The Na$^+$/H$^+$ exchanger is phosphorylated in human platelets in response to activating agents

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α-Thrombin, phorbol esters (PMA) and 1,2-diacylglycerol (DAG), three activators of the amiloride-sensitive Na$^+$/H$^+$ exchange in human platelets, rapidly increase the intracellular pH and the level of phosphorylation of the Na$^+$/H$^+$ exchanger protein (NHE1). This stimulatory effect is suppressed by staurosporine, a potent kinase inhibitor, and increased by okadaic acid, a potent inhibitor of phosphatase 1 and 2A. The modulations of NHE1 phosphorylation by these factors correlate well with their effects on platelet pH. Thus, we conclude that in platelets (i) Na$^+$/H$^+$ exchange is mediated by NHE1, and (ii) platelet activating agents stimulate NHE1 via the modulation of the kinase/phosphatase equilibrium.

Platelet; Thrombin; Na$^+$/H$^+$ exchanger; Phosphorylation; Okadaic acid

1. INTRODUCTION

An amiloride-sensitive Na$^+$/H$^+$ exchange [1] is present in the plasma membrane of blood platelets where it has been described to play a major role in the regulation of intracellular pH [2,3], cell volume [4] and platelet activation by α-thrombin [5–8] and other agonists [9]. Indeed, treatment of human platelets by α-thrombin [3,5–8] and by direct kinase C activators [7,8,10,11] such as phorbol esters or 1,2-diacylglycerol analogs, stimulate Na$^+$/H$^+$ exchange activity within seconds. Recently, using a specific polyclonal antibody [12], we demonstrated in CCL39 fibroblasts [12] and A431 epithelial cells [12] that Na$^+$/H$^+$ exchange is achieved by a phosphorylated transmembrane glycoprotein of 110 kDa referred to as NHE1 (Na$^+$/H$^+$ exchanger 1) [12,13]. In G0-arrested fibroblasts, the stimulation of the Na$^+$/H$^+$ exchange activity by either mitogens (EGF, α-thrombin, serum) [12], kinase C activator (phorbol esters) [12] or phosphatase 2A and 1 inhibitor (okadaic acid) [14] is associated with a parallel increase in the level of phosphorylation of NHE1, suggesting that phosphorylation of NHE1 triggers Na$^+$/H$^+$ exchange activation.

In the present report, using the anti-NHE1 polyclonal antibody mentioned above, we now demonstrate that NHE1 is present in human blood platelets and that its level of phosphorylation in these cells parallels the modulation of the Na$^+$/H$^+$ exchange activity by pla-

telet activators (α-thrombin and kinase C activators), kinase inhibitor (staurosporine) and phosphatase 1 and 2A inhibitor (okadaic acid).

2. MATERIALS AND METHODS

2.1. Chemicals
[^{32}P]Orthophosphate (10 mCi/ml) was from Amersham. Albumin (bovine, essentially fatty acid-free from fraction V), sn-1,2-dioctanoyl-glycerol (DAG), apyrase, prostaglandin E1 and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Staurosporine was from Calbiochem and the inhibitor R59022 from Janssen. Protein-A sepharose 6-MB was from Pharmacia and the detergent Nikkol BL-8SY (octo-ethyleneglycol mono-n-dodecyl-ether) from Nikko Chemicals (Japan). Highly purified human α-thrombin was generously provided by Dr J.W. Fenton II (New York State Department of Health, Albany, NY) and okadaic acid by Dr P. Cohen (Department of Biochemistry, University of Dundee, Dundee) and Y. Tsukitani (Fugisawa Chemicals, Tokyo).

2.2. Platelet preparation
Venous blood was drawn from healthy human volunteers, and anticoagulated with acid-citrate-dextrose (65 mM citric acid, 11 mM glucose and 85 mM trisodium citrate) at a volume ratio of 6:1 (blood:anticoagulant), platelet-rich plasma was obtained by centrifugation at 120 × g for 10 min and had a pH 6.5. Then this plasma was supplemented with prostaglandin E1 (2.8 μM) and apyrase (2 U/ml) and centrifuged at 1000 × g for 10 min. The platelets were resuspended (about 1–2 × 10^9/ml) in an isotonic medium A (140 mM NaCl, 5 mM KCl, 10 mM glucose, 20 mM HEPES, pH 6.8, 0.5 mg/ml albumin and apyrase) and gel-filtered through a Sepharose 2B column (10 × 0.76 cm) [11,15].

