Stimulation of Mitogen-activated Protein Kinase and Na⁺/H⁺ Exchanger in Human Platelets

DIFFERENTIAL EFFECT OF PHORBOL ESTER AND VASOPRESSIN*

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Platelets are terminally differentiated cells that exhibit rapid phosphorylation of many proteins upon agonist-induced activation. Thus, platelets are a good model system to study signal transduction events that are not regulated by gene expression of proteins.

Mitogen-activated protein kinases (MAPKs)1 comprise a family of 40–45-kDa protein serine/threonine kinases that are activated by many extracellular stimuli, including growth factors and hormones. MAPKs require phosphorylation on both threonine and tyrosine residues in the sequence Thr183-Glu185 to become active (1, 2). This dual phosphorylation reaction is carried out by a single, specific enzyme, MAPK kinase (MEK) (3). Recently, a selective inhibitor of MEK, PD98059, was identified (4) and used to evaluate the role of the MAPK pathway in insulin action (5) and neuronal differentiation (6). Two forms of MAPK, 42 and 44 kDa, were identified in sheep platelets (7) and in human platelets (8, 9). Stimulation of these proteins was observed during platelet activation by thrombin (8, 9), phorbol 12-myristate 13-acetate (PMA), and platelet-activating factor (PAF) (7).

The Na⁺/H⁺ exchange system is ubiquitous and is present in plasma membranes of essentially all mammalian cell types. The first Na⁺/H⁺ exchanger isoform, referred to as NHE-1 (10), is the predominant species in nonepithelial cells. This form of the antiporter was found in human platelets (11) and is thought to be primarily responsible for intracellular pH homeostasis. Immunoprecipitation studies of NHE-1 demonstrate that the antiporter is phosphorylated in unstimulated cells (11–14). It was suggested that growth factor-activated NHE-1 is controlled by phosphorylation of the exchanger protein, since parallel to the induced pH change, increased phosphorylation of the NHE-1 was observed (12, 15). Recently, the properties of the NHE-1 were further analyzed, using deletion variants expressed in the exchanger-deficient mutant cell line PS120 (16). It was found that deletion of all major phosphorylation sites including growth factor-sensitive ones reduced growth factor-induced cytoplasmic alkalization by only 50%. Therefore, it was suggested that growth factor activation of the NHE-1 occurs at least in part by a mechanism that does not involve phosphorylation of the exchanger. In agreement with a NHE-1 phosphorylation independent mechanism, activation of the antiporter during volume regulation was not associated with increased phosphorylation (14). The understanding of the Na⁺/H⁺ exchange regulation mechanism, by extracellular signals, is still far from complete. The MAPK pathway, which is essential for the propagation of growth factor signals, was suggested to be a good candidate for mediation of NHE activation (17). In agreement, it was found that the protooncogene product p39 c-Mos kinase activates Raf-1 kinase and p45 MAPK (18–20) and up-regulates the Na⁺/H⁺ exchange in Xenopus oocytes (21). In addition, long-term expression of c-Ha-Ras stimulates Na⁺/H⁺ and Na⁺/H⁺−dependent Cl⁻-HCO₃⁻ exchange in NIH-3T3 fibroblasts (22).

In previous studies, we showed that PMA (23–26) and arginine vasopressin (AVP) activate the NHE, in human platelets, by two different pathways (26). Recent observations demonstrate that AVP activates MAPKs in vascular smooth muscle cells (VSMC) (2) and induces tyrosine phosphorylation of protein(s) in human platelets (27). Thus, it was interesting to investigate whether AVP and PMA affect the Na⁺/H⁺ exchange...
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change, in human platelets, due to a possible mediation of MAPKs. In this study, we report that in human platelets the phosphorylation and activation of the 42- and 44-kDa MAPK variants are stimulated by PMA and AVP through two different signaling pathways. Parallel to this activation of MAPKs, PMA and AVP stimulate Na+/H+ exchange, while only PMA induces NHE-1 phosphorylation. We suggest a possible role for MAPKs in NHE activation by both agonists through a mechanism that does not involve phosphorylation of the exchanger. Our data also suggest that phosphorylation of the NHE-1, induced by protein kinase C (PKC) activation, may cause additional activation of the transporter.

