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PROTEIN PHOSPHORYLATION AND ACTIVATION OF HUMAN PLATELETS BY SODIUM FLUORIDE

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SUMMARY: The ability of sodium fluoride (NaF) and thrombin to stimulate aggregation and protein phosphorylation in intact human platelets was measured and compared. When platelets were stimulated by NaF, phosphorylation of the 20 KDa protein was transient and after 5-10 min returned to the same level as that of unstimulated cells. On the other hand, 47 KDa protein was slowly phosphorylated without obvious dephosphorylation. The slow activation of the 47 KDa protein phosphorylation correlated well with the time required for the aggregation and secretion. Phosphoamino acid analysis showed that the phosphorylated amino acids of the 47 KDa protein from platelets activated by NaF and thrombin were slightly different. These results suggest that different stimuli may lead to the same protein phosphorylation by different biochemical mechanisms of action. © 1988 Academic Press, Inc.

Platelets respond to external stimuli with a multitude of cellular and biochemical responses including: shape change, aggregation, secretion and clot retraction. It has also been suggested that the phosphorylation of certain cellular proteins in platelets is closely associated with the activation of platelets and subsequent these responses. Platelets stimulated by thrombin or collagen are accompanied with enhanced ^{32}Pi incorporation into two endogenous proteins of 47 KDa and 20 KDa (1,2). Activation of protein kinase C may be responsible for the phosphorylation of the 47 KDa protein (3), whereas the 20 KDa protein, identified as the myosin light chain, is phosphorylated by Ca^{2+} and calmodulin-dependent protein kinase (4,5). However, the exact roles of

The abbreviation used is: SDS, sodium dodecyl sulfate.

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protein phosphorylation in platelet have not yet been clearly established.

The addition of sodium fluoride (NaF) to platelets induces dense granule release (6-9). However, the mechanism of NaF induced platelet activation is unknown. In this study, we have investigated the phosphorylation of 47 KDa and 20 KDa proteins in platelets activated by NaF and thrombin. The difference in protein phosphorylation induced by these agonists may provide important clues as to the function of these proteins in platelet activation.

EXPERIMENTAL PROCEDURES

Materials: NaF was obtained from Wako Chemicals. Thrombin was purchased from Sigma. Carrier-free (^{32}P)orthophosphoric acid was from ICN. Other chemicals were reagent grade.

Preparation of human platelets: Venous blood was collected by forearm venepuncture from normal healthy volunteers who had not taken medication for at least the previous 2 weeks. Washed human platelets were prepared by the method of Haslam and Lynham (2). Washed platelets (3 x 10^9 cells/ml) were labeled with 1 mCi of carrier-free ^{32}Pi (2). The platelets were then washed in Tyrode's solution (with phosphate) containing both 0.35 % bovine serum albumin and apyrase (0.6 ADPase units/ml) and were finally suspended at a count of 4 x 10^8/ml in albumin-free Tyrode's solution containing apyrase and 1 mM $CaCl_2$.

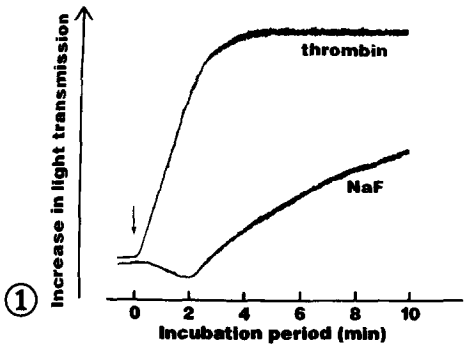
Measurement of protein phosphorylation: Aliquots of ^{32}P-labeled platelet suspension were used for measurement of agonist-stimulated protein phosphorylation. Samples (final volume 0.3 ml) of platelets were added to plastic tubes with buffer or agonist, and the reactions terminated after designated times by taking a 30 µl of aliquot and adding it to 15 µl of 3 times concentrated electrophoresis sample buffer. The proteins were separated using 12.5 % SDS-polyacrylamide gel electrophoresis (10), and changes in ^{32}P-phosphorylation state were visualized by autoradiography, and quantitated by densitometric tracing at 440 nm using a chromatogram scanner (Toyo Scientific Industry Co., Model DMU-33C).

