Defects of thrombin-induced protein phosphorylation in platelets from stroke-prone spontaneously hypertensive rats

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Aggregation and secretion of washed platelets from stroke-prone spontaneously hypertensive rats (SHRSP) were greatly reduced by the development of the hypertension compared with those of platelets from age-matched normotensive Wistar-Kyoto rats (WKY). Concomitantly, thrombin-induced phosphorylation of the 47 kDa protein in SHRSP platelets was significantly decreased. However, TPA-induced aggregation, secretion and 47 kDa protein phosphorylation in SHRSP platelets were similar to those in WKY platelets. These results suggest that protein kinase C activity and its substrate were normally present in SHRSP platelets and that defects in the receptor-mediated activation of protein kinase C. This defective protein phosphorylation may be an underlying mechanism for the dysfunction of SHRSP platelets.

(Stroke-prone spontaneously hypertensive rat) Platelet aggregation 12-O-Tetradecanoylphorbol-13-acetate Serotonin Secretion Protein phosphorylation

1. INTRODUCTION

We have shown that thrombin-, collagen-, ADP- and ionophore A23187-induced aggregation, as well as thrombin-induced serotonin release, in washed platelets from SHRSP were greatly reduced with the development of hypertension compared with those of platelets from age-matched normotensive WKY [1,2]. To clarify the mechanism for dysfunctions in SHRSP platelets, we examined platelet-protein phosphorylation which played an important role in stimulus-response coupling [3].

In human platelets stimulated with thrombin or other physiological stimuli, there was a rapid and transient rise in 1,2-diacylglycerol and intracellular Ca^{2+} concentration. On the other hand, two endogenous proteins with approximate molecular masses of 47 kDa and 20 kDa are rapidly and heavily phosphorylated in parallel with physiological responses [3]. The phosphorylation of 20 kDa protein (myosin light chain) is calmodulin dependent and requires mobilization of Ca^{2+}. The 47 kDa protein is phosphorylated by phospholipid-dependent and Ca^{2+}-activated protein kinase (protein kinase C), its activation is associated with the transient appearance of diacylglycerol in the membrane. Diacylglycerol produced by the receptor-activated hydrolysis of phosphatidylinositol, acts as a signal molecule by enhancing the Ca^{2+} sensitivity of protein kinase C. Several phorbol esters such as TPA (a potent tumor promotor) directly activate protein kinase C by increasing the affinity for Ca^{2+}.

Here it is demonstrated that thrombin-induced protein phosphorylation (47 kDa) was significantly decreased in SHRSP platelets which showed

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Abbreviations: SHRSP, stroke-prone spontaneously hypertensive rats; WKY, Wistar-Kyoto rats; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; $[Ca^{2+}]_i$, cytoplasmic free calcium concentration

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hypoaggregation and reduced secretory responses. Similar abnormalities of platelets in a patient have been cited as a new congenital defect of platelets by Hardisty et al. [4].

2. MATERIALS AND METHODS

[^{14}C]Serotonin (58.5 mCi/mmol) was from NEN (Boston, MA) and [^{32}P]orthophosphate (carrier free) from Japan Isotope (Tokyo), ionophore A23187 from Calbiochem (La Jolla, CA), TPA from Sigma (St. Louis, MO) and thrombin from Midori Cross (Osaka).

2.1. *Experimental animals and preparation of washed platelets*

SHRSP and WKY, maintained by brother-sister breeding, were killed at 11–14 weeks old. Washed platelets were prepared according to Baenziger and Majerus [5] or Feinstein et al. [6].

2.2. *Measurement of platelet aggregation*

Platelet aggregation was measured by the turbidometric method described in [7].

2.3. *Assay of [^{14}C]serotonin release*

The assay was performed by a modification of the methods of Hofmann et al. [8] and Holmsen et al. [9]. [^{14}C]Serotonin prelabelled platelets in resuspending buffer [5] (4×10^8 cells/ml) were incubated with thrombin at 37°C in the presence of 1.5 mM Ca^{2+}. The percentage of release of [^{14}C]serotonin was calculated according to the following equation: release (%) = (St–Sc)/(T–Sc) [T; total radioactivity in the whole mixture, St and Sc; radioactivity in the supernatant with and without thrombin].

