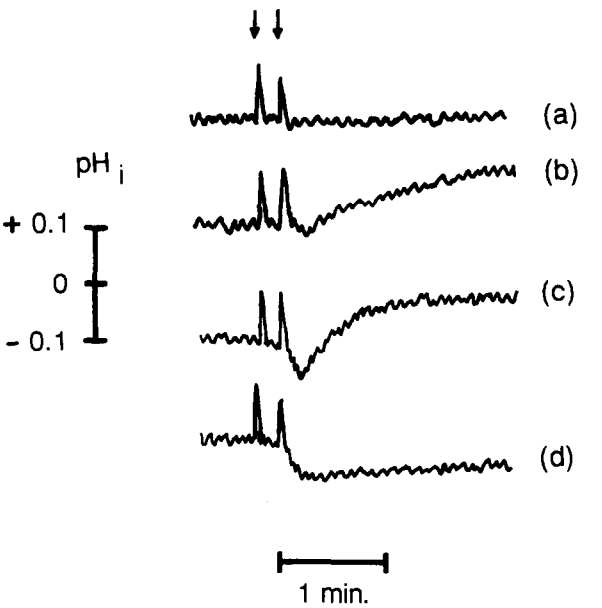

Fig.1. Changes in the cytoplasmic pH induced by adrenaline and thrombin in the absence or presence of EIPA. BCECF-loaded platelets were stimulated with (a) adrenaline (10 μM), (b) thrombin (0.10 U/ml), (c) adrenaline (10 μM) and thrombin (0.10 U/ml), or (d) EIPA (10 μM) plus adrenaline (10 μM) and thrombin (0.10 U/ml). The agonists were added as indicated by the arrows.

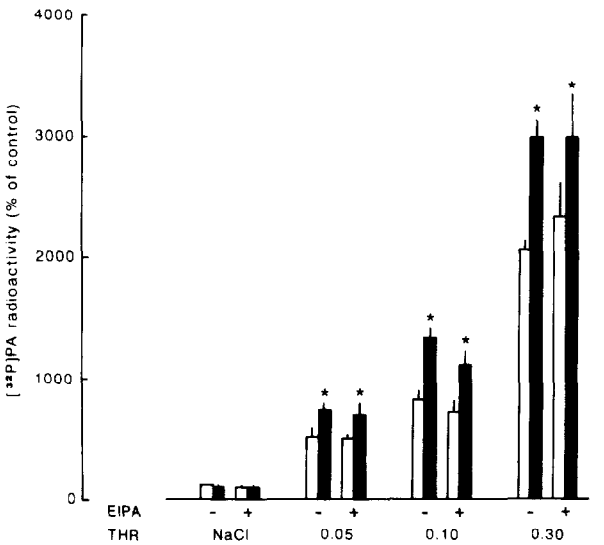

Fig.2. The effect of EIPA on [^{32}P]PA formation induced by thrombin alone and by adrenaline plus thrombin. [^{32}P]P_i-prelabelled platelets were stimulated with saline or increasing concentrations of thrombin (U/ml) with (closed bars) or without (open bars) adrenaline (10 μM) in the absence (−) or presence (+) of EIPA (10 μM). The paired Student t-test was used to determine whether or not adrenaline significantly enhanced the thrombin-induced [^{32}P]PA formation ($n = 6$; $P <$ 0.05 indicated by asterisks).

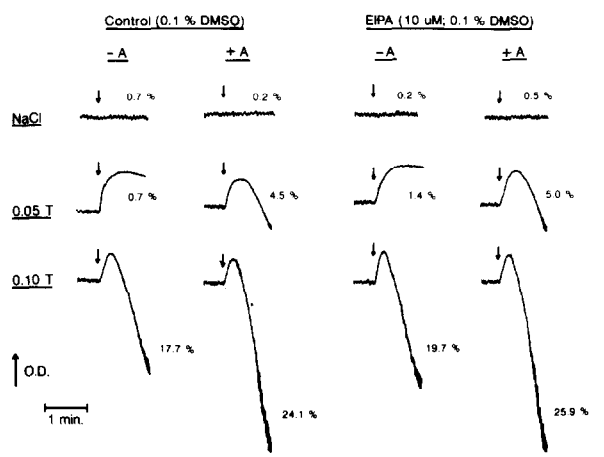

Fig.3. The effect of EIPA on the potentiation by adrenaline on thrombin-induced aggregation and dense granule secretion. Platelet suspensions were stirred at 400 rpm and stimulated with saline or thrombin (T; 0.05 or 0.10 U/ml) with (+) or without (−) adrenaline (A; 10 μM) in the absence or presence of EIPA (10 μM). The degree of secretion is indicated as a percentage of the total content of ADP plus ATP in the platelets.

strongly inhibited by the Na^+/H^+-antiport blocker EIPA (10 μM; fig.1d).