Platelet aggregation: In another set of experiments, 1.9 ml of platelet suspension (4 x 10^8/ml) in Tyrode's solution containing 1 mM $CaCl_2$ was stirred at 37° C in a platelet aggregometer (Bryston Manufacturing LTD.). Aggregating agents were added after stirring for 1 min and aggregation was recorded the increase in transmission.

Phosphoamino acid analysis: The proteins were eluted from the excised gels as in (11). One-dimensional high voltage electrophoresis was performed at 3,000 volts for 1 h in the presence of pyridine acetate buffer (5 % acetic acid and 0.5 % pyridine, v/v) as in (12).

RESULTS

Stimulation of human platelets with NaF induced a slow aggregation with a lag period of 2 min (Fig. 1). In contrast,



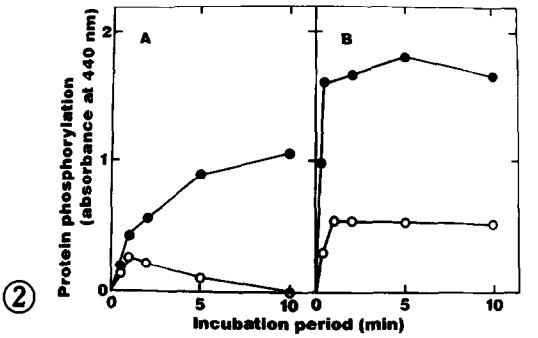


Fig. 1. NaF and thrombin-induced aggregation of human platelets. Aliquots (1.9 ml) of washed human platelets were placed in aggregometer tubes and NaF (30 mM) or thrombin (100 mU/ml) was added. The aggregation was recorded as an increase in light transmission through the platelet suspension. The arrow indicates the addition of the agonists.

Fig. 2. Time courses of phosphorylation of 47 KDa and 20 KDa proteins in platelets induced by NaF (30 mM) or thrombin (100 mU/ml). The proteins were separated by SDS-polyacrylamide gel electrophoresis. The gels were stained, destained, dried and subjected to autoradiography. The extent of phosphorylation was quantitated as in "EXPERIMENTAL PROCEDURES". A, with NaF; B, with thrombin. ●, 47 KDa protein; ○, 20 KDa protein.

thrombin caused a rapid aggregation with little or no detectable lag period. The maximal intensity of aggregation induced by NaF was about 60 % of that induced by thrombin. This observation was compatible with the data reported previously (6,13).

Thrombin is known to induce the phosphorylation of two main proteins in human platelets, 47 KDa and 20 KDa (1,2), which are substrates for protein kinase C (3) and myosin light chain kinase (4,5), respectively. Fig. 2 shows the degree of phosphorylation of the 47 KDa and 20 KDa proteins in human platelets induced by NaF and thrombin as a function of activation time. NaF induced the phosphorylation of 47 KDa and 20 KDa proteins clearly in distinct time dependent manner from that induced by thrombin (Fig. 2). After 1 min following the treatment of NaF, the 20 KDa protein was maximally phosphorylated and after 5-10 min returned to the same level as that of unstimulated cells (Fig. 2A). In the case of the phosphorylation of 47 KDa protein, however, this protein was gradually phosphorylated with the time for half-

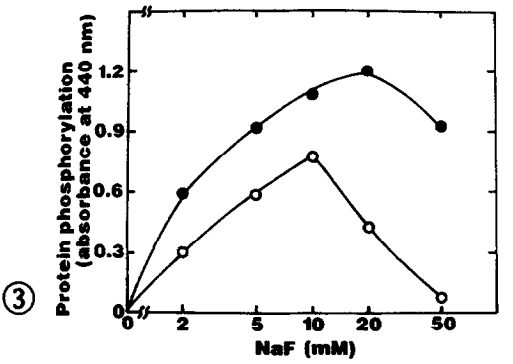
maximal phosphorylation being about 3 min, without obvious dephosphorylation even after 10 min (Fig. 2A). In contrast, the effect of thrombin on the phosphorylation of both 47 KDa and 20 KDa proteins was rapid with the time for half-maximal phosphorylation of these proteins being about 10 s (Fig. 2B).