2.3. [^{32}P]Orthophosphate platelet labeling
Gel-filtered platelets (1–2 × 10^9/ml) were incubated for 90 min in medium A containing 500 μCi/ml [^{32}P]orthophosphate and gel-filtered again through a sepharose 2B column. Then, just prior to stimulation with agonists, the pH of the assay media was increased to 7.2 with HEPES. After 5 min stimulation at 37°C with agonists, cells were immediately frozen in liquid nitrogen.

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2.4. Immunoprecipitation of NHE1 and electrophoretic analysis

10⁸ frozen platelets in buffer A (pH 7.2) were mixed with an equal volume of ice-cold buffer B (1% Nikkol, 50 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 3 mM KCl, 20 mM sodium pyrophosphate, 10 mM ATP, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM o-phenantroline, 1 mM iodoacetamide), sonicated for 40 s, left on ice for 15 min, and then centrifuged for 15 min at 100 000 × g. An aliquot of the supernatant (C) was kept for further electrophoretic analysis. The supernatants were adsorbed to protein A-sepharose 6-MB beads for 1 h and then incubated for 2 h at 4°C with affinity-purified RP-C28 antibodies [12]. A 50% suspension of protein A sepharose beads (50 µl) (previously incubated with a Nikkol extract of Na⁺/H⁺ antiporter deficient mutant PS120 [16] was added, and the mixture was incubated for 1 h at 4°C in a rotating shaker. The beads were then washed 6 times with buffer A containing 1% Nikkol and 1 mg/ml bovine serum albumin. Immunoprecipitated proteins and aliquots of the supernatants (C) were solubilized by boiling in Laemmli sample buffer. Samples were analysed by SDS-PAGE electrophoresis on 7.5% polyacrylamide gels under reducing conditions. Molecular weight markers were from Sigma: Phosphorylase b (97.4 kDa); Bovine albumin (66 kDa); Egg albumin (45 kDa); Pepsin (34 kDa); Trypsinogen (24 kDa). Phosphoproteins were visualized by autoradiography.

3. RESULTS AND DISCUSSION

3.1. NHE1 is present in human blood platelets

The presence of the amiloride-sensitive Na⁺/H⁺ exchanger (NHE1) in Human blood platelets was investigated using the immunopurified rabbit polyclonal anti-human NHE1 antibody RPc-28 [12]. This antibody was previously demonstrated to be able to detect phosphorylated NHE1 in other cell types [12]. Thus, platelets were labeled with [³²P]orthophosphate, solubilized with 1% detergent (Nikkol) solution, and the presence of NHE1 detected by immunoprecipitation with RPc-28 antibody. The analysis of immunoprecipitated phosphoproteins by SDS-PAGE and autoradiography revealed a single phosphorylated protein with an apparent molecular weight of 105–110 kDa (Figs. 1 and 3), a size similar to that detected in fibroblasts and epithelial cells [12]. This molecular weight and the ex-
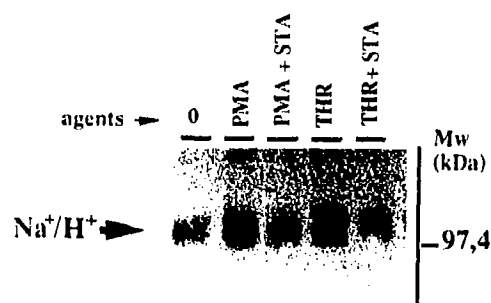

Fig. 1. Effect of phorbol ester (PMA) α-thrombin and staurosporine on the phosphorylation of NHE1 in human platelets. 10⁸ ³²P-labeled intact platelets were treated (0 denotes non-treated platelets) for 5 min with 100 nM PMA or 1 U/ml α-thrombin, with or without a pretreatment with 100 nM staurosporine for 20 s. Cells were then lysed with 1% Nikkol, and NHE1 was immunoprecipitated with the anti-NHE1 antibody RP-c28 from the detergent-extracted proteins. Immunoprecipitated NHE1 was analysed by SDS-PAGE and autoradiography. Na⁺/H⁺ → indicates immunoprecipitated NHE1.

treme specificity of RP-c28 antibodies for NHE1 [12], led us to conclude that NHE1 is present in human platelets.

