Diacylglycerol mediates the thrombin-induced, protein kinase C and Ca^{2+} independent activation of the Na^+/H^+ exchanger in platelets

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Received 6 November 1996

Abstract Treatment of aspirinated platelets with the electroneutral K^+/H^+ exchanger nigericin induces a decrease in intraplatelet pH as measured with the intracellular fluorescent indicator BCECF. Under these conditions, the proton permeability of the plasma membrane is unaffected. The addition of thrombin induces a rapid partial recovery of pH_i, which is completely abolished by the Na^+/H^+ exchanger inhibitor NHA. The effect is also evident in the presence of the PKC inhibitors GF 109203X or staurosporine and in the absence of both external (EGTA-chelated) and internal (BAPTA-chelated) Ca^{2+}. This makes the thrombin-induced activation of the exchanger independent of the involvement of the hitherto described activators, namely PKC and the increase in $[Ca^{2+}]_i$, as well of the recently reported activator arachidonic acid [Cavallini, L., Coassin, M., Borean, A., and Alexandre, A. (1996) Biochem. J. 319, 567–574], whose production requires a high $[Ca^{2+}]_i$. The thrombin-dependent recovery of pH_i is prevented by the phospholipase C inhibitor ET 18 O-CH_3 and is mimicked by the addition of the permeable diglyceride dioctanoyl glycerol (DiC_8) exogenously supplied. The effect of thrombin and DiC_8 is unaffected by inhibition of diacylglycerol lipase and diacylglycerol kinase. These experiments identify diglyceride as a novel activator of the Na^+/H^+ exchanger in platelets.

Key words: Diglyceride; Na^+/H^+ exchanger; Platelet activation

1. Introduction

Thrombin-induced alterations of pH_i in human platelets appear to comprise two phases, an initial transient acidification, followed by a second phase of prolonged alkalinization [1–5]. In the presence of inhibitors of the Na^+/H^+ exchanger, or in Na^+-free media, the increase in pH_i is replaced by a long-lasting acidification, whose origin is not totally clear. It has been ascribed to increased H^+ production because of increased metabolic demands [6,1], or to stimulation of the plasma membrane-associated Ca^{2+}-ATPase, which has been shown (in erythrocytes) to exchange external H^+ for internal Ca^{2+} [7,8]. More recently, it was recognized that ArA, whose production is stimulated by thrombin, activates a H^+ conductance pathway across the plasma membrane, which leads to cytosolic acidification in the presence of negative plasma membrane potentials [9].

The prevailing alkalinization observed after thrombin in uninhibited platelets, which reflects the activation of the Na^+/H^+ exchanger, completely masks the effect of the pH_i-decreasing process(es) [1–5,10]. The activation of the exchanger has been shown to be mediated by the stimulation of PKC [1–5,9,11–15], as well as by the increase in $[Ca^{2+}]_i$ [9,10,16]. We recently reported that ArA operates as a novel independent activator of the antiporter [9].

The present study analyzes in greater detail the properties of the Na^+/H^+ exchanger during thrombin-dependent platelet activation. It is shown that yet another activator can be detected, under conditions where all the others are not operative. The new activator is identified as diglyceride produced by the activity of phosphatidylinositol-specific phospholipase C.

2. Materials and methods

2.1. Materials

Hirudin, apyrase, ionomycin, Tg, NHA, BSA fraction V essentially fatty acid free, GF 109203X, Quin 2-AM and staurosporine were purchased from Sigma (St. Louis, MO, USA); BCECF-AM, BAPTA-AM, INDO 1AM, ET 18-OCH_3, R 599949 and RHC-80267 were from Calbiochem, PGI_2 from Cascade Biochem Ltd., UK; [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid was from Amersham. All other reagents were of analytical grade.

2.2. Platelet preparation

Platelet-rich plasma (PRP) and washed platelets were prepared and treated with aspirin as previously reported [17] from fresh blood drawn from healthy volunteers and mixed with acid citrate-dextrose anticoagulant supplemented with hirudin (50 mU/ml) apyrase (80 mU/ml) and PGI_2 (0.2 μg/ml).