The effect of NaF concentration on the phosphorylation of 47 KDa and 20 KDa proteins was then tested. The effect of NaF on the phosphorylation of these proteins was dependent upon the concentration. NaF concentration that produced maximal phosphorylation was 20 mM for 47 KDa protein and 10 mM for 20 KDa protein, respectively (Fig. 3).

In order to determine the nature of the phosphorylated amino acids, 47 KDa protein was eluted from the SDS-polyacrylamide gel and the acid hydrolysates of the polypeptides were subjected to high voltage paper electrophoresis (Fig. 4). In NaF induced phosphorylation of 47 KDa protein, the major phosphorylated amino acid was phosphoserine. No phosphotyrosine could be detected with this stimulant. While in the case of 47 KDa protein from thrombin stimulated platelets not only serine but threonine was also phosphorylated to a small extent. This suggests that the mechanism of phosphorylation of the 47 KDa protein by NaF and thrombin appears to be different.

DISCUSSION

In the present study we have demonstrated that NaF induced activation of platelets accompanies by the time-dependent incorporation of ^{32}Pi into 47 KDa and 20 KDa proteins. When platelets are stimulated by NaF, the 20 KDa protein is phosphorylated and then rapidly dephosphorylated. Under the same condition, however, the 47 KDa protein is slowly phosphorylated without obvious dephosphorylation. It is important to note that the slow time course of the 47 KDa protein phosphorylation



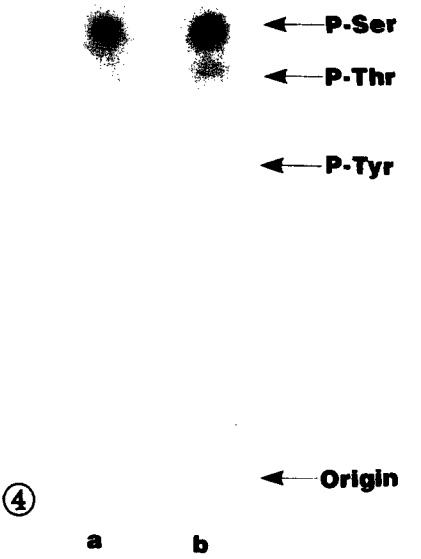


Fig. 3. NaF induced phosphorylation of 47 KDa and 20 KDa proteins in platelets as a function of NaF concentration. Aliquots of ^{32}P-labeled platelets (4 x 10^8/ml) were activated with various concentrations of NaF for 5 min. The proteins were separated and analyzed as in the legend to Fig. 2. ●, 47 KDa protein; O, 20 KDa protein.

Fig. 4. Phosphoamino acid analysis of phosphorylated 47 KDa protein from NaF or thrombin activated platelets. Phosphorylated proteins from activated platelets were analyzed by SDS-polyacrylamide gel electrophoresis. The 47 KDa protein was localized by autoradiography. The proteins were eluted from the excised gels, hydrolyzed in the presence of 6N HCl for 2 h at 110° C and the hydrolysates were subjected to high-voltage paper electrophoresis followed by autoradiography. Lane a, with NaF; lane b, with thrombin. The radioactivities of these spots corresponding to phosphoserine and phosphothreonine of the protein from NaF-activated platelets and those from thrombin-activated ones were 2,050 cpm, 450 cpm, 2,500 cpm and 1,490 cpm, respectively. The standards used were 10 µg of each phosphoserine, phosphothreonine and phosphotyrosine.

correlates well with that of aggregation. These observations strongly suggest that the phosphorylation of the 47 KDa protein may be involved in platelet activation by NaF. Daniel et al. reported that ADP caused the shape change and the 20 KDa protein phosphorylation without secretion or the 47 KDa protein phosphorylation in human platelets (14). Moreover, NaF is known to induce a slow secretion (about 15 min) of adenine nucleotides and serotonin (6,8,9,13). Along with these reports, our data would be consistent with the view of others (2,15) that the 47 KDa protein phosphorylation is closely associated with secretion.

The phosphorylated amino acids of the 47 KDa protein from platelets activated by NaF and thrombin were slightly different. A similar observation was also reported that the pattern of the 47 KDa protein phosphorylation induced by wheat germ agglutinin was different from that stimulated by thrombin (16). Thus, the distinct biochemical mechanisms of action elicited by different agonists may respond to multiple cellular responses of platelets.

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