3.2. NHE1 is phosphorylated in response to platelet activating agents

The next objective of this study was to assess the effect of platelet-activating agents on the phosphorylation of NHE1. Unactivated ³²P-labeled platelets contain weakly phosphorylated NHE1 (Fig. 1) as well as weakly phosphorylated P47 protein (or pleckstrin) (Fig. 2) [17,18]. P47 is a major and specific substrate for kinase C in platelets [18] which is easily detectable among total phosphoproteins (Figs. 2 and 4). When added for 5 min to these 'resting' platelets, in a concentration range that is known to produce a maximum activation of Na⁺/H⁺ exchange, α-thrombin (1 U/ml) induces a marked increase in the level of phosphorylation of NHE1 (Fig. 1) and of P47 proteins (Fig. 2). This phosphorylation of NHE1 occurs within seconds following stimulation (We clearly observed PMA-enhanced phosphorylation within 20 s, data not shown), in a time-dependent manner which correlates well with an increase in intracellular pH [11] as previously reported [3,5–7]. In many reports it is suggested that this α-thrombin-dependent activation of the platelet Na⁺/H⁺ exchange is mediated via G-protein-
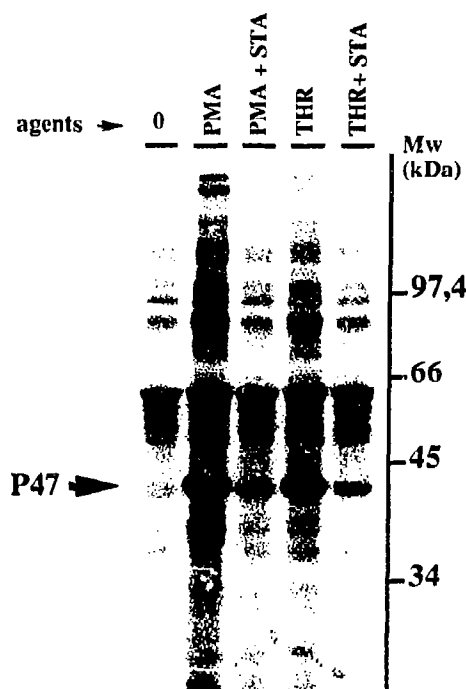

Fig. 2. Effect of phorbol ester (PMA), α-thrombin and staurosporine on the phosphorylation of total detergent (1% Nikkol)-extracted proteins. Platelets were treated as described in Fig. 1. Cells were then lysed with 1% Nikkol and aliquots of detergent-extracted proteins were analysed by SDS-PAGE and autoradiography. P47→ indicates P47 protein (pleckstrin), the major substrate of protein kinase C.
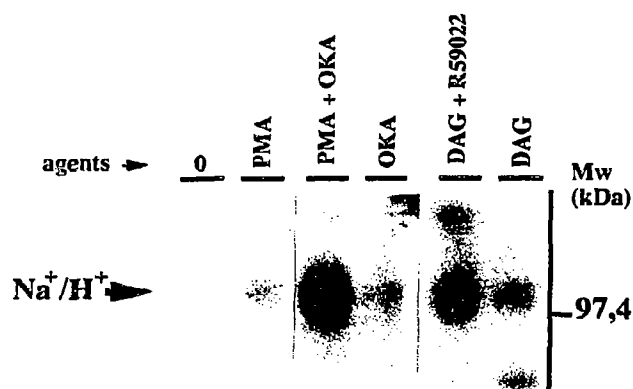
Fig. 3. Effect of phorbol ester (PMA), 1,2-diacylglycerol (DAG), Janssen inhibitor R59022 and okadaic acid on the phosphorylation of NHE1. 10^8 ^{32}P-labeled intact platelets were treated (0 denotes non-treated platelets) for 5 min with 100 nM PMA, 10 μM DAG, 1 μM okadaic or with a combination of 100 nM PMA and 1 μM okadaic acid, 10 μM DAG and 10 μM inhibitor R59022. NHE1 immunoprecipitation was performed as described in Fig. 1. Na$^+$/H$^+$ → indicates immunoprecipitated NHE1.