2.3. Determination of cytosolic pH

The intracellular pH was determined with the fluorescent pH indicator BCECF exactly as described in [9]. The pH-sensitive fluorescent probe BCECF-AM (5 μM) was added to aspirinated PRP and loading was performed for 30 min at 25°C. The pellet was resuspended in Tyrode buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM Na-HEPES, 10 mM glucose, pH 7.4) in the presence of 40 mU/ml apyrase and 5 mU/ml hirudin. EGTA (0.2 mM) was added at the beginning of incubation. The platelets were used at a concentration of $0.5–1\times10^8$/ml. Fluorescence was measured at 25°C in a thermostated, magnetically stirred cuvette, in a Shimadzu RL-5000 spectrofluorimeter with excitation and emission wavelengths set at 503 and 530 nm, respectively.

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Abbreviations: ArA, arachidonic acid; BAPTA-AM, 1,2-bis-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetraacetoxymethyl ester; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein triacetoxymethyl ester; BSA, bovine serum albumin; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; ET 18-OCH_3, 1-*O*-octadecyl-2*O*-methyl-*rac*-glycero-3-phosphorylcholine; DiC_8, 1,2-dioctanoyl-*sn*-glycerol; GF 109203X, bisindolylmaleimide; INDO 1AM, 1-(2-amino-5-(6-carboxyindol-2-yl)phenoxy)-2-(2′-amino-5′-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester; NHA 5-(*N*,*N*-hesamethylene)amiloride; PGI_2, prostaglandin I_2 or prostacyclin; PKC, protein kinase C; PMA, phorbol, 12-myristate, 13-acetate; PRP, platelet-rich plasma; R 599949 diacylglygerol kinase inhibitor II, 3-(2-(4-(bis(4-fluorophenyl)methylene)-1-piperidinyl)ethyl)-2,3-dihydro-2-thioxo-4(1H)-quinazolinone; RHC-80267, 1,6-bis(cyclohexylaminocarbonylamino)hexane; Tg, thapsigargin.

PII S0014-5793(96)01364-6

2.4. Chelation of internal Ca^{2+}

Chelation of internal Ca^{2+} was achieved by addition to PRP of 50 μM BAPTA-AM 20 min after BCECF-AM, and prolonging the incubation for 20–30 min more. Alternatively, chelation of internal Ca^{2+} was performed with Quin 2-AM (see below).

2.5. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ measurement was performed in INDO 1AM-loaded platelets as reported in [18] with excitation and emission wavelengths set at 340 and 400 nm, respectively.

2.6. PLA_2 activity

PLA_2 activity was measured as [^{3}H]arachidonic acid release. Washed aspirinated platelets resuspended in Tyrode medium (buffered at pH 6.4 with citric acid) at a concentration of 3×10^9/ml were loaded with 3 μCi of carrier-free [^{3}H]arachidonic acid for 90 min at room temperature, in the presence of hirudin (50 mU/ml), apyrase (80 mU/ml) and PGI_2 (0.2 μg/ml). At 60 min, one aliquot was withdrawn and treated with 100 μM Quin-AM for 30 min. After 5-fold dilution in the same medium, containing 3 mg/ml of BSA, and centrifugation at $700\times g$ for 12 min, the pellet was washed again and the cells resuspended in Tyrode buffer at pH 7.4 supplemented with 0.2 mM EGTA, at a concentration of 5×10^8/ml. Platelets were equilibrated for 5 min at 25°C before addition of 0.3 U/ml thrombin. At the indicated times, aliquots were withdrawn and the reaction stopped by dilution with the same volume of an ice-cold mixture of 75 mM Na-phosphate buffer (pH 7.4) containing 4 mM EDTA and 6% (v/v) glutardialdehyde. After centrifugation at $10000\times g$ for 3 min, the released [^{3}H]arachidonate was measured in the supernatants by liquid-scintillation counting (United Technologies Insta-gel). Values of [^{3}H]arachidonate release were expressed as % of total counts of the corresponding suspension solubilized with 1% Triton X-100 and subtracted from the basal value before stimulation.