EXPERIMENTAL PROCEDURES

Materials

Bovine serum albumin (fraction V), AVP, PMA, staurosporine, and MNB were obtained from Sigma. Genistein was obtained from Indofine Chemical Co. The specific MEK inhibitor, PD98059, was kindly provided by Dr. A. R. Saltiel, Parke-Davis Pharmaceutical Research Division, Ann Arbor. The specific V1 (Phe2Orn8 Vasotocina) and V2 receptor antagonists (10

Methods

Preparation and Activation of Human Platelets—Venous blood was drawn from healthy volunteers, aged 22–40, who had not received any medication during the previous 14 days. The blood was anticoagulated with 1:100 sodium citrate solution comprising 65 mM citric acid, 11 mM glucose, and 85 mM trisodium citrate, at a volume ratio of blood:anti-coagulant of 6:1. Platelet rich plasma (PRP) was obtained by centrifugation at 200 g for 5 min. Platelet-rich plasma (PRP) was used to prepare the platelet suspension comprising 500,000 platelets per mm

Preparation of NHE Assay—To prepare the NHE assay, platelet extracts were diluted 15-fold in buffer containing 25 mM Tris HCl, pH 7.0, and incubated for 30 min with 10 mM MgCl2, 25 μM ATP, and 5 μM GST-NHE-1 fusion protein. After incubation, the reaction was stopped by the addition of a 10-fold excess of 50 mM EGTA and 1 mM DTT. The reaction mixture was centrifuged at 15,000 rpm for 15 min, and the supernatant was used as a source of NHE-1 activity for the assay. The activity of NHE-1 was determined by incubating the supernatant with 25 mM Tris HCl, pH 7.0, for 30 min at 37°C. The reaction was then stopped by the addition of 10 mM NaCl and 10 mM NaOH. The activity of NHE-1 was determined by measuring the increase in the concentration of NaCl in the reaction mixture. The activity of NHE-1 was expressed as pmol of NaCl transported per min per mg of protein.

RESULTS

Identification of MAPKs in Human Platelets—Antibody mapping against MAPK carboxyl-terminal peptide (residues 349–361)
was used for Western immunoblots of human platelet extracts. As shown in Fig. 1A, the major MAPK variant, both in human platelets and in rat VSMC, is the 42-kDa protein. Longer exposures revealed, in both cell types, the existence of lower amounts of the 44-kDa MAPK variant (data not shown). Addition of the carboxyl-terminal peptide of MAPK (5 μg/ml) abolished the observed chemiluminescence of the MAPK bands (Fig. 1B). These results emphasize the specificity of the antibody toward the 42- and 44-kDa variants of MAPK in human platelets.

Phosphorylation of MAPK by PMA and AVP in Human Platelets—Exposure of human platelets to maximal stimulating concentration of PMA (5 × 10⁻⁷ M, Fig. 2, lane b) or AVP (10⁻⁶ M, lane c), for 1 min, is associated with a slight electrophoretic retardation in MAPK mobility on SDS-PAGE, as compared to control platelets (lane a). This mobility shift is a characteristic of MAPK when it becomes phosphorylated and activated (31). Longer exposures revealed a mobility shift for the 44-kDa MAPK variant also (data not shown).

Activation of MAPK by PMA and AVP in Human Platelets—In previous studies, it was shown that MAPK can phosphorylate MBP, a commercially available substrate, at a significant rate (2, 7, 9). In the procedure presented in Fig. 3, MAPK activity was determined in MBP-containing polyacrylamide gels. The results illustrate that renatured 42- and 44-kDa variants of MAPK, obtained from PMA- and AVP-stimulated platelets (Fig. 3A and B, respectively), expressed time-dependent and transient stimulation of MBP kinase activity. The MAPK variants appeared as a doublet on the MBP-containing polyacrylamide gels, with higher activity of the 42-kDa protein. These results are in agreement with higher amounts of this variant in human platelets (Fig. 1). The effect of PMA is rapid, reaching almost maximal phosphorylation of MBP within 30 s (Fig. 3A). This maximal level of phosphorylation starts to decline only after 2 min. In AVP-stimulated platelets, the MBP kinase activity reaches a maximum at 1 min (Fig. 3B) and then returns to almost the basal level after 2 min of hormone addition.

MAPK stimulation by PMA and AVP is concentration-dependent. Stimulatory saturation is apparent at 3 × 10⁻⁷ M PMA (Fig. 4A) and 10⁻⁷–10⁻⁶ M AVP (Fig. 4B), while half-maximal effect is attained at 10⁻⁷ M PMA and 0.9 × 10⁻⁹ M AVP. The MAPK effect is in agreement with considerable stimulation of 42-kDa MAPK in sheep platelets (7).