coupled receptors and subsequent protein kinase C activation [8,11]. Indeed, phorbol esters (PMA) [7,11] and 1,2-diacylglycerol analogs (DAG) [7,10,11] have been reported to be potent activators of the Na$^+$/H$^+$ exchange in human platelets. In Fig. 1 and Fig. 3, we now demonstrate that PMA and DAG both stimulate NHE1 and P47 phosphorylation to a similar extent to that
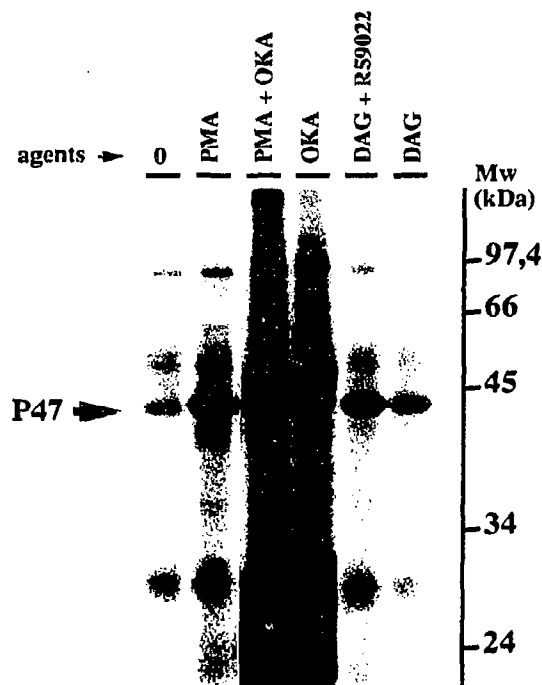

Fig. 4. Effect of phorbol ester (PMA), 1,2-diacylglycerol analog (DAG), inhibitor R59022 and okadaic acid on the phosphorylation of total detergent (1% Nikkol)-extracted proteins. Platelets were treated as described in Fig. 3 and proteins analysed as described in Fig. 2. P47→ indicates P47 protein (pleckstrin), the major substrate of protein kinase C.

elicited by α-thrombin. The effect of DAG appears to be strongly increased in the presence of a diacylglycerol kinase inhibitor (R59022) [19] (Figs. 3 and 4). Indeed, this addition probably avoids the rapid conversion of DAG to non-active forms [19]. Conversely, staurosporine, a potent but non-specific inhibitor of kinase C [20] which has been described to curtail DAG and PMA-induced Na$^+$/H$^+$ exchange activation in platelets [11], completely abolishes PMA and α-thrombin-stimulated phosphorylation of NHE1 (Fig. 1) and of P47 proteins (Fig. 3) [21].

In view of the results described above, we conclude that NHE1 and P47 protein phosphorylation seem to be stimulated and inhibited in the same manner. This analogy suggests that protein kinase C is involved in the regulation of the Na$^+$/H$^+$ exchange in human platelets via direct or indirect stimulation of NHE1 phosphorylation.

3.3. NHE1 is phosphorylated in response to okadaic acid, a phosphatase inhibitor

Like most phosphoproteins, the level of phosphorylation of the exchanger probably results from a dynamic equilibrium between kinase and phosphatase activities. Indeed, okadaic acid, a potent inhibitor of protein phosphatase 1 and 2A [22], activates the Na$^+$/H$^+$ exchange in fibroblast [15] and in human platelets [11]. As shown in Fig. 3, alone, this inhibitor weakly stimulates the phosphorylation of NHE1 in platelets. This effect of okadaic acid on intact platelets, is probably due to the fact that the toxin produces a large increase in the total phosphorylation of cellular protein [22] (Fig. 4), presumably by inhibiting phosphatases PP1 and PP2A in vivo. This also suggests that the kinase(s) which phosphorylates NHE1 is partly active in the absence of platelet activating agents. As the toxin also strongly increases the NHE1 phosphorylation induced by PMA (Fig. 3), it is tempting to suggest that okadaic acid acts at two levels, on NHE1 itself and on the kinase activating cascade mimicking platelet activating agents [23].

In conclusion, the ability of platelet activating agents as well as okadaic acid to stimulate phosphorylation of NHE1, concomitant with the activation of Na$^+$/H$^+$ exchange, strongly supports the conclusion that NHE1 phosphorylation is likely to be the mechanism that controls the amiloride-sensitive Na$^+$/H$^+$ exchange in human platelets.

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