3.2. *The potentiation by adrenaline of thrombin-induced [^{32}P]PA formation, aggregation and dense granule secretion is not blocked by EIPA*

Fig.2 shows that the presence of EIPA (10 μM) had no inhibitory effect on the potentiation by adrenaline of thrombin-induced phospholipase C activation measured as [^{32}P]PA formation. Similarly, the potentiating action of adrenaline on thrombin-induced final platelet responses (aggregation and dense granule secretion) persisted during blockage of the Na^+/H^+ exchange by EIPA (fig.3). The results were independent of whether or not the platelets had been preincubated with BCECF.

4. DISCUSSION

It has been well documented that the platelet-stimulating effect of adrenaline is mediated via alpha$_2$-adrenergic receptors [8]. Activation of these receptors in human platelets inhibits the adenylate cyclase system through coupling via a G_i-protein [9], but this effect is probably not involved in platelet stimulation [10–12]. Moreover, it is clearly established that adrenaline potentiates phospholipase C activation induced by several platelet agonists [5,13–15], although there is no conclusive evidence that adrenaline stimulates PLC directly. As a consequence, the recent demonstration that adrenaline may induce cytoplasmic alkalinization via activation of the Na^+/H^+ exchange mechanism [1,2] offered a challenging proposal: this rise in pH_i was shown to trigger a sequence of events, involving arachidonate liberation by phospholipase A_2, followed by formation of platelet-stimulating prostaglandins and thromboxanes by the cyclooxygenase pathway, which finally activated PLC.

In the present paper we show that adrenaline may potentiate thrombin-induced cytoplasmic alkalinization via the Na^+/H^+ antiport mechanism. Adrenaline by itself did not activate the Na^+/H^+ exchange, which is in contrast to the results obtained by Sweatt et al. [1,2]. This discrepancy may be explained by the use of cyclooxygenase inhibitor, removal of extracellular

ADP by CP/CPK as well as the nominal absence of fibrinogen in our experiments [13,16,17].

The presence of the Na^+/H^+ antiport blocker EIPA strongly inhibited the rise in pH_i induced either by thrombin alone or by the association of adrenaline and thrombin. However, EIPA did not alter the potentiating action of adrenaline on thrombin-induced PLC activation measured as [^{32}P]PA formation and final platelet responses. Also, it was recently demonstrated that adrenaline may reverse thrombin-mediated homologous desensitization in human platelets even in the presence of EIPA [3,4]. Therefore, the mechanism involved in the potentiation by adrenaline of thrombin-induced responses does not require activation of the Na^+/H^+ exchange mechanism, even at low concentrations of thrombin.

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REFERENCES

[1] Sweatt, J.D., Blair, I.A., Cragoe, E.J. and Limbird, L.E. (1986) J. Biol. Chem. 261, 8660–8666.
[2] Sweatt, J.D., Connolly, T.M., Cragoe, E.J. and Limbird, L.E. (1986) J. Biol. Chem. 261, 8667–8673.
[3] Crouch, M.F. and Lapetina, E.G. (1988) Biochem. Biophys. Res. Commun. 151, 178–186.
[4] Crouch, M.F. and Lapetina, E.G. (1988) J. Biol. Chem. 263, 3363–3371.
[5] Steen, V.M., Tysnes, O.B. and Holmsen, H. (1988) Biochem. J. 253, 581–586.
[6] Horne, W.C., Norman, N.E., Schwartz, D.B. and Simons, E.R. (1981) Eur. J. Biochem. 120, 295–302.
[7] Steen, V.M. and Holmsen, H. (1985) Thrombos. Haemostas. 54, 680–683.
[8] Grant, J.A. and Scrutton, M.C. (1979) Nature 277, 659–661.
[9] Aktories, K. and Jakobs, K.H. (1981) FEBS Lett. 130, 235–238.
[10] Connolly, T.M. and Limbird, L.E. (1983) J. Biol. Chem. 258, 3907–3912.
[11] Motulsky, H.J., Shattil, S.J., Ferry, N., Rozansky, D. and Insel, P.A. (1986) Mol. Pharmacol. 29, 1–6.
[12] Thompson, N.T., Scrutton, M.C. and Wallis, R.B. (1986) Eur. J. Biochem. 161, 399–408.
[13] Banga, H.S., Simons, E.R., Brass, L.F. and Rittenhouse, S.E. (1986) Proc. Natl. Acad. Sci. USA 83, 9197–9201.
[14] Bushfield, M., McNicol, A. and MacIntyre, D.E. (1987) Biochem. J. 241, 671–676.
[15] De Chaffoy de Courcelles, D., Roevens, P., Van Belle, H. and De Clerck, F. (1987) FEBS Lett. 219, 283–288.
[16] Plow, E.F. and Marguerie, G.A. (1980) J. Biol. Chem. 255, 10971–10977.
[17] Figures, W.R., Scearce, L.M., Wachtfogel, Y., Chen, J., Colman, R.F. and Colman, R.W. (1986) J. Biol. Chem. 261, 5981–5986.