As shown in Fig. 5, the AVP effect is mediated through a V₁ receptor, since only the V₁ receptor-specific antagonist (32) (lane 4) decreased significantly the AVP-activated MBP phosphorylation (lane 2). In addition, only the V₁ receptor-specific agonist (lane 6) mimics the AVP-activated MBP phosphorylation (lane 2). These observations are consistent with the V₁ receptor existence (33) and with AVP-V₁ receptor-dependent tyrosine phosphorylation of protein(s) in human platelets (27).

Do MAPKs Mediate PMA and AVP Activation of the NHE?—We compared the effect of staurosporine, a potent and relatively specific inhibitor of PKC (34, 35), genistein, a relatively specific tyrosine kinase inhibitor (36) and MEK inhibitor (4–6) on MAPK (Fig. 6) and NHE (Table I) activities stimulated by PMA or AVP. The results show that both PMA and AVP (treatments 2 and 6, respectively) markedly increase the MAPK kinase activity of the 42- and 44-kDa variants of MAPK and the Na⁺/H⁺ exchange rate. Both PMA-induced MAPK and Na⁺/H⁺ exchange activities are reduced by staurosporine (treatments 3) and MEK inhibitor (treatments 5), but are not affected by genistein (treatments 4). In contrast, both activities of the AVP-induced MAPK and NHE are not affected by staurosporine (treatments 7), but are reduced by genistein (treatments 8) and MEK inhibitor (treatments 9).

Does MAPK Mediation Require Direct Phosphorylation of the NHE-1?—Antibody against the NHE-1 carboxyl-terminal peptide (residues 801–815), prepared in our laboratory, was used for Western immunoblots of human platelet extracts. As was previously reported (11), human platelets contain this antiporter with an apparent molecular mass of 100–115 kDa (Fig. 7A). Addition of the carboxyl-terminal peptide of the NHE-1 (5 μg/ml) abolished the observed chemiluminescence of the NHE-1 protein band (Fig. 7B). These results emphasize the specificity of the antibody toward this antiporter.

Immunoprecipitations of NHE-1-phosphorylated protein, using this antibody, are shown in Fig. 8. As reported for fibroblasts (12, 13), MGH-U1 cells (14), and platelets (11), the antiporter is phosphorylated in unstimulated cells (lane a). Surprisingly, the degree of phosphorylation of the NHE-1, in platelets stimulated for 1 min with AVP (lane b), was indistinguishable from that observed in control platelets (lane a). The phosphorylation of the NHE-1 protein in AVP-treated platelets did not increase up to 5 min (data not shown). In contrast, treatment of platelets with PMA resulted in increased phosphorylation of the NHE-1 (lane c). This phosphorylation was...
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**Fig. 4.** The effect of PMA and AVP concentrations on MAPK activity. Platelets were activated by different concentrations of PMA (A) or AVP (B) for 1 min. MBP kinase activity was determined as described in Fig. 3. Shown are graphic presentations obtained by scanning films and integrating the specific phosphorylated MAPK protein bands, using ImageQuant Personal Densitometer.

**Fig. 5.** The effect of V₁ and V₂ agonists and antagonists on MAPK activity. Platelets were activated for 1 min by 10⁻³ M AVP (lanes 2–4), V₂ (lane 3) and V₁ (lane 4) antagonists (10⁻⁶ M) were simultaneously added with AVP. Platelets were also exposed to V₂ (lane 5) and V₁ (lane 6) agonists (10⁻³ M). Control platelets are presented in lane 1. MBP kinase activity was determined as described in Fig. 3.

**Fig. 6.** The effect of staurosporine, genistein, and MEK inhibitor on PMA- and AVP-elevated MAPK activity. Platelets were activated for 1 min by 5 x 10⁻⁷ M PMA (lanes 2–5) or by 10⁻⁷ M AVP (lanes 6–9). Staurosporine at 5 x 10⁻⁷ M was added to the cells in lanes 3 and 7, genistein at 2 x 10⁻⁵ M was added to the cells in lanes 4 and 8, and MEK inhibitor at 2.5 x 10⁻⁵ M was added to the cells in lanes 5 and 9. Control platelets are presented in lane 1. MBP kinase activity was determined as described in Fig. 3, representative of three experiments.

DISCUSSION

Agonist stimulation of platelets results in activation of many protein kinases. In this study, we have examined the stimulation of MAPKs during PMA- and AVP-induced platelet activation. Two forms of MAPK, 42 and 44 kDa, were identified in human platelets. The change in the electrophoretic mobility of MAPKs (Fig. 2) indicates the conversion of these proteins to their phosphorylated and activated forms by PMA and AVP. Accordingly, the in-gel MBP kinase activity of both MAPKs (Fig. 2) indicates the conversion of these proteins to their phosphorylated and activated forms by PMA and AVP. The presented values are the means ± S.E. of 4 different experiments.