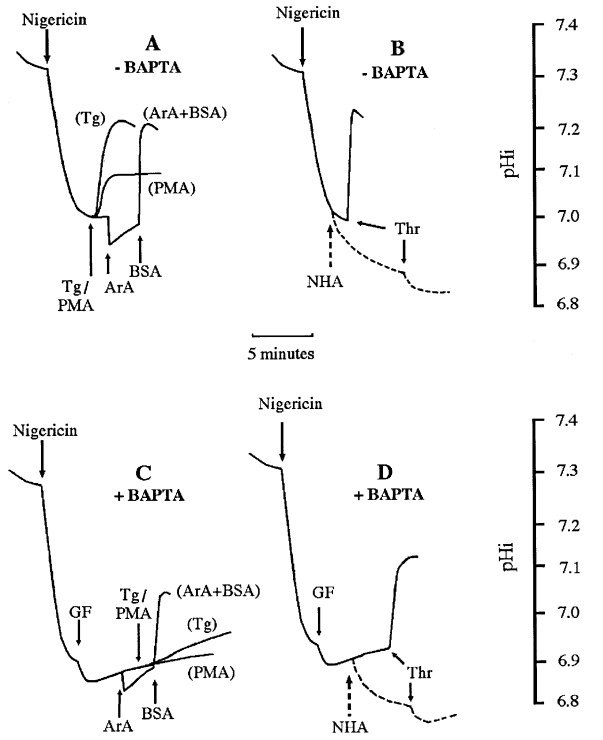

Fig. 1. Effect of Tg, PMA, ArA and thrombin on nigericin-acidified cytosol, in control and BAPTA-loaded, GF 109203X-treated platelets. BCECF-loaded platelets untreated (A,B, − BAPTA) or co-loaded with BAPTA (C,D, +BAPTA) were resuspended in Na^+ Tyrode as described in Section 2. Nigericin (Nig) was 25 ng/ml and GF 109203X (GF) was 2 μM (B,D). Further additions: Tg (0.4 μM), PMA (50 nM), ArA (8 μM), followed by BSA (0.3 mg/ml) in A,C; thrombin (Thr, 0.3 U/ml) and NHA (20 μM, dotted line) in B,D.

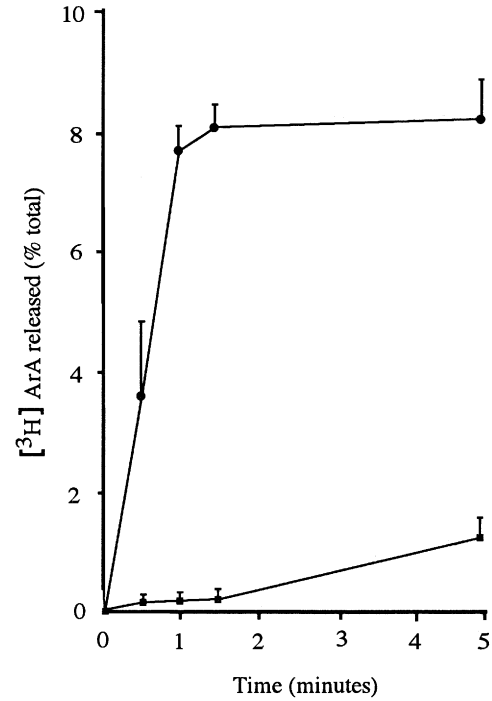

Fig. 2. Chelation of cytosolic Ca^{2+} prevents thrombin-induced production of ArA. [^{3}H]ArA-labelled platelets, untreated (●) or co-loaded with Quin 2 (■), were stimulated with thrombin (added at time zero). The points are taken from duplicate determinations from two platelet preparations. See Section 2 for details.