2.4. *Analysis of platelet-protein phosphorylation*

^{32}P-prelabelled platelets (0.2 ml) which were prepared according to Feinstein et al. [6] and suspended in 25 mM Tris-HCl buffer containing 137 mM NaCl, 5.4 mM KCl, 0.2% dextrose, pH 7.4, to make 5×10^8 cells/ml, were incubated with $CaCl_2$ (10 μl, final concentration 1.5 mM) and aggregating agents (20 μl) while stirring at 37°C. The reaction was terminated by adding a SDS stopping solution (9% SDS, 6% mercaptoethanol, 15% glycerol, 186 mM Tris-HCl, pH 6.8, with a small amount of bromphenol blue) and then the mixture was boiled for 2 min. The protein was subjected to SDS-polyacrylamide gel electrophoresis by the method of Laemmli [10] and ^{32}P content of the protein bands were analyzed by densitometry of autoradiographs (Chromoscan 200, Joice Loebie, Gateshead, England). Results are expressed as relative protein phosphorylation to phosphorylation observed in unstimulated condition.

3. RESULTS

3.1. *Thrombin-induced aggregation and [^{14}C]serotonin release*

When platelets from SHRSP at hypertensive ages and age-matched WKY were stimulated with thrombin, SHRSP platelets aggregated and released [^{14}C]serotonin at a significantly reduced rate compared with WKY platelets as shown in fig.1. Hypoaggregation and reduction of [^{14}C]serotonin release in SHRSP platelets were observed at all the concentrations examined (fig.1b and d). Aggregation was affected more than the release in SHRSP platelets.

3.2. *Protein phosphorylation by thrombin*

Since aggregation as well as secretion paralleled with protein phosphorylation [11], thrombin-induced protein phosphorylation was examined. ^{32}P-prelabelled platelets from SHRSP and WKY were incubated at various times with 0.15 U/ml thrombin and the protein was subjected to SDS-PAGE. Gels were stained with Coomassie brilliant blue R and the dried gels were analyzed by autoradiography. In the autoradiogram the band of 47 kDa protein was barely visible in the sample incubated without thrombin, while dark bands appeared when incubated with thrombin. The bands of SHRSP platelets were lighter than those of WKY platelets. In contrast, the 20 kDa protein bands were already observed in unstimulated samples and became slightly darker in the stimulated samples. Fig.2 shows the time courses and concentration-dependency curves of phosphorylation of the 47 kDa proteins of SHRSP and WKY platelets by thrombin.

When ^{32}P-prelabelled platelets were incubated with 0.15 U/ml thrombin, phosphorylation of protein occurred instantly and reached a maximum at 30 s. Compared with WKY platelets, there was