**Fig. 7.** Western Immunoblots for NHE-1 in human platelets. Western immunoblotting of human platelets, with specific anti-NHE-1 carboxyl terminus peptide antibody in the absence (A) or in the presence (B) of the NHE-1 carboxyl-terminal peptide (5 μg/ml).

**Fig. 8.** Effect of PMA and AVP on the phosphorylation of the NHE-1 immunoprecipitation of ³²P-labeled platelets with specific anti-NHE-1 carboxyl terminus peptide. Extracts were prepared from control platelets (lane a) or from platelets that were activated by 10⁻⁷ M AVP (lane b) or by 5 x 10⁻⁷ M PMA (lanes c–e) for 1 min. Staurosporine at 5 x 10⁻⁷ M was added to the cells in lane d, genistein at 2 x 10⁻⁵ M to the cells in lane e, and MEK inhibitor at 2.5 x 10⁻⁵ M to the cells in lane f. The rate (at pH 7.0) of BCECF-labeled platelet alkalinization following acidification in NaCl-sodium propionate solution was calculated. The rate of alkalinization is shown in Fig. 7.

**Table 1.** The effect of staurosporine, genistein, and MEK inhibitor on AVP- and PMA-elevated Na⁺/H⁺ exchange rate. The rate (at pH 7.0) of BCECF-labeled platelet alkalinization following acidification in NaCl-sodium propionate solution was calculated. The rate of alkalinization is shown in Fig. 7.
human platelets, induced by 10^{-7} \text{ m} \text{ PMA}, were below the detection range in the work of Nakashima et al. (9). PMA concentration-dependent studies indicate that a concentration of 10^{-7} \text{ m} is sufficient to induce 50% of maximal MAPK stimulation (Fig. 4A). These results are in agreement with a significant stimulation of cytosolic MBP and S6 kinase activities, in sheep platelets, using 2 \times 10^{-7} \text{ m} \text{ PMA} (7).

The activation of MAPKs in response to PMA and AVP is transient (Fig. 3). Activation of MAPKs requires both threonine and tyrosine phosphorylation, which is catalyzed by the single dual-specific enzyme-MAPK kinase (3). Therefore, it is likely that MAPK dephosphorylation is regulated by the activity of a dual specificity protein phosphatase, capable of dephosphorylating threonine and tyrosine residues (31, 39–42). Recently 3CH134, a dual specificity protein phosphatase, was established as a dynamically regulated, immediate gene product. This protein phosphatase was suggested to have a role in attenuating signaling pathways initiated by angiotensin II in VSMC (42) and by serum in fibroblasts (31). Platelets are nonproliferative, terminally differentiated cells that do not contain a nucleus or active machinery for protein synthesis (43, 44). Thus, deactivation of MAPKs following agonist-dependent activation, does not involve induction of protein phosphatase expression.

Several lines of evidence indicate a possible regulatory role for MAPKs in NHE activation in human platelets. 1) Both PMA and AVP rapidly increase, in a similar time frame, the phosphorylation and activation of the MAPKs and the activity of the NHE. When platelets are suspended in NaCl-sodium propionate solution, a rapid intracellular acidification takes place followed by alkalization process that represents the activity of the NHE. If the NaCl-sodium propionate solution contains PMA or AVP, the platelets respond by an increase in the ratio of Na\(^+\)/H\(^+\) exchange that can be measured within 10 s (26). The results in Fig. 3 indicate that both PMA and AVP induce an increase in the in-gel MBP kinase activity within this period of time. 2) The concentration dependence is very similar for activation of both MAPKs (Fig. 4) and NHE (24). Maximal activation of the PMA and AVP effects are observed at 3 \times 10^{-7} \text{ m} and 10^{-6}-10^{-7} \text{ m}, respectively. The concentrations for half-maximal effect for MAPK and NHE activation are: (a) 10^{-7} \text{ M} and 1.2 \times 10^{-7} \text{ M} PMA, respectively, and (b) 0.9 \times 10^{-9} \text{ M} and 0.3 \times 10^{-9} \text{ M} AVP, respectively. 3) Both PMA-induced MAPK activity and Na\(^+\)/H\(^+\) exchange are inhibited by staurosporine and MEK inhibitor but are not affected by genistein. In contrast, both AVP-induced MAPK activity and Na\(^+\)/H\(^+\) exchange are inhibited by genistein and MEK inhibitor but are not affected by staurosporine.