3. Results and discussion

A useful system to study the mechanisms of activation of the Na^+/H^+ exchanger is to induce a controlled decrease in cytosolic pH (0.2–0.3 pH units) by adding small amounts of the electroneutral K^+/H^+ exchanger nigericin to platelets suspended in standard Na^+ Tyrode. Under these conditions, the subsequent addition of agents that promote the activation of the exchanger is expected to induce the recovery of pH_i. The effect of established activators of the antiporter is reported in Fig. 1A. As shown, the recovery may be promoted by increasing $[Ca^{2+}]_i$ with the inhibitor of the endomembrane Ca^{2+}-ATPase Tg (the effect of Tg is also dependent in part on the production of ArA [9]). Alternatively, the recovery may be promoted by the activation of PKC, with the phorbol ester PMA. Finally, as recently shown [9], exogenous ArA is capable of activating a H^+ conductance pathway across the plasma membrane as well as the Na^+/H^+ antiporter; the effect of ArA on the antiporter is best evident when excess ArA is removed by BSA [9]. The effects of Tg, PMA and ArA are sensitive to the inhibitor of the Na^+/H^+ exchanger, the amiloride derivative NHA [9]. Fig. 1B demonstrates that rapid recovery of pH_i previously decreased by nigericin is also promoted by thrombin. The effect of thrombin is mediated by the Na^+/H^+ exchanger, as it is sensitive to NHA.

If a similar experiment is conducted in platelets co-loaded with the Ca^{2+} chelator BAPTA, to prevent the increase in $[Ca^{2+}]_i$, and in the presence of the PKC inhibitor bisindolylmaleimide GF 109203X [19] or of staurosporine (2 μM, not shown), Tg and PMA lose their ability to promote the recovery of pH_i and only ArA (plus BSA) is still somewhat active (Fig. 1C; see also [9]). Interestingly, the NHA-sensitive recov-

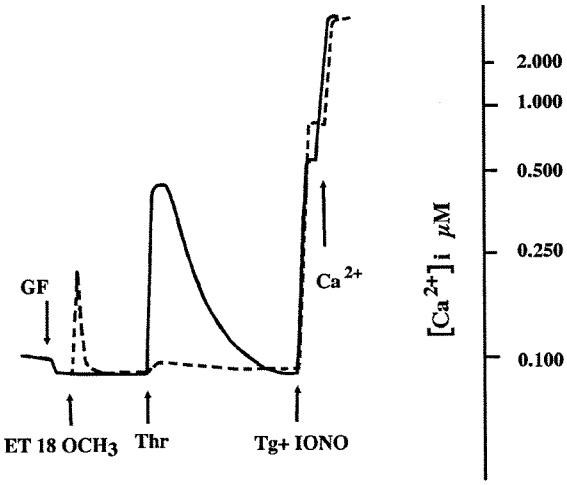

Fig. 3. Inhibition by ET 18-OCH_3 of thrombin-induced Ca^{2+} release from intracellular stores. INDO 1AM-loaded platelets were treated with thrombin (0.2 U/ml) in the absence (full line) and presence (dotted line) of ET 18-OCH_3 (ET, 8 μM). When indicated Tg was 0.4 μM, ionomycin 100 nM, and free Ca^{2+} 0.8 mM. The traces are representative of duplicate experiments with at least 5 different preparations with similar results.

ery of pH_i induced by thrombin is almost unaffected (Fig. 1D). This finding excludes the increase of $[Ca^{2+}]_i$ and the activation of PKC as mediators of the thrombin effect. Also, the production of ArA, which is readily detectable upon supplementing thrombin in the absence of a cytosolic Ca^{2+} chelator, becomes undetectable when the agonist-induced increase in cytosolic Ca^{2+} is prevented by loading with the Ca^{2+} chelator Quin 2 (Fig. 2). This finding also excludes ArA as the mediator of the thrombin-induced activation of the exchanger and shows that thrombin operates by yet another mechanism.

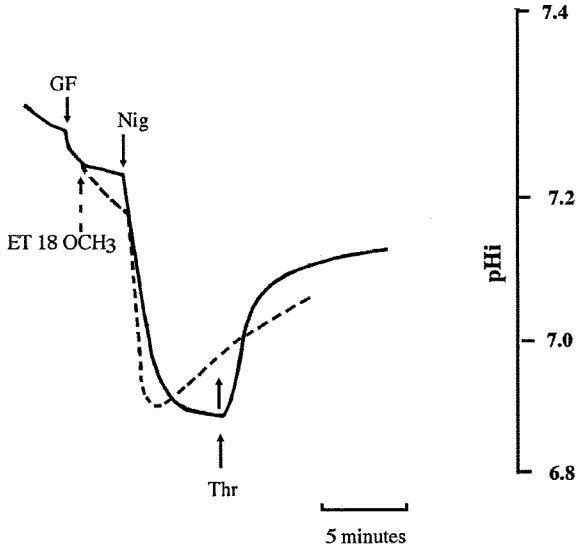

Fig. 4. Thrombin-induced recovery of pH_i is sensitive to ET 18-OCH_3. BCECF-loaded platelets were co-loaded with BAPTA. When indicated ET 18-OCH_3 (broken line) was 8 μM. The traces are representative of duplicate experiments with at least 3 different preparations with similar results.

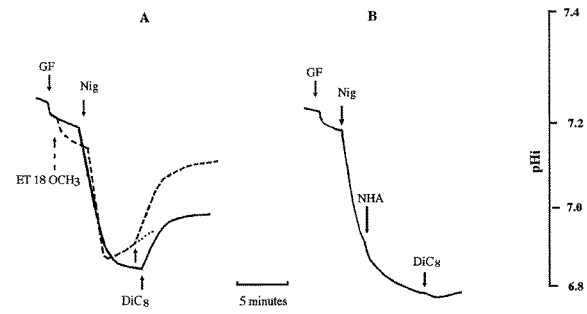

Fig. 5. Recovery of pH_i is promoted by the permeant diglyceride DiC_8. BCECF-loaded platelets were co-loaded with BAPTA. When indicated DiC_8 was 20 μM. Other additions: ET 18-OCH_3 (A, broken line) 8 μM; NHA 20 μM (B). The traces are representative of duplicate experiments with 3 different preparations.

To gain further insight into the mode by which thrombin activates the Na^+/H^+ exchanger under the conditions of Fig. 1C, we tried to determine whether its effect depends on the operation of the phosphatidylinositol-specific phospholipase C. To this end, we used the phospholipase C inhibitor ET 18-OCH_3 [20]. An experiment showing the efficacy of ET 18-OCH_3 as an inhibitor of the thrombin-induced increment of $[Ca^{2+}]_i$ is reported in Fig. 3. ET 18-OCH_3 induces a transient small increase in $[Ca^{2+}]_i$ and prevents, as expected, the release of Ca^{2+} from the stores upon addition of thrombin. Ca^{2+} is then regularly released by the combined addition of Tg and a small concentration of ionomycin [18,21,22].

As shown in Fig. 4, although ET 18-OCH_3 per se affects pH_i to some extent, mainly by promoting a slow recovery after acidification with nigericin, it clearly inhibits the sharp recovery of pH_i induced by thrombin. This is an indication that phospholipase C may be required for induction of the thrombin effect.

This finding led us to hypothesize that diglyceride may be the mediator of the thrombin activation of the Na^+/H^+ exchanger. As shown in Fig. 5, the membrane-permeable diglyceride DiC_8 does indeed activate a NHA-sensitive recovery of pH_i in BAPTA-loaded, protein kinase C-inhibited platelets. As may be expected, the effect of DiC_8 is not prevented by ET 18-OCH_3.

The activation of the Na^+/H^+ exchanger by thrombin, or by the diglyceride DiC_8 does not depend on the formation of phosphatidic acid or other diacylglycerol metabolites as it is unaffected by the diacylglycerol kinase inhibitor II R 599949 (3 μM) and the inhibitor of the diacylglycerol lipase RHC 80267 (20 μM) (not shown).

The findings reported in this study demonstrate that diglyceride functions as a previously unidentified activator of the Na^+/H^+ exchanger in platelets. The action of diglyceride, either endogenously produced or supplied as the membrane-permeable DiC_8, is independent of the intervention of previously recognized activators, including the elevation of $[Ca^{2+}]_i$, the activation of PKC or the production of ArA. Like the mentioned activators, diglyceride appears to operate by increasing the activity of the exchanger when the intraplatelet pH is moderately decreased.

Acknowledgements: This work was supported by 40% and 60% MURST funds, C.T. CNR No 95.02102.CT04 and by Centro di Studio delle Biomembrane CNR.

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