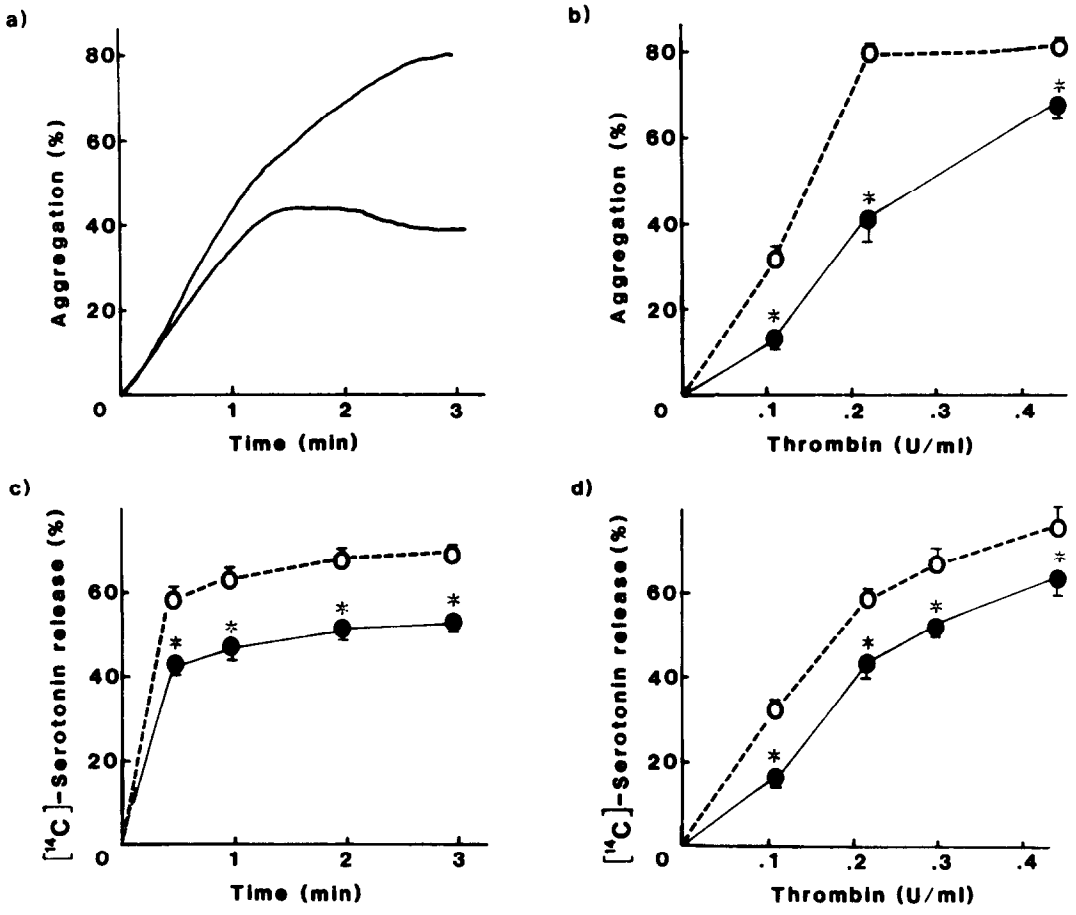

Fig.1. Time courses and concentration-dependency curves of thrombin-induced aggregation (a,b) and [^{14}C]serotonin release (c,d) in platelets from SHRSP (—●—) and WKY (- - ○ - -). Washed platelets (4×10^8 cells/ml) were stimulated with thrombin (0.22 U/ml in a, 0.30 U/ml in c) for 3 min in b and d. Results are expressed as mean ± SD for the number of platelet preparations ($n = 5$ in b, $n = 3$ in c, $n = 4$ in d). * Significance $P < 0.001$.

a marked decrease in phosphorylation of the 47 kDa protein in SHRSP platelets. The phosphorylation of the 20 kDa protein was similar in the platelets from the two strains. The 47 kDa protein was dose-dependently phosphorylated up to 0.4 U thrombin/ml in SHRSP platelets. The phosphorylation was a maximum at 0.2 U thrombin/ml in WKY platelets. Simultaneous measurement of aggregation in a ^{32}P-prelabelled platelet preparation showed significantly attenuated responses in SHRSP platelets compared with those in WKY platelets (see fig.2, legend).

3.3 *Effects of TPA on protein kinase C*

Since there was a marked decrease in thrombin-induced phosphorylation of the 47 kDa protein of SHRSP platelets the effects of TPA, a direct activator of protein kinase C, were examined (fig.3). TPA at concentrations less than 20 nM did not induce aggregation (fig.3a) or [^{14}C]serotonin secretion (not shown), while at the higher concentrations, aggregation was induced slowly with time as shown in fig.3b. In contrast to thrombin stimulation, the aggregation response to TPA (80 nM) was similar in SHRSP and WKY platelets. Fig.3c and d depicts the effect of TPA on 47 kDa protein phosphorylation. The TPA effect on phosphorylation was seen at a concentration as low as 0.5 nM and it peaked at 30 s–1 min. Again there was no difference in the magnitude of 47 kDa pro-

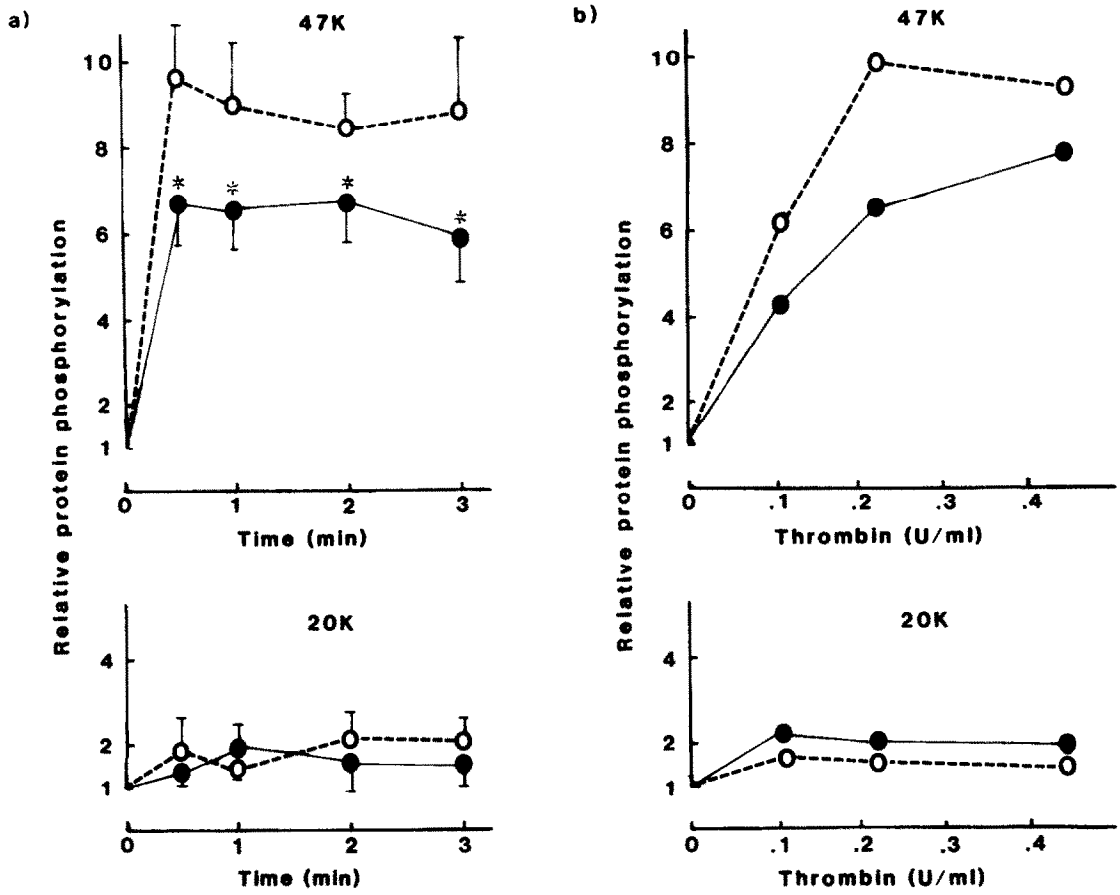

Fig.2. Time courses (a) and concentration-dependency curves (b) of thrombin-induced protein phosphorylation in platelets from SHRSP (—●—) and WKY (- -○- -). ^{32}P-prelabelled platelets in 25 mM Tris-HCl buffer (pH 7.4) were stimulated with thrombin (0.15 U/ml in a) for 3 min in b. Phosphorylated protein was analyzed by autoradiography as described in section 2. Results are expressed as mean ± SD in a ($n = 4$) and mean of duplicates in b. Aggregation responses simultaneously measured with the ^{32}P-prelabelled platelets are: a, 31% in SHRSP and 73% in WKY; b, (thrombin 0.11 U/ml) 9% in SHRSP and 33% in WKY, (thrombin 0.22 U/ml) 59% in SHRSP and 82% in WKY, (thrombin 0.44 U/ml) 86% in SHRSP and 89% in WKY. * Significance $P < 0.05$.

tein phosphorylation between platelets from the two strains. These results suggest that the activity of protein kinase C and the amount of the substrate protein (47 kDa) were comparable in SHRSP and WKY platelets, but the receptor-mediated activation of protein kinase C seems to be impaired in SHRSP platelets.

4. DISCUSSION

We found in this study that thrombin-induced phosphorylation of the 47 kDa protein was significantly reduced in SHRSP platelets accompanying hypoaggregation and reduced serotonin release. The phosphorylation of the protein, however, was comparable to that in WKY platelets when stimulated with TPA which activated protein kinase C directly in vivo and in vitro. The possibility that a reduction in protein phosphorylation might result from a reduced production of [^{32}P]-ATP in SHRSP platelets would be excluded by the fact that TPA-induced phosphorylation of the 47 kDa protein was similar in platelets from the two strains; there was no difference in thrombin-induced phosphorylation of the 20 kDa protein. The defective phosphorylation of the 47 kDa pro-

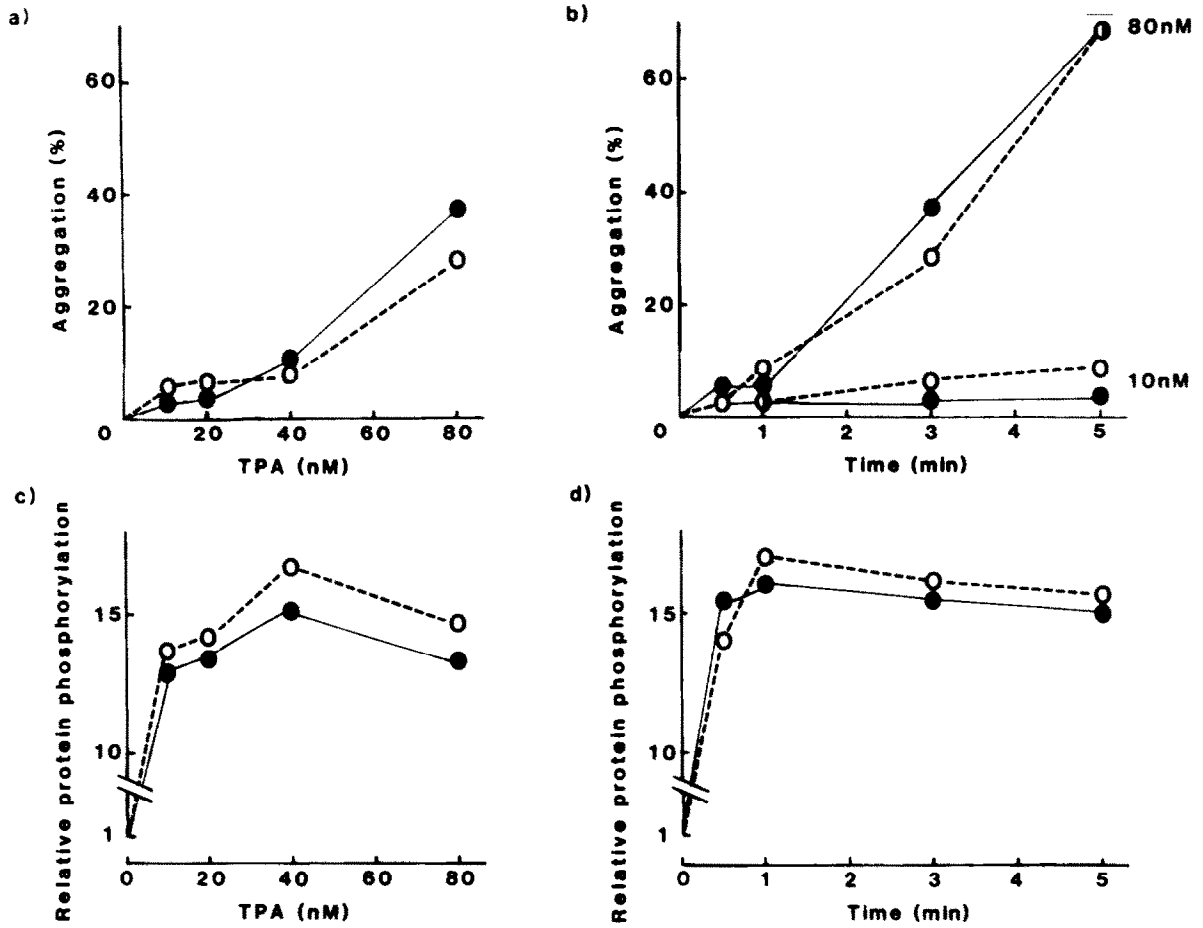

Fig.3. Concentration-dependency curves and time courses of TPA-induced aggregation (a,b) and 47 kDa protein phosphorylation (c,d) in platelets from SHRSP (—●—) and WKY (- -○- -). ^{32}P-prelabelled platelets in 25 mM Tris-HCl buffer (pH 7.4) were stimulated with TPA for 5 min in a and 1 min in c. Each point represents mean of triplicates.

tein may be influenced by an impairment of receptor-mediated formation of intracellular signals such as diacylglycerol and Ca^{2+} or an impairment in activation of protein kinase C by these activators. Since decreased responses in SHRSP platelets were observed with several stimuli other than thrombin, defects will not be in specific receptors. Therefore, it is of interest to note that thrombin-induced increase in $[Ca^{2+}]_i$, measured by using quin 2, was delayed in SHRSP platelets; the time to peak was twice as long as in SHRSP platelets. Thrombin-induced uptake of $^{45}Ca^{2+}$ was also delayed in SHRSP platelets [13]. Additionally, the content of phosphatidylinositol in SHRSP platelets was lower than in WKY platelets. The contents in the erythrocytes from SHR are reportedly changed [14].

Phosphatidylinositol and phosphatidylinositol phosphates play a central role in stimulus-response coupling [3,15]. It remains to be explored how the decrease in phosphatidylinositol content of SHRSP platelets influences signal transduction and how the delay of thrombin-induced $[Ca^{2+}]_i$ increase is associated with defective protein phosphorylation and consequent physiological responses. This study will contribute towards understanding a link of defective phosphorylation of platelet protein to genetic dysfunction of platelets.

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REFERENCES

[1] Tomita, T., Umegaki, K. and Hayashi, E. (1984) Stroke 15, 70–75.
[2] Umegaki, K., Nakamura, K. and Tomita, T. (1985) Blut, in press.
[3] Nishizuka, Y. (1984) Nature 308, 693–698.
[4] Hardisty, R.M., Machin, S.J., Nokes, T.J.C., Rink, T.J. and Smith, S.W. (1983) Br. J. Hematol. 53, 543–557.
[5] Baenziger, N.L. and Majerus, P.W. (1974) Methods Enzymol. 31, 149–155.
[6] Feinstein, M.B., Egan, J.J. and Opas, E.E. (1983) J. Biol. Chem. 258, 1260–1267.
[7] Tomita, T., Umegaki, K. and Hayashi, E. (1983) J. Pharmacol. Methods 10, 31–44.
[8] Hofmann, S.L., Prescott, S.M. and Majerus, P.W. (1982) Arch. Biochem. Biophys. 215, 237–244.
[9] Holmsen, H. and Dangelmaier, C.A. (1981) J. Biol. Chem. 256, 10449–10452.
[10] Laemmli, U.K. (1970) Nature 277, 680–685.
[11] Verhoeven, A.J.M., Gorter, G., Mommersteeg, M.E. and Akkerman, J.W.N. (1985) Biochem. J. 228, 451–462.
[12] Tomita, T., Umegaki, K., Nakamura, K. and Hayashi, E. (1984) Thromb. Res. 35, 219–230.
[13] Umegaki, K., Ikeda, M. and Tomita, T. (1985) Thromb. Res., in press.
[14] Koutouzov, S., Marche, P., Girard, A. and Meyer, P. (1983) Hypertension 5, 409–414.
[15] Berridge, M.J. and Irvine, R.F. (1984) Nature 312, 315–321.