These observations, summarized in Fig. 9, indicate the existence of: 1) distinct pathways for PMA and AVP stimulation of the NHE; 2) a possible regulatory role for MAPKs in NHE activation by both PMA and AVP; 3) MEK and MAPK as a part of a converging signaling pathway leading to the activation of the NHE; 4) PKC but not tyrosine kinase involvement, upstream to MAPKs, in the signaling pathway initiated by PMA; 5) tyrosine kinase but not PKC involvement, upstream to MAPKs, in the signaling pathway initiated by AVP.

Epidermal growth factor, \(\alpha\)-thrombin, serum, and okadaic acid stimulate Na\(^+\)/H\(^+\) exchange activity in fibroblasts in a time-dependent manner that correlates with increased phosphorylation of the NHE-1 (12, 13). Activation and phosphorylation of the Na\(^+\)/H\(^+\) antiport by okadaic acid were also demonstrated in lymphocytes (15). These results suggest that the proximate step in Na\(^+\)/H\(^+\) exchange activation is mediated by growth factor-activable NHE-1 kinases(s). Unlike the effects of growth promoters, activation of the antiport during volume regulation was not associated with increased phosphorylation of the NHE-1 (14). Furthermore, deletion of all major phosphorylation sites, mapped to the cytoplasmic tail between amino acids 636 and 815, still preserves 50% of the growth factor-induced cytoplasmic alkalization (16). These results support the existence of a mechanism that does not require direct phosphorylation of the NHE-1. In this study, we are demonstrating that MAPK may be a part of a converging signaling pathway leading to the activation of Na\(^+\)/H\(^+\) exchange by PMA and AVP. We anticipated that if phosphorylation of the NHE-1 is essential for its activation, by MAPK downstream kinases, then both PMA and AVP will increase its phosphorylation. Our results demonstrate that treatment of platelets with PMA resulted in increased phosphorylation of the NHE-1. In contrast, exposure of platelets to AVP did not change the phosphorylation of the antiporter (Fig. 8). Therefore, we propose that the control of the Na\(^+\)/H\(^+\) exchange by the MAPK pathway, in human platelets, probably does not require direct phosphorylation of the NHE-1. Both PMA and AVP activate Na\(^+\)/H\(^+\) exchange in human platelets, with higher activation induced by PMA (Table I and Ref. 26). On the other hand, both PMA and AVP phosphorylate and activate MAPKs to the same degree (Figs. 2 and 3). Nevertheless, Na\(^+\)/H\(^+\) exchange activation induced by AVP is completely abolished by the MEK inhibitor while Na\(^+\)/H\(^+\) exchange activation induced by PMA is inhibited only by 50%. Interestingly, only PMA induces NHE-1 phosphorylation that is inhibited by staurosporine but is not affected by MEK inhibitor (Fig. 8). Taken together, these data support the existence of an additional distinct mechanism for PMA activation of the antiporter. In an earlier study, we investigated the phosphorylation of pleckstrin, a major substrate for PKC in human platelets (26), and a similar pattern of phosphorylation was observed. We propose, as illustrated in Fig. 9, that PKC activated by PMA can lead to Na\(^+\)/H\(^+\) exchange activation through two separate mechanisms. One mechanism involves the phosphorylation of the NHE-1 and the other one involves MEK and MAPK activation but does not require the phosphorylation of the NHE-1.

The binding of AVP to the V\(_1\) receptor is known to induce the rapid hydrolysis of inositol phospholipids to generate the intracellular second messengers diacylglycerol, which activates PKC, and inositol 1,4,5-trisphosphate that mobilizes Ca\(^{2+}\) (45).
Interestingly, tyrosine phosphorylation induced by neuromodulator toxins in glomerular mesangial cells, was mediated through PKC-dependent and -independent pathways (46). In addition, PKC was not responsible for the rapid stimulation of p125^{FAK} tyrosine phosphorylation by bombesin, in Swiss 3T3 cells (47). Furthermore, PAF-induced activation of 42-kDa MAPK variant in sheep platelets was relatively low, it was achieved through PKC-dependent and independent mechanisms (7).

The AVP-induced stimulation of MAPKs, although mediated through a V_{1A} receptor (Fig. 5), does not involve activation of PKC since the AVP-stimulated MAPK activity was not inhibited by staurosporine. These results are consistent with our previous study (26) in which NHE activation by AVP through a V_{1A} receptor was not mediated by PKC. The present study provides evidence that, in human platelets, AVP stimulates the NHE through a signal transduction pathway that is independent of PKC. MAPK-induced activation of the NHE probably does not involve the phosphorylation of the antipporter. Further studies are needed for the discovery of additional elements in the signaling pathways leading to the activation of the NHE by extracellular